



Original Article

IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS  
Copyright © 2018 by Iran University of Medical Sciences

Iranian J Pharmacol Ther. 2018 (March);16:1-8.



## On-demand cellular uptake of cysteine conjugated gadolinium based mesoporous silica nanoparticle with breast cancer-cells

Hojjatollah Nazari<sup>1,2</sup>, Khadijeh Ashtari<sup>1</sup>, Masoud Soleimani<sup>3</sup>, Kowsar Bagherzadeh<sup>4</sup>, Sepideh Safari<sup>4</sup>,  
Bitamehravi<sup>1,5\*</sup>

<sup>1</sup> Department of Medical Nanotechnology, Faculty of Advanced Technology in Medicine, Iran University of Medical Sciences, Tehran, Iran

<sup>2</sup> Stem Cell Technology Center, Tehran, Iran

<sup>3</sup> Department of Hematology, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran

<sup>4</sup> Razi Drug Research Center, Iran University of Medical Sciences, Tehran, Iran

<sup>5</sup> Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran

### Please cite this article as:

Nazari H, Ashtari Kh, Soleimani M, Bagherzadeh K, Safari S, Mehravi B. On-demand cellular uptake of cysteine conjugated gadolinium based mesoporous silica nanoparticle with breast cancer-cells. *Iranian J Pharmacol Ther.* 2018 (March);16: 1-8.

### ABSTRACT

Design, synthesis, and conjugation of mesoporous silica nanoparticles (MSNs) with biomolecules is a matter of growing interest to enhance selective uptake of contrast agents like gadolinium (Gd<sup>3+</sup>) by cancer cells. Here, by targeting xc-cystine/glutamate antiporter system in breast cancer cells, conjugation of MSN-Gd<sup>3+</sup> with cysteine is used to enhance cancer cellular uptake of Gd<sup>3+</sup>. Reactions designed to make different covalent bonds between MSNs and cysteine to investigate impact of cysteine conjugation of MSNs-Gd<sup>3+</sup> on uptake of Gd<sup>3+</sup> by breast cancer cells. Cysteine amino acids were attached to the surface of MSNs via its three functional groups using three different conjugation methods. Therefore, the external surface of MSNs were first modified by three different linkers to create amine, epoxy, and isocyanate groups on the surface of MSNs, then pores loaded with Gd<sup>3+</sup> complexes and reacted with thiol, epoxy, and amine groups of cysteine, respectively (nanoprobe A, B and C). The nanoprobees were characterized using different techniques, including (scanning electron microscope) SEM, Brunauer–Emmett–Teller BET, dynamic light scattering (DLS) and Fourier Transform Infrared (FTIR). The intracellular uptake of nanoprobees by human dermal fibroblasts (HDF) and human breast adenocarcinoma cells (MCF-7) was investigated using inductively coupled plasma atomic emission spectrometer (ICP-AES). Results demonstrated that accumulation of Gd<sup>3+</sup> in cancer cells is highly related to method of cysteine conjugation. The amount of Gd<sup>3+</sup> was taken by cancer cells increased 7 folds, when thiol group of cysteine was responsible to make covalent bond with MSNs, in other words when the zwitterionic form of cysteine was on the surface (nanoprobe B). The average intracellular uptake of Gd<sup>3+</sup> by cancer cells was 0.5±0.09 pg/cell. On the other hand, uptake of Gd<sup>3+</sup> delivered by nanoprobe B into cancer cells was up to 4.7 times higher than normal cells. No appreciable cytotoxicity was seen using HDF and MCF-7 cell lines. This study provides MRI nanoprobees using suitable conjugation of Gd<sup>3+</sup> based MSNs with cysteine for next studies about MR imaging of cancer.

Conflicts of Interest: Declared None

Funding: Iran University of Medical Sciences

### Keywords

Breast Cancer,  
Cysteine,  
Gadolinium,  
Mesoporous Silica Nanoparticle

### Corresponding to:

Bitamehravi,  
Radiation biology research center,  
Iran University of Medical  
Sciences, Tehran, Iran

Email:  
[mehravi.b@iums.ac.ir](mailto:mehravi.b@iums.ac.ir)

Received: 31 Oct 2017

Revised: 30 Nov 2017,

Accepted: 6 Feb 2018

### INTRODUCTION

Targeting specific biological molecules, including transporters which are overexpressed on the surface of cancer cells leads to enhanced accumulation of contrast agents [1-8] and avoids the common limitations such as

toxicity [9], lack of selectivity and contrast in soft tissues. Surface modification of nanoparticles with agents that have a high affinity for specific markers overexpressed by cancer cells improves cellular uptake of contrast agents, as well as

MR imaging accuracy [6, 10].

Different kinds of nanomaterials such as dendrimers, liposomes, peptides, and mesoporous silica nanoparticles (MSNs) have been used as carriers for contrast agents [1-5]. MSNs have been extensively investigated for improving Gd<sup>3+</sup> based MRI contrast agents due to their unique properties, including biocompatibility, high-surface area, ease of synthesis and their ability of being bio-conjugated [5, 8, 11-14]. MSNs pores consist of honeycomb-like channels [15] that enable them to encapsulate relatively large amounts of contrast agents. Several researches have investigated impact of external surface conjugation of MSNs by biologic targeting agents such as antibodies [16-18], peptides [19], aptamers [20], glucosamine [8], hyaluronic acid [6], folic acid [21], and methionine [14] to increase cellular uptake and specific accumulation of contrast agents.

Recent studies in cancer biology show that not only sugars like glucose [22], but also amino acids are necessary to support the high metabolic demand of cancer cells. Different strategies are currently under investigation for using amino acid metabolism and corresponding transporters and enzymes to enhance uptake of cargos [23]. Amino acids have been used as passive/metabolic targeting agents in the diagnosis of cancer cells [3, 8-11]. Some amino acid transporters have been used for targeted drug delivery and imaging due to the overexpression of some transporters such as methionine transporter [14]. Therefore, conjugation of nanoparticles with amino acids can improve cancer cells nanoparticles uptake.

Cysteine is a non-essential amino acid necessary for the biological synthesis of glutathione (GSH), which is crucial for cell homeostasis [24]. Studies demonstrated that cysteine uptake by cancer cells is increased because of their rapid proliferation and increased antioxidant demands [24]. xc-cystine/glutamate antiporter system imports the oxidized form of cysteine through the cells [25]. Studies reported that cystine/glutamate antiporter is overexpressed in some cancers like breast cancer. Cancer cells growth, malignant progression, and GSH-mediated resistance of cancer cells to anticancer drugs are among the functions of these antiporters [24]. The impressive potential of xc-cystine/glutamate antiporter system as a target for cancer therapeutic and prevention purposes have been under investigation.

Cysteine can be attached to the external surface MSNs via its three functional groups, resulting in different surface properties and nanoparticle-antiporter interactions. Therefore, it was suggested that the conjugation method can influence the cellular uptake of nanoparticle. To make amine, epoxy and isocyanate groups on the MSNs surface, 3-Aminopropyl trimethoxysilane (APTES), 3-Glycidyloxypropyltrimethoxysilane (GPTS) and 3-(triethoxysilyl) propyl isocyanate (TSPI) were used, respectively.

This paper design reactions to make different covalent bonds between MSNs and cysteine to investigate impact of cysteine conjugation of MSNs-Gd<sup>3+</sup> on selective intracellular uptake of Gd<sup>3+</sup> by breast cancer cells.

## MATERIAL AND METHODS

### Materials

GdCl<sub>3</sub>·6H<sub>2</sub>O (99%), anhydrous ethanol (EtOH, 99.5%), cetyltrimethylammonium bromide (CTAB, 98 %), tetraethyl orthosilicate (TEOS, 98%), 3-(trimethoxysilylpropyl) diethylenetriamine (99.9%), sodium hydroxide, and toluene (99.8%) were provided from Acros (Belgium) and were used without further purification. Dialysis bags with a 500 Da cutoff were purchased from Spectrum Labs (Torrance, CA, USA). (3-Aminopropyl) triethoxysilane (APTES, 99%), 3-(triethoxysilyl)propyl isocyanate (TSPI), 3-Glycidyloxypropyltrimethoxysilane (GPTS), 3-(trimethoxysilylpropyl) diethylene triamine, L-cysteine Methyl sulfoxide (DMSO, 99%), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Na<sub>2</sub>HPO<sub>4</sub> (Sodium phosphate dibasic 99%), penicillin-streptomycin, and fetal bovine serum were prepared from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA). The rest of materials were supplied from Merck (Frankfurt, Germany). Dulbecco's modified eagle medium (DMEM) and Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F-12) were purchased from Thermo Fisher Scientific, Inc (Schwerte, Germany).

### Instrumentation

Zeta-potential of Cys-MSN-Gd<sup>3+</sup> was measured by dispersing in a water-soluble solution at pH=7-8 using Zetasizer analyzer (Malvern Instruments, Worcestershire, UK). The cellular uptake of gadolinium (Gd<sup>3+</sup>) ions was measured by an inductively coupled plasma atomic emission spectrometer (ICP-AES) (Optima 2300; PerkinElmer, Waltham, MA, USA). Fourier transform infrared spectroscopy (FTIR) spectra were obtained by Equinox 55 spectrometer in the range of 400-4000 cm<sup>-1</sup>. The suspensions were sonicated with a Sonicator (Branson Ultrasonics). The textural properties of Cys-MSN-Gd<sup>3+</sup> were evaluated using nitrogen adsorption isotherms at 77 K using a Belsorp Mini device (Toyonaka City, Japan). The absorbance of MTT was assessed using BioTek absorbance microplate readers at 450 nm (ELX800; BioTek Instruments, Inc, Winooski, VT, USA). Brunauer-Emmett-Teller surface area was calculated from adsorption data in the range of intermediate partial pressures (0.05 ≤ P/P<sub>0</sub> ≤ 0.25). Pore volume and size was measured using the Barrett-Joyner-Halenda (BJH) method.

### Synthesis of MSNs-Cys

**MSNs Synthesis:** The synthesis of MSNs was based on Stober method and Mehravi et.al. previous reports [10]. In brief, CTAB (1.0 g) and sodium hydroxide (4.0 mL, 2 M) were dissolved in distilled water (480.0 mL) and the reaction solution was heated up to 80 °C. TEOS (5.0 mL) was added to the mixture in drops over 15 min. After stirring for 3 hours at 80 °C, MSNs were purified by filtration and washed twice with distilled water and ethanol. A white powder with high efficacy was thus obtained (>95 %) [5, 14].

### Synthesis of Si-DTTA Complex

In order to synthesis (trimethoxysilylpropyl)-diethylenetriamine tetraacetate (Si-DTTA) complex, 3-(trimethoxysilylpropyl) diethylene triamine (0.4 g) and bromoacetic acid (1.0 g) were dissolved in distilled water (2.0 mL) and NaOH (4.0 mL, 2 M) and the solution was heated up to 50°C. Sodium hydroxide (6.0 mL, 2 M) was added dropwise over 20 min. The reaction solution was vigorously stirred for three hours at 50°C. Then, the solvent was removed under low pressure to get a viscous, yellowish oil. A hygroscopic powder in high efficacy (90%) was obtained after three times washing with pure ethanol [10].

#### *Synthesis of Gd<sup>3+</sup>-Si-DTTA*

Si-DTTA (100.0 mg) was dissolved in distilled water (4.0 mL) by stirring at room temperature (25±1). Next, GdCl<sub>3</sub> (300 µL, 50 M) was added to the reaction solution in drops. The pH of solution was raised to 9 by addition of sodium hydroxide 1 M. Then, the solution was stirred for three hours at 24°C and then concentrated to 1.0 mL [10, 26, 27].

#### *Synthesis of MSN-Gd<sup>3+</sup>-APTES-Cys (Nanoprobe A)*

After dispersing MSN (1.0 g) in anhydrous ethanol (100 mL), the solution was sonicated for 10 min. APTES (1.0 mL) was added drop-wise to the solution within approximately 15 min and the reaction mixture was stirred for 12 hours. The resulting solution was centrifuged at 5,000 rpm for 25 min and the residues were re-dispersed in water to remove unreacted APTES [10, 14]. After washing with water and ethanol, a white powder with high yield (>90%), was obtained. The surfactant template was then extracted with a 1% wt solution of NaCl in methanol [5]. MSN-APTES (400.0 mg) were refluxed with Gd<sup>3+</sup>-Si-DTTA complex (1.0 mL, 0.1 M) in toluene for 48 hours [10]. The resulting Cys-MSN-Gd<sup>3+</sup> were centrifuged (8000 rpm, 15 min) and then washed three times using water and pure ethanol. Nanoparticles were dialyzed against phosphate-buffered saline (PBS) to make sure that no free complex has remained. MSN-Gd<sup>3+</sup>-APTES (270 mg) was dispersed in 0.01 M MES buffer (pH=5.5). Cysteine (733.0 mg) was added, and the mixture was sonicated for 5 min. The sulfo-NHS (250.1 mg) and EDC (151.2 mg) were added into the solution under stirring. The reaction was allowed to continue at high speed for 3 days in 24 °C.

#### *Synthesis of MSN-Gd<sup>3+</sup>-GPTS-Cys (Nanoprobe B)*

The GPTS functionalization of silica particles was achieved according to Chu et al method [28]. MSN (9.7 g) was added to 56 mL of 1:1 mixture (w/w) of water and ethanol. The pH was decreased to 4.0 by the addition of nitric acid (1 M) while stirring, and the solution was sonicated for 10 min. GPTS (3 mL) was added drop-wise to the mixture with vigorous stirring, and the reaction was allowed to proceed (24 hours, 24 °C). After 24 hours, a small amount of concentrated ammonium hydroxide was added to the solution to increase the pH to 7.5-8. The solution (MSN-GPTS) was then dialyzed in a 500 Da MW cutoff dialysis

membrane and against 66% ethanol in water for 8 hours, and then against pure water for 20 hours to purify the MSN-GPTS [29]. At the end, a white powder remained in high efficacy (>90%). The surfactant template was extracted and loaded with Gd<sup>3+</sup>-Si-DTTA complex as mentioned. MSN-Gd<sup>3+</sup>-GPTS (300 mg) was dispersed in BICINE buffer (75 mL, pH 9, 0.1 M). Cysteine (733 mg) was added in excess, and the reaction solution was sonicated for 5 min. The reaction solution was heated in an oil bath (65°C) equipped under vigorous stirrer for 24 hours. MSN-GPTS-Cys were purified and washed according to reference [29].

#### *Synthesis of MSN-Gd<sup>3+</sup>-TSPI-Cysteine (Nanoprobe C)*

MSN (500 mg) and TSPI (0.25 mL) were dispersed in anhydrous toluene (80 mL) and the reaction solution was stirred for 20 hours. MSN-TSPI was collected, three times washed with anhydrous toluene and dried in an oven at 70 °C [30]. A white powder remained in high efficacy (>90%). The surfactant template was extracted and MSN loaded with Gd<sup>3+</sup>-Si-DTTA complex as mentioned. MSN-Gd<sup>3+</sup>-TSPI (300 mg) were dispersed in BICINE buffer (75 mL, pH 9, 0.1 M). The (protected) cysteine was added in excess (733.0 mg), and the solution was sonicated for 5 min. The reaction solution was heated up to 65 °C in oil bath under high stir. The reaction was allowed to proceed for 24 hours.

#### *Separation of precipitated Cystine from Nanoprobos*

To remove the cystine dimers formed by the excess cysteine in solution, the solution was centrifuged at 4100 rpm for 5 min, which precipitated cystine at the bottom of the tube and a mostly clear supernatant with a slight cloudiness (due to silica nanoparticles) was obtained. The supernatant was then dialyzed using 500 Da MW cutoff spectrum. The particles were then washed four more times [29].

#### *Cell Culture*

Human dermal fibroblast (HDF) and human breast adenocarcinoma (MCF-7) cell lines were prepared from the National Cell Bank of Iranian Biological Research Center. HDF was cultured at 37°C and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM) and MCF-7 was cultured at 37°C and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12). The cell culture mediums were supplemented with 10% FBS and 1% penicillin-streptomycin.

#### *Cell Viability Test*

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the viability of cells interacting with nanoprobos. MCF-7 and HDF cell lines (1×10<sup>4</sup> cells per well) were incubated with different concentrations of MSN, nanoprobos, and Magnevist (500, 100, 50, 10, 5 and 1 µg/mL) in 96-well microplates for 48 hours with untreated cells as control. The concentrations were tested in triplicate and the cells were washed well with PBS before adding MTT (20 µl of 5 mg/mL MTT solution)

to each. After four hours, the absorbance of formazan was measured at 450 nm [10].

### Intracellular Uptake of Cys-MSN-Gd<sup>3+</sup>

Intracellular uptake of nanoprobe was assessed using the protocol of Mehravi et. al. [10]. MCF-7 and HDF cell lines were re-plated into 24-well plates at a concentration of  $8 \times 10^4$  cells per well and incubated at 37°C and 5% CO<sub>2</sub> for 24h. Finally, the intracellular uptake of Gd<sup>3+</sup> ions was quantified using ICP-AES in triplicate and the mean  $\pm$  SD of the results was calculated.

The cells were in interaction with various total concentrations (1, 5, 10, 50 and 100  $\mu$ g/mL) of nanoprobe in 0.5 mL of cell culture in the wells. The cells were incubated for 0.5, 1, 2, 3, 4 and 6 hours at 37°C with 5% CO<sub>2</sub>. The cells were detached via 200  $\mu$ L trypsin, centrifuged at 1000 rpm for 5 min, and reconstituted in 100  $\mu$ L of phosphate-buffered saline. The cells were washed more three times with phosphate-buffered saline to detach the remained nanoprobe on the surface of the cells. Cellular uptake of Gd<sup>3+</sup> ions was quantified using ICP-AES. The average amount of Gd<sup>3+</sup> ions taken up by each cell was determined based on Gd<sup>3+</sup> ions concentration and the total number of cells. These assessments were performed in triplicate and the mean and standard deviation of results were calculated.

### Statistical analysis

One-way ANOVA was used for multigroup comparisons with  $p < 0.05$ . Results are expressed as mean  $\pm$  standard deviation ( $n=3-5$ ).

## RESULTS

### Size and morphology

The investigation of nanoprobe morphology and size, using scanning electron microscopy demonstrated no change in morphology of MSNs after cysteine conjugation, surfactant extraction and grafting of Gd<sup>3+</sup>-Si-DTTA-chelated molecule. Nanoprobe were spherical in terms of morphology with average size of  $88.0 \pm 2.2$  nm (Fig. 1).

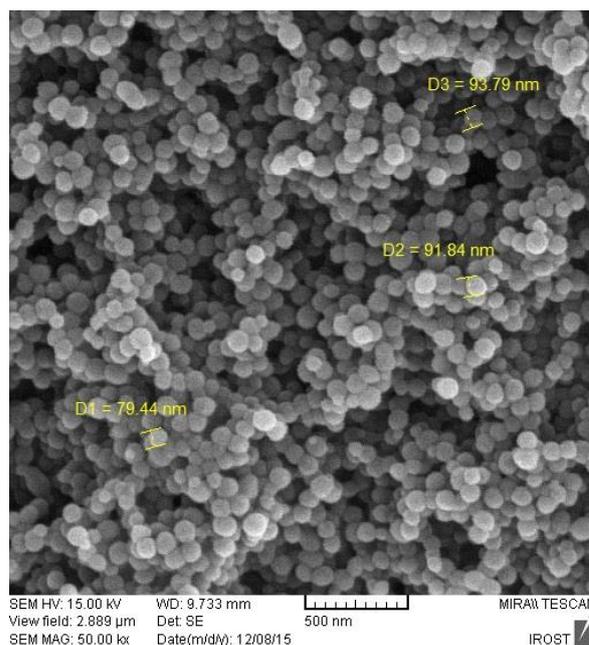


Figure 1. SEM images of nanoprobe

### Porosity of MSN and Nanoprobe

Nitrogen adsorption and desorption measurements revealed a high porosity in the surfactant-extracted MSNs. The surface area, average pore diameter, and total pore volume of MSN was 975 m<sup>2</sup>/g, 2.7 nm, and 0.55 cm<sup>3</sup>/g, respectively. Meanwhile, a significant reduction in surface area to 29.52 m<sup>2</sup>/g, a mean pore diameter of 1.7 nm, and a total pore volume of 0.082 cm<sup>3</sup>/g was observed after loading Gd<sup>3+</sup> complex (Table 1).

### Zeta potential measurements

The measured zeta potentials for MSN, MSN-APTES, MSN-GPTS, MSN-TSPI, MSN-APTES-Cys, MSN-GPTS-Cys, and MSN-TSPI-Cys are shown in Table 2.

Table 1. Porosity of MSN and Nanoprobe

Samples	Surface Area (m <sup>2</sup> /g) multipoint	Total Pore Volume (cm <sup>3</sup> /g) Desorption Isotherm	Average Pore Diameter (nm) Desorption Isotherm
Extracted MSN	975	0.55	2.7
MSN-Gd <sup>3+</sup> .Cysteine	29.52	0.082	1.7

Table 2. Zeta potential of mesoporous silica nanoparticles modified with linkers and conjugated with cysteine

Sample	Zeta potential (mv)
MSN	-9.62
MSN-APTES	25.0
MSN-APTES-Cysteine (Nanoprobe A)	13.2
MSN-GPTS	-33.3
MSN-GPTS-Cysteine (Nanoprobe B)	5.77
MSN-TSPI	-20.1
MSN-TSPI-Cysteine (Nanoprobe C)	-14.3

Note: There is remarkable difference between zeta potentials.

Abbreviations: MSN, Mesoporous silica nanoparticle; MSN-APTES, Mesoporous silica nanoparticle modified with NH<sub>2</sub> group, MSN-GPTS, Mesoporous

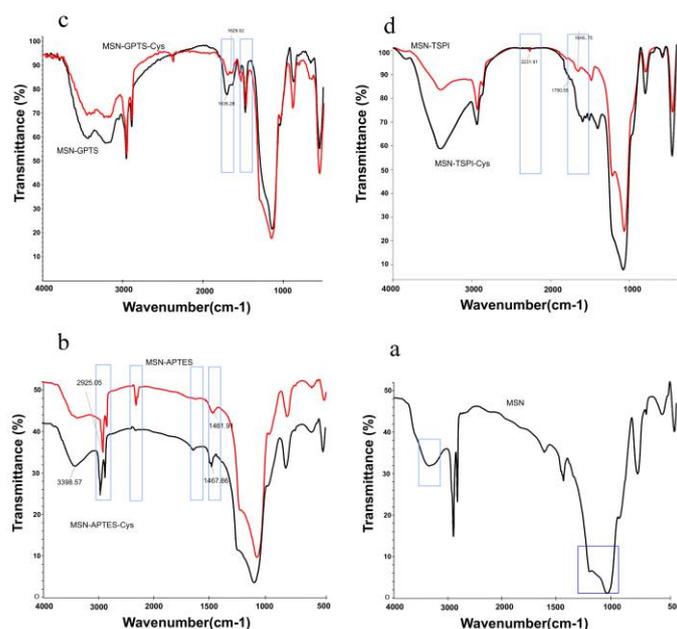


Figure 2. Fourier transform infrared spectroscopy analysis of MSN and nanoprobes

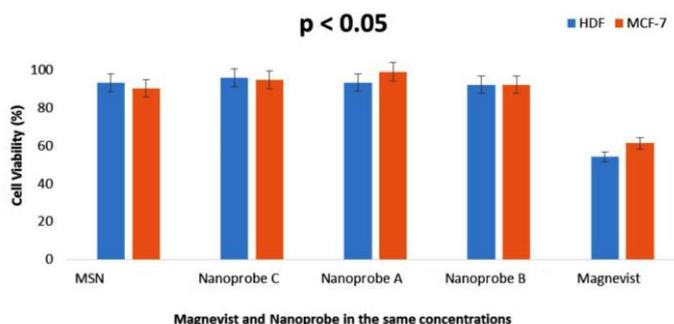


Figure 3. MTT results of HDF (human dermal fibroblast cell line) and MCF-7 (human breast adenocarcinoma cell line) treated with cysteine decorated MSNs. Note: The in vitro cytotoxicity of MSNs, cysteine decorated MSNs, and Magnevist was examined at concentration of 100  $\mu\text{g}/\text{mL}$ . Abbreviations: MSN, decorated Mesoporous silica nanoparticles; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide).

#### Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) in the region of 400-4000  $\text{cm}^{-1}$  was used to confirm the process of functionalizing and cysteine conjugation of MSNs (Fig. 2).

#### Cell viability

The viability of HDF and MCF-7 cell lines (using MTT assay) are shown in Figs. 3 and 4. Results shown cysteine decorated MSNs-labeled cells had lower toxicity in comparison with Magnevist at a concentration of 100  $\mu\text{g}/\text{mL}$ . Also, no significant toxicity was observed at different concentrations (1, 5, 10, 50, 100 and 500  $\mu\text{g}/\text{mL}$ ) for 48 hours ( $p < 0.05$ ).

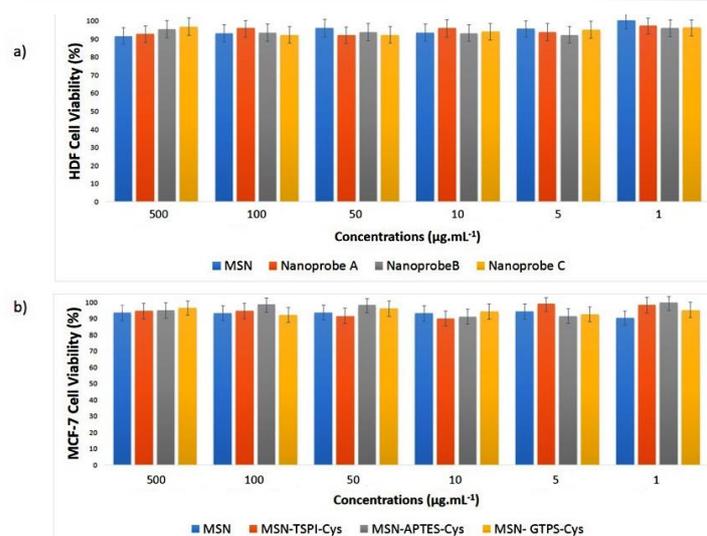
#### Cellular uptake

ICP-AES results indicated that MSN-GPTS-Cysteine loaded with  $\text{Gd}^{3+}$ -DTTA (Nanoprobe B) was taken considerably more than other nanoprobes. The average

uptake of  $\text{Gd}^{3+}$  internalized into cancer cells using nanoprobe B was  $0.5 \pm 0.09$   $\text{pg}/\text{cell}$ . Results also demonstrated that the uptake of  $\text{Gd}^{3+}$  using nanoprobe B into cancer cells was up to 4.7 times more than normal cells. Data confirms the important role of cysteine conjugation using GPTS (Fig. 5).

#### DISCUSSION

Surface modification of nano-carriers with specific biomolecules have been widely used to enhance cellular uptake of different cargos to the cells. The golden principle of choosing cysteine for surface modification is that cancer cells take up more cysteine than normal cells because of higher cellular metabolic rate and overexpression of xc-cysteine/glutamate antiporter system [24]. Conjugation of cysteine can help to enhance cellular uptake of cargos such as  $\text{Gd}^{3+}$ , but probably some specific functional groups of cysteine can be responsible for enhancing the cellular uptake. APTES, GPTS and TSPI linkers have been used for



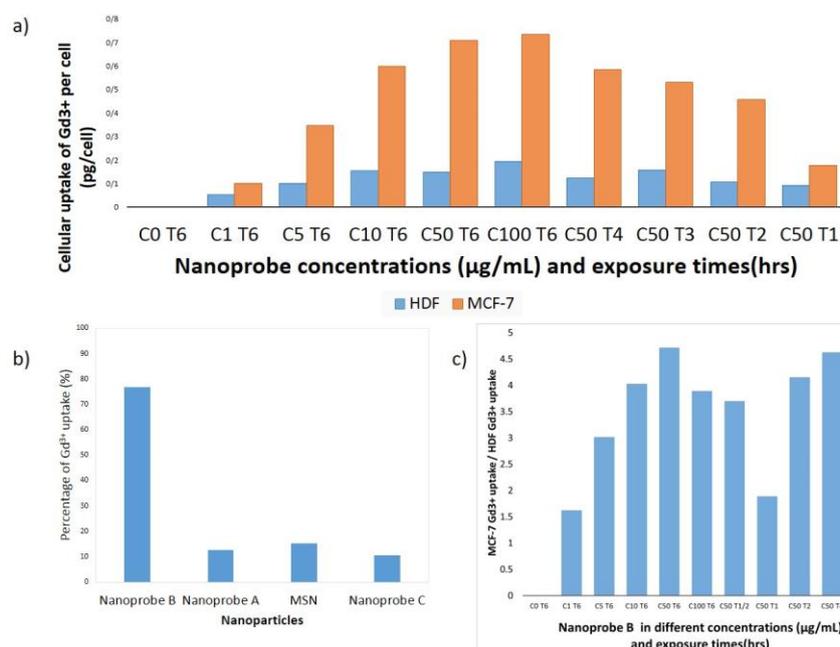
**Figure 4.** (a) MTT results of 48 hours of cysteine decorated MSNs exposure to t HDF (human dermal fibroblast cell line), (b) MTT results of 48 hours of cysteine decorated MSNs exposure to the MCF-7 (human breast adenocarcinoma cell line). Note: The in vitro cytotoxicity of MSNs, cysteine decorated MSNs was examined at concentration of 1 µg/mL, 5 µg/mL, 50 µg/mL, 100 µg/mL and 500 µg/mL. There is no significant difference in HDF cell viability. Abbreviations: MCF-7, human breast adenocarcinoma cell line; HDF, human dermal fibroblast MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide).

conjugation of cysteine onto the surface of MSN-Gd<sup>3+</sup> to find the most effective conjugation method. Other researches have also conjugated biomolecules for targeting the over expression of methionine transport system [14, 31], glucose transporters [10] and other receptors [32] in cancer cells, using one type of conjugation. This fact leads to ignoring the possibility of different kinds of biomolecule- receptors

interactions.

In this study, cysteine is involved in a series of reactions to share its carboxyl, thiol and amine groups to make covalent bonding with active groups of MSN-Gd<sup>3+</sup>-APTES, MSN-Gd<sup>3+</sup>-GPTS and MSN-Gd<sup>3+</sup>-TSPI, respectively.

Results revealed no changes in MSN morphology, which was noticed by SEM after the conjugation of cysteine onto



**Figure 5.** (a) Gd<sup>3+</sup> uptake by MCF-7 is more than HDF in different times and concentrations, (b) Percentage of Nanoprobe uptake by MCF-7 cell line, (c) ratio of MCF-7 Gd<sup>3+</sup> uptake to HDF in different times and concentrations. Note: The intracellular uptake of CSN was measured at concentrations of 0, 1, 5, 50 and 100 µg/mL, in 0.5-6 hours' exposure to HDF and MCF-7 cell lines. There is significant difference in Gd<sup>3+</sup> uptake by HDF and MCF-7 cells. Abbreviations: HDF, human dermal fibroblast; MCF-7, human breast adenocarcinoma cell line; Gd<sup>3+</sup>, Gadolinium; C, concentration (µg/mL); T, time (hours).

its surface, extraction of the surfactant, or attachment of  $Gd^{3+}$ -DTTA-chelated complexes. Nanoprobes had a spherical morphology ( $88.0 \pm 0.1$  nm in diameter). The high porosity of the surfactant-extracted MSNs (surface area of  $975 \text{ m}^2/\text{g}$ ) and the average pore diameter were noticeably decreased upon successful grafting of  $Gd^{3+}$ -DTTA-chelated molecule to the internal surface of MSNs (Table 1). The zeta potential of the synthesized MSN was  $-9.62$  mv and the infrared spectrum demonstrated a broad transmittance peak at  $3180\text{--}3600 \text{ cm}^{-1}$ , corresponding to the silanol-OH bond, as well as the strong transmittance peak of siloxane (Si-O-Si) group at  $1077 \text{ cm}^{-1}$ , which is in agreement with previous studies [8, 14, 33].

FTIR spectrum, the surface charge property and zeta potential results confirmed the desired step-by-step surface modifications. In the developmental modification of Nanoprobe A, the zeta potential of MSN ( $-9.62$  mv) related to its hydroxyl groups was increased to  $+25$  mv after being functionalized with APTES, which can be interpreted by the existence of amine groups on the surface of nanoparticle. Also, the FTIR spectrum showed a new transmittance peak at  $2361 \text{ cm}^{-1}$  corresponding to amine ( $NH_2$ ) bond and a transmittance peak of siloxane (Si-O-Si) group at  $1062 \text{ cm}^{-1}$ , as well as an amide carbonyl ( $-NH-C=O$ ) stretch mode at about  $1655$  and  $1501 \text{ cm}^{-1}$ . In the next step, after formation of an amide bond with cysteine using EDC/sulfo-NHS, the zeta potential decreased to  $+13.2$  mv because of thiol negative charge of cysteine on the surface. These results indicate cysteine successful conjugation to MSN- $Gd^{3+}$ -APTES (Fig. 2c).

With respect to the development of Nanoprobe B, the zeta potential of MSN ( $-9.62$  mv) decreased to  $-33.3$  mv after being functionalized with GPTS because of the presence of epoxy groups on the surface. When GPTS was grafted onto MSNs, the spectrum showed new transmittance peaks at  $927 \text{ cm}^{-1}$  and  $801 \text{ cm}^{-1}$  corresponding to the epoxy ring; moreover, a transmittance peak was visible at  $1063 \text{ cm}^{-1}$  related to siloxane (Si-O-Si) group. After conjugation with cysteine, the reaction between epoxy groups of MSN- $Gd^{3+}$ -GPTS with thiol group of cysteine, the zeta potential increased to  $+5.77$  mv. Cysteine took its zwitterionic mode because of amine and thiol groups that were accessible on the surface of nanoprobe, which is also reported in the literature [29]. Also, the spectrum showed a new transmittance peak at  $3100\text{--}3500 \text{ cm}^{-1}$  corresponding to carboxylic acid ( $-COOH$ ) group of cysteine (Fig. 2a).

In developmental synthesis of Nanoprobe C, TSPI functionalization decreased MSN zeta potential ( $-9.62$  mv) to  $-20.1$  mv due to its isocyanate groups. Also, the spectrum showed a new transmittance peak at  $2231 \text{ cm}^{-1}$  corresponding to carbonyl ( $N=C=O$ ) bond and a transmittance peak of siloxane (Si-O-Si) group at  $1064 \text{ cm}^{-1}$ . By conjugation of MSN- $Gd^{3+}$ -TSPI with cysteine, the zeta potential increased to  $-14.3$  mv. Also the spectrum showed new transmittance peaks at about  $1592$  and  $1553 \text{ cm}^{-1}$  related to amide carbonyl ( $-NH-C=O$ ) stretch mode indicating that cysteine was incorporated into MSN- $Gd^{3+}$ -

TSPI (Fig. 2b).

Breast cancer cells (MCF-7) and normal cells (HDF) viability in interaction with nanoprobes demonstrated no toxic elements. This can be due to covalent attachment of  $Gd^{3+}$  complex inside MSN pores.

The cellular uptake of nanoprobes by breast cancer was highly dependent upon cysteine free groups available on the surface of nanoprobes. Among the three types, nanoprobe B had the highest level of intracellular uptake by cancer cells. This can be explained by accessibility of free amine and epoxy groups of cysteine, which present a zwitterionic mode of cysteine which is similar to the oxidized form of cysteine (the substrate of xc-cystine/glutamate antiporter system). The cellular uptake of  $Gd^{3+}$  by cancer cells, via Nanoprobe B was about 5 times more than nanoprobes A, C and also MSN- $Gd^{3+}$ . The result also showed that the uptake of  $Gd^{3+}$  by cancer cells (Fig. 5a) was significantly (4.7 times) more than those of HDF cell lines (Fig. 5c). According to references, some nanoparticles have been conjugated with cysteine, but their cellular uptake were not investigated [34]. Clearly, targeting xc-cystine/glutamate antiporter system using appropriate cysteine conjugation method on MSN- $Gd^{3+}$  appears to be an efficient route to improve cellular uptake of  $Gd^{3+}$  by cancer cells.

## CONCLUSION

Hence, the present investigation suggest that attaching the zwitterionic form of cysteine on the surface of MSN- $Gd^{3+}$ , clearly enhance the uptake of  $Gd^{3+}$  by cancer cells. This design and synthesis method is promising to invent new cellular imaging nanoprobes for early detection of cancer cells in next studies. However, more investigations are still needed regarding the interaction of these nanoparticles with xc-cystine/glutamate antiporter system and the internalization pathway via these transporters.

## ACKNOWLEDGMENTS

This paper is a major part of MSc thesis in Medical Nanotechnology by Hojjatollah Nazari. Financial support was provided by Iran University of Medical Sciences.

## CONFLICT OF INTEREST

The authors declare that this research does not have any conflict of interest with anyone or any institute.

## REFERENCES

- Bulte JW, Arbab AS, Douglas T, Frank JA. Preparation of magnetically labeled cells for cell tracking by magnetic resonance imaging. *Methods Enzymol* 2004;386: 275-299.
- Bulte JW, Kraitchman DL. Iron oxide MR contrast agents for molecular and cellular imaging. *NMR Biomed* 2004;17(7):484-499.
- Ferrari M. Cancer nanotechnology: opportunities and challenges. *Nature Rev Cancer* 2005;5(3):161-171.
- Na HB, Song IC, Hyeon T. Inorganic nanoparticles for MRI contrast agents. *Advanc Mater* 2009;21(21):2133-2148.
- Taylor KM, Kim JS, Reiter WJ, An H, Lin W, Lin W. Mesoporous silica nanospheres as highly efficient MRI contrast agents. *J Am Chem Soc* 2008;130(7):2154-2155.

6. Lee T, Lim EK, Lee J, Kang B, Choi J, Park HS, et al. Efficient CD44-targeted magnetic resonance imaging (MRI) of breast cancer cells using hyaluronic acid (HA)-modified  $MnFe_2O_4$  nanocrystals. *Nanoscale Res Lett* 2013;8(1):1.
7. Nasongkla N, Bey E, Ren J, Ai H, Khemtong Ch, Setti Guthi J, et al. Multifunctional polymeric micelles as cancer-targeted, MRI-ultrasensitive drug delivery systems. *Nano Lett* 2006; 6(11):2427-2430.
8. Mehravi B, Ahmadi M, Amanlou M, Mostaar A, Ardestani MS, Ghalandarlaki N. Cellular uptake and imaging studies of glycosylated silica nanoprobe (GSN) in human colon adenocarcinoma (HT 29 cell line). *Int J Nanomedicine* 2013;8:3209.
9. Kuo PH, Kanal E, Abu-Alfa AK, Cowper SE. Gadolinium-based MR contrast agents and nephrogenic systemic fibrosis 1. *Radiology* 2007;242(3):647-649.
10. Mehravi B, Ahmadi M, Amanlou M, Mostaar A, Ardestani MS, Ghalandarlaki N. Conjugation of glucosamine with Gd3+-based nanoporous silica using a heterobifunctional ANB-NOS crosslinker for imaging of cancer cells. *Int J Nanomedicine* 2013;8:3383-94.
11. Gerion D, Herberg J, Bok R, Gjersing E, Ramon E, Maxwell R, et al. Paramagnetic silica-coated nanocrystals as an advanced MRI contrast agent. *J Physic Chem C* 2007;111(34):12542-12551.
12. Hu H, Arena F, Gianolio E, Boffa C, Di Gregorio E, Stefania R, et al. Mesoporous silica nanoparticles functionalized with fluorescent and MRI reporters for the visualization of murine tumors overexpressing  $\alpha v \beta 3$  receptors. *Nanoscale* 2016;8(13):7094-7104.
13. Feldmann V, Engelmann J, Gottschalk S, Mayer HA. Synthesis, characterization and examination of Gd [DO3A-hexylamine]-functionalized silica nanoparticles as contrast agent for MRI-applications. *J Colloid Interface Sci* 2012;366(1):70-79.
14. Mehravi B, Ardestani MS, Damercheli M, Soltanghorae H, Ghanalardlaki N, Alizadeh AM, et al. Breast Cancer Cells Imaging By Targeting Methionine Transporters with Gadolinium-Based Nanoprobe. *Mol Imaging Biol* 2014;16(4):519-528.
15. Slowing II, Vivero-Escoto JL, Wu CW, Lin VS. Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers. *Adv Drug Deliv Rev* 2008;60(11):1278-1288.
16. Tsai CP, Chen CY, Hung Y, Chang FH, Mou CY. Monoclonal antibody-functionalized mesoporous silica nanoparticles (MSN) for selective targeting breast cancer cells. *J Mater Chem* 2009;19(32):5737-5743.
17. Chen F, Hong H, Zhang Y, Valdovinos HF, Shi S, Kwon GS, et al. In vivo tumor targeting and image-guided drug delivery with antibody-conjugated, radiolabeled mesoporous silica nanoparticles. *ACS Nano* 2013;7(10):9027-9039.
18. Mehravi B, Mirzaei M, Ardestani MS, Amanlou M, Soltanghorae H, Ghalandarlaki N, et al. Synthesis and development of msn-gd3+-c595 as mr imaging contrast agent for prostate cancer cell imaging. *Curr Mol Imaging* 2014;3(1):43-51.
19. Pan L, He Q, Liu J, Chen Y, Ma M, Zhang L, et al. Nuclear-targeted drug delivery of TAT peptide-conjugated monodisperse mesoporous silica nanoparticles. *J Am Chem Soc* 2012;134(13):5722-5725.
20. Li LL, Yin Q, Cheng J, Lu Y. Polyvalent Mesoporous Silica Nanoparticle Aptamer Bioconjugates Target Breast Cancer Cells. *Adv Healthc Mater* 2012;1(5):567-572.
21. Khosravian P, Shafiee Ardestani M, Khoobi M, Ostad SN, Abedin Dorkoosh F, Akbari Javar H, et al. Mesoporous silica nanoparticles functionalized with folic acid/methionine for active targeted delivery of docetaxel. *OncoTargets Ther* 2016;9:7315.
22. Calvaresi EC, Hergenrother PJ. Glucose conjugation for the specific targeting and treatment of cancer. *Chem Sci* 2013;4(6):2319-2333.
23. Munoz-Pinedo C, El Mjijad N, Ricci J. Cancer metabolism: current perspectives and future directions. *Cell Death Dis* 2012;3(1):e248.
24. Lo M, Wang YZ, Gout PW. The x c- cystine/glutamate antiporter: a potential target for therapy of cancer and other diseases. *J Cell Physiol* 2008;215(3):593-602.
25. Bannai S. Exchange of cystine and glutamate across plasma membrane of human fibroblasts. *J Biol Chem* 1986;261(5):2256-2263.
26. Lu J, Liang M, Sherman S, Xia T, Kovochich M, Nel AE, et al. Mesoporous silica nanoparticles for cancer therapy: energy-dependent cellular uptake and delivery of paclitaxel to cancer cells. *Nanobiotechnology* 2007;3(2):89-95.
27. Jesús M, Penadés S. Glyconanoparticles: types, synthesis and applications in glycoscience, biomedicine and material science. *Biochim Biophys Acta (BBA)* 20061760(4):636-651.;
28. Chu L, Daniels M, Francis L. Use of (glycidoxypropyl) trimethoxysilane as a binder in colloidal silica coatings. *Chem Mater* 1997;9(11):2577-2582.
29. Rosen JE, Gu FX. Surface functionalization of silica nanoparticles with cysteine: a low-fouling zwitterionic surface. *Langmuir* 2011;27(17):10507-10513.
30. Zhang Y, Yuan Q, Chen T, Zhang X, Chen Y, Tan W. DNA-capped mesoporous silica nanoparticles as an ion-responsive release system to determine the presence of mercury in aqueous solutions. *Analytic Chem* 2012;84(4):1956-1962.
31. Chen H, Li B, Ren X, Li S, Ma Y, Cui S, et al. Multifunctional near-infrared-emitting nano-conjugates based on gold clusters for tumor imaging and therapy. *Biomaterials* 2012;33(33):8461-8476.
32. Ghalandarlaki N, Latifi N, Mirzaei M, Joghatei MT, Safari S, Ashtari Kh, et al. In vitro Evaluation of Gadolinium-Silica Mesoporous Nanoparticles-Monoclonal Antibody: Potential Nanoprobe for Prostate Cancer Cell Imaging. *Curr Mol Imaging* 2015; 4(1):35-42.
33. Suwalski A, Dabboue H, Delalande A, Bensamoun SF, Canon F, Midoux P, et al. Accelerated Achilles tendon healing by PDGF gene delivery with mesoporous silica nanoparticles. *Biomaterials* 2010; 31(19):5237-5245.
34. Dolci S, Lerardi V, Remskar M, Jaglicic Z, Pineider F, Boni A, et al. Chemical-physical properties, morphology, and magnetic investigations on new cystine functionalized ultra-small superparamagnetic iron-oxide nanoparticles. *J Mater Sci* 2013;48(3): 1283-1291.