Using a bifunctional polymer for the functionalization of Fe₃O₄ nanoparticles

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A bifunctional maleimido-tetra(ethylene glycol)-poly(glycerol monoaCRYlate) (MAL-TEG-PGA) polymer
was synthesized and used as a linker to couple functional biomolecules to iron oxide nanoparticles. The
cell-penetrating peptide Tat was chosen as a model ligand and successfully conjugated to the surface
of Fe₃O₄ nanoparticles using MAL-TEG-PGA. The Tat-conjugated Fe₃O₄ nanoparticles can be prepared
simply by applying the linker to the iron oxide nanoparticles and then coupling the Tat peptide to the
maleimide terminus or by coating the nanoparticles with a pre-coupled linker. Cell-uptake studies dem-
onstrated that the Tat peptide was an efficient functional biomolecule to translocate iron oxide nanopar-
ticles into the cell nucleus. Tat-conjugated nanoparticles thus prepared may be useful for drug or gene
delivery.

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1. Introduction

Magnetic iron oxide nanoparticles (MIONs) have attracted a great deal of attention for their potential applications in biomedical
cal fields such as cellular labeling, tracking, imaging, drug delivery,
and tumor treatment using hyperthermia [1–4]. For these applica-
tions, the ability to disperse the nanoparticles in water and to mod-
ify their surfaces with functional biomolecules (e.g. for targeting
and therapeutics) is crucial to their widespread use. The stabiliza-
tion of MIONs in aqueous solution can be achieved by modifying
their surface with small molecular surfactants or polymers [5].
The biocompatibility and toxicity of MIONs are important criteria
for their biomedical application. Thus, MIONs are often coated with
biocompatible and functional polymers to protect the iron oxide
core from agglomeration, to provide chemical handles for conjuga-
tion with biomolecules, and to reduce non-specific cell interac-
tions. A large number of biocompatible polymers that might per-
form these coating functions have been investigated, including
natural polymers (dextran [6,7], chitosan [8], starch [9], and gelatin
[10], etc.) and synthetic polymers (polyethylene glycol [11,12],
polyacrylic acid [13], and other copolymers [14–16], etc.).
Coating MIONs with biocompatible polymers followed by mod-
ification with biomolecules improves detection abilities and spe-
cific binding to target cells. Many strategies have been developed
to conjugate functional biomolecules to the polymeric coatings
on MION surfaces. These techniques are a prerequisite for enhanc-
ing the functionalized properties of MIONs [17] and can be catego-
rized as either covalent linkage strategies [18] or physical
interactions [19]. Compared with physical interactions, covalent
linkage strategies are more effective due to their low sensitivity
to environmental conditions and high control over the molecular
orientation of bound ligands. A number of functional biomolecules
have been covalently conjugated to the polymeric coatings on
MIONs surfaces, including antibodies [20,21], proteins [22], pep-
tides [23,24], and other targeting ligands [25]. Covalent linkage
chemistry techniques include direct nanoparticle conjugation and
linker strategies.

Direct conjugation methods are not the preferred approach for
the attachment of biomolecules. MIONs may crosslink when a bio-
molecule, such as a peptide or protein, contains multiple amino
functional groups. Many biomolecules are not natively reactive
with nanoparticles and require initial modification prior to conjuga-
tion, but this modification often leads to a loss of bioactivity [26].
Linker strategies typically involve complicated multi-step synthe-
sis and modification procedures because functionalization requires
stepwise MION modification to attach a functional ligand to the
surface [23,27–29]. Therefore, the use of a polymer linker that
coordinates strongly to the iron oxide surface and also conjugates
directly with biomolecules at the desired location would simplify
the functionalization process and allow biomolecules immobilized
on the nanoparticles to retain their functional characteristics to a
large extent.

Here we report the development of a novel bifunctional
polymer anchored to the nanoparticle surface via anchoring groups
at one end and covalently linked to the functional biomolecule at

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the other end. Poly(glycerol monacrylate) (PGA) was used as the terminal anchoring block because it has been shown to coordinate tightly to the surface of Fe₃O₄ nanoparticles through its 1,2-diol groups [30,31]. A maleimide at the other terminus can be coupled to thiol-containing ligands such as peptides with cysteine residues. Functionalized iron oxide nanoparticles can be prepared by applying the linker to the iron oxide nanoparticles and then coupling the ligand to the maleimide terminus or by coating nanoparticles with a pre-coupled linker. To demonstrate the effectiveness of the surface modification, we coupled the widely used Tat cell-penetrating peptide to iron oxide nanoparticles. The Tat peptide is a sequence (residues 48–57) derived from the HIV-1 Tat protein that confers the ability to translocate across the plasma membrane [32,33]. A variety of cargos have been transported using the Tat peptide, including polymers [34], liposomes [35], and nanoparticles [36,37]. The Tat peptide sequence used in the current study was GRKKRRQRRRGGG (the italicized amino acids correspond to residues 48–57 of the HIV-1 Tat protein), which features a cysteine residue covalently linked to the maleimide. Iron oxide nanoparticles modified with the Tat peptide were efficiently taken up into the cell nucleus.

2. Experimental

2.1. Materials

Solketal (2,2-dimethyl-1,3-dioxane-4-methanol, 97%, Acros Organics), 2-bromoisobutyryl bromide (97%, Acros Organics), 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA, 98%, Alfa Aesar) and anisole (98%, Alfa Aesar) were used as received. Solketal acrylate (SA) was synthesized as previously described [38]. Cuprous bromide (CuBr, AR, Shanghai National Drug Chemical Plant, China) was purified by overnight treatment with glacial acetic acid followed by washing with absolute ethanol and ethyl ether and overnight drying under vacuum at room temperature. Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 without phenol red, and fetal bovine serum (FBS) were purchased from Gibco (Uxbridge, UK). HeLa cell lines were obtained from the Cell Resource Center (IBMS, CAMS/PUMC). All other chemicals were commercially available and used without further purification.

2.2. Synthesis of the Tat peptide

A modified Tat peptide containing the translocation sequence of the HIV-1 Tat protein was synthesized on a solid-support (Rink amide MBHA resin) using Fmoc chemistry and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyloxazolin hexafluorophosphate (HBTU)/N-hydroxysuccinimidazole (HOBr) as an activating agent. The sequence of the peptide was Gly-Arg-Lys-Glu-(Arg)₃-Gly-(Arg)₃-Gly-Cys-Gly-NH₂ (the italicized amino acids correspond to residues 48–57 of the HIV-1 Tat protein). The peptide was cleaved using tri-fluoroacetic acid/thioanisole/ethanol and purified using C18 reversed-phase HPLC. MALDI-TOF MS [M+H⁺]: 1655.9 (calc.), 1655.0 (found). For fluorescein isothiocyanate (FITC)-labeled Tat peptides, the peptide sequence is FITC-NH₂-Gly-Arg-Lys-Glu-(Arg)₃-Gly-(Arg)₃-Gly-Cys-Gly-NH₂. MALDI-TOF MS [M+H⁺]: 2115.4 (calc.), 2114.7 (found).

2.3. Synthesis of MAL-TEG-PGA (Scheme 1)

Initiator (0.20 g, 0.41 mmol), CuBr (58 mg, 0.41 mmol), SA (3.04 g, 16.3 mmol), and anisole (1.5 mL) were placed in a Schlenk flask. The flask was evacuated and purged with argon for three cycles. PMDETA (87 µL, 0.41 mmol) was added using a syringe that had been purged with argon. The flask was immersed in an oil bath preheated to 65 °C. The polymerization was allowed to proceed for 3 h under stirring. The resulting mixture was diluted with 20 mL of THF and filtered through a column packed with neutral alumina to remove the catalyst. The filtrate was concentrated and precipitated into petroleum ether to give polymer 2. A solution of 2 (2.0 g) in toluene (20 mL) was heated to reflux. After 7 h, the solvent was removed under reduced pressure to give 3. Polymer 3 was dissolved in 1.0 M HCl/dioxane (1:3, v/v), and the reaction mixture was stirred at room temperature for 24 h. The product was dialyzed (MWCO 3500) against deionized water and then lyophilized to obtain polymer 4 (MAL-TEG-PGA).

2.4. Synthesis of Tat-TEG-PGA

MAL-TEG-PGA (0.5 g, 0.11 mmol) was dissolved in deionized water, and the Tat peptide (0.23 g, 0.14 mmol) was added under stirring. The reaction mixture was stirred at room temperature for 3 h, dialyzed (MWCO 3500) against deionized water, and lyophilized to obtain the Tat-TEG-PGA.

2.5. Preparation of Fe₃O₄-PGA-TEG-Tat

Path (1): MAL-TEG-PGA (0.12 g) was dissolved in 3.0 mL of deionized water. The solution was purged with argon to remove oxygen. After 30 min, HClO₄-stabilized ferrofluid (0.4 mL) was added with vigorous stirring, and the reaction mixture was stirred at room temperature overnight. Fe₃O₄-PGA-TEG-MAL was magnetically recovered and washed three times with deionized water. The Tat peptide (2 mg, 0.0012 mmol) was added to 3 mL of the aqueous dispersion of Fe₃O₄-PGA-TEG-MAL. After sonication for 30 min, the reaction mixture was stirred at room temperature overnight. The polymer-coated nanoparticles were separated with a magnet and washed three times with deionized water. Finally, Fe₃O₄-PGA-TEG-Tat was dispersed in deionized water after being sonicated for 20 min. Path (2): Tat-TEG-PGA (0.12 g) was dissolved in 3.0 mL of deionized water. The pH of the solution was adjusted to ~3 with hydrochloric acid. The solution was purged with argon to remove oxygen. HClO₄-stabilized ferrofluid (0.4 mL) was added with vigorous stirring. After sonication for 30 min, the reaction mixture was stirred at room temperature overnight. The polymer-coated nanoparticles were separated with a magnet and washed three times with deionized water. Finally, Fe₃O₄-PGA-TEG-Tat was dispersed in deionized water after being sonicated for 20 min.

2.6. Cell culture

HeLa cells were cultured in DMEM containing 10% FBS and supplemented with penicillin (100 units mL⁻¹), streptomycin (100 µg mL⁻¹) and nonessential amino acids (0.1 mM). The culture was maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

2.7. Cell uptake of nanoparticles

HeLa cells were seeded in 6-well plates on glass coverslips at a density of 5 × 10⁵ cells/well. After 24 h, nanoparticles at a final concentration of 100 µg mL⁻¹ were co-cultured with cells at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) at pre-set intervals and subsequently fixed with 4.0% formaldehyde at room temperature for 15 min. The formaldehyde solution was discarded, and the cells were washed twice with 2 mL of PBS. The fixed cells were incubated with 2% potassium ferrocyanide and 6% hydrochloric acid (1:1) for 30 min and counterstained with nuclear fast red. The coverslips were washed twice with PBS.
and placed onto the glass microscope slides. Sample uptake was visualized using an optical microscope.

2.8. In vitro cytotoxicity assays

HeLa cells were seeded in a 96-well plate at a density of $5 \times 10^3$ cells/well in DMEM containing 10% FBS at 37°C and 5% CO$_2$. After 24 h of culture, the medium in the wells was replaced with 200 µL of fresh medium containing nanoparticles with iron concentrations ranging from 0 to 200 µg mL$^{-1}$. After incubation for 24 h, the medium was removed, and the cells were washed three times with PBS buffer to remove the nanoparticles. A 20 µL portion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)/phenazine methosulfate (PMS) solution (20:1, v/v, CellTiter 96 $^\text{A}$Queous kit) was added to each well of a 96-well plate containing cells in 100 µL of RPMI 1640 without phenol red. After incubation for 1 h at 37°C in a humidified 5% CO$_2$ atmosphere, the absorbance at 490 nm was recorded using an ELISA plate reader. The spectrophotometer was calibrated to zero absorbance using culture medium without cells. The relative cell viability compared to control wells containing medium without nanoparticles was calculated by $A_{\text{test}}/A_{\text{control}}$, where $A_{\text{test}}$ and $A_{\text{control}}$ are the average absorbances of the test and control samples, respectively.

2.9. Confocal image analysis

For confocal laser scanning microscopy (CLSM) studies, HeLa cells were seeded onto 22 mm round glass coverslips, placed in a 6-well plate, and grown overnight. The medium was removed, and the cells were washed twice with PBS. The cells were treated with FITC-labeled nanoparticles for 1, 12, or 24 h at 37°C and 5% CO$_2$. The cells were washed three times with PBS and fixed with 4.0% formaldehyde at room temperature for 15 min. After washing with PBS, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, 1 µg mL$^{-1}$) for 15 min. Coverslips were placed onto glass microscope slides, and the cells were examined using a Carl Zeiss LSM 510 META laser-scanning confocal microscope (405/ 488 nm excitation).

Scheme 1. Reaction scheme for polymer synthesis and conjugation. Reagents and conditions: (a) CuBr/PMDETA, SA, anisole, 65°C, 3 h; (b) toluene reflux, 7 h; (c) 1.0 M HCl/ dioxane (1:3, v/v), 25°C, 24 h; (d) Tat peptide, deionized water, 3 h.

Fig. 1. ATRP of SA in anisole from initiator 1. [SA]$_0$:[I]$_0$:[CuBr]$_0$:[PMDETA]$_0$ = 40:1:1:1. (A) Kinetic plot. (B) Experimental $M_n$ and PDI (from GPC) versus conversion.
2.10. Characterization

$^1$H NMR spectra were obtained using a Japan ECA-400 NMR instrument operating at 400 MHz. Gel permeation chromatography (GPC) was performed with a Waters 515 HPLC pump, a Waters 2414 refractive index detector, and a combination of column Styragel HT-2, HT-3, and HT-4; the effective molar mass ranges were 100–10,000, 500–30,000, and 5000–600,000, respectively. Linear polystyrene standards were applied for calibration. The eluent was THF at a flow rate of 1.0 mL min$^{-1}$ at 35 °C. High performance liquid chromatography (HPLC) studies were performed using a Shimadzu analytical HPLC system equipped with an LC-10AT vp plus pump and an SPD-10A vp plus UV-Vis detector. The analytical column was an Agilent Zorbax SB-C18 (5 μm particles, 4.6 × 150 mm). Two eluents were used: A contained 0.1/100 (v/v) trifluoroacetic acid (TFA)/H$_2$O, and B contained 0.1/70/30 (v/v/v) TFA/CH$_3$CN/H$_2$O. B was increased from 5% to 70% over 12 min at a flow rate of 1 mL min$^{-1}$. The chromatograms were recorded using a UV detector set at 210 nm. A Bruker Reflex III mass spectrometer (Bruker Daltonics, Inc., USA) was used to obtain matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra. α-Cyano-4-hydroxycinnamic acid was used as a matrix, and a mixture of TFA/CH$_3$CN/H$_2$O was used as a solvent. Fourier transform infrared (FTIR) spectra were obtained using a Nicolet A-370 FTIR spectrometer. The crystal structure of the iron oxide nanoparticles was obtained from a powder X-ray diffraction (XRD) pattern recorded using a Rigaku D/max-2500 diffractometer with a monochromatized X-ray beam of nickel-filtered Cu Kα radiation (λ = 1.54056 Å) at a 4° min$^{-1}$ scan rate. The continuous scan mode was used to collect 2θ data from 3° to 80°. Thermogravimetric analysis (TGA) was performed on a TA Instruments TGA Q50 from 25 to 800 °C in an air flow at a heating rate of 10 °C min$^{-1}$. Transmission electron microscopy (TEM) images were taken on a JEM-2010F transmission electron microscope. Dynamic light scattering (DLS) and zeta potential measurements were performed at 25 °C using a Malvern Nano Series ZS instrument equipped with a 532 nm laser at a fixed scattering angle of 90°. The aqueous dispersions of magnetic nanoparticles (∼100 μg mL$^{-1}$ Fe) were filtered

![Fig. 2. $^1$H NMR spectra of (A) polymer 2 in CDCl$_3$, (B) polymer 3 in CDCl$_3$, and (C) MAL-TEG-PGA in D$_2$O. Spectra (A) and (B) show peaks at 0.8 ppm due to petroleum ether from the purification process.](image)

![Fig. 3. HPLC analyses of the reaction solution under the following molar ratios of the Tat peptide to MAL-TEG-PGA: (a) 0.1; (b) 0.5:1; (c) 1:1; (d) 1.2:1; (e) 1.5:1. (f) HPLC analyses of the mixture solution after dialysis. The purified Tat peptide exhibits a retention time of 8.22 min.](image)
through a 0.45 μm cellulose membrane filter before analysis. The nanoparticles used for the FTIR, TGA, and XRD analyses were obtained by lyophilization.

3. Results and discussion

Scheme 1 illustrates the synthetic pathway for synthesis of MAL-TEG-PGA. Using compound 1 as an initiator, the atom transfer radical polymerization (ATRP) of SA was carried out to produce polymer 2. The conversion of monomer, molecular weight and molecular weight distribution were monitored by $^{1}$$H$ NMR and GPC. Typical kinetic and evolution of polymer molecular weight versus conversion data are described in Fig. 1. The plot of ln[(M<sub>i</sub>0)/(M<sub>i</sub>)] versus polymerization time yields a straight line, as shown in Fig. 1A, suggesting a constant concentration of growing radicals during the polymerization. Fig. 1B shows a linear increase in molecular weight versus monomer conversion, which indicates that the polymerization is a controlled radical process. Furthermore, the molecular weight distributions remain narrow with increasing

![Scheme 1](image)

**Fig. 4.** (A) Preparation of Fe<sub>3</sub>O<sub>4</sub>-PGA-TEG-Tat; (B) Photographs indicating attraction of polymer-coated nanoparticles dispersed in water by a magnet (left). After the supernatant was discarded, polymer-coated nanoparticles were redispersed in water (right).

![FTIR spectra](image)

**Fig. 5.** (A) Surface modification of Fe<sub>3</sub>O<sub>4</sub> nanoparticles via path (1). FTIR spectra of (a) HClO<sub>3</sub>-stabilized Fe<sub>3</sub>O<sub>4</sub> nanoparticles, (b) Fe<sub>3</sub>O<sub>4</sub>-PGA-TEG-MAL, (c) Fe<sub>3</sub>O<sub>4</sub>-PGA-TEG-Tat and (d) the Tat peptide; (B) Surface modification of Fe<sub>3</sub>O<sub>4</sub> nanoparticles via path (2). FTIR spectra of (a) HClO<sub>3</sub>-stabilized Fe<sub>3</sub>O<sub>4</sub> nanoparticles, (b) Tat-TEG-PGA and (c) Fe<sub>3</sub>O<sub>4</sub>-PGA-TEG-Tat.
conversion (PDI = 1.05–1.11). All these facts suggest a “living”/controlled nature for ATRP of SA initiated by the initiator 1.

The formation of polymer 2 can be confirmed by the $^1$H NMR spectrum, as shown in Fig. 2A. In addition to the characteristic signals of the poly(solketal acrylate) block, we found the characteristic signals of the furan-protected maleimide at $\delta = 6.51$ (a), 5.26 (b), and 2.85 (c) ppm. The furan protective group was removed through a Retro-Diels–Alder reaction to produce polymer 3. Successful deprotection was confirmed by the disappearance of signals at $\delta = 2.85$ and 5.26 ppm in the $^1$H NMR spectrum, as shown in Fig. 2B. The acetonide protective groups were readily cleaved to generate the diol groups by treating polymer 3 with 1.0 M HCl/dioxane (1:3, v/v). In comparison with the $^1$H NMR spectrum in Fig. 2B, the characteristic signals of the two methyl protons from the acetonide protective groups at $\delta = 1.35$ and 1.42 ppm were absent in Fig. 2C, demonstrating the complete hydrolysis of the acetonide groups in the poly(solketal acrylate) segments. A Retro-Diels–Alder reaction and acidic hydrolysis to remove the furan and acetal groups yielded polymer 4 (MAL-TEG-PGA).

The formation of 5 (Tat-TEG-PGA) by conjugation of 4 with the Tat peptide was evaluated using HPLC and MALDI-TOF mass spectrometry. Fig. 3 shows a comparison of the spectra of the reaction solutions, prepared using molar ratios of the Tat peptide to MAL-TEG-PGA of 0:1 (a); 0.5:1 (b); 1:1 (c); 1.2:1 (d); and 1.5:1 (e) in 1 mL of water. HPLC spectra were recorded after the reaction mixture was stirred for 3 h. The most noticeable changes are a clear shift toward a lower retention time for the conjugate along with the disappearance of MAL-TEG-PGA. The MAL-TEG-PGA peak disappeared completely when the molar ratio of the Tat peptide to MAL-TEG-PGA reached 1.2:1. The retention times of MAL-TEG-PGA and the conjugate were 11.0 min and 10.2 min, respectively. Purification of the Tat-TEG-PGA conjugate was achieved by dialysis (MWCO 3500) against deionized water for 1 day (Fig. 3f). The Tat-TEG-PGA conjugate was further characterized using MALDI-TOF mass spectrometry. The observed $M_n$ for MAL-TEG-PGA was 4391 Da [M+Na$^+$]. The $M_n$ value of Tat-TEG-PGA based on the MAL-TEG-PGA was calculated as 6023 Da [M+H$^+$]. The conjugate gave a series of peaks centered at 6022 Da, which correspond to the [M+H$^+$] ion of Tat-TEG-PGA (Supplementary material, Fig. 5).

HClO$_4$-stabilized Fe$_3$O$_4$ nanoparticles were synthesized using a previously reported method [39]. The Tat peptide was introduced onto the surface of the nanoparticles through one of two approaches (Fig. 4A). In the first approach, the nanoparticles were first coated with MAL-TEG-PGA through the PGA segment, and the Tat peptide was then conjugated to the maleimide terminus of the linker. The final product was separated from the reaction medium using a permanent magnet. After the supernatant was discarded, the nanoparticles were washed three times with deionized
water and dispersed in water (Fig. 4B). In the second approach, Tat-TEG-PGA was directly applied to the nanoparticles through coordination of the 1,2-diols of PGA simply by mixing Tat-TEG-PGA and HClO₄-stabilized Fe₃O₄ nanoparticles in aqueous media.

Modification of the nanoparticle surface was confirmed using FTIR spectroscopy. For path (1) (Fig. 5A), the nanoparticles were first coated with MAL-TEG-PGA. After removing unbound MAL-TEG-PGA, a peak remained at 1728 cm⁻¹ (ester carbonyl stretching) in the spectrum of the iron oxide nanoparticles, suggesting that MAL-TEG-PGA was bound to the particle surface. The Tat peptide was then attached to the nanoparticles through the terminal maleimide of MAL-TEG-PGA. Following conjugation, peaks appeared at 1651 and 1539 cm⁻¹, indicating that the Tat peptide was linked to the surface. In path (2) (Fig. 5B), the nanoparticles absorbed very strongly at approximately 580 cm⁻¹, which can be attributed to Fe-O stretching in the iron oxide [16]. After modification with Tat-TEG-PGA, the characteristic peaks of Tat-TEG-PGA were observed at 1726, 1651, and 1121 cm⁻¹, corresponding to stretching bands for C=O, C=N, and C–O.

The crystalline nature of the Fe₃O₄-PGA-TEG-Tat nanoparticles formed via path (2) was determined using powder XRD (Supplementary material, Fig. S2). The peak position and relative intensity match well with the standard XRD data for magnetite (ICPDS card, file No. 19-0629). The compositions of Fe₃O₄ and the Tat peptide within the conjugate were analyzed by dissolving a portion of the conjugate using 3 M HCl, reducing ferric ions to ferrous ions using hydroxylamine hydrochloride, and spectrophotometrically measuring the total amount of iron ions using 1,10-phenanthroline as a color indicator [40]. The Fe₃O₄ content of the conjugate was calculated from the Fe content. In parallel, TGA was employed to determine the number of Tat-TEG-PGA assemblies in an equal amount of conjugate (Supplementary material, Fig. S3). Using these results, the number of Tat peptide molecules per Fe₃O₄ nanoparticle was estimated to be approximately 96 (denoted Fe₃O₄-PGA-TEG-Tat96) in conjugates formed via path (2). In contrast, the amount of MAL-TEG-PGA per Fe₃O₄ in path (1) was also evaluated and estimated to be 362 (denoted Fe₃O₄-PGA-TEG-MAL362). A comparison between path (1) and path (2) reveals a difference in the amount of coated polymer on the Fe₃O₄ nanoparticles. This may be because Tat peptides interfere with the coordinated interaction between the PGA block and the Fe₃O₄ nanoparticles in path (2).

Fig. 6 contains TEM images of the Fe₃O₄-PGA-TEG-MAL362 (Fig. 6A) and Fe₃O₄-PGA-TEG-Tat96 (Fig. 6B) particles. No obvious differences were observed in particle core shape, size, or dispersion. The majority of the Fe₃O₄-PGA-TEG-MAL362 and Fe₃O₄-PGA-TEG-Tat96 nanoparticles were roughly spherical or ellipsoidal, with a core diameter of approximately 12.8 nm. DLS measurements indicated the hydrodynamic size of Fe₃O₄-PGA-TEG-MAL362 in water to be 47.7 nm (PDI 0.129) and Fe₃O₄-PGA-TEG-Tat96 to be 67.5 nm (PDI 0.148) (Fig. 6C and D). The increased size (≈19.8 nm) of the Fe₃O₄-PGA-TEG-Tat96 particles can be attributed to the surface-bound Tat peptide (MW = 1654 Da) and associated water molecules. The aqueous dispersion of Fe₃O₄-PGA-TEG-Tat96 had a positive zeta potential (+19.3 mV) due to the positive charges located on the Tat peptide (eight amino group residues per peptide molecule), while the aqueous dispersion of Fe₃O₄-PGA-TEG-MAL362 had a negative zeta potential (−7.4 mV). Compared to the positive surface charge in water, the surface charge of Fe₃O₄-PGA-TEG-Tat96 was reversed to a negative value (−7.4 mV) in DMEM/FBS. At the same time, the average size of Fe₃O₄-PGA-TEG-Tat96 in DMEM/FBS increased to 180.2 nm. The change in surface charge and particle size should be the result of absorption of proteins from
the medium. The nanoparticle size and surface charge remained stable over 24 h in the complete cell culture medium (Table 1).

The cellular uptake of FeO₄₋ₓPG-TET-MAL362 and FeO₄₋ₓPG-TET-Tat96 was compared in a HeLa cancer cell line. The uptake of the nanoparticles was assessed histologically using Prussian blue staining (Fig. 7). After 1 h of incubation, the cells were washed with PBS buffer three times to remove residual nanoparticles in solution. The cell outer surfaces were visibly stained with FeO₄₋ₓPG-TET-Tat96, and a small number of the nanoparticles were accumulated in a unique intracellular region. After 12 h of incubation, a greater number of FeO₄₋ₓPG-TET-Tat96 nanoparticles were transported and accumulated to this intracellular location, and fewer nanoparticles were located on the cell surface. After 24 h, the majority of the FeO₄₋ₓPG-TET-Tat96 nanoparticles had been transported and accumulated in the cell nucleus. In contrast, almost no FeO₄₋ₓPG-TET-MAL362 nanoparticles were observed in the nucleus (Supplementary material, Fig. S4). The zeta potentials of the FeO₄₋ₓPG-TET-Tat96 particles and the FeO₄₋ₓPG-TET-MAL362 particles in the complete cell culture medium are not significantly different, so the influence of surface charge on cellular uptake can be ruled out. Although the mechanism of Tat-mediated penetration into the nucleus is unclear, these results indicate that the Tat peptide can mediate the entrance of iron oxide nanoparticles into the cell nucleus and may be useful for applications requiring nuclear delivery. The cytotoxicity of FeO₄₋ₓPG-TET-MAL362 and FeO₄₋ₓPG-TET-Tat96 was assessed using a CellTiter 96® AQueous non-radioactive cell proliferation assay (MTS, Promega). For both types of nanoparticles, cell viability was comparable to the nanoparticle-free control assay after 24 h of incubation, indicating that the internalization of the nanoparticles at the present dose results in very low cytotoxicity (Fig. 8).

Confocal microscopy experiments further confirmed that Tat peptides can mediate the entrance of FeO₄₋ₓPG nanoparticles into the cell nucleus. A FITC-labeled Tat peptide was synthesized and conjugated onto the surface of FeO₄₋ₓPG nanoparticles via path (2) (denoted FeO₄₋ₓPG-TET-FITC). The FeO₄₋ₓPG nanoparticles conjugated with a FITC-labeled tripeptide (FITC-NH(CH₂)₂CO-GGH-NH₂) were used as control samples (denoted FeO₄₋ₓPG-TET-MAL-FITC). The cell uptake of the nanoparticles was evaluated using CLSM analysis (Fig. 9). There were almost no nanoparticles in the cell nucleus after 24 h incubation with FeO₄₋ₓPG-TET-MAL-FITC (Fig. 9C). In contrast, more FeO₄₋ₓPG-TET-FITC nanoparticles were clearly visible in the nucleus after 12 h (Fig. 9E), consistent with Prussian blue staining results. These results reconfirmed that FeO₄₋ₓPG nanoparticles can be transported into the cell nucleus by surface conjugation of Tat peptides.

4. Conclusions

The MAL-TET-PGA polymer has been designed and synthesized to couple peptides to FeO₄₋ₓ nanoparticles. Two synthetic pathways were successfully employed to prepare Tat-conjugated FeO₄₋ₓ nanoparticles. This methodology should lead to a variety of bimodal conjugates, such as FeO₄₋ₓPG-TET-Tat, that are suitable for further investigation in biomedical applications. Moreover, Tat-conjugated nanoparticles were delivered into the cell nucleus, demonstrating the potential of using the FeO₄₋ₓPG-TET-Tat platform for nuclear drug delivery.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.reactfunctpolym.2012.01.003.

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