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⁸⁹Zr Labeled Fe₃O₄@TiO₂ Nanoparticles: *In Vitro* Affinities with Breast and Prostate Cancer Cells

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Abstract

In this study, Fe₃O₄@TiO₂ nanoparticles were synthesized as a new Positron Emission Tomography/Magnetic Resonance Imaging (PET/MRI) hybrid imaging agent and radiolabeled with ⁸⁹Zr. In addition, Fe₃O₄ nanoparticles were synthesized and radiolabeled with ⁸⁹Zr. Df-Bz-NCS was used as bifunctional ligand. The nanoconjugates were characterized with transmission electron microscopy, scanning electron microscopy, and dynamic light scattering. Radiolabeling yields were 100%. Breast and prostate cancer cell affinities and cytotoxicity were determined using *in vitro* cell culture assays. The results demonstrated that Fe₃O₄@TiO₂ nanoparticles are promising for PET/MR imaging. Finally, unlike Fe₃O₄ nanoparticles, Fe₃O₄@TiO₂ nanoparticles showed a fluorescence spectrum at an excitation wavelength of 250 nm and an emission wavelength of 314 nm. Therefore, in addition to bearing the magnetic properties of Fe₃O₄ nanoparticles, Fe₃O₄@TiO₂ nanoparticles display fluorescence emission. This provides them with photodynamic therapy potential. Therefore multimodal treatment was performed with the combination of PDT and RT by using human prostate cancer cell line (PC3). The development of ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄@TiO₂ nanoparticles as a new multifunctional PET/MRI agent with photodynamic therapy and hyperthermia therapeutic ability would be very useful.

Graphical Abstract

Graphical Representation of ⁸⁹Zr labeled Fe₃O₄@TiO₂ nanoparticles as a new PET/MR hybrid imaging agent.

Keywords

$\text{Fe}_3\text{O}_4@ \text{TiO}_2$; ^{89}Zr ; Breast Cancer Cells; Prostate Cancer Cells; Imaging

INTRODUCTION

Recently, theranostic nanoparticles have emerged as a highly promising field within oncology. Superparamagnetic iron oxide (SPIO) nanoparticles are of particular interest due to their combined imaging and therapeutic potential. These nanoparticles can be tailored to enable magnetic resonance imaging (MRI) contrast as well as magnetic hyperthermia.^[1–4] SPIO nanoparticles can also be loaded with a therapeutic drug to be delivered to the site of interest. For example, Fe_3O_4 nanoparticles loaded with doxorubin have been shown to both enable MRI imaging as well as treat liver cancer.^[5] The conjugation of Fe_3O_4 nanoparticles with titanium dioxide (TiO_2) have recently been shown to enable photodynamic therapy (PDT) and magnetic hyperthermia in addition to enabling MRI contrast.^[3,6] Further, SPIO nanoparticles, when radiolabeled with positron emission tomography (PET) radionuclides, are potentially useful for hybrid imaging approaches such as PET/MRI.

Ferahem is a commercial iron oxide-based nanoconjugate and consists of a superparamagnetic Fe_3O_4 core and a shell of carboxymethyldextran polymer. It has 5874 iron atoms and 8752 oxygen atoms in its core. Its coated chemical formula is $\text{C}_{11719}\text{H}_{18682}\text{O}_{9933}\text{Na}_{414}$.^[7] The conjugation of Fe_3O_4 nanoparticles with titanium dioxide (TiO_2) have recently been shown to enable photodynamic therapy (PDT) and magnetic hyperthermia in addition to enabling MRI contrast.^[3,6] TiO_2 nanoparticles are inorganic photosensitizers and are typically used in PDT.^[8,9] Under the irradiation of ultraviolet light, the free radicals and singlet oxygen produced by TiO_2 nanoparticles can produce light toxicity and destroy the tissues and cancer cells. DFO (Desferrioxamine B) is a nice chelator

that is a clinically approved and used drug for the treatment of Fe (III) overload for a long time.^[10,11] As a hexadentate ligand with a hard base donor atom, DFO strongly binds Fe (III) over a wide pH range (1–11)^[12] and given the ability of Zr to choose hard, anionic donor groups and complexing with monohydroxamates^[13,14], it has also been estimated that they effectively bind Zr (IV) ions. Progress in ligand development of DFO-based radiopharmaceuticals and current status of clinical trials are summarized in recent reviews^[14,15,16]. We synthesized 1-(4-isothiocyanatophenyl) derivative of DFO in this work.

PET/MRI using ⁸⁹Zr-labeled iron oxide nanoparticles has been demonstrated to be feasible *in vitro* and *in vivo*.^[17] Zirconium (⁸⁹Zr) is a PET radionuclide of increasing interest due to its suitable decay characteristics and half-life ($t_{1/2}$ =78 h). It is considered appropriate for antibody-based immuno-PET due to its longer half life compared with other PET radionuclides such as ⁶⁸Ga or ¹⁸F. The relatively low decay energy of the emitted positrons enables high-resolution imaging. The use of ⁸⁹Zr-labeled radiopharmaceuticals in humans are increasing especially in the form of ⁸⁹Zr-labeled antibodies in immunoPET applications; ⁸⁹Zr-labeled radiopharmaceuticals are used less commonly for cell labeling.^[18]

This study involved the synthesis of ⁸⁹Zr-labeled Fe₃O₄@TiO₂ nanoparticles for PET/MR imaging, PDT, and hyperthermia therapy. The nanocomposites were characterized with transmission electron microscopy (HRTEM), scanning electron microscopy (SEM), and dynamic light scattering (DLS). Breast and prostate cancer cell affinities and cytotoxicity were determined using *in vitro* cell culture assays.

METHODS

Chemicals and Equipment

⁸⁹Zr (1.427 GBq/mL in 1 mol/L oxalic acid) was manufactured by BV Cyclotron VU, (Amsterdam, Netherlands) and supplied by Perkin Elmer, Inc. (Massachusetts, United States). Acetonitrile, HCl, ethanol, NH₃, acetone, phosphate buffer, DMSO, isopropanol, and methanol were purchased from Merck (Darmstadt, Germany). Protamine sulfate was obtained from APP pharmaceuticals (Illinois, United States). Heparine was obtained from Fresenius Kabi (Illinois, United States). MCF7 (ER+ human breast adenocarcinoma cell line), MDA-MB-231 (ER– human breast cancer cell line), EMT6 (mouse mammary carcinoma cells), PC3 (AR– human prostate cancer cell line), and Du145 (AR– human prostate cancer cell line) were supplied by the American Type Culture Collection (Virginia, United States). Minimum essential amino acid, dulbecco's MEM, L-glutamine, sodium bicarbonate, sodium pyruvate, fetal bovine serum, penicillin/streptomycin, tripan blue, trypsin (EDTA), and Minimum Essential Medium (MEM Eagle) were supplied from Lonza Chemicals, Inc. (Basel, Switzerland). All other chemicals were supplied from Sigma-Aldrich (Missouri, United States).

The following equipment from Ege University Institute of Nuclear Sciences were used: high performance liquid chromatography (HPLC) SPD-10AV UV/vis detector system with LC-10ATvp pump (Shimadzu Corporation, Kyoto, Japan), Hypersil™ ODS C18 HPLC column (Thermo Fisher Scientific, Darmstadt, Germany), CTO-10ASVP column oven (Shimadzu Corporation, Kyoto, Japan), SIL-20A HT automatic sampler (Shimadzu

Corporation, Kyoto, Japan), Thermo Scientific Varioskan Flash Multimode Microplate Reader (Thermo Fisher Scientific, Darmstadt, Germany), AR-2000 radio TLC (Thin Layer Radiochromatography) imaging scanner (Eckert & Ziegler, Berlin, Germany), inverted microscope (Leica Microsystems, Wetzlar, Germany), and FreeZone 2.5 lyophilizer (Labconco Corporation, Missouri, United States). The Institute of Technology, IZTECH Izmir provided: Philips XL-30 FEG SEM (Phillips, Amsterdam Netherlands). Mu la Sıtkı Koçman University, Research and Application Centre for Research Laboratories provided: JEM 2100F high resolution transmission electron microscopy (HRTEM) (Jeol, Tokyo, Japan).

Synthesis of Fe₃O₄ Nanoparticles

2M FeCl₃ (12 mL) was prepared in 2M HCl and diluted with 100 mL distilled water. Freshly prepared 0.08 M Na₂SO₃ (50 mL) was added slowly under nitrogen gas. 25% NH₃ (8 mL) was added with slow stirring under nitrogen gas. The solution temperature was maintained at 70°C for 15–30 minutes. Then it was cooled to below 45°C. The black magnetic precipitates were removed with an external magnet and were washed 3 times with ethanol (100 mL). The precipitates were redistributed in 100 mL of ethanol.

Synthesis of Fe₃O₄@TiO₂ Nanoparticles

An Fe₃O₄/TiCl₄ solution was prepared in a 1:1 (Fe:Ti) ratio and 10 mL of methanol was added dropwise and the mixture was incubated at room temperature for 1 h. After the gas outlet was exhausted and color conversion was observed, 160 mL of distilled water was added (H₂O/Ti, 16:1). The resulting solution was further incubated by adding 10 mL of n-dodecylamine and stirring at 100°C for 1 h. Upon completion of the incubation, a white stock solution was obtained. The stock solution was washed with acetone and the unbound dodecylamine was removed from the medium. For the washing step, acetone (99%) was added to the solution, vortexed for 1 minute at 1000 rpm, centrifuged at 1500 rpm for 5 minutes, and the supernatant was discarded. This procedure was repeated 3 times. Consequently, 2 mL of the stock solution was dispensed in 10 mL of oleic acid. The mixture was placed in a porcelain crucible. The total solution was placed in a furnace programmed with Step 1: heating rate of 5°C/min, Step 2: 2 h incubation at 250°C, and Step 3: cooling rate of 1°C/min. Upon completion of this programme, the sample was washed with acetone to remove excess oleic acid. The obtained precipitate were Fe₃O₄@TiO₂ nanoparticles which were dispersed in acetone (stored at 4°C).

Characterization of Synthesized Fe₃O₄@TiO₂

The hydrodynamic diameters and the zeta potential of the synthesized nanoparticles and conjugates were measured by DLS using a Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK) which measures the particle size distribution as well as the electrophoretic mobility of the dispersed particles. The measurement was repeated three times and the results were expressed as mean standard deviation.

The particle size and the morphology of the nanoparticles were obtained using SEM, EDX and HRTEM.

The fluorescence spectra of $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles were recorded using the fluorimetric analyzer.

Synthesis of p-isothio cyanato-benzyl-desferri oxamine (Df-Bz-NCS)

Df-Bz-NCS ((1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetroxo-27-[N-acetylhydroxyamino)-6,11,17,22-tetraazaheptaicosane] thiourea) was synthesized as previously described in the literature.^[19] 1 mmol (6.5 mg) of desferroxamine mesylate was dissolved in 1 mL isopropanol/water (1:1). 0.013 mol (2.5 mg) 1,4 phenylene isothiocynate and 1.5 μL triethylenamine were dissolved in 0.5 mL of chloroform and subsequently added to the desferroxamine mesylate solution while stirring. Upon completion, the reaction mixture was extracted with 0.1 M HCl. The lower organic layer was concentrated in vacuo to remove the chloroform but not the isopropanol. The product was lyophilized and about 6 mg of the product was obtained as white powder and was analysed with HPLC with a C18 column to confirm purity (85% yield). Mobile phase: 0–10 min 40/60 A/B, and 10–15 min 90/10 A/B. Flow rate: 1 mL/min. Wavelength: 275 nm. A=Acetonitrile (ACN), B=Water ^{13}C NMR and ^1H NMR chemical shift data (ppm) were used to confirm the structure of the synthesized product. ^1H NMR ($\text{D}_6\text{-DMSO}$): δ (ppm) 1.21 (–CH₂, 6H), 1.38 (–CH₂, 4H), 1.95 (–CH₃, 3H), 2.27 (–CH₂, 4H), 2.99 (CH₂, 4H), 3.45 (CH₂, 8H), 7.35 (2,6-ArH, J = 8.9 Hz, 2H), 7.75 (3,5 = ArH, J = 8.9 Hz, 2H), 7.75 (N-H, 2H), 7.88 (N-H, 1H), 9.59 (N-OH, 4H). ^{13}C NMR: δ (ppm) 19.18 (CH₃), 22.34, 22.35, 22.44, 24.88, 24.90, 24.94, 24.96, 24.98, 26.42, 26.88, 27.66, 28.78, 28.79, 28.83, 37.28, 37.92, 42.57, 42.60, 42.63, 45.67, 45.97, 121.92, 123.50, 124.98, 131.58, 138.17 (N=C=S), 169.00 (C=O), 170.20 (C=O), 170.84 (C=O), 179.02 (C=S). The structure was also confirmed by the theoretical NMR data using the ACD/CHEMSKETCH software program.

Radiolabeling with ^{89}Zr

250 μg Df-Bz-NCS in 20 μL DMSO and 40 $\mu\text{g}/10 \mu\text{L}$ Fe_3O_4 or $\text{Fe}_3\text{O}_4@\text{TiO}_2$ were incubated at 37°C (30 min) and subsequently combined with 0.55 MBq (15 μCi)/200 μL ^{89}Zr solution at room temperature (1 h). Prior to the labeling, the pH was adjusted from 3.5 to 7 with 160 μL 0.1 M NaOH + 40 μL 1 M oxalic acid.

Quality Control Studies using Thin Layer Radiochromatography (TLRC)

In quality control studies of ^{89}Zr nanocomplexes ($^{89}\text{Zr-Fe}_3\text{O}_4$ or $^{89}\text{Zr-Fe}_3\text{O}_4@\text{TiO}_2$) using TLRC, cellulose TLC strips measuring 10 cm $\times$ 1.5 cm and three different bath solutions (ACN, PBS (pH=7), 0.1 M DTPA) were used. The bath solutions were placed in glass tanks for 20 min, and then 2 μL of $^{89}\text{Zr-Fe}_3\text{O}_4$ or $^{89}\text{Zr-Fe}_3\text{O}_4@\text{TiO}_2$ was dropped to the TLRC strips (two strips each) and added to glass tanks. The strips were removed from the bath solutions, the last point was labeled, and the strips were stored until dry. After drying the TLRC strips, the radiochromatogram was obtained from each strip with a Bioscan AR2000 TLRC scanner. The radioactivity R_f values and binding efficiencies of radioactive materials were calculated from the radiochromatograms. R_f values of unbound ^{89}Zr and ^{89}Zr nanocomplexes were calculated in TLRC quality control studies.

Quality Control Studies using High Performance Liquid Chromatography (HPLC)

The Shimadzu HPLC SPD-10AV UV/vis detector system with LC-10Atp pump and the SIL-20A HT automatic sampler (Shimadzu Corporation, Kyoto, Japan), and the Inertsil® ODS-3, column (GL Sciences Inc, Tokyo, Japan) were used. The mobile phase consisted of: ACN 8%, TFA-H₂O 0.15%. Flow rate=1 mL/min, wavelength=215 nm, temperature=26°C. The Raytest (1×1) NaI detector connected to the HPLC system was used to obtain the radiochromatogram.

Stability Testing

For stability studies, samples were incubated in PBS buffer (pH=7) for up to 24 h at 21°C–25°C and then analyzed using the TLRC scanner to determine radiolabeling yields.

Ligand Exchange Stability Test with DTPA

⁸⁹Zr-labeled nanocomplexes were incubated in DTPA (0.1 M) at room temperature for 1 h and 2 h in PBS medium.

Pyrogenicity Test

Pyrogenicity test was performed using the Limulus Amoebocyte Lysate (LAL) test. The LAL test is accepted as a standard method according to the United States Pharmacopeia bacterial endotoxin test guidelines and the US Food and Drug Administration good manufacturing practice guidelines. LAL reagents and test samples were incubated at 37 °C for 60 min. A positive response indicates that the amount of endotoxin is greater than or equal to λ . The endotoxin limit was accepted as 5 endotoxin units (EU)/kg for parenteral drugs and 0.2 EU/kg for intrathecal drugs. Serums of medical devices should not exceed 0.5 EU/mL.^[20]

In Vitro Studies

Commercial cells with different expression properties for use in cell culture and which may also be tumorigenic in Balb C mice were selected. MCF7, EMT6, and MDA-MB-231 cells were cultured in MEM Eagle, 2 mM glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS). PC3 and Du145 cells were produced in media consisting of Dulbecco's MEM, 2 mM glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, and 10% FBS. After the cells covered the flasks to 80%, they were separated from the flask using a solution of 0.25% (w / v) trypsin-EDTA and the cells were frozen at –80° C in 10% DMSO.

Cell Viability and Cytotoxicity

A conventional WST-8 setup was established to investigate the cell viability responses and IC50 (concentration range causing 50% mortality) values of the synthesized nanoparticles in a dose-dependent way by using the standard protocol in our previous works^[21]. The cells (MCF7, EMT6, MDA-MB-231, PC3, and Du145) were sustained in MEM and were prepared from confluent monolayer cultures and then plated. The cell suspension was prepared at 10⁵ cells/mL per well in 96-well microplates. To each well, 100 μ L of cell suspension and Fe₃O₄ or Fe₃O₄@TiO₂ samples were added at 7 different concentrations

(0.5–1000 ng/mL). Cells and reagent-free media were used as negative controls. In the study, each parameter was studied in triplicate. Cells were incubated at 37 °C in 5% CO₂ for up to 24 h. At the end of incubation, 10 µl of WST solution was added to each buffer and incubated for 4 h, after which the absorbance value (OD) of each buffer at 450 nm wavelength and 690 nm reference interval was read using a spectrophotometer. Negative control was accepted as zero absorbance. Cell viabilities (%) were calculated by using the following formula; [measured absorbance value/absorbance value of negative control) × 100].

Cell Incorporation Studies of ⁸⁹Zr nanocomplexes

MCF7, MDA-MB-231, EMT6, PC3, and Du145 cells were seeded to determine the binding efficiency of ⁸⁹Zr, ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄, and ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄@TiO₂. After the cells had grown to capacity, the cells were distributed into 24-well plates. The amount of ligand used for each well on the plate was determined to be 10 µg/mL and ⁸⁹Zr specific activity to be used was determined to be 1 µCi/mL. To determine the level of binding of ⁸⁹Zr at the cell level, 0.5 mL of medium containing 1 µCi/mL ⁸⁹Zr was added to each well. To determine the level of binding of ⁸⁹Zr nanocomplexes, before the addition of medium containing ⁸⁹Zr nanocomplexes to the wells, the media on the cells were discarded and the wells were washed three times with PBS to remove dead cells from the media. After washing, each cassette on the 24-well plates was loaded with 0.5 mL medium containing ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄ or ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄@TiO₂. Samples containing ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄, and ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄@TiO₂ were removed after 120 min and then washed three times with PBS; thereafter, 0.2 mL RIPA solution was added to remove the cells from the surface. 0.1 mL of ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄ or ⁸⁹Zr-Df-Bz-NCS-Fe₃O₄@TiO₂ was placed in a vial containing a 0.9 mL scintillation cocktail, and the radioactivity counts (A1) for both were determined using a liquid scintillator. The amount of protein was determined using the BCA assay with 25 mL of RIPA-treated cells. Binding ratios were calculated using the protein values. The control group counts were subtracted from these values and the binding ratios percentage were calculated. The mean ± SD percent viable cells was compared with the buffer control (n=3).

Photodynamic Therapy and Radiotherapy

PC3 cells grown in the appropriate medium for this study were seeded 4×10³ in each well of 96 well plates. The Fe₃O₄@TiO₂ aliquots of were added at 50 ng and 500 ng per well. The preparations were exposed to UVC (energy 200 to 280 nm) for 5 minutes at a distance of about 10 cm. Thereafter, the samples were irradiated at a distance of 10 cm at a dose of 5 Gy radiation dose in 2 minutes, then incubated at 37 °C for 72 hours at 5% CO₂. After 72 hours WST test procedure was applied and absorbance values were calculated for each well similarly with our previous work [22].

Viability (%) values were calculated with the formula [(measured absorbance value / control value) × 100]. Absorbance of the negative control is assumed to be zero.

Statistical Analysis

The results were represented as the mean standard deviation (SD). The statistical analysis was performed with one-way ANOVA, followed by Tukey's multiple comparison tests using GRAPHPAD PRISM software. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Characterization

Continuing our studies on the application of several radionuclides such ^{18}F , $^{99\text{m}}\text{Tc}$, and $^{125/131}\text{I}$ radiolabeled SPIO nanoparticles,^[4,23,24] herein we report the preparation and characterization of multifunctional $^{89}\text{Zr}\text{-Fe}_3\text{O}_4\text{@TiO}_2$ nanoparticles which we achieved by applying Df-Bz-NCS as a bifunctional ligand. $^{89}\text{Zr}\text{-Fe}_3\text{O}_4\text{@TiO}_2$ nanoparticles are promising as a new PET/MRI hybrid imaging agent.

The molecular structure of Df-Bz-NCS was confirmed in ^{13}C NMR and ^1H NMR studies. The hydrodynamic diameters of the Fe_3O_4 and $\text{Fe}_3\text{O}_4\text{@TiO}_2$ nanoparticles were determined to be 49.13 and 91.85 nm, respectively (Figure 1). According to SEM images, Fe_3O_4 and $\text{Fe}_3\text{O}_4\text{@TiO}_2$ nanoparticles are very homogeneous and have a smooth surface and dimensions from 13–24 nm and 50–89 nm, respectively (Figure 2). The morphology and dimensions of core-shell nanoparticles were further determined using HRTEM (Figure-3). Dimensions of $\text{Fe}_3\text{O}_4\text{@TiO}_2$ nanoparticles are below 10 nm and they have rather polyhedral shapes. While the nanoparticle diameters are comparable to those obtained from SEM, the shape and size are better appreciated in HRTEM because the solubilities in the HRTEM are better than SEM. In HRTEM images, the magnetic core is visible as a dark spot inside the bright spherical Schiff base complex grafted to O bond;Ti material. Our results agree with previous data presented in literature.^[24, 25]

The elemental composition of synthesized catalysts was examined using EDX analysis. Atomic ratios are listed in Table 1. In agreement with the TEM observations, EDX results demonstrated that nanocomposite was composed of the elements of Fe, Ti and O. Ti/Fe ratio is 2, O/Ti ratio is 10,74, Ti/Fe ratio is 2 of $\text{Fe}_3\text{O}_4\text{@TiO}_2$ -nanocomposites, consistent with the theoretical data.^[26]

Some studies demonstrated that Fe_3O_4 nanoparticles could be radiolabeled with $^{89}\text{Zr}^{4+}$ ions without chelating agent (using either oxalate or chloride salt formulations) under aqueous conditions (pH 8.0) upon heating and stirring the reaction mixture at 120 °C for less than 1 h. Boros et al. radiolabelled the FDA-approved, carbohydrate coated commercial Fe_3O_4 nanoparticles called ferumoxytol (Feraheme®) with a range of more commonly available radiometal isotopes, including ^{89}Zr and ^{64}Cu for PET and ^{111}In for SPECT.^[27] Particles were heated in an aqueous solution with the radiometal ions (80–120°C) at a pH between 7–9.^[27–29] However the nature of the chemical interaction between the metal isotopes and iron oxide nanoparticles remained unidentified in these reports.

The authors preferred to use DFO chelating agent to label ^{89}Zr with $\text{Fe}_3\text{O}_4\text{@TiO}_2$ nanoparticles in their work similar to other reports.^[30,31] Graphical abstract and figure 4 summarize the water-soluble carbodiimide chemistry employed to link the $\text{Fe}_3\text{O}_4\text{@TiO}_2$

nanoparticles. There is no other reports regarding ^{89}Zr labeling of $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles according to our knowledge.

Finally, unlike Fe_3O_4 nanoparticles, $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles showed a fluorescence spectrum at an emission wavelength of 250 nm and an emission wavelength of 314 nm (Figure 5). Therefore, in addition to bearing the magnetic properties of Fe_3O_4 nanoparticles, $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles display fluorescence emission. This provides them with PDT potential. The development of $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles as a new multifunctional PET/MRI agent with PDT and hyperthermia therapeutic ability would be very useful. In cancer diagnosis and therapy, UV light can be directed to cancer cells using UV light sources. Then, using an intra-operative PET/MRI technique, real-time MRI and PET and in situ PDT can be realized. Thus, $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanocomposites could have important applications in the future as a multifunctional probe allowing PET/MR imaging, PDT, and hyperthermia therapy of cancer.^[6]

Both TLRC and HPLRC were used to verify the radiochemical purity (RCP) and ^{89}Zr radiolabeling efficiency of $\text{Fe}_3\text{O}_4@\text{TiO}_2$. TLRC chromatograms of $\text{Fe}_3\text{O}_4@\text{TiO}_2$ mobile phase ACN and 0.1 M DTPA are given in Figure 6 and R_f values are given in Table 2. Both Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{TiO}_2$ can be radiolabeled with ^{89}Zr with a high yield and are well separated with TLRC and HPLC (Figure 6, Figure 7, Table 2, Table 3). The results show that the radiolabeling yields are 100%. The peak purity analysis shows that the picked peaks are over 99.9% pure.

Stability Results

$^{89}\text{Zr}-\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles remained stable (over 85% radiolabeling yield) at room temperature for 24 h.

Ligand Exchange Stability Test with DTPA

$^{89}\text{Zr}-\text{Df-Bz-NCS}-\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles were incubated in DTPA (0.1M) at room temperature for 1 h and 2 h in PBS and showed stability at 100% efficiency in working conditions (Figure 8). $^{89}\text{Zr}-\text{Df-Bz-NCS}-\text{Fe}_3\text{O}_4$ showed a radiolabeling yield of 100% up to 4 h which dropped to 50% at 8 h. Our results on $^{89}\text{Zr}-\text{Fe}_3\text{O}_4$ nanoparticles agree with Boros et al.^[27] who previously reported that $^{89}\text{Zr}-\text{Df-Bz-NCS}-\text{Fe}_3\text{O}_4$ can be radiolabeled with ^{89}Zr with 93% radiolabeling yield. However, as we found that $^{89}\text{Zr}-\text{Df-Bz-NCS}-\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles were more stable than $^{89}\text{Zr}-\text{Fe}_3\text{O}_4$ after 8 h and remained stable for up to 72 h, we speculate that the Df-Bz-NCS bifunctional ligand supplied more stability to the structure in $^{89}\text{Zr}-\text{Fe}_3\text{O}_4@\text{TiO}_2$ than in $^{89}\text{Zr}-\text{Df-Bz-NCS}-\text{Fe}_3\text{O}_4$, and for this reason $^{89}\text{Zr}-\text{Df-Bz-NCS}-\text{Fe}_3\text{O}_4@\text{TiO}_2$ would be thermodynamically and kinetically more stable in vitro than $^{89}\text{Zr}-\text{Df-Bz-NCS}-\text{Fe}_3\text{O}_4$.

Here, we describe the synthesis of a bifunctional version of DFO, DFO-Bz-NCS. This bifunctional chelator is prepared starting from the commercially available DFO, which makes DFO*-based chelating agents attractive candidates for commercialization and wide spread use in clinical nuclear medicine. Mejis et al. ^{89}Zr (IV) demonstrated its high stability in human serum by making the first assessment of desferrioxamine B (DFO) for chelation [32].

Apriogenicity Test

$^{89}\text{Zr-Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles also passed the apriogenicity tests.

Cell Viability and Cytotoxicity

Breast and prostate cancer cell viabilities and cytotoxicities were determined by in vitro cell culture assays using MCF7, EMT6, MDA-MB-231, PC3, and Du145 cells (Figure 9, Table 4). In the study, IC50 (concentration range causing 50% mortality) values were colorimetrically determined by WST-8 test. The IC50 values for $\text{Fe}_3\text{O}_4@\text{TiO}_2$ were well below the recommended 500 mg dose.

$\text{Fe}_3\text{O}_4@\text{TiO}_2$ NPs decreased cell viability when applied in treatment. Mitochondria JC-1 staining assay showed that mitochondrial membrane permeability injury occurred in both $\text{Fe}_3\text{O}_4@\text{TiO}_2$ and TiO_2 NPs treated cells according to some other reports [33]. $\text{Fe}_3\text{O}_4@\text{TiO}_2$ NPs induced slightly higher cytotoxicity than Fe_3O_4 NPs in all cell lines according to cell viability assays. Su et al reported that under a low dose (6.25g/cm(2)), $\text{Fe}_3\text{O}_4@\text{TiO}_2$ and TiO_2 NPs had any significant toxicity on HL7702 cells. [34] There is no significant toxicity under 10 $\mu\text{g/mL}$ both for $\text{Fe}_3\text{O}_4@\text{TiO}_2$ NPs or Fe_3O_4 NPs in our work.

Cell Incorporation Results

MCF7, EMT6, MDA-MB-231, PC3, MCF7, and Du145 cells were used to determine the binding efficiency of $^{89}\text{Zr-Df-Bz-NCS-Fe}_3\text{O}_4@\text{TiO}_2$. $^{89}\text{Zr-Df-Bz-NCS-Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles were found to bind to breast and prostate cells at different ratios under working conditions (Figure 10). Thus, according to these results, they can be used for PET and MR imaging.

Photodynamic and Radiotherapy Potentials

TiO_2 has a potential as photosensitizer for cancer cells.[20] For photodynamic therapy, the cells were irradiated at a distance of 10 cm using an UV beam source emitting light at a wavelength of 250–400 nm at a power of 4 (J cm-2). X-radiation was made in the same geometry as photodynamic therapy. While photodynamic therapy is carried out through a selective reactive effect on a single molecule, the radiolysis mechanisms through ionized, excited, and radical series formed during the water's radiolysis are more effective in radiotherapy.[34]

When $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoconjugate was used alone no significant change was observed in the concentration of 50 –500 ng/mL. Compared to non-irradiated control group, irradiation with $\text{Fe}_3\text{O}_4@\text{TiO}_2$ has more cytotoxic effect on PC-3 cancer cells. The cell viability decreased from 96% to 72% when we use 500 ng/mL $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoconjugate. When the cells irradiated without $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoconjugate at the same conditions there was no harmful effect.

Used radiation dose is far below the harmful lethal dose for cells, it can generate effective therapy on the target without side effects of radiotherapy and chemotherapy when it is directed to cancer cells with nanoconjugate radiation and light effect.

$\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles have been demonstrated to be nontoxic, easy to prepare, and highly stable in biological systems.^[36] Additionally, they have potential for enabling PDT. [3,8,9,28] TiO_2 is a widely used photosensitizer and can generate oxidative radicals by light photons or ultrasound waves which have good penetration ability. TiO_2 has been applied as photosensitizer for cancer cells recently.^[35] Several kinds of photosensitizers such as porphyrin and its derivatives,^[22,34] 5,10,15,20- tetrakis(m-hydroxyphenyl)chlorin, [37] 5-aminolevulinic acid,^[38,39] and TiO_2 [40,41] have been explored for PDT treatment of cancers. However, the biological half-life of TiO_2 nanoparticles are longer in the body than that of traditional organic photosensitizers, and they are nontoxic and stable under no light irradiation. Besides being used as a sonosensitizer for sonodynamic therapy, TiO_2 can also be used for the loading of an anticancer drug.^[36]

$\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles can also enable the monitoring of lymph node imaging. Previously, it has been reported in the literature that Fe_3O_4 nanoparticles with dimensions of around 30 nm can identify sentinel lymph node involvement.^[42,43] Fe_3O_4 nanoparticles are held by macrophages and transported by the lymphatic system to the lymph node. The hypothesis that lymph node flow is different in metastases has been supported by MRI and PET studies using the ^{89}Zr radionuclide.

Conclusion

This work addresses a new, multi-functional imaging agent that can enable multifunctional hybrid PET/MRI and PDT activity in cancer treatment. The multifunctional module is based on ^{89}Zr -Df-Bz-NCS- $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles. ^{89}Zr -Df-Bz-NCS-labeled nanoconjugates can be prepared as radiolabeled theranostics resulting in radioimmunoconjugates with high radiochemical purity and optimal immunoreactivity. This makes Df-Bz-NCS a candidate successful bifunctional chelate for clinical ^{89}Zr labeled theranostics.

These nanoparticles might allow lesions to be identified with higher accuracy and sensitivity. We strongly believe that the results presented herein complete the knowledge on the ^{89}Zr -Df-Bz-NCS linked nanoconjugates interactions, providing the speciation of complexes together with their stabilities, and might be useful when designing more effective PET chelators.

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Figure 1.
DLS size graphics a Feraheme (a) and Fe₃O₄@TiO₂ (b).

Figure 2.
SEM images of Feraheme (a) and Fe₃O₄@TiO₂ (b).

Figure 3.
HRTEM images of Feraheme (**a**) and $\text{Fe}_3\text{O}_4@\text{TiO}_2$ (**b**).

Figure 4.
Synthesis and radiolabeling of ^{89}Zr - $\text{Fe}_3\text{O}_4@\text{TiO}_2$

Figure 5.
Fluorescence emission spectra of of $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles.

Figure 6.
TLRC chromatograms of $\text{Fe}_3\text{O}_4@\text{TiO}_2$ mobile phase ACN (**a**) and 0.1M DTPA (**b**).

Figure 7.
HPLC radiochromatograms: ^{89}Zr (a), ^{89}Zr -Ferrahem (b), ^{89}Zr - Fe_3O_4 @ TiO_2 (c)

Figure 8.
Stability graphic of $^{89}\text{Zr-Fe}_3\text{O}_4@\text{TiO}_2$ and $^{89}\text{Zr-Feraheme}$.

Figure 9.
Cell Viabilities

Figure 10.

In vitro cell incorporation graphic of EMT6, MCF-7, MDA, PC3, Du145 cells incubated with $^{89}\text{Zr}\text{-Fe}_3\text{O}_4\text{@TiO}_2$ nanoparticles of different concentrations for 120 minutes.

Table 1Atomic (%) and Atomic Ratios of Fe₃O₄@TiO₂ NPs according to EDX Data

Atomic (%)			Atomic Ratio		
Ti	Fe	O	O/Ti	O/Fe	Ti/Fe
8,17	4,08	87,75	10,74	21,48	2,00

Table 2.TLRC R_f values of ⁸⁹Zr, **⁸⁹Zr- Feraheme** and ⁸⁹Zr-Fe₃O₄@TiO₂ (n=10)

TLRC R _f values			
Mobile phase	⁸⁹ Zr	⁸⁹ Zr-Feraheme	⁸⁹ Zr-Fe ₃ O ₄ @TiO ₂
ACN	0.058±0.01	0.039±0.010	0.045±0.00
0.1M DTPA	0.869±0.08	0.541±0.023	0.931±0.01

Table 3HPLC retention times values of ^{89}Zr , **^{89}Zr - Feraheme** and $^{89}\text{Zr-Fe}_3\text{O}_4@\text{TiO}_2$ (n=10)

Mobile phase	HPLRC retention times (min.)		
ACN 8%,	^{89}Zr	^{89}Zr - Feraheme	$^{89}\text{Zr-Fe}_3\text{O}_4@\text{TiO}_2$
TFA- H_2O 0.15%	2.691	3.070	3.899

Table 4IC₅₀ values of Fe₃O₄@TiO₂ ve Feraheme

IC ₅₀ value (ng/mL)		
Cell	Fe ₃ O ₄ @TiO ₂	Feraheme
MCF7	739,1±14	5066±21
MDA-MB-231	361,1±05	1211±90
EMT6	407,3±60	193,9±16
Du145	392,4±50	392,4±50
PC3	829,2±50	1813±50