A novel biocompatible nanoprobe based on lipoproteins for breast cancer cell imaging

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ABSTRACT

Objective(s): Contrast-enhanced magnetic resonance imaging (MRI) of breast cancer provides valuable data on the disease state of patients. Biocompatible nanoprobes are expected to play a pivotal role in medical diagnosis in the future owing to their prominent advantages. The present study aimed to introduce a novel biocompatible nanoprobe based on lipoproteins for breast cancer cell imaging.

Materials and Methods: In this study, a biocompatible nanoprobe based on high-density lipoprotein was synthesized successfully. Scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), and dynamic light scattering (DLS) were used for material characterization, and cellular uptake and in-vitro and in-vivo imaging were investigated using the nanoprobe.

Results: The nanoprobe could significantly reduce the relaxation time in the phantom and cancer cells with no toxicity in the studied cells. In addition, the nanoprobe demonstrated proper cellular uptake in the cancer cells. The in-vivo tumor images were obtained 30, 60, and 120 minutes after the injection of the nanoprobe (5.0 µmol/kg) via the tail vein, and the results indicated that the synthesized nanoprobe could be introduced as a potential MRI contrast agent.

Conclusion: Future developments may allow the application of this nanoparticle to be used in pathological and physiological processes in preclinical models.

Keywords: Breast Cancer, Contrast Agent, High-density Lipoprotein, Nanoprobe

INTRODUCTION

Cancer is considered to be a global, complex disease associated with high mortality in different countries. According to the World Health Organization (WHO), 17 million individuals are expected to be at the risk of cancer by 2020 [1-2].

Diagnosis of cancer in the early stages is highly effective in the prevention of disease expansion, and diagnostic methods play a pivotal role in the successful treatment of cancer [3-4]. Despite the availability of numerous diagnostic techniques, magnetic resonance imaging (MRI) has gained special attention in clinical and research settings as a noninvasive and relatively inexpensive modality. MRI has numerous benefits, while it requires proper contrast agents to compensate for its low sensitivity and increase its relaxation time.

Nanotechnology has unique properties and is effective in the signal enhancement of various imaging modalities. The nanoparticles that are based on natural materials for biocompatibility could be considered a major strategy for the elimination of the restrictions that are detected in the other contrast agents used for imaging [5-8].

Among various properties that are considered in the design of appropriate nanoprobes, the main influential factors include a proper complex with high stability in the circulatory system and ability to deliver large amounts of ion-targeted...
gadolinium-based MRI toward cancer cells [9-10].

Lipoproteins are composed of one water-soluble protein and other water-insoluble lipids, which lead to the selectivity of the behavior of the cell membrane. High-density lipoprotein (HDL) is composed of smaller lipid nanoparticles (diameters: 5-112 nm) compared to low-density lipoprotein (LDL). HDL becomes stable through interacting with apolipoprotein A-1 proteins, which contains 243 amino acids and two amphipathic α-helix structures [11]. To date, HDL has been used to convey various types of drugs [12-15]. One of the first studies using HDL as a nanocarrier for disease treatment was conducted at Van Berkel Laboratory in the Netherlands [16]. According to the obtained results, lipophilic antiviruses and the analogous of dioleyl iododeoxyuridine and 9-(2-phosphonylmethoxyethyl) adenine could be delivered to liver cells. The other studies in this regard have employed HDL as a nanocarrier for chemotherapy [17-24]. In the present study, a human HDL nanoprobe was synthesized successfully, and the obtained results indicated that the nanoprobe had a spherical shape with the proper size to enter cells with no cell toxicity. Moreover, cell reabsorption and relaxation time were evaluated in an animal model at various times. The synthesized nanoprobe could be introduced as an appropriate biocompatible contrast agent for breast cancer cell imaging.

MATERIALS AND METHODS

Experimental materials
The experimental materials required for this study were obtained from Sigma-Aldrich Chemical Co. (USA), including HDL (high-density from human plasma lyophilized powder; Sigma-Aldrich Co., USA), 3-(trimethoxysilylpropyl)diethylenetriamine, bromoacetic acid (≥99.0%), ethanol, oleic acid (≥99.0%), ethyl(dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysulfosuccinimide (Sulfo-NHS) (≥98.0%), gadolinium(III) chloride (GdCl₃) (3-aminopropyl)triethoxysilane (APTES) (99%), dimethyl sulfoxide (DMSO), and sodium hydroxide (NaOH). Neuberger chambers and dialysis bags (cutoff: 12,000 Da) were also obtained from the Spectrum Lab (USA). MCF-7 (human breast cancer cell) and MCF-10A cell lines (non-tumorigenic epithelial cell) were purchased from Pasteur Institute (Tehran, Iran), and BALB/c mice were supplied from the Cancer Research Center at Tehran University of Medical Sciences (Tehran, Iran).

Synthesis of the nanoprobe, chelator, and Si-DTTA
For the synthesis of the chelator, 0.4 gram of 3-(trimethoxysilylpropyl)diethylenetriamine and one gram of bromoacetic acid were dissolved in two milliliters of distilled water, and two molar of sodium chloride (NaOH) solution was added to the resultant solution, with the temperature increased to 50°C. Following that, NaOH (6 ml; 2 M) was added and stirred for three hours at the same temperature. Afterwards, the solvent was removed under pressure, and an oily substance was obtained and washed three times with distilled water and ethanol (compound A).

Conjugation of Si-DTTA to oleic acid
Initially, a mixture of oleic acid in distilled water was obtained and stirred for 15 minutes. Following that, the crosslinker agents (EDC/sulfo-NHS) were added to the resultant structure, and compound B was obtained with the addition of APTES and after stirring for two hours. Compound A was added to compound B, and the mixture was stirred for one hour. The final stages involved centrifugation and dialysis (cutoff: 12,000 Da).

Conjugation of HDL to the chelator
After the dissolution of compound C in DMSO, HDL (500:1) was added to the obtained solution and stirred for four hours. After 16 hours of dialysis, compound D was obtained.

Synthesis of the nanoprobe
To synthesize the nanoprobe, 100 grams of compound D was dissolved in distilled water and stirred at room temperature. Afterwards, 0.5 molar of gadolinium chlorides was added to the solution, and the pH of the medium was set at nine. After stirring for three hours at room temperature, the solution was dialyzed in order to separate the unloaded Gd³⁺.

Characterization
The morphology, size, and zeta potential of the synthesized nanoprobe were characterized using SEM (HITACHI S-4160), DLS and a zeta potentiometer (Malvern Instruments, UK), respectively. In addition, FTIR (Thermo Nicolet, Nexus 870 FTIR, USA) was utilized to determine the chemical structure, and Gd³⁺ concentration was detected by inductively coupled plasma optical emission spectrometry (ICP-AES; Optima 2300, Perkin-Elmer).

In-vitro analysis
MTT assay
The MTT assay was used to evaluate the cytotoxicity of the nanoprobe on the MCF-10A and MCF-7 cell lines. In total, 5×10⁵ MCF-10A
and MCF-7 cells were cultured in 200 microliters of Dulbecco’s modified eagle medium and 10% of fetal bovine serum (FBS). After 24 hours, the culture medium was replaced with 10% FBS. Following that, 10, 50, and 100 μg/ml of the nanoprobe and 100 μg/ml of HDL were incubated for 48 hours. At the next stage, 20 microliters of the MTT solution was added to each well and incubated at the temperature of 37°C for four hours. During incubation, MTT reduced by the mitochondrial dehydrogenase enzyme, and blue formazan crystals were produced. The absorbance of the specimen was read using the ELISA reader (ELX800, Biotek, USA) at the wavelength of 570 nanometers [25].

**Cellular uptake**

Initially, the cells were put in contact with a specific concentration of the nanoprobe at 0.5, two, four, and six hours at the temperature of 37°C. Following that, various concentrations of the nanoprobe were added to each well, and the cells were incubated for six hours in the same conditions.

The cells were washed with phosphate buffered saline (PBS; 500 μl) and centrifuged at 1,000 rpm for four minutes in 100 microliters of PBS. The cell reabsorption of the Gd³⁺ ions was determined via ICP-AES. Based on the concentrations of the Gd³⁺ ions and total cell count, the mean cellular absorption of the Gd³⁺ ions was calculated by the counting of each cell. The measurements were performed in triplicate, and the obtained mean values were reported.

**Relaxation time (T₁ and T₂)**

**Protocol**

At this stage, MRI (1.5 T) with the head coil located in Vali-e Asr Hospital Imaging Center of Tehran University of Medical Sciences was applied for the evaluation of the relaxation time. In order to obtain T₁, a multipoint method was applied. The T₁ exponential curve was achieved in terms of the intensity of the signal in the echoes of an ecosystem 32 (1st echo) echo sequence with equivalents (2 ms) and 3,000 ms (matrix=256×256, slice thickness=1.5 mm, non-averaged). By fitting a linear curve on the logarithm, the intensity of the signal was calculated in terms of T₁ with the slope of the line or -1/T₁ assessed in the Excel environment. In addition, T₁ maps were achieved using a flash protocol with the repeated times of 20, 50, 100, 200, 400, 600, 1,000, 2,000, and 3,000 milliseconds (TE: 12 ms; matrix=256×256; slice thickness=1.5 mm, non-averaged).

**In-vitro studies**

At this stage, 2×10⁵ MCF-7 cells were cultured at the temperature of 37°C and 5% CO₂ for 24 hours.

The culture medium was discarded afterwards, and the nanoprobes with various concentrations of Gd³⁺ (0.086, 0.0645, 0.043, 0.0215, 0.01075, and 0.00535 mM) was added to each well and incubated for two hours. The cells were washed twice with PBS (0.5 ml), and 0.5 milliliter of trypsin was added to each well; after 5-10 minutes, the cells were detached and suspended. MRI was performed, and image analysis was analyzed in the DICOM software version 1.3.0.5. (MEDAV GmbH Company). In addition, the ICP method was used to determine the gadolinium concentration in the cell. To this end, the cell was dissolved in concentrated nitric acid (1 ml). After centrifugation (1,500 rpm) for five minutes at the temperature of -7°C, the cell was diluted to distilled water (1:5), and after 10 minutes of sonication, ICP was applied.

**In-vivo studies**

To evaluate the efficacy of the synthesized nanoprobe in animal breast cancer cell imaging, in-vivo tests were carried out. Each BALB/c rat was anesthetized via the intraperitoneal injection of ketamine/xylazine (10 mg/kg of body weight), and Murine mammary adenocarcinoma cells were injected intraperitoneally at the right flank. After approximately four weeks, tumor growth was tracked. To this end, the nanoprobe was injected into normal and cancerous animals at the desired concentration, and images were obtained based on the T1-weighted sequence using an MRI (1.5 T) device. The process was performed immediately after the injection (repetition time: 1,120 ms, echo time: 24 ms, field of view: 60×60 mm, matrix size: 512×512, contiguous slice thickness: 2 mm).

**Statistical analysis**

Multigroup comparison of the means was performed using one-way analysis of variance (ANOVA). In all the statistical analyses, the P-value of less than 0.05 was considered significant, and the obtained results were expressed as mean and standard deviation (n=3-5).
RESULTS

Synthesis of the nanoprobe

Fig 1 depicts the SEM results of the synthesized HDL and spherical morphology of the nanoprobe. As can be seen, the spherical shape of HDL had no changes after the synthesis of the nanoprobe. Table 1 shows the mean diameter and zeta potential of HDL and the nanoprobe. Accordingly, the mean diameter of HDL and the nanoprobe was 12 and 55 nanometers, while the zeta potential was estimated at -8.88 and -18.4 mV, respectively.

Table 1. Properties of Synthesized Nanoprobe and HDL (Zeta Potential and DLS)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta Potential (mV)</th>
<th>Average Diameter (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL</td>
<td>-8.88</td>
<td>12</td>
<td>0.4</td>
</tr>
<tr>
<td>Nanoprobe</td>
<td>-18.4</td>
<td>55</td>
<td>0.43</td>
</tr>
</tbody>
</table>

In-vitro studies

MTT assay

The cytotoxicity studies of the nanoprobe on normal and cancer cells are shown in Fig 3. As is observed, the diverse concentrations of the nanoprobe had no toxicity on either of the mentioned cells; therefore, it could be concluded that these nanocarriers were compatible with the cells (P<0.05).

Cellular uptake of the nanoprobe

Figs 4 and 5 depict the cellular uptake of the synthesized nanoprobe at various times (0.5, 2, 4, and 6 hours) and its concentration at six hours, respectively. According to the obtained results, cellular uptake increased with time, with a regular trend achieved with concentration.
In-vitro MRI experiment

An in-vitro MRI experiment was performed in order to achieve adequate concentrations of the internalized nanoprobe for the MRI visualization of the cancer cells. To this end, the MCF-7 cells were incubated for one hour in the presence of the nanoprobe (100 μM). According to the findings, the cells that were labeled with the nanoprobe displayed a strong, intense signal on the T1-weighted spin-echo image. On the other hand, the untreated cells demonstrated the efficient uptake of the nanoprobe.

Figs 6 and 7 show the findings of the current research on T₁/T₂ relaxation times. It is notable that the spin-echo and gradient-echo protocol results needed to be further processed in order to derive the data in Tables 1-B and 1-C. Accordingly, the synthesized nanoprobe significantly decreased the T₁ and T₂ relaxation times compared to water (P<0.001).

In-vivo tumor imaging

The efficacy of the synthesized nanoprobe as an in-vivo MRI contrast agent was evaluated, and the tumor images were obtained 30, 60, and 120 minutes after the injection of the nanoprobe (5.0 μmol/kg) via the tail vein. In addition, the in-vivo imaging indicated that the synthesized nanoprobe could be introduced as a potential MRI contrast agent (Fig 8).

DISCUSSION

The present study aimed to synthesize a novel nanoprobe as an invasive contrast agent for MRI imaging. To this end, a biocompatible lipoprotein nanoprobe was selected as a carrier of Gd³⁺ in breast cancer diagnosis. The morphology and size of the synthesized nanoprobe were characterized using SEM (Fig 1) and DLS (Fig 2). The SEM results confirmed the spherical shape of the nanoparticles, while DLS determined the average diameter to be 50 nanometers. Moreover, the surface charge of -18.4 mV was obtained using the zeta potentiometer.
Biocompatibility and cytotoxicity are important parameters in the investigation of nanoscale compounds. The cytotoxic activity of the HDL nanoparticles loaded with Gd$^{3+}$ (nanoprobe) on the MCF-7 and MCF-10A cells was also investigated. According to the obtained results, the nanoprobe caused no toxicity in the normal and cancer breast cells (concentration up to 100 μg/ml) (Fig 3).

Nanoprobe reabsorption in cancer cells is considered critical in the design of nanoprobes for imaging processes. As can be seen in Fig 4, the absorption rate of the synthesized nanoprobe in the cancer cells was high and had an increasing trend with time. As is depicted in Fig 5, the absorption rate of the nanoprobe increased at higher concentrations. Furthermore, the ICP test results indicated that the intracellular penetration of the synthesized nanoprobe was high, resulting in the high accumulation of the nanoprobe inside the cells. Previous findings have indicated that the HDL carriers of Gd$^{3+}$ could successfully target the cancer cells, thereby leading to the release of Gd$^{3+}$ into malignant tumor cells [26].

The main objectives of the current research were MRI imaging using HDL nanoparticles and determining the efficacy of the applied nanoprobe. MRI imaging was performed with $T_1$ and $T_2$ relaxation times after tumor growth. Excellent contrast and high-resolution images were observed owing to the higher accumulation of this compound in the cancer cells compared to the normal cells. Moreover, the obtained results indicated that the synthesized nanoprobe reduced $T_1$ and $T_2$ relaxation times more significantly compared to water, thereby increasing the contrast (Figs 6 & 7).

The in-vitro studies of the BALB/c mice demonstrated the distinct increment of the contrast and high resolution in the treatment of the Gd$^{3+}$-loaded HDL nanoparticles. Furthermore, the intravenous injection of the nanoprobe in the cancer model of mice showed that this substance could provide appropriate contrast between the tumor and surrounding tissues, with the tumor detected visibly. This phenomenon was attributed to the ability of the nanoprobe to enter the cancer cells due to the high expression of its receptors in these cells (Fig 8).

According to the findings of the current research, the synthesized nanoprobe was well adapted to the body, and the molecules of contrast compositions were largely accumulated in the cancer tissues, representing proper performance in the selective imaging of breast cancer cells. Use of HDL nanoparticles as a molecular contrast agent is a noninvasive approach to investigating the changes on a molecular level, which is a promising and highly sensitive method. In the present study, the tumor-oriented MRI contrast agent based on the nanoprobe targeted to the HDL receptors, which were overexpressed in the tumor cells, indicating the ability of the nanoprobe to enhance the $T_2$-weighted images.

In a study in this regard, the efficacy of a nanoprobe was evaluated as an in-vivo MRI contrast agent, and the tumor images were obtained a few minutes after the tail vein injection of the nanoprobe at the concentration of 5.0 μmol/kg. In another research conducted by Rijpkema et al., the MRI data were obtained from the HDL-containing contrast compositions for the detection of human tumors. According to the results of the mentioned study, the rate of the contrast absorbance after transfusion increased significantly by HDL and was clearly visible after the injection [27]. This finding is consistent with the results of the present study.

CONCLUSION

In this study, an HDL-based nanostructure system was successfully designed as a probe for the detection of breast cancer. In general, HDL nanoparticles in the form of a nanoprobe could be a promising protocol for cancer diagnosis in-vivo.

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