Published in final edited form as:

J Mater Chem. 2009 January 1; 19(35): 6400–6406. doi:10.1039/b902373a.

Conjugating Methotrexate to magnetite (Fe₃O₄) nanoparticles via trichloro-s-triazine^{†,‡}

Kaylie L. Young, Chenjie Xu, Jin Xie, and Shouheng Sun

Department of Chemistry, Brown University, Providence, Rhode Island, 02912, USA.

ssun@brown.edu; Fax: +1 401-863-9046; Tel: +1 401-863-3329

Abstract

Monodisperse Fe₃O₄ nanoparticles (NPs) originally synthesized with a hydrophobic oleylamine capping ligand were made water soluble and conjugated to the anticancer drug Methotrexate (MTX) using a new chemistry based on the readily available linker trichloro-s-triazine (TsT). This new linker is much more versatile than those that currently exist for attaching biomolecules to magnetic NPs. The MTX-conjugated NPs were found to be stable under physiological conditions for over 72 hours and MTX was shown to maintain its anticancer activity after conjugation to the NP surface. Through cell viability studies and intracellular uptake studies, MTX-conjugated NPs were shown to have targeting specificity for a tumor cell line (9L rat glioma) over a healthy cell line (Cultured Pulmonary Artery Endothelial). Additionally the MTX-conjugated NPs were visualized inside 9L cells using fluorescence microscopy to help elucidate their path within a cell after internalization.

Introduction

Magnetic nanoparticles (NPs) with diameters below 20 nm exhibit interesting size-dependent magnetic properties, including the phenomenon of superparamagnetism.^{1,2} Superparamagnetic magnetite NPs are ideal candidates for biomedical applications because they are not subject to strong magnetic interactions between one another in the dispersion state, facilitating their long-term stability in biological systems, and they generate a large magnetic signal under an external magnetic field.³ Due to their low toxicity⁷ and stable magnetic properties, magnetite (Fe₃O₄) NPs have been investigated for potential applications in bio-separation, bio-sensing, drug delivery, magnetic fluid hyperthermia, and magnetic resonance imaging (MRI) contrast enhancement.^{3,5–8}

Linking hydrophilic macromolecules, especially biomolecules, to Fe₃O₄ NPs is a vital step for producing water-based NPs to use in the above-mentioned applications.⁵ The synthesis procedures for monodisperse Fe₃O₄ NPs often result in a hydrocarbon-based capping. As a result, the as-synthesized NPs are only soluble in non-polar or weakly polar organic solvents.⁹ Existing linker chemistries to conjugate macromolecules to Fe₃O₄ NPs are hard to work with and often not economically available.⁵ Thus two of the major challenges in this field include (i) the ability to make high quality monodisperse water-soluble Fe₃O₄ NPs and (ii) the lack of readily available linker chemistry that provides an easy and inexpensive way of attaching functional molecules to Fe₃O₄ NPs. Previously we reported an approach to conjugate Fe₃O₄

[†]This paper is part of a *Journal of Materials Chemistry* theme issue on inorganic nanoparticles for biological sensing, imaging, and therapeutics. Guest editor: Jinwoo Cheon.

[‡]Electronic supplementary information (ESI) available: Synthesis of Fe₃O₄ nanoparticles, synthesis of TsT-PEG-TsT precursor, conjugation of Methotrexate to Fe₃O₄ nanoparticles and MALDI mass spectrometry data. See DOI: 10.1039/b902373a

Correspondence to: Shouheng Sun.

NPs with poly(ethylene glycol) (PEG)-based hydrophilic macromolecules *via* the organic linker trichloro-s-triazine (TsT).⁵ Building upon our previous work, we created a new inexpensive linking chemistry based on PEG and TsT, allowing for the facile conjugation of biomolecules to Fe₃O₄ NPs.

The proposed ligand combination layer consists of dopamine (DA), PEG, and two trichloro-s-triazine (TsT) molecules, as shown in Scheme 1. DA has the ability to replace the original capping ligand, oleylamine, on the Fe₃O₄ NPs surface and has been shown to serve as a robust anchor on the surface of Fe₃O₄ NPs. Spectroscopic studies suggest that catechol acts as a chelating agent, forming tight bonds with iron oxides by converting the under-coordinated cationic iron surface sites to a bulk-like lattice structure.¹⁰ PEG is a hydrophilic and biocompatible polymer often used to increase the stability of nanomaterials in biological systems. It is commonly regarded as a non-specific interaction reducing agent and has been widely used to extend the circulation time of proteins and nanomaterials *in vivo* as it can prevent aggregation and absorption by the reticulo-endothelial system (RES).¹¹ PEG chains are commercially available in many molecular weights and with various functional groups. TsT, a symmetrical heterocyclic compound, is a readily available organic linker molecule containing three acyl-like chlorines which show different reactivities toward nucleophiles in aqueous solution.^{5,12} The functionality provided by the second TsT molecule in the ligand allows for the conjugation of various functional molecules to the NPs.

The chemotherapy drug Methotrexate (MTX) was chosen to conjugate to Fe₃O₄ NPs with the DA, PEG, and TsT-based ligand (Scheme 1). MTX is an analogue of folic acid that inhibits dihydrofolate reductase (DHFR), a critical enzyme in the folic acid cycle and key to regulating homeostasis, leading to reduced cell viability and cell death. As folate receptors are overexpressed on the cell membranes of many types of cancer cells, MTX has proven to be an effective targeting agent.^{13,15} MTX is one of the most widely used drugs for the treatment of many forms of cancer including tumors of the brain, breast, ovary, and several leukemias. However, the clinical application of this drug is limited by its low solubility, short half-life in the bloodstream and rapid diffusion throughout the body.^{14,16} In the past, several groups have incorporated Methotrexate into magnetic liposomes and dendrimers to overcome this issue, but the overall size is much larger than the system presented here.¹⁷

The primary objectives of the present studies are (i) to present a newly designed simple linker chemistry that makes Fe₃O₄ NPs soluble in an aqueous environment and allows for the conjugation of functional biomolecules, (ii) to demonstrate that the ligand is stable under physiological conditions, and (iii) to demonstrate that MTX maintains its anti-tumor activity after conjugation to the NPs. The conjugation of MTX to Fe₃O₄ NPs provides the potential for a multifunctional entity which can take advantage of the superparamagnetic nature of the NP core for imaging purposes while relying on the MTX to be the targeting and therapeutic agent.

Experimental

Reagents were purchased from Sigma-Aldrich and solvents were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ) unless otherwise noted. NPs were imaged with transmission electron microscopy (TEM, Philips EM 420) at 120 kV. UV-Visible spectra were measured with a Hewlett-Packard 8452 Diode Array Spectrometer. Molecular weights were measured with matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Voyager DE Pro, Applied Biosystems). Iron concentrations were identified with inductively coupled plasma atomic emission spectroscopy (ICP-AES, JY 2000).

Modification of Fe₃O₄ NPs

Monodisperse oleylamine-coated Fe₃O₄ NPs (8 nm in diameter) were synthesized by a previously reported one-pot high temperature method and stored in hexane (see ESI[†]).⁵ The Fe₃O₄ NPs were conjugated with MTX following the reaction sequence outlined in Scheme 1. To prepare the TsT-PEG-TsT precursor, polyethylene glycol (PEG, MW = 6000) was activated using trichloro-s-triazine by following a protocol based on those of Abuchowski *et al.* and Gotoh *et al.* (see ESI[†]).¹² To modify the capping ligand on the Fe₃O₄ NPs, 100 mg of dopamine hydrochloride (DA) dispersed in 2 mL N,N-dimethylformamide (DMF) was added to 800 μ L of the oleylamine-capped 8 nm Fe₃O₄ NPs dispersed in 2 mL chloroform (CHCl₃) and sonicated for 30 minutes. Then 5 mL of hexane was added to the mixture and a permanent magnet was used to separate the magnetic NPs out of solution. After repeating the wash step two more times, the DA-capped NPs were redispersed in a 1:1 mixture of DMF and CHCl₃. Next, 130 mg of the TsT-PEG-TsT precursor and 10 mg of sodium carbonate was added and stirred for 24 hours. The TsT-PEG-TsT-DA-capped NPs were separated from the excess TsT-PEG-TsT precursor as above and redispersed in a 2:1 mixture of DMF and CHCl₃. 150 μ L of ethylene diamine (NH₂-C₂H₄-NH₂) was added and the mixture was stirred for 24 hours. The NH₂-TsT-PEG-TsT-DA-capped nanoparticles were purified as above and dried under a gentle stream of nitrogen. The modified Fe₃O₄ NPs were redispersed in deionized water. To remove any remaining excess ethylene diamine, the NH₂-terminated NPs were dialyzed for 24 hours using molecular porous membrane tubing (MWCO = 12–14,000, Spectrum Laboratories, CA).

Conjugation of Methotrexate to NH₂-terminated NPs

Methotrexate (MTX) was conjugated to the amine-terminated NPs through NHS/EDC coupling chemistry (see ESI[†]). The MTX conjugation reaction may occur through either the α or β carboxylic acid groups on the glutamic acid residue.¹⁴ To remove the excess MTX, the NPs were separated with a permanent magnet and washed with deionized water. The samples were further purified using Centriprep 15 mL centrifugal filter devices (Amicon Bioseparations-Millipore). The final MTX-conjugated NPs were stored in a 20 mL glass vial covered in aluminium foil to avoid light exposure.

Characterization of MTX-conjugated NPs

To determine the concentration of MTX in the MTX-conjugated NPs sample, a standard linear-fit curve of free MTX in water was created by plotting the UV-Vis absorbance at 304 nm of several MTX solutions of known concentrations. The concentration of MTX in the MTX-conjugated particles was then determined by subtracting out the background absorbance of a sample of NH₂-terminated NPs with a normalized amount of iron at 304 nm. The accuracy of this method was confirmed using the method developed by Kohler *et al.*¹³

Stability under physiological conditions

To test the stability of both the NH₂-terminated NPs and the MTX-conjugated NPs, the particles were dispersed in a PBS (Dulbecco's Phosphate Buffered Saline, Atlanta Biologicals) solution with 10% fetal bovine serum (FBS, Atlanta Biologicals) and kept in an incubator at 37 °C and 5% CO₂. The hydrodynamic diameters of NPs were measured using a Dynamic Light Scattering (Malvern Instruments, Zetasizer Nano Series, Nano-S90) instrument over the course of 72 hours. Data was collected for the hydrodynamic diameter of the particles at t = 0, 1, 2, 4, 8, 16, 24, 48, and 72 hours.

[†]Electronic supplementary information (ESI) available: Synthesis of Fe₃O₄ nanoparticles, synthesis of TsT-PEG-TsT precursor, conjugation of Methotrexate to Fe₃O₄ nanoparticles and MALDI mass spectrometry data. See DOI: 10.1039/b902373a

Cell culture

9L rat glioma cells which have an overexpression of the folate receptor and mammalian cultured pulmonary artery endothelial (CPAE) cells with little folate receptor were chosen to examine the targeting ability of MTX-conjugated NPs. 9L cells and CPAE cells were grown in 75 cm² canted neck polystyrene culture flasks (Corning) in an incubator kept at 37 °C and 5% CO₂. The medium used for culture was DMEM (Dulbecco's Modified Eagle Medium with D-glucose, L-glutamine, and sodium pyruvate, Atlanta Biologicals) with 10% FBS and 5% antibiotics. The cells were allowed to grow to 80–90% confluency before they were split or harvested for experimental purposes.

Cell viability studies

1×10^5 9L or CPAE cells were seeded into 25 cm² polystyrene culture flasks (Corning) containing 3 mL of DMEM and placed in an incubator for 24 hours. Solutions of MTX-conjugated NPs and NH₂-terminated NPs with iron concentrations of 0.01 and 0.005 mg Fe/mL were incubated with the cells for 48 hours. The flasks were washed twice with PBS to remove any dead cells and 1 mL of 0.25% Trypsin 1× (Gibco) was added. After collecting the cells from the flask, the cells were counted using a hemacytometer (Hausser Scientific). As a control, cells were incubated with DMEM. Cells were also incubated with MTX drug solutions with concentrations corresponding to those in the MTX-conjugated NP samples. Each iron concentration was repeated three times and the cells were counted three times. The average percentage of living cells was calculated by comparison with the control.

Intracellular uptake

The intracellular uptake of the MTX-conjugated NPs and the NH₂-terminated NPs was quantified by measuring the concentration of iron within the cells for each sample using ICP-AES. 1.5×10^4 cells were seeded into 25 cm² polystyrene culture flasks (Corning) containing 3 mL of DMEM and placed in an incubator for 24 hours. Solutions of MTX-conjugated NPs and NH₂-terminated NPs with iron concentrations of 0.01 and 0.005 mg/mL were incubated with the cells for 4 hours. The NP solutions were then removed from the flasks and 1 mL of 0.25% Trypsin 1× was added to detach the cells. The cells were counted using the hemacytometer. After counting, the cell suspensions were placed in 1.5 mL Eppendorf tubes and centrifuged at 10,000 RPM for 5 minutes. The supernatant was removed and 4–5 drops of concentrated HNO₃ was added to break down the cells. The iron uptake (pg per cell) was calculated by dividing the total amount of iron by the total number of cells in each sample.

Tagging NPs with fluorescein isothiocyanate (FITC)

NH₂-NPs were dispersed in a 0.1 M Na₂CO₃/NaHCO₃ buffer with pH = 9. The total number of ligands on the surface of each particle was estimated and the amount of FITC necessary to block 10% of these ligands (2.177×10^{-7} g/mL) was dissolved in DMSO. The FITC solution was then added to the NP solution dropwise and stirred overnight under aluminium foil to prevent bleaching of the FITC.¹² Excess FITC was removed by dialysis with molecular porous membrane tubing (MWCO = 12–14,000). MTX was then conjugated to the FITC-conjugated NPs using the procedure described previously.

Particle internalization study

9L cells were seeded in five small glass bottom culture dishes (MatTek Corporation, MA) for 24 hours. Then 200 µL of DMEM solutions of MTX&FITC-conjugated NPs with a concentration of 0.005 mg Fe/mL were added to each dish and placed in the incubator. Over the course of 60 minutes the dishes were removed from the incubator, washed 3 times with PBS, and the cells were fixed with 4% paraformaldehyde in PBS. The samples were then

imaged using a Nikon Eclipse TE2000-U Fluorescence Microscope. The acquisition time was 5 ms for the bright field and 500 ms for the fluorescence field.

The early/sorting endosomal marker Rab5 was used to further investigate the path of MTX-conjugated NPs in 9L cells.¹⁷ A Rab5 plasmid was transfected into the 9L cells and as a result the early/sorting endosomes within the 9L cells fluoresced red under a fluorescence microscope. 9L cells were cultured in a Petri dish in DMEM medium. The DMEM was removed and the cells were incubated with 1 mL of OPTI-MEM for 40 minutes. To transfect the cells, a solution containing 6 μ L of the plasmid, 24 μ L of the commercial transfection reagent Lipofectamine 2000 (Invitrogen), and 1.170 mL of OPTI-MEM was incubated with the cells for 4 hours. The OPTI-MEM was then removed and 2 mL of DMEM was added. After overnight culturing, the cells were incubated with NPs and the imaging study described above was repeated.

Results and discussion

Fe₃O₄ NPs were synthesized according to a previously reported method.⁵ The TEM image in Fig. 1 (A) shows that as synthesized Fe₃O₄ NPs are nearly monodisperse with an average diameter of 8 nm. The NPs were coated with a hydrophobic layer of oleylamine. To functionalize these NPs with DA-PEG-TsT as illustrated in Scheme 1, the oleylamine ligand is replaced by DA through a ligand exchange reaction, resulting in DA-capped NPs (a). The TsT-PEG-TsT precursor reacts with the amine group of dopamine to form TsT-PEG-TsT-DA capped NPs (b). By controlling the ratio of TsT-PEG-TsT and dopamine added, one end of TsT-PEG-TsT reacts with one DA-capped NP, leaving the other TsT end free for further functionalization. Then ethylene diamine is added to provide an amine group to the free TsT for further functionalization (c). Once two of the chlorines of TsT have been coupled to nucleophilic groups, it is very difficult for the third one to react.¹ Thus only one ethylene diamine will react with each TsT. Finally, MTX was conjugated with NH₂-terminated NPs through EDC/NHS chemistry. A TEM image of the final MTX-conjugated NPs, shown in Fig. 1 (B), indicates that the NPs do not change in size or morphology during the modification steps.

The MTX-conjugated NPs were analyzed by UV-Visible Spectroscopy and MALDI Mass Spectrometry to confirm that MTX was successfully conjugated to the surface of the Fe₃O₄ NPs. Fig. 2 is the UV-Visible spectra of the MTX-conjugated NPs, the NH₂-terminated NPs, and an aqueous solution of free MTX. MTX has a characteristic UV absorbance at 304 nm.¹³ The NH₂-terminated NPs do not have such an absorbance peak. The MTX-conjugated NPs show the characteristic absorbance peak at 304 nm, confirming the presence of MTX on the NP surface. The MALDI spectra gave an average molecular weight of 6,224 for the NH₂-terminated NPs and 6,590 for the MTX-conjugated NPs, with a difference of 366 between the two (Fig. S1, ESI[†]). The majority of the molecular weight is attributed to the PEG chain, which has an average molecular weight of 6,000. While there is variability in the weight of PEG chains, the difference of 366 provides further evidence that MTX is attached to the NPs.

The stability of the MTX-NPs was tested in a PBS buffer solution with 10% FBS over the course of 72 hours at an incubation temperature of 37 °C to mimic physiological conditions. Dynamic light scattering (DLS) was used to track the size change of the NPs during the incubation. The DLS stability data for both the NH₂-terminated and MTX-conjugated NPs is shown in Fig. 3. The hydrodynamic diameter, including the Fe₃O₄ core and the ligand coating, of both NPs started at 37 nm and leveled out at about 50 nm after 24 hours, indicating very little particle aggregation. The slight increase in size was most likely due to adsorption of FBS molecules onto the NPs. In the DLS experiments, the NH₂-terminated NPs were measured to have slightly smaller hydrodynamic diameters compared with the MTX-conjugated NPs, further supporting the conjugation of MTX.

To demonstrate that MTX retained its anti-tumor activity after conjugation to the Fe_3O_4 NPs, the conjugates were incubated with 9L rat glioma cells, a type of robust tumor cell with a high metabolic activity leading to the overexpression of the folate receptor on the cell surface.^{13–15} The toxicity of the MTX-conjugated NPs was compared to the toxicity of the NH_2 -terminated NPs, with equivalent iron concentrations as determined by ICP, and the toxicity of free MTX at concentrations equal to those of the MTX-conjugated NPs as determined by UV-Vis. The Fe_3O_4 NPs were incubated with the 9L cells at two different iron concentrations (0.01 mg/mL and 0.005 mg/mL) for 48 hours and the live cells were counted to determine the cell viability. The cells were counted using a hemocytometer because our data indicated that commercial cytotoxicity assays were inaccurate in assessing the viability of 9L cells in the presence of MTX. The 9L cell viability data, shown in Fig. 4 (A), indicates dose-dependent toxicity with the highest concentration of iron and the highest concentration of MTX being the most toxic. The MTX-conjugated NPs killed 60% and 70% of the 9L cells at iron concentrations of 0.005 mg/mL and 0.01 mg/mL respectively over the course of 48 hours. This is in stark contrast to the NH_2 -terminated NPs, which were observed to be non-toxic to the 9L cells, killing less than 10% at an iron concentration of 0.01 mg/mL over 48 hours. This is in agreement with the literature.⁴ Interestingly, the MTX-conjugated Fe_3O_4 NPs were observed to be slightly less toxic than the equivalent concentration of free MTX drug. This is likely caused by the uptake difference as the bulkier MTX- Fe_3O_4 NPs may not be taken up as easily as the free MTX at the same concentration, or by the fact that the MTX may need to be cleaved from the NPs before it shows the toxicity.

To demonstrate the specificity of the MTX-conjugated Fe_3O_4 NPs for cancer cells, the cell viability experiments were repeated with CPAE (pulmonary artery endothelial cells) cells. The CPAE cell viability data, shown in Fig. 4 (B), indicates that the MTX-conjugated NPs were much less effective in reducing cell viability in CPAE cells compared with 9L cells. At each iron concentration tested, less than 30% of the CPAE cells died when incubated with MTX-conjugated NPs for a 48 hour period. The solutions of free MTX showed similar results. The NH_2 -terminated NPs were found to be relatively nontoxic to the CPAE cells, with only a slightly higher toxicity when compared with NH_2 -terminated NPs in 9L cells. This is thought to be due to a higher level of nonspecific uptake in the CPAE cells. The viability experiments with 9L cells and CPAE cells reinforce the validity of the intracellular trafficking model proposed by Kohler *et al.* in which MTX internalizes *via* the folate receptor and reduced folate carrier.¹⁴ Since 9L cells overexpress the folate receptor and CPAE cells do not, free MTX and MTX conjugated to Fe_3O_4 NPs can more efficiently internalize and cause reduced cellular viability in 9L cells. Thus the data in Fig. 4 demonstrates the potential for the MTX-conjugated NPs to be used as a targeting agent as well as a cytotoxic entity.

The targeting specificity of the MTX-conjugated NPs was further investigated through cellular uptake studies of the Fe_3O_4 NPs conjugated with MTX compared to those without MTX. The intracellular iron uptake for 9L and CPAE cells incubated with solutions of 0.01 and 0.005 mg Fe/mL MTX-conjugated NPs and NH_2 -terminated NPs is shown in Fig. 5. The uptake data demonstrates the specificity of the MTX-NPs for a cancer cell line over a healthy cell line. At an Fe concentration of 0.01 mg/mL, the uptake of the MTX-conjugated NPs into 9L cells is almost twice that of the uptake of the NH_2 -terminated NPs. At an Fe concentration of 0.005 mg/mL, the uptake of the MTX-conjugated particles into 9L cells is four times that of the NH_2 -terminated particles. The uptake data for the CPAE cells does not show a preference for uptake of either type of NP, but instead the NPs seem to be nonspecifically internalized. The uptake data in Fig. 5 supports the trends seen in the cell viability data in Fig. 4. The MTX-conjugated NPs target the 9L cells resulting in more particles being internalized, thus leading to a reduced cell viability. However, it must not be ruled out that the difference in uptake between the 9L and CPAE cells could partially be due to differences in metabolic activity of the two cell types.¹⁴ The cell viability experiments combined with the uptake studies provide

evidence that our newly developed linker chemistry does not alter the biological activity of the conjugated drug.

To visualize the location of the NPs inside the cells after internalization, the green fluorescent probe Fluorescein Isothiocyanate (FITC) was used to label the MTX-Fe₃O₄ NPs. Because MTX possesses primary amine groups, FITC was conjugated to the NH₂-terminated NPs before MTX was conjugated to the particles to prevent FITC from binding to MTX and affecting its ability to reduce cell viability. A small amount of FITC was added to block approximately 10% of the NH₂ groups on the NP surface so that the remaining ligands were available to attach to MTX. NPs conjugated with MTX and FITC were incubated with 9L cells and imaged over the course of 60 minutes using a fluorescence microscope.

In our preliminary data the MTX-conjugated NPs were observed in cellular components believed to be early endosomes following their uptake into 9L cells. To confirm that the cellular compartments containing the NPs were early endosomes, the early/sorting endosomal marker Rab5 was used to label the early endosomes.¹⁸ The 9L cells were transfected with a Rab5 plasmid causing their early endosomes to fluoresce red. These transfected 9L cells were incubated with NPs conjugated with MTX and FITC and images were collected over the course of 60 minutes. In order to capture both the green fluorescence of the FITC and the red fluorescence of the Rab5, one image of the cells was taken with the green channel of the fluorescence microscope and a corresponding image of the same cells was taken with the red channel of the fluorescence microscope. These images were then overlaid using software.

The color green indicates the location of the FITC-conjugated NPs and the color red indicates the location of the early endosomes. The color yellow indicates overlap of the NPs and the early endosomes. Fig. 6 shows images of the transfected 9L cells collected at 15, 30, and 60 minutes of incubation with the MTX-conjugated NPs. While it would be ideal to monitor the same cells over the course of the experiment, this was not possible due to the fact that the cells has to be fixed with paraformaldehyde in order to be imaged. Thus cells that were representative of the entire sample were chosen to be imaged. Focusing on the overlay images in the third row of Fig. 6, after 15 minutes of incubation the NPs, indicated by green, are in the cytoplasm and some are in the early endosomes, indicated by yellow. After 30 minutes, the majority of the particles are in the early endosomes as yellow is the main color observable. This experiment provides further evidence to support the first step of the intercellular trafficking model proposed by Kohler *et al.* in which the NPs are transported to early endosomes following their uptake.¹⁴ After 60 minutes, the large amount of red fluorescence from empty endosomes suggests that the NPs are no longer in the early endosomes. Kohler *et al.* suggest that the endosomes containing the MTX-conjugated NPs then fuse with lysosomes where proteases cleave the peptide bond between MTX and the rest of the NP ligand thereby releasing the MTX drug into the cell.¹⁴

Conclusions

Monodisperse magnetite NPs have been conjugated with the anticancer drug Methotrexate using a new linker trichloro-s-triazine (TsT). UV-Vis spectroscopy and MALDI mass spectrometry were used to confirm that MTX was immobilized on the NP surface. Both the NH₂-terminated and MTX-conjugated Fe₃O₄ NPs were found to be stable under physiological conditions. The MTX-conjugated NPs showed specificity and high toxicity to 9L rat glioma cells rather than to CPAE cells, while the NH₂-terminated NPs were found to be nontoxic to both cell lines. Finally, the intracellular trafficking for MTX-conjugated NPs was investigated by attaching the fluorescent probe FITC to the Fe₃O₄ NPs and visualizing the particles in 9L cells transfected with a Rab5 plasmid under a fluorescence microscope. These fluorescence experiments confirmed that the particles enter the early/sorting endosomes following their

uptake into target cells. We hope to further extend this system to conjugate other types of biomolecules, such as peptides, proteins, and DNAs to Fe₃O₄ NPs for biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported by NIH/NCI 1R21CA12859.

References

1. Jun YW, Seo JW, Cheon J. *Acc Chem Res* 2008;41(2):179. [PubMed: 18281944]
2. Xu C, Sun S. *Dalton Trans.* 2009;10.1039/b900272n
3. Xu C, Sun S. *Polym Int* 2007;56:821.
4. Lewinski N, Colvin V, Drezek R. *Small* 2008;4(1):26. [PubMed: 18165959]
5. Xie J, Xu C, Xu Z, Hou Y, Young KL, Wang SX, Pourmand N, Sun S. *Chem Mater* 2006;18:5401. [PubMed: 18176627]
6. Pankhurst QA, Connolly J, Jones SK, Dobson J. *J Phys D: Appl Phys* 2003;36:R167.
7. McNeil S. *J Leukocyte Biol* 2005;78:585. [PubMed: 15923216]
8. Xu C, Xie J, Kohler N, Walsh EG, Chin YE, Sun S. *Chem Asian J* 2008;3:548. [PubMed: 18080259]
9. Sun S, Zeng H. *J Am Chem Soc* 2002;124:8204. [PubMed: 12105897] Sun S, Zeng H, Robinson DB, Raoux S, Rice PM, Wang SX, Li G. *J Am Chem Soc* 2004;126:273. [PubMed: 14709092] Xie J, Peng S, Brower N, Pourmand N, Wang SX, Sun S. *Pure Appl Chem* 2006;78:1003.
10. Xu C, Xu K, Gu H, Zheng R, Liu H, Zhang XX, Guo Z, Xu B. *J Am Chem Soc* 2004;126:9938. [PubMed: 15303865]
11. Xie J, Xu C, Kohler N, Hou Y, Sun S. *Adv Mater* 2007;19:3163.
12. Hermanson, GT. *Bioconjugate Techniques*. Academic Press; New York: 1996.
13. Kohler N, Sun C, Fichtenholtz A, Gunn J, Fang C, Zhang M. *Small* 2006;2(6):785. [PubMed: 17193123]
14. Kohler N, Sun C, Wang J, Zhang M. *Langmuir* 2005;21:8858. [PubMed: 16142971]
15. Dhar S, Liu Z, Thomale J, Dai H, Lippard S. *J Am Chem Soc* 2008;130:11467. [PubMed: 18661990] Quintana A, Raczka E, Piehler L, Lee I, Myc A, Majoros I, Patri AK, Thomas T, Mule J, Baker J Jr. *Pharm Res* 2002;19(9):1310. [PubMed: 12403067]
16. American Cancer Society website. www.cancer.org
17. Viroonchatapan E, Ueno M, Sato H, Adachi I, Nagai H, Tazawa K, Horikoshi I. *Pharm Res* 1995;12(8):1176. [PubMed: 7494831] Zhu L, Huo ZL, Wang LL, Tong X, Xiao Y, Ni KY. *Int J Pharm* 2009;370(1–2):136. [PubMed: 19114095] Kojima C, Kono K, Maruyama K, Takagishi T. *Bioconjugate Chemistry* 2000;11(6):910. [PubMed: 11087341]
18. Suvorova E, Gripenrot J, Miettinen Heini. *Traffic* 2005;6:100. [PubMed: 15634211]

Fig. 1. TEM images of (A) oleylamine-coated 8 nm Fe₃O₄ NPs in hexane and (B) MTX-conjugated Fe₃O₄ NPs in water.

Fig. 2. UV-Visible absorption spectra of free MTX, NH₂-terminated NPs, and MTX-conjugated NPs in water. MTX has a characteristic absorbance at 304 nm.

Fig. 3.
Hydrodynamic diameter of NH₂-terminated NPs and MTX-conjugated NPs under physiological conditions over the course of 72 hours measured by DLS.

Fig. 4. 9L (A) and CPAE (B) cell viability data for MTX-conjugated NPs, NH₂-terminated NPs of equivalent iron concentrations, and aqueous solutions of MTX corresponding to the concentration of MTX in the MTX-conjugated NPs samples. Iron concentrations of 0.005 mg/mL and 0.01 mg/mL were tested.

Fig. 5.

Intracellular uptake of MTX-conjugated NPs and NH₂-terminated NPs in 9L and CPAE cells for iron concentrations of 0.005 mg/mL and 0.01 mg/mL after 4 hours of incubation. The black bars represent 9L cells and the grey bars represent CPAE cells.

Fig. 6.

Fluorescence images of 9L cells transfected with Rab5 to dye the early/sorting endosomes red. The transfected cells were then incubated with MTX&FITC-conjugated NPs for 15 minutes (column A), 30 minutes (column B), and 60 minutes (column C). The first row (1) shows the green channel of the fluorescence image. This green fluorescence is due to the FITC conjugated to the NPs. The second row (2) shows the red channel of the fluorescence image. This red fluorescence comes from the early/sorting endosomes that fluoresce red due to the Rab5. The third row (3) shows the overlap of the red and green channels. The color yellow indicates red and green overlap.

Scheme 1.

Chemical modification of Fe_3O_4 NPs for their coupling with MTX *via* TsT.