A specific tumor-targeting magnetofluorescent nanoprobe for dual-modality molecular imaging

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Poly(acrylic acid) was decorated onto Fe₃O₄ resulting in a highly water-soluble superparamagnetic iron oxide. The Poly(acrylic acid) iron oxide (PAAIO) complexes possess specific magnetic properties in the presence of an external magnetic field and are attractive contrast agents for magnetic resonance imaging (MRI). The free carboxylic groups of PAAIO exposed on the surface allow for covalent attachment of a fluorescent dye, Rhodamine 123 (Rh123) to form PAAIO-Rh123, which permits applications in fluorescence imaging. PAAIO-Rh123 is therefore a dual-modality molecular probe. In order to endow specific properties to compounds that target cancer cells and to prevent recognition by the reticuloendothelial system (RES), folic acid-linked poly(ethylene glycol) (FA-PEG) was further conjugated onto PAAIO-Rh123. The amounts of Rh123 and FA-PEG on the modified iron oxides were quantitatively determined by elemental analysis. The iron content was determined by inductively coupled plasma-optical emission spectrometer (ICP-OES). The particle diameters were characterized by dynamic light scattering (DLS) and transmission electron microscope (TEM). Superparamagnetism was confirmed by the superconducting quantum interference device (SQUID) magnetometer. The cellular internalization efficacy of the modified iron oxides was realized in folate-overexpressed FR(+) and folate-deficient FR(−) KB cells by flow cytometry and confocal laser scanning microscopy (CLSM). The quantitative amount of iron internalized into different harvested KB cells was measured by ICP-OES. The T₂-weighted MR images were tested in FR(+) KB cells.

1. Introduction

Superparamagnetic iron oxide nanoparticles (SPION) with multi-functional properties have been extensively studied as anti-cancer drug carriers for drug delivery systems [1–3] and as contrast agents for magnetic resonance imaging (MRI) [4–7]. SPION are more efficient than gadolinium diethylenetriaminopentaacetic acid (Gd-DTPA) as MRI contrast agents due to the decreased toxicity and improved biocompatibility, good blood retention, and increased contrast enhancement [8]. Indeed, SPION have emerged as an important research subject that has resulted in the improvement of functional properties such as spatial resolution, three-dimensional anatomic details, specific localization at target sites, and others.

Several recent reviews have discussed design strategies, physicochemical characteristics, and biomedical applications of modified SPION [6,9–11]. One design strategy is metal substitution. When Fe is replaced by other metals such as Mn [12] or Co [13,14], the likely result is an increase in superparamagnetism. For example, the system generated by loading 41.7 wt% of MnFe₂O₄ and 40.9 wt% Fe₂O₄ magnetic iron oxides inside micelles possesses a greater saturated magnetic field than Fe₃O₄ [12]. Another important improvement strategy is surface coatings. A proper surface coating, such as organic polymers [15] and inorganic silica materials [16], allows SPION to be homogenously dispersed into ferrofluids, and drugs can be encapsulated in the coating materials for therapeutic applications [9]. Without a coating, SPION is hydrophobic and toxic in character, which could limit its biomedical applications. Finally, targeting ligands attached to SPION surfaces can trigger active delivery to specific cancer tissue sites [17]. Targeting ligands include small molecules (such as folic acid to bind folate receptors), peptides (such as RGD sequence to bind αvβ3 integrins) [8], and monoclonal antibodies (such as Herceptin to bind Her-2/neu...
The targeted SPION localized to specific cells has been shown to prolong the duration of action of the SPION and increase the residence time at target sites. The folate-mediated delivery system has been the subject of numerous studies regarding active targeting in oncologic imaging [6]. It has been shown that aggressive or undifferentiated tumors at an advanced stage, such as tumors of the breast, brain, colon, endometrium, lungs, ovaries, and myeloid cells of hematopoietic origin [20,21], have an increase in folate receptor (FR) density. In contrast, except cells of the choroids plexus, placenta, and low levels in lung, thyroid, and kidneys [11], normal tissues are severely restricted in possessing folate receptors. Thus, folic acid has emerged as a choice ligand for the highly selective delivery of imaging and therapeutic agents to cancer sites [15,20,22,23].

In addition to the recognition of molecular receptors due to the targeting ligand, PEGylated SPION is a common sterically stabilized motif that can prolong the circulation of SPION in the blood pool, and can prevent protein binding and recognition by the reticuloendothelial system (RES) [24–26]. PEGylation of SPION is very important for its in vivo administration. Due to its long circulation time, PEGylation also increases PEGylated SPION’s cytotoxicity which promotes the formation of reactive oxygen species (ROS) by the reaction of oxygen with human tissues. The ROS is a crucial element triggering proinflammatory singling [6]. This situation mediates SPION-induced macrophage cytotoxicity. Thus, a PEGylated SPION that specifically targets a cell is needed to reduce this side effect and increase its utility.

In this study, a folate receptor targeted SPION for dual-modality imaging probes was synthesized. A highly water-soluble SPION was used as a magnetofluorescent nanoprobe, by combining high spatial resolution magnetic resonance (MR) and quantitative photonic fluorescence imaging. Optical fluorescence imaging is more sensitive than MRI, but the penetration depth in the tissue using optical methods is limited to centimeters in depth because of the properties of refraction and absorption of light in the living organism. On the other hand, MRI provides high spatial resolution and unlimited depth penetration [27]. Therefore, the combination of a multifaceted molecular imaging technique can offer synergistic advantages over a single modality alone. Jennings and Long [28] published a recent review article titled, “Two is better than one—probes for dual-modality molecular imaging”, which highlights the importance of using a double molecular imaging technique for today’s clinical applications.

Rhodamine 123 (Rh123) was adapted as a model fluorescent probe. The probe was attached via chemical reaction between the folate receptor (FA) and anti-biofouling PEG segments were linked to the surface. The modified PAAIO was analyzed by FTIR and UV–vis spectrophotometry (Varian Cary Eclipse, CA, USA). The absorbance wavelength was set in the range from 200 to 500 nm for UV–vis and the excitation and emission wavelength was set at 480 and 525 nm for Rh123 fluorescence spectrum. The bond formation between PAAIO and FA-PEG was confirmed by Fourier transform infrared (FTIR), and was carried out using a Perkin–Elmer-2000 spectrometer. Dried samples were pressed with KBr powder into pellets. Sixty-four scans were signal-averaged in the range from 4000 to 4000 cm

A qualitative measurement of the FA and Rh123 groups bound to the PAAIO was visualized by cryo-TEM (Jeol JEM-1200, Tokyo, Japan). A carbon coated 200 mesh copper specimen grid (Agar Scientific Ltd, Essex, UK) was glow-discharged for 1.5 min. One drop of the sample solution was deposited on the grid and left to air-dry. The grid was then examined with an electron microscope. The magnetic properties were measured using a magnetic properties measurement system (MPMS) from Quantum Design (MPMS-XL 7), which utilizes a superconducting quantum interference device (SQUID) magnetometer at fields ranging from −15 to 15 kOe at 25 °C. X-ray diffraction spectroscopy (XRD) measurements were performed on a Rigaku 2KW spectrometer (Tokyo, Japan) with the following operation conditions: 40 kV and 30 mA with a Cu Kα1 radiation at λ = 1.54184 Å. The relative intensity was recorded in the scattering range from 25 to 65° at a rate of 2°/min. Iron contents in PAAIO and its modifiers were determined using an inductively coupled plasma-optical emission spectrometer (ICP-OES) positioned at 239.562 nm (Optima 7000DV, Perkin Elmer, Boston, MA, USA). Transversal T2 (relaxivities were carried out using magnetic resonance (MR) spectroscopy (GE Medical System, Milwaukee, WI, USA) equipped with a 1.5 T MR scanner. Samples containing different concentrations of PAAIO-Rh123 (1.0 × 10−6 to 3.3 × 10−5 M) and PAAIO-Rh123-FA-PEG (3.0 × 10−5 to 6.1 × 10−5 M) were prepared in DD water. The transversal relaxation times were measured using a standard fast spin echo with the following parameters: TR = 2500 ms/79.4 ms, ET = 10 ms, FOV = 260 × 260 mm2, matrix size = 256 × 128. The T2-weighed images were acquired using a fast gradient echo pulse sequence (TR/TEflip angle 150 ms/4.2 ms/30°).

2. Materials and methods

2.1. Materials

Folic acid, iron(III) chloride anhydrous (FeCl3, Fw 162.21 g/mol), and sodium hydroxide were acquired from TCI (Tokyo, Japan). Poly(ethylene glycol) (PEG, Mn 1000) was obtained from Sigma Chemical Company (St. Louis, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Beit Haemek, Israel). RPMI 1640 and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was acquired from Sigma Biomedicals (Eschwege, Germany). All other unstated chemicals were obtained from Sigma Chemical Company (St. Louis, USA), and used without further purification.

2.2. Synthesis of iron oxide nanoparticles

According to the literature, a one-step synthesis of a highly water-soluble super (PAAIO) was synthesized by reaction of magnetite (Fe3O4) nanocrystals with polyacrylic acid (PAA) [15,29]. PAAO (100 mg) was dissolved in 80 mL of double deionized (DD) water and added to a one neck flask. Next, 10 mg of 3-dimethylaminopropyl)-N'-ethycarbodiimide hydrochloride (EDAC) dissolved in 10 mL DD water was added to the flask. The solution was adjusted to pH 7.4 with 0.1 N NaOH and stirred for 1 h at rt. One mg of Rh123 in 10 mL DD water was added to the above solution, and the reaction was stirred for 6 h at rt in the dark. The reaction mixture was transferred into a dialsis tube (Spectrum, Millipore, MWCO 10 K) and dialyzed for 2 d against DD water, which was changed every 3–6 h. Rh123 bound to PAAIO was recovered by lyophilization. The resulting product, PAAIO-Rh123, was stored at −20°C for further use.

Poly(ethylene glycol)-folic acid adduct (FA-PEG) was synthesized via 1,1-Carboxydiimide (CDI) mediated ester formation [30]. Briefly, 30 mg of EDAC was added to a solution of 100 mg of PAAIO-Rh123 in 100 mL of DD water. The reaction was adjusted to pH 7.0 and stirred for 1 d at rt. Next, 20 mg of FA-PEG in 10 mL DMSO/DD water (1/1 v/v), was added into the above solution and the reaction was allowed to sit in the dark for 1 d at rt. The solution was poured into a dialysis membrane MWCO (35,000) and dialyzed against DD water, which was changed every 3–6 h for 2 d. The aqueous solution was freeze-dried and the resulting product PAAIO-Rh123-FA-PEG was stored at −20°C for future use.

2.3. Characterizations of iron oxide nanoparticles

A qualitative measurement of the FA and Rh123 groups bound to the PAAIO surface was carried out using a UV–visible spectrophotometer (Agilent 8453, CA, USA) and fluorescence spectrophotometer (Varian Cary Eclipse, CA, USA). The absorbance wavelength was set in the range from 200 to 500 nm for UV–vis and the excitation and emission wavelength was set at 480 and 525 nm for Rh123 fluorescence spectrum. The bond formation between PAAIO and FA-PEG was confirmed by Fourier transform infrared (FTIR), and was carried out using a Rigaku 2KW spectrometer (Tokyo, Japan) with the following operation conditions: 40 kV and 30 mA with a Cu Kα1 radiation at λ = 1.54184 Å. The relative intensity was recorded in the scattering range from 25 to 65° at a rate of 2°/min. Iron contents in PAAIO and its modifiers were determined using an inductively coupled plasma-optical emission spectrometer (ICP-OES) positioned at 239.562 nm (Optima 7000DV, Perkin Elmer, Boston, MA, USA). Transversal T2 (relaxitivity) was measured using a fast spin echo with the following parameters: TR = 2500 ms/79.4 ms, ET = 10 ms, FOV = 260 × 260 mm2, matrix size = 256 × 128. The T2-weighed images were acquired using a fast gradient echo pulse sequence (TR/TEflip angle 150 ms/4.2 ms/30°).
washed with 10 mL of 0.1M PBS. The cells were detached by 1 mM trypsin and then incubated at rt for 24 h. PAAIO-Rh123 and PAAIO-Rh123-FA-PEG were then added at a concentration of 100 μg/mL in the same culture medium used to culture KB cells, and then incubated separately for 0.5, 3.0 and 6.0 h. The culture mediums were aspirated and the cells were washed and detached by 1× trypsin. The cells were resuspended in 2 mL of 0.1% PBS and 1×10^5 cell counts were immediately analyzed using a flow cytometer (Beckman Coulter, California, USA). The cellular uptake of iron oxides was quantified by ICP-OES, where 2×10^5 cell counts from each sample were analyzed for iron content. The centrifuged cell pellets were dissolved in 37% HCl and were incubated for 1 h at 70°C. The samples were diluted to a final volume of 3 mL for analysis. The iron content of the samples were calculated using Fe(NO3)3 calibration curve.

2.4.4. Confocal microscopy measurements

FR(+) KB cells (2×10^5 cells) were seeded into a 12-well culture plate containing one glass coverslip/well and incubated for 24 h at 37°C. The medium was removed and replaced with 1 mL of 0.1% PBS containing PAAIO-Rh123 or PAAIO-Rh123-FA-PEG and then incubated at rt for 30 min. The coverslips with cells were treated with 1 mL of 3.7% formaldehyde in 0.1% PBS and allowed to sit at rt for 30 min. The cells were treated with 1 mL/well of Triton X-100 and incubated for 10 min. After three washings with 0.1% PBS, the cells were then incubated with 0.5 mL/well of DAPI for 10 min at 37°C. An Olympus Fv 500 CLSM (Tokyo, Japan) was used for cell imaging. The emission wavelength was set to 565 nm.

2.5. In vitro MRI

A clinical 3.0 T MR scanner (Sigma, GE Medical System, Milwaukee, WI, USA) was used to measure T2-weighted signal intensities using iron concentrations ranging from 0 to 30 μg/mL in FA-deficient RPMI medium. FR(+) KB cells (5×10^5 cells) were seeded into a 6-well culture plate 1 d before adding the various concentrations of PAAIO-Rh123 or PAAIO-Rh123-FA-PEG. The addition of the samples was followed by incubation at 37°C for 3 h. The medium was dispensed and the cells were washed three times with 0.1% PBS containing 2% FBS. The T2-weighted images were acquired using a fast gradient echo pulse sequence (TR/TE/flip angle 3000/90/10).

3. Results and discussion

3.1. Synthesis and compositions of PAAIO-Rh123 and PAAIO-Rh123-FA-PEG

A highly water-soluble PAAIO was synthesized via a one-step solvothermal reaction according to a method described previously [15]. In order to maintain equivalent amounts of Rh123 in PAAIO-Rh123 and PAAIO-Rh123-FA-PEG, PAAIO-Rh123 was synthesized as depicted in Scheme 1, and then reacted with FA-PEG. Since the fluorescence intensity directly correlates to the internalization ability of iron oxide particles into cells, this procedure is important to minimize incorrect explanations of cellular uptake, due to

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>N</th>
<th>C</th>
<th>H</th>
<th>PAAIO Rh123 FA-PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAAIO</td>
<td>30.40±0.19</td>
<td>6.43±0.05</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PAAIO-Rh123</td>
<td>0.26±0.01</td>
<td>25.47±0.02</td>
<td>4.91±0.02</td>
<td>96.6</td>
</tr>
<tr>
<td>PAAIO-Rh-FA-PEG</td>
<td>3.09±0.02</td>
<td>28.84±0.02</td>
<td>4.92±0.05</td>
<td>67.2</td>
</tr>
</tbody>
</table>
variable amounts of Rh123. Qualitative confirmation of the Rh123 molecules on the surface of PAAIO was observed by fluorescence spectrophotometer (Supporting information S1a). Moreover, a profound UV–vis absorbance peak at ~280 nm appears in PAAIO-Rh123-FA-PEG (S1b), which is a characteristic absorbance peak of FA-PEG [30]. The IR absorbance peaks (S2) for FA-PEG were observed at 1652 cm\(^{-1}\) (C=O), 1560 cm\(^{-1}\) (N–H), and 1405 cm\(^{-1}\) (C–N). Since pure PAA has a strong C=O IR absorbance peak at 1718 cm\(^{-1}\) [29], the shift in the absorbance peak of PAAIO-Rh123 (shifted to 1638 cm\(^{-1}\)) may be attributed to strong hydrogen bonding between carboxylic acid groups which is influenced by the presence of a hydrophobic molecule [34]. The band at 591 cm\(^{-1}\) has been reported for Fe–O [23]. PAAIO-Rh123-FA-PEG appears as a combination of peaks due to both FA-PEG and PAAIO characteristics.

To quantitatively determine the composition of Rh123 and FA-PEG on the modified iron oxides, PAAIO, PAAIO-Rh123, and PAAIO-Rh123-FA-PEG were subjected to elemental analysis. Table 1 shows the average contents of C, H, and N % from three measurements. The N% was used to calculate the weight percents of Rh123 and FA-PEG. The molecular weights of PAAIO, Rh123, and FA-PEG are taken as 2231.6, 380.8, and 1441.1 g/mol, respectively. The N content of PAAIO-Rh123 and PAAIO-Rh123-FA-PEG were measured to calculate the molar fractions of Rh123 and FA-PEG (S3). Their transformed weight percents are also summarized in Table 1.

The weight percent of Rh123 was controlled at 1 wt% in feed but the measured values of PAAIO-Rh123 and PAAIO-Rh123-FA-PEG were 3.4 and 2.4 wt% respectively. As indicated in Table 1, the weight percent of FA-PEG in PAAIO-Rh123-FA-PEG was controlled at 20 wt% in feed but 30.4 wt% was measured. The increase in Rh123 and FA-PEG content of iron oxides after purification implies that the purification by dialysis removed the unmodified PAAIO.

Using surface plasmon resonance (SPR), Bae and Kataoka [35] have tested the minimum amount of FA on polymeric micelles needed to produce a receptor recognition. The authors found that the amount of FA causes little difference in receptor recognition as long as the polymeric micelles possess an FA content of more than 10 wt%. Therefore, using 30 wt% of FA in PAAIO-Rh123-FA-PEG should be sufficient to trigger cellular uptake via FR-mediated endocytosis, and will thus provide a high efficacy in cancer cell targeting delivery.
3.2. Characterization of iron oxides

PAAIO displays the characteristic X-ray diffraction pattern of Fe₃O₄ at 2θ of 30.2, 35.5, 43.2, 53.3, 57.1, and 62.8°, and is the same as listed on the ASTM XRD standard card (19-0629). The signal intensity of PAAIO-Rh123-FA-PEG when decorated with FA-PEG is reduced (S4), but the peaks are in the same position. The average hydrodynamic diameter measured at a concentration of 0.1 mg/mL by DLS for PAAIO, PAAIO-Rh123, and PAAIO-Rh123-FA-PEG was 35.6 ± 0.5 nm (PDI = 0.24), 36.8 ± 0.4 nm (PDI = 0.25), and 85.8 ± 7.4 nm (PDI = 0.53), respectively. As shown in Fig. 1, the particle diameters were smaller (~10 nm for all three samples) when measured by cryo-TEM. It is suspected that the discrepancy of particle size measured by DLS and TEM is more profound in PAAIO-Rh123-FA-PEG because of the decorated hydrophilic polymer layer on the surface. Since TEM images are taken in a dried state, polymer coatings that result in differences in measured particle diameters have often been reported [36,37].

PAAIO has been synthesized via a two-step method by Ma et al. [7]. Accordingly, Fe₃O₄ was prepared by treatment of FeCl₃·6H₂O and FeCl₂·4H₂O with ammonium hydroxide. Following purification, Fe₃O₄ was further reacted with PAA oligomer to form PAAIO. The PAAIO prepared by Ma et al. was 9.6 ± 2.6 nm in diameter, as measured by TEM. The average particle diameter was controlled by changing the amount of NaOH or the reaction time in the one-step synthesis in the presence of PAA [29]. The particle diameters of 2.9, 6.6, and 11.3 nm were measured from TEM images. Since particle sizes of less than 10 nm in diameter will be rapidly excreted by the human renal system [38], the iron oxides at a particle diameter of 10–30 nm has been reported to be the most suitable for biomedical applications [39]. As can be seen in Fig. 1, there are insignificant changes in the TEM images of PAAIO (Fig. 1a) and PAAIO-Rh123 (Fig. 1b), and PAAIO-Rh123-FA-PEG (Fig. 1c). The larger particle diameters, with a high PDI value by DLS, indicates serious aggregation is occurring after grafting of FA-PEG onto PAAIO. Aggregation may be occurring due to the crosslinking of the PEG residue.

As measured by ICP-OES, the iron content in PAAIO, PAAIO-Rh123, and PAAIO-Rh123-FA-PEG were found to be 1.808, 1.547, and 0.945 ppm, respectively. These values were used to normalize the Fe content in the SQUID measurements. The saturation magnetization values (Fig. 2) of PAAIO, PAAIO-Rh123, and PAAIO-Rh123-FA-PEG were 78, 60, and 57 emu/g Fe, respectively. For clinical applications as targeted contrast agents for MRI, it is critical that the iron oxides retain their magnetic properties after polymer coatings. As expected, the modified iron oxides are superparamagnetic at rt and show negligible hysteresis. PAAIO has a remanent magnetization (Mr) of 0.84 emu/g Fe and coercivity (Hc) of 11.85 Oe, while the modified iron oxides have a Mr of 0.48 emu/g Fe and an Hc of 12.45 Oe (see Fig. 2 inset).

3.3. Cytotoxicity and cellular uptake

To examine the acute toxicity of Fe₃O₄, PAAIO, PAAIO-Rh123, and PAAIO-Rh123-FA-PEG in FR(+) and FR(−) KB cells, the iron oxide nanoparticles were tested at the concentration range of 5–1000 μg/mL. The amount of viable cells was determined by the

![Fig. 3. Cell viability of iron oxides incubated in different harvested KB cells. (a) FR(+) KB cells, and (b) FR(−) KB cells.](image-url)
MTT assay. In both types of KB cells, PAAIO is non-toxic in all tested concentrations. The cell viability decreases, however, with an increase in concentration of Fe_{3}O_{4}, PAAIO-Rh123, and PAAIO-Rh123-FA-PEG for both cell types. Fig. 3 demonstrates that the cell viability decreases with an increase in concentration. In FR(+) KB cells, cell viability of less than 80% was observed at a concentration of greater than 500 \( \mu \)g/mL. Conversely, in FR(-) KB cells, cell viability of less than 80% was observed at 250 \( \mu \)g/mL. As commonly known, SPION is toxic without a coating material \[9\]. Here, we indeed observe the reduced cytotoxicity after PAA decoration on the iron oxide surface. The cytotoxicity found in PAAIO-Rh123 and PAAIO-Rh123-FA-PEG may be due to the grafted Rh123 molecule. In unpublished work, we have also observed that the cytotoxicity of chitosan increases dramatically when a fluorescent dye such as FITC is used.

FR(+) and FR(-) KB cells were harvested to study the cellular uptake efficacy of iron oxides with and without FA-PEG. Using flow cytometry, PAAIO-Rh123-FA-PEG displayed a profound time-dependent increase of fluorescence intensity relative to the control group in FR-positive KB cells. On the other hand, PAAIO-Rh123 displayed an insignificant increase in fluorescence intensity with increasing incubation time. On the contrary, for FR-deficient KB cells, the cellular uptake of both PAAIO-Rh123 and PAAIO-Rh123-FA-PEG increases with increasing incubation time. It was
determined that PAAIO-Rh123 with smaller particle sizes (~35 nm) results in efficient cellular internalization in cells with a low expression of folate receptors, and the summarized results are shown in Fig. 4. A small targeting molecule, folic acid, indeed triggers the recognition of folate-binding proteins and enhances the internalization of PAAIO-Rh123-FA-PEG, even though its particle size is larger than PAAIO-Rh123. This result indicates that the cellular uptake of nanoparticles may depend more strongly on mediated-ligands rather than on nanoparticle sizes.

In a parallel experiment, the amount of iron within the cells was quantitatively determined by ICP-OES. Fig. 5 shows the Fe uptake in FR(+) and FR(−) KB cells at different incubation time periods. When PAAIO-Rh123-FA-PEG was incubated in FR(+) KB cells, the cellular uptake of Fe was found to increase with increasing incubation time. With PAAIO-Rh123, the increase in the internalized Fe amount into FR(+) KB cells is insignificant with an extended incubation time period. There is an approximately 2.5 fold increase in Fe uptake of PAAIO-Rh123-FA-PEG compared with PAAIO-Rh123 with 6 h of incubation. On the contrary, the Fe uptake of both iron oxides in FR(−) KB cells is similar for all three incubation time periods.

The cellular uptake images of the modified iron oxides into FR(+) KB cells were visualized by CLSM (Fig. 6) and were performed under similar conditions as the aforementioned flow cytometry experiments. Both PAAIO-Rh123 and PAAIO-Rh123-FA-PEG show a similar tendency in the confocal images. The red fluorescent color is seen surrounding the nucleus surface at 30 min, then appears inside the nucleus at 3 h, and finally increases in intensity inside the nucleus at 6 h. Under the same incubation conditions, a larger increase in fluorescence intensity for PAAIO-Rh123-FA-PEG was observed than for PAAIO-Rh123. This enhancement of cellular uptake of PAAIO-Rh123-FA-PEG over PAAIO-Rh123 is in agreement with the previous flow cytometry findings. Thus, PAAIO-Rh123-FA-PEG is a potential fluorescent probe to trace cancer cells that overexpress folate receptors. In addition to Rh123, any fluorescent dye that possesses amino functional groups can be conjugated to PAAIO via similar chemistry as described above.

3.4. In vitro MR imaging

Transversal ($r_2$) relaxivities in DD water were carried out using 1.5 T MR scanner. Transversal relaxation rates ($1/T_2$) were plotted as a function of iron concentration and $r_2$ relaxivities were obtained by the slope of the fitting straight line. Transversal relaxivities determined as above were found to be: $r_2 = 216.06 \text{ mM}^{-1} \text{s}^{-1}$ for PAAIO-Rh123 and $r_2 = 105.39 \text{ mM}^{-1} \text{s}^{-1}$ for PAAIO-Rh123-FA-PEG, respectively (55). The high $r_2$ values imply a good potential application as contrast agents of modified iron oxides.

In vitro MR imaging was conducted using similar conditions as the cellular uptake experiments. This was done to evaluate the potential of PAAIO-Rh123-FA-PEG as targeted MR contrast agents to cancer cells that overexpress folate receptors. FR(+) KB cells cultured with PAAIO-Rh123 or PAAIO-Rh123-FA-PEG at various iron concentrations were incubated for 3 h at 37°C. The $T_2$-weighted MR phantom images are shown in Fig. 7. The images of the cells incubated with PAAIO-Rh123-FA-PEG displays a better negative contrast enhancement (signal darkening) over those cells incubated with PAAIO-Rh123. The enhancement of the MR images of the cells after incubation with the modified iron oxides is defined by the following equation [40]:

![Fig. 6. Confocal microscopy analysis of PAAIO-Rh123 and PAAIO-Rh123-FA-PEG at a concentration of 100 μg/mL internalized into FR(+) KB cells for three incubated time periods at 37°C.](image-url)
the observed enhancement for PAAIO-Rh123 was

$$\text{Enhancement (\%)} = \frac{S_{\text{Sample}} - S_{\text{Control}}}{S_{\text{Control}}} \times 100\%$$  

where $S_{\text{Sample}}$ and $S_{\text{Control}}$ are the signal intensities of MR images of the cells incubated with and without samples in the cells. The enhancement results are summarized in Table 2. At Fe concentrations of less than 10 $\mu$g/mL, the MRI signal enhancement shows little difference between PAAIO-Rh123 and PAAIO-Rh123-FA-PEG. Nevertheless, the rapid and efficient folate receptor-mediated endocytosis leads to a distinguishable darkening of the MR images of the cells incubated with PAAIO-Rh123-FA-PEG at Fe concentrations of higher than 15 $\mu$g/mL. The MRI signal enhancement decreases from −45.20% for PAAIO-Rh123, to −55.66% for PAAIO-Rh123-FA-PEG. The more significant decrease in MRI intensity observed with PAAIO-Rh123-FA-PEG versus PAAIO-Rh123 occurs at the Fe concentration of 30 $\mu$g/mL. Under these conditions, the observed enhancement for PAAIO-Rh123 was −67.52%, and −83.42% for PAAIO-Rh123-FA-PEG. The $T_2$-weighted MR phantom images of PAAIO-Rh123-FA-PEG, shown in Fig. 7, displays an increase in negative contrast enhancement as compared to PAAIO-Rh123. These results are also consistent with the cellular uptake results obtained above.

In our previous studies [15], FA–Pluronic F127 decorated onto PAAIO (PF127-PAAIO, −29.75%) also showed a larger MR contrast enhancement than without FA (PF127-PAAIO, −10.37%) even at a low Fe concentration of 6 $\mu$g/mL. The observable difference, however, between PAAIO-Rh123 (−22.11%) and PAAIO-Rh123-FA-PEG (−27.96%) is insignificant at a concentration of 5 $\mu$g/mL. The increased concentration needed to visualize the particles of PAAIO-Rh123. This situation may compensate the mechanism activated by the FA-mediated endocytosis. However, at the same Fe concentration of 30 $\mu$g/mL, PAAIO-Rh123-FA-PEG was found to be a better MRI contrast agent (−83.42% enhancement) than FA-PF127-PAAIO (−66.34% enhancement).

### 4. Conclusions

This report introduces a simple chemical conjugation method that incorporates a fluorescent dye onto a highly water-soluble iron oxide PAAIO, and its illustration as a potentially dual-modality probe for fluorescence and MR imaging. A specific targeting ligand FA was grafted onto the surface of PAAIO that recognizes folate-binding proteins overexpressed on KB cell surfaces. PEG was introduced to enhance the circulation time under physiological conditions. As verified by flow cytometry, CLSM, $T_2$-weighted MRI phantom imaging, and iron content, the PAAIO-Rh123-FA-PEG moiety showed an increase in cellular internalization in FR(+) KB cells, whereas in FR(−) KB cells, the efficiency of cellular uptake is dependent on the particle size. The designed PAAIO-Rh123-FA–PEG possessed good magnetic properties. As such, our future studies will include focusing the PAAIO-Rh123-FA-PEG towards specific tumor sites using an imposing external magnetic field, which will hopefully improve the efficacy of therapeutic agents towards tumor tissues.

### Acknowledgements

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### Appendix

Figures with essential color discrimination. All figures and Scheme of this article may be difficult to interpret in black and white. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2009.11.026.

#### Appendix. Supplementary data

The supplementary materials can be viewed at doi:10.1016/j.biomaterials.2009.11.026.
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