

Aptamer-conjugated Magnetic Nanoparticles as Targeted Magnetic Resonance Imaging Contrast Agent for Breast Cancer

Abstract

Early detection of breast cancer is the most effective way to improve the survival rate in women. Magnetic resonance imaging (MRI) offers high spatial resolution and good anatomic details, and its lower sensitivity can be improved by using targeted molecular imaging. In this study, AS1411 aptamer was conjugated to Fe₃O₄@Au nanoparticles for specific targeting of mouse mammary carcinoma (4T1) cells that overexpress nucleolin. *In vitro* cytotoxicity of aptamer-conjugated nanoparticles was assessed on 4T1 and HFFF-PI6 (control) cells. The ability of the synthesized nanoprobe to target specifically the nucleolin overexpressed cells was assessed with the MRI technique. Results show that the synthesized nanoprobe produced strongly darkened T₂-weighted magnetic resonance (MR) images with 4T1 cells, whereas the MR images of HFFF-PI6 cells incubated with the nanoprobe are brighter, showing small changes compared to water. The results demonstrate that in a Fe concentration of 45 µg/mL, the nanoprobe reduced by 90% MR image intensity in 4T1 cells compared with the 27% reduction in HFFF-PI6 cells. Analysis of MR signal intensity showed statistically significant signal intensity difference between 4T1 and HFFF-PI6 cells treated with the nanoprobe. MRI experiments demonstrate the high potential of the synthesized nanoprobe as a specific MRI contrast agent for detection of nucleolin-expressing breast cancer cells.

Keywords: Breast, contrast agent, early detection of cancer, humans, magnetic resonance imaging, magnetic resonance spectroscopy, molecular imaging, nanoparticles

Introduction

Breast cancer is the most frequently diagnosed tumor in women and is the second leading cause of cancer-related death in this group.^[1] Early diagnosis of the disease is the most effective way to improve the survival rate.^[2] Positron emission tomography (PET), single photon emission computed tomography (SPECT), ultrasound, and magnetic resonance imaging (MRI) are the commonly used imaging modalities for the detection of breast cancer.^[3] PET and SPECT are nuclear medicine imaging modalities, and although they have high sensitivity, they lack good resolution, use ionizing radiation, and need radiotracers.^[4] MRI has been widely used in clinical oncology imaging and offers high spatial resolution and also good anatomical and functional details without using ionizing radiation; however, its sensitivity is lower than PET and SPECT.^[5,6]

To improve the sensitivity of MRI for molecular and cellular imaging, magnetic contrast agents are often used.^[7] Molecular imaging provides detailed image of what is happening inside the body at the molecular level. One of the magnetic nanoparticles that has been used is the superparamagnetic iron oxide nanoparticles (SPIONs).^[8-17] These kind of nanoparticles have gained significant attention because of its low toxicity, biodegradability, long blood half-time, and high relaxivity.^[12,13] SPIONs can reduce T₂ relaxation time of water, and therefore, they are called negative T₂ contrast agents.^[11,4]

American Cancer Society recommended breast MRI as a screening approach, adjunct to mammography, for the early detection of breast cancer.^[15] To improve the sensitivity and specificity of magnetic resonance (MR) cancer imaging, MR molecular imaging can be used. In this method, biomarker target-specific imaging nanoprobe are utilized.^[16-18]

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To construct target-specific nanoprobes, surface of the nanoparticles can be modified by target ligands such as peptides, antibodies, and aptamers.^[19] Aptamers are single-stranded oligonucleotides that can strongly bind to their targets with high affinity and specificity.^[20,21] Aptamers that have been called chemical antibodies have many unique advantages over antibodies. For example, they have lower immunogenicity, better tissue penetration, more stability, can be easily synthesized, and are inexpensive.^[20,22-25] Therefore, aptamers offer better targeting efficacy and cost-effectiveness.

Nucleolin is a protein that is overexpressed on the cell surface of many tumors such as in breast cancer, lymphocytic leukemia, and hepatocellular, prostate, and renal carcinoma.^[26-28] Therefore, nucleolin can act as a biomarker for targeting breast cancer. AS1411 is an aptamer that binds to nucleolin with high affinity and specificity.^[29] As a result of overexpression of nucleolin in cancer cells, compared with normal cells,^[29] it can be useful for molecular imaging and targeted drug delivery.

There are some studies that have used antibodies and peptides for targeted MR molecular imaging of breast cancer.^[4,19,30,31] Nevertheless, to the best of authors' knowledge, there is no study about the use of aptamers for targeted MR molecular imaging of breast cancer. According to the mentioned many advantages of aptamers over antibodies and peptides, aptamers were used along with magnetic nanoparticles to design and apply aptamer-conjugated superparamagnetic nanoparticles for targeted MRI of breast cancer.

The aim of this study is to investigate the cytotoxicity effects of the prepared nanoprobe, which was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and finally development of aptamer-conjugated magnetic nanoparticles as an MRI contrast agent for breast cancer cell detection.

Materials and Methods

Materials and instruments

Tri(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Sigma. Fe₃O₄@Au nanoparticles were prepared at the Department of Chemistry, Isfahan University. The AS1411 aptamer had the sequence of 5'-GGTGGTGGTGGTTGTGGTGGTGGTGGTTT-3'-OH-SH. The size and morphology of the nanoparticles were studied using TEM imaging (Philips-EM208S).

Preparation of aptamer-conjugated Fe₃O₄@Au nanoparticles (nanoprobe)

AS1411 aptamer was conjugated on the surface of Fe₃O₄@Au nanoparticles for specific detection of breast cancer. The aptamer-conjugated Fe₃O₄@Au nanoparticles (nanoprobe) were prepared via the gold-sulfur chemistry. The S-S bond of the aptamer was deprotected by the addition

of 20 µL of 0.5 mM TCEP to 100 µL aliquot of aptamer in dark for 1 h. Subsequently, 1 mg of the Fe₃O₄@Au nanoparticles was dissolved in 3 mL PBS, then treated aptamer was added, and the mixture was stirred for 3 h at room temperature in the dark. After magnetic separation with an external magnet, the nanoprobe was washed three times using PBS (pH 7.4) to remove the unbound SH-aptamers. Finally, the nanoprobe was resuspended in 1.0 mL PBS (pH 7.4) buffer and stored at 4°C.

Cell lines

4T1, mouse mammary carcinoma, and HFFF-PI6, human foreskin fibroblast, cell lines were purchased from the National Cell Bank of Iran (Pasteur Institute, Iran). The cells were cultured in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) and incubated at 37°C in a humidified incubator with 5% CO₂.

In Vitro cytotoxicity

In vitro cytotoxicity of the nanoprobe was evaluated by using thiazolyl tetrazolium (MTT) assay of 4T1 and HFFF-PI6 cells. About 2 × 10⁴ cells per well were seeded into 96-well cell culture plates and incubated for 24 h in a humidified incubator with a CO₂ concentration of 5% to allow adherence of the cells. Then, the cells were incubated with different iron concentrations of the nanoprobe ranging from 10 to 60 µg/mL for 24 h.

After 24 h incubation, the medium was removed, 100 µL fresh medium and 20 µL MTT (5 mg/mL) was added into each well, and the plate was returned to the incubator. After 4 h, the culture medium was carefully removed, and 100 µL dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals for 10 min. The absorbance at 570 nm was measured using an ELISA reader (Synergy H1, BioTek). Experiments were performed in triplicate, and cell survival was determined as a percentage of viable cells in comparison with control wells.

MR imaging

The potential of the nanoprobe as contrast agent for MRI was investigated *in vitro* after incubation with 4T1 and HFFF-PI6 cells. 4T1 and HFFF-PI6 cells were seeded into a 6-well plate at a density of 1 × 10⁶ cells/well. After incubation at 37°C in a humidified incubator with 5% CO₂ for 24 h, the medium was replaced with a fresh medium containing the nanoprobe with different Fe concentrations ([Fe] = 10, 20, 30, and 45 µg/mL). It should be noted that one well was left untreated and was considered as control. After 4 h incubation, the cells were washed three times with PBS solution. Subsequently, the cells were trypsinized, centrifuged, and fixed into 1 mL prepared gelatin solution. Then, the Eppendorf tubes were placed on ice powder until solidity was obtained.

MRI was performed using a 1.5 T MRI clinical scanner (Symphony, Siemens, Germany) located in Isfahan city (Isfahan MRI Center) with T₂-weighted spin-echo sequence. Imaging parameters were as follows: repetition time, 3000 ms; echo time, 33 ms; field of view, 201 mm × 229 mm; slice thickness, 3 mm.

Statistical analysis

Data are presented as the mean ± standard deviation and analysis was performed using *t*-test. A value of *P* < 0.05 was considered statistically significant.

Results

TEM imaging was used to determine the size and morphology of prepared Fe₃O₄@Au nanoparticles. Figure 1 shows that the nanoparticles had a spherical morphology with size of less than 50 nm.

The biocompatibility of the nanoprobe was assessed by MTT assay. Figure 2 shows the result of MTT assay at different iron concentrations for 24 h. The results are presented in terms of percentage cell viability. Materials with cell viability more than 80% can be considered as being biocompatible.^[32]

Figure 2(a) shows that for 4T1 cells, at concentration of 60 μg/mL, the nanoprobe has cytotoxicity effects. In addition, for 4T1 cells at concentrations from 10 to 45 μg/mL, cell toxicity was low or moderate. MTT results for HFFF-PI6 cells are shown in Figure 2(b); it shows that up to 60 μg/mL, the nanoprobe had no cytotoxicity effects.

Figure 3 shows T₂-weighted images of 4T1 and HFFF-PI6 cells after 4 h incubation with the nanoprobe at different Fe concentrations. It is clear that 4T1 cells are darker than HFFF-PI6 cells. Moreover, by increasing Fe concentration, signal intensity decreased in 4T1 cells compared to HFFF-PI6 cells. The nanoprobe produced darkened T₂-weighted MR images with higher concentrations of the nanoprobe with 4T1 cells, whereas the MR images of HFFF-PI6 cells incubated with the nanoprobe were brighter, showing small changes compared to water.

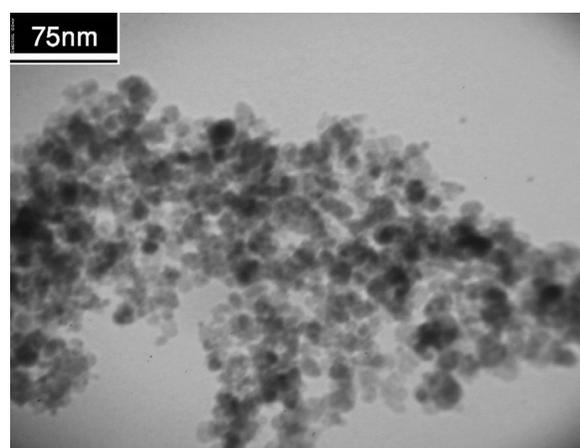


Figure 1: TEM image of Fe₃O₄@Au nanoparticles

Figure 4 shows MR signal intensity as a function of Fe concentration for 4T1 and HFFF-PI6 cells treated with the nanoprobe. It is clear that signal intensity decreases as Fe concentration increases for both cells. The results demonstrate that in a Fe concentration of 45 μg/mL, the nanoprobe reduced by 90% MR image intensity in 4T1 cells compared with the 27% reduction in HFFF-PI6 cells. Statistical analysis reveal that for all Fe concentrations, difference in signal intensity between 4T1 and HFFF-PI6 was statistically significant (*P* < 0.05).

Discussion

Figure 1 shows the TEM image of Fe₃O₄@Au nanoparticles and it reveals that the size of the nanoparticles is less than

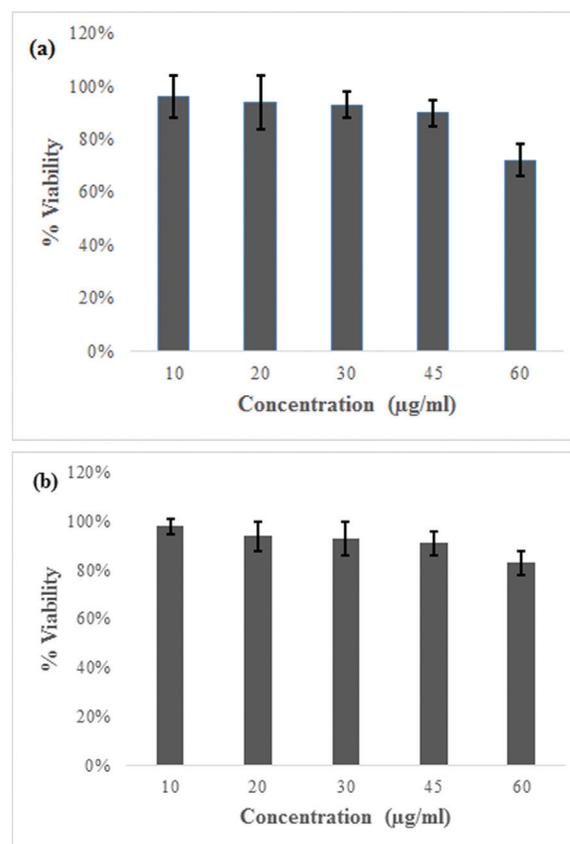


Figure 2: Representation of cytotoxicity of aptamer-conjugated Fe₃O₄@Au nanoparticles (nanoprobe) on (a) 4T1 and (b) HFFF-PI6 cells, with different iron concentrations

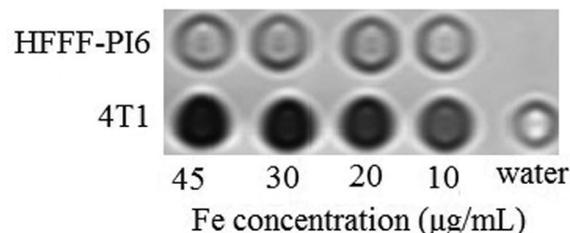


Figure 3: T₂-weighted imaging of 4T1 and HFFF-PI6 cells after 4 h incubation with nanoprobe at Fe concentrations of 0, 10, 20, 30, and 45 μg/mL

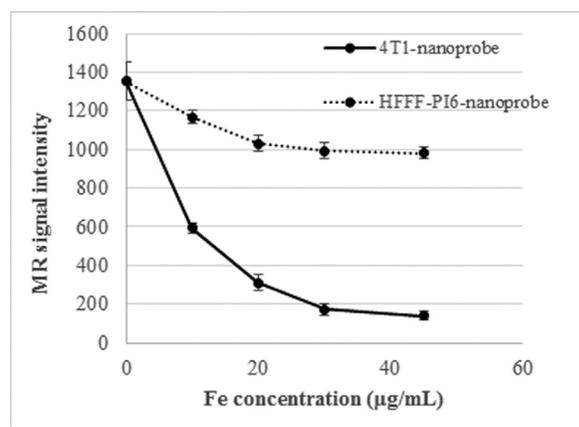


Figure 4: MR signal intensity as a function of Fe concentration for 4T1 and HFFF-PI6 cells treated with nanoprobe

50 nm. The TEM image shows some aggregated or interconnected particles, which could be because of the TEM sample preparation process. As shown in Figure 2, the nanoprobe displayed good biocompatibility and no significant toxicity was observed on 4T1 and HFFF-PI6 cells up to 45 and 60 µg/mL, respectively.

Li *et al.*^[33] investigated the biocompatibility of magnetic iron oxide nanoparticles on human cervical cancer cell line (Hela) and immortalized normal human retinal pigment epithelial cell line (RPE). Their results showed that cytotoxicity effects of magnetic nanoparticles are not the same as that for the two studied cells, implying that cytotoxicity is cell-type specific, which is in good agreement with the results here.

Then, the ability of the synthesized nanoprobe to target specifically to the nucleolin overexpressed cells was assessed with MRI technique. Results of Figures 3 and 4 suggest that the nanoprobe specifically and selectively binds to the 4T1 cells, because of overexpression of nucleolin, and has very low accumulation in HFFF-PI6 cells, implying potential ability of the nanoprobe as an MRI contrast agent.

Analysis of MR signal intensity [Figure 4] using *t*-test showed statistically significant signal intensity difference between 4T1 and HFFF-PI6 cells treated with the nanoprobe.

Yang *et al.*^[4] developed new targeted iron oxide nanoparticles using a recombinant peptide containing the amino-terminal fragment of urokinase-type plasminogen activator (uPA) conjugated to magnetic iron oxide nanoparticles amino-terminal fragment conjugated-iron oxide (ATF-IO). This nanoprobe targets uPA receptor, which is overexpressed in breast cancer tissues. Results of the MRI study showed strong contrast by a 3 T MRI scanner. Their results suggested that uPA receptor-targeted ATF-IO nanoparticles have potential as targeted molecular agents for MRI of breast cancer. Rasanah *et al.*^[30] modified magnetic nanoparticles with Trastuzumab antibody to act as a new contrast agent for breast cancer MRI. T₂-weighted MR image and signal intensity of SKBr3 (breast cancer cell line) showed

darkened and low-signal intensity compared to control, which showed its capability for targeted molecular MRI.

Our study with aptamer-conjugated magnetic nanoparticles is in good agreement with other studies that used antibody and peptide.^[4,30] Nevertheless, significant advantages of aptamers such as lower immunogenicity, high stability, and cost-effectiveness can make them more interested in related applications.

Conclusion

It can be concluded that thiolated AS1411 aptamer conjugated to Fe₃O₄@Au nanoparticles can be used for specific targeting of 4T1 cells overexpressing nucleolin on the cell surface. Results of the MRI study show that the designed nanoprobe can bind specifically to breast cancer and has very low accumulation in normal cells, confirming its capability as targeted contrast agent for MRI of breast cancer.

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Conflicts of interest

There are no conflict of interest.

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