Synthesis of Radioiodinated Thymoquinone Glucuronide Conjugated Magnetic Nanoparticle \((^{125}\text{I}-\text{TQG-Fe}_3\text{O}_4)\) and its Cytotoxicity and In Vitro Affinity

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ABSTRACT

The aim of the current study is to develop solid, semisolid or liquid form of radionuclide labeled thymoquinone glucuronide conjugated magnetite nanoparticles those targets to the tumor for diagnosis and therapy of cancer.

For this purpose, thymoquinone (TQ), a molecule present in the seeds of Nigella \((\text{Nigella sativa})\) used to enhance the affinity of the drug to the tumor cells. TQ was isolated from its microsomes and an enzymatic method applied to synthesize beta glucuronic acid derivatized thymoquinone glucuronide (TQG). TQG attached magnetic nanoparticles \((\text{TQG-Fe}_3\text{O}_4)\). TQG-\text{Fe}_3\text{O}_4 were radiolabeled with \(^{125}\text{I}\) and \((^{125}\text{I}-\text{TQG-Fe}_3\text{O}_4)\) its cytotoxic effect and in vitro affinity was investigated. The IC\text{50} values of TQG-\text{Fe}_3\text{O}_4 were found 27.31, 18.68, 11.88 (µg/mL) respectively 24, 48 and 72 hours against A549 cell line by WST-8 test as a colorimetric way. Incorporation ratios of TQG-\text{Fe}_3\text{O}_4 with A549 cells is the highest levels. It is seen that TQG-Fe\text{3O}4 could inhibit the apoptosis on A549 cells but, there is no apoptotic effect of the samples on BEAS-2B cells. Size distribution, cellular uptake and toxicity characteristics of TQG-\text{Fe}_3\text{O}_4 in this study maintains a useful targeted delivery system in lung cancer diagnosis and therapy.

Keywords: Nigella thymoquinone (TQ), glucuronide derivative, beta glucuronidase, radiolabeling with \(^{125}\text{I}\), magnetic nanoparticles, Fe\text{3O}4.

INTRODUCTION

\textit{Nigella sativa} is an annual flowering plant, which is native to India and Pakistan and commonly used as a spice. The oil obtained from the seeds of this plant has traditionally used for the treatment of arthritis, pulmonary disease and hy-
percholesterolemia, especially in Arabian culture. Biological activity of Nigella seeds originates from thymoquinone derivatives, which is dominant (30-48%) compound of volatile oil content. A limited number of clinical trials show that, there is no side effects of thymoquinone on human, even at high dosages of this compound. Therefore, it is highly desirable to form a novel compound, which is hydrolyzed in tumor by beta-glucuronidase with glucuronic acid conjugation of thymoquinone.

It is well-known that, some cancer cells are very rich for beta glucuronidase. Information is available about lung cancer cells have high beta glucuronidase activity.

Labeling glucuronide derivatives with technetium-99m (99mTc) or radioiodine with the advantage of higher expression of beta glucuronidase enzymes in tumor tissues rather than normal tissues, have been studied by our group for more than a decade. Stachulski and Meng reported that glucuronide derivatives of the cytotoxic molecules used in the cancer therapy are significantly reducing the use of glucuronide derivative molecule increased day by day. Different studies uracil-O-glucuronide are showing that affinity of this molecule in cancer cells is significantly higher than normal cells. For example, production of uracil-O-glucuronide (UOG) significantly increased in HuTu 80 small bowel cancer cells in comparison with normal human intestinal epithelial cells.

Beta-glucuronidase is present in higher levels than in normal tissue, also led this enzyme in tumor tissue to be more active in more acidic environments such as the and relatively easy to measure to some cytotoxic molecules glucuronide derivative of the birth of the idea that can be a good anticancer agents. Nanoparticles with magnetic properties can be used in imaging, diagnosis and treatment of various diseases and to separate of biological materials. Patient-related, magnetic controlled drug targeting system is reported by Lubbe et al. for the first time. Lubbe and colleagues also states that, directing iron nanoparticles to tumor cells is feasible under 0.5-0.8 Tesla magnetic field strength. Mohapatra et al. reported that folic acid-conjugated magnetic nanoparticles retained specifically by folate receptor containing cancer cells. Magnetic nanoparticles were synthesized by precipitation reaction of Fe²⁺/Fe³⁺ and following surface modification with 2-carboxyethyl phosphonic acid. Resulting nanoparticles with a free carboxyl group attached to the folic acid and fluorescein isothiocyanate (FITC) using 2,2- (ethylenedioxy) - bisethylamine.

Moritake et al. silanized 3nm sized magnetite nanoparticles with (3-aminopropyl) triethoxysilane and reported that it may also be suitable to attach pharma-
ceuticals and biomolecules after functionalization of surface with amino groups. It is reported that extreme small particles without any other modification may penetrate to inside of cells due to increasing endocytic binding, because of their cationic structures. The cells that bind nanoparticles have continued to proliferate and have not shown any extinguisher effect on mitosis. In addition, when the particles injected under ear skin, they magnetized under outer magnetic field\textsuperscript{19}.

Thymoquinone compound radiolabeled with \textsuperscript{99m}Tc is the only thymoquinone derivative that be usable over gamma scintigraphy\textsuperscript{17}.

The aim of current study is as follows:

- Synthesis of a thymoquinone glucuronide derivative,
- Conjugation with magnetic nanoparticles (Fe\textsubscript{3}O\textsubscript{4}, nm size),
- Radiolabeling with \textsuperscript{125}I and also other iodine radioisotopes,
- Development of formulations of generated solid radiolabeled nanoparticles showing the magnetic properties in solid, semi-solid and liquid forms.
- Determination of \textit{in vitro} affinity by using Human lung adenocarcinoma cells (A549) and normal human lung epithelial cells (BEAS-2B).

**MATERIAL METHOD**

**Materials**

Na\textsuperscript{125}I is obtained from the Institute of Isotopes Co. Ltd., Budapest. BEAS-2B and A549 cell lines were obtained from American Type Culture Collection, Rockville, MD, USA and Bio-engineering Department of Ege University, Izmir, Turkey, respectively. WST-8 assay, iodogen (1,3,4,6-tetrachloro-3\alpha,6\alpha-diphenylglycouril), ferrous chloride (FeCl\textsubscript{3}) and ferric chloride tetrahydrate (FeCl\textsubscript{2}.4H\textsubscript{2}O) were purchased from Sigma-Aldrich. All other chemicals purchased from Merck and Lonza. All solvents were reagent grade and, are used without further purification.

**Equipment**

Transmission electron microscopy (TEM, JEOM JEM 2100F HRTEM), X-ray diffraction (XRD, Philips X’Pert Pro), dynamic light scattering (DLS) (Malvern Nano-ZS) were used for characterization of synthesized Fe\textsubscript{3}O\textsubscript{4} nanoparticles. Bioscan AR-2000 Imaging Scanner and Packard Tri-Carb-1200 Liquid Scintillation Spectrometer used for Thin Layer Radio Chromatography (TLRC) method and incorporation studies, respectively.
Glucuronidation of Thymoquinone (TQ)

Obtaining of UDP-glucuronyl transferases enzyme (UDPGT) from A549 Cells

Microsome preparates separated from human lung carcinoma cells (A549) used as a UDP-glucuronyl transferase (UDPGT) source, which is required for glucuronidation of thymoquinone. A549 cells were grown in MEM supplemented with 10 % FBS, 2.0 mM glutamine, 0.1 mM nonessential amino acid, 1.5 g/L sodium bicarbonate and 1 mM sodium pyruvate. Mediums removed and cells washed 3 times with PBS. Cells were detached from the surface and cell pellets washed with sucrose/hepes 3 times with centrifugation (4 °C, 1000 rpm and 10 min) in every step. Then cells ruptured with homogenizator for 4 minutes at ice bath. Homogenate was centrifuged at 4 °C, 10500 rpm for 10 minutes and supernatant was centrifuged at 4 °C, 28000 rpm for 10 minutes by ultracentrifuge. After centrifugation supernatant was removed and enzymes were solubilized with pH 7 buffer (0.2 M potassium phosphate, 2 mM mercaptoethanol and 0.4% Triton X100) and stirred for 30 min. at 4 °C. Solubilized enzymes were centrifuged at 4 °C, 28000 rpm for 10 minutes. Protein concentration of the supernatant, which contains microsomal samples determined with bicinchoninic acid method using Thermo Varioskan Flash multimode microplate reader. Absorbance reading was performed at 260 nm and protein concentration was obtained as 3.44 mg/mL.

Enzymatic Synthesis of Thymoquinone Glucuronide (TQG)

34.4 mg microsomal enzyme in 5 mL of 50 mM Tris Buffer (pH = 8) which contains 6 mM CaCl$_2$, 10 mM UDPGA and 1 mM DTT were stirred at 37 °C for 10 min in a water bath. Then thymoquinone in 10 mg/mL of DMSO was added and incubated at the same temperature for 18 hours. After incubation acetonitrile (300 µL) was added to the solution and centrifuged at 2000 rpm for 20 min. Purity analysis of product was conducted by HPLC (Schimadzu LC10 ATVP).

Preparation of Magnetic Nanoparticle Conjugated TQ and TQG

Synthesis of Magnetic (Fe$_3$O$_4$) Nanoparticles

One hundred mL of ferrous salt solution in acidic environment was prepared using 2 M FeCl$_3$ (12 mL) and 2 M HCl, Then, 0.08 M Na$_2$SO$_3$ (50 mL) was added slowly under nitrogen gas. Ammonia (NH$_3$) (25%, 8 mL) was added to the mixture under nitrogen gas. The mixture waited at 70 °C for 15-30 minutes. Then the temperature of the mixture was cooled under 45° C. Black colored particles were collected with a magnet and washed with ethanol for 3 times. Particles were stored in ethanol at 4° C.
Coating of Fe$_3$O$_4$ Nanoparticles with Silica

Five mL tetra ethyl ortho silicate (TEOS) and NH$_3$ (10%, 5 mL) was added into the magnetic particle solution. The reaction was carried under at 40°C for 12 hours under magnetic stirring and was washed with methanol 3 times.

Amino Silane Conjugation of Silica Coated Fe$_3$O$_4$ Nanoparticles

Amino silane agent [N-(2-aminoetil) -3-aminopropil-trimetoksisilan] was added into the magnetic nanoparticles in ethanol and solution was kept in an ultrasonic bath for 5 minutes. After uniform dispersing, the reaction was carried out at 60 °C for 12 minutes and washed with ethanol 3 times. The resulting precipitates were collected, dried under vacuum and stored at 4°C.

Immobilization of TQ and TQG to Fe$_3$O$_4$ Nanoparticles (TQ-Fe$_3$O$_4$ and TQG-Fe$_3$O$_4$)

Twenty-five mg/mL of silanized Fe$_3$O$_4$ nanoparticles was washed with 0.1 M phosphate buffer solution (PBS pH=7.0 25°C). Then, nanoparticles were incubated with glutaraldehyde (2.5%) at 4°C in an ultrasonic bath for 4 hours. The suspension was centrifuged and washed with 0.1 M PBS. TQ (4 mg) and TQG (4 mg) were dissolved in 1 mL 0.1 M PBS, which contains 0.15 M NaCl and 0.005 M EDTA (pH=7.2). After 12 hours of incubation particles were washed with 0.1 M borate tampon which contains (pH=9.2) 0.5 mg/mL NaBH$_4$ and kept at 4°C for 30 minutes. Particles washed with 0.1 M PBS (pH=7.0 25°C) and resuspended in 1 mL 0.5M MES (pH=6.6).

Inactive iodination of TQG [Inactive iodine (127I) labeled TQG (127I-TQG)]

Cold-iodination of TQG was performed to gain insight about the molecular structure of radioiodinated TQG. With this purpose, TQG was iodinated within active iodine (127I) by using stoichiometrically equivalent amounts of potassium iodide and oxidizing agent iodogen to prove the identity of the radiolabeled compound.

Quality Control Studies of TQ and TQG by using High Performance Liquid Chromatography (HPLC)

TQ and enzymatically glucuronidated TQG were analyzed with HPLC. A low-pressure gradient HPLC system (LC-10ATvp quaternary pump, SPD-M20A DAD detector, a syringe injector equipped with a 500 IL loop, CTO-10AS column oven, SIL 20A-HT auto sampler, FRC-10A fraction collector and 5μ C18-ODS column (250 x 4.6 mm ID) was used for HPLC analysis.1mL/min was flow rate of the mobile phase and readings were collected at 254 nm. Mobile phase of analysis was water/methanol/isopropanol (50:45:5). HPLC chromatogram of TQ and TQG was shown in Figure 1.
**Figure 1.** HPLC chromatogram of the TQ and TQG.

**Structural Analysis of TQG and $^{127}$I-TQG**

Structural analysis of TQG and $^{127}$I-TQG were performed by Liquid Chromatography-Mass Spectroscopy (LC-MS/MS) at the Laboratory of LC-MS at Ege University, Ege University, Center for Drug R&D and Pharmacokinetic Applications (ARGEFAR). m/z values of the TQG and $^{127}$I-TQG, and also with other fragments were given in Table 1.
Table 1. LC-MS/MS m/z values of the TQG and $^{127}$TQG compounds and some fragments.

<table>
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<th>Fragment</th>
<th>m/z</th>
<th>Fragment</th>
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<tr>
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<td><img src="image" alt="Diagram J" /></td>
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<tr>
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<td><img src="image" alt="Diagram L" /></td>
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<td>M</td>
</tr>
<tr>
<td>F</td>
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<td>G</td>
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<td>368.33</td>
<td>O</td>
</tr>
<tr>
<td>H</td>
<td><img src="image7.png" alt="Image" /></td>
<td>354.43</td>
<td>P</td>
</tr>
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</table>
**Characterization Studies**

Characterization studies of Fe$_3$O$_4$ nanoparticles, silica coated Fe$_3$O$_4$ nanoparticles, silane conjugated Fe$_3$O$_4$ nanoparticles were done by using Dynamic Light Scattering Analysis, Transmission Electron Microscope (TEM) and Energy-dispersive X-ray Spectroscopy (EDX) methods (Figure 2).

![Figure 2.](image)

(a) DLS analysis diagram (b) EDX and (c) TEM image of silane conjugated Fe$_3$O$_4$ nanoparticles.

**Radiolabeling with $^{125}$I Procedure**

Iodogen coated tubes were prepared as containing 250 µg of iodogen. The solvent was allowed to evaporate forming a thin solid layer on the wall of the reaction vial. Tubes were kept at 4 °C. 50 µg of samples (TQ, TQG, TQ-Fe$^3$O$_4$ and TQG-Fe$^3$O$_4$) were added to prepared iodogen coated tubes and then 0.1 mCi/0.1 mL $^{125}$I was added. The mixture was incubated for 30 min. at airtight conditions.

**Quality control studies of radiolabeling compounds**

The thin layer radio chromatography (TLRC) was used to determine the radiolabeling compounds ($^{125}$I-TQ, $^{125}$I-TQG, $^{125}$I-TQ-Fe$^3$O$_4$ and $^{125}$I-TQG-Fe$^3$O$_4$).

10 cm × 1.5 cm sized cellulose TLC strips were used as stable phase and 4 developing baths were used as mobile phase. The content of the developing baths is: n-butanol / bidistilled water / acetic acid (4:2:1) (TLRC1), n-butanol / ethyl alcohol / 0.2 N NH$_3$OH (5:2:1) (TLRC2), isopropanol / n-butanol / 0.2 N NH$_3$OH (4:2:1) (TLRC3) and isopropanol / n-butanol / 0.2 N NH$_3$OH (2:1:1) (TLRC4). Two µL of radio ligand was applied to Thin Layer Chromatography (TLC) strips and placed to the solutions. After mobile phase reached the end-
point, TLC strips were removed from bath solutions and left to dry. Radiochromatograms of TLC strips were obtained by Bioscan AR2000 TLRC scanner. Rf values of radiolabeled compounds and radiolabeling yields were calculated. Because of the higher separation efficiency TLRC1 developing bath was used in determining radiolabeling yield. Rf values of radioactive components were given in Table 2.

**Table 2.** Rf values of 125I, Oxi, 125I-TQ, 125I-TQG, 125I-TQ-Fe3O4 and 125I-TQG-Fe3O4 at TLRC1 solution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf</th>
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<tr>
<td>125I-TQ</td>
<td>0.39</td>
</tr>
<tr>
<td>125I-TQG</td>
<td>0.87</td>
</tr>
<tr>
<td>125I-TQ-Fe3O4</td>
<td>0.45</td>
</tr>
<tr>
<td>125I-TQG-Fe3O4</td>
<td>0.15</td>
</tr>
<tr>
<td>125I-TQG-Fe3O4</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Cellulose TLC strips, n-butanol / bidistilled water / acetic acid (4:2:1) (TLRC 1)

**Cell Culture Studies**

Human lung adenocarcinoma cells (A549) were grown in MEM supplemented with 10 % FBS, 2.0 mM glutamine, 0.1 mM nonessential amino acid, 1.5 g/L sodium bicarbonate and 1 mM sodium pyruvate and; normal human lung epithelial cells (BEAS-2B) were grown in Clonetics BFGM BulletKit. All cells were grown at 37 °C in a humidified incubator equilibrated with 5.0% CO2. The cells were maintained in exponential growth by sub-culturing with trypsin-EDTA (0.25% by w/v in Hank’s balanced salt solution). The cells were then pelleted and resuspended in cell medium. In cell culture studies, samples that are conjugated to magnetic nanoparticles were exposed to a constant magnetic field (1.17-1.21 Tesla) while studies. Control and other nonmagnetic samples were not treated with the external magnetic field.

**Cytotoxicity Studies**

Cytotoxicity studies of TQ, TQG, Fe3O4, TQ-Fe3O4, TQG-Fe3O4, TQ-Fe3O4 (MF) and TQG-Fe3O4 (MF) were performed on A549 cells. Each parameter was performed in two different days and repeated three times. IC50 values of samples were calculated by WST-8 test as a colorimetric way. The cell suspension was prepared as 10^5 cells/ml for each well at 96-well plate. One hundred µL of cell suspension and 5 different concentrations of sample were added to each well. Cell and medium without regent was used as negative control. Cells were incubated at 37 °C in a humidified incubator equilibrated with 5.0% CO2 for 72 hours. After incubation 10 µl of WST solution was added to the wells and incubated for 4 hours. Absorbance values (OD) were obtained at 450 nm wavelength and 690 nm reference range by spectrophotometric method. Zero absorbance was ap-
plied as negative control. Cytotoxicity values (%) were calculated by using this formula;

\[
\text{% Cytotoxicity} = \left( \frac{\text{measured optical density value}}{\text{control value}} \right) \times 100
\]

**Apoptosis Study**

Apoptotic effects of samples were evaluated on A549 and BEAS-2B cells by using the Dead Ende Fluoro-metric TUNEL System (Promega) according to the manufacturer’s instructions as described below. Minimum 300 cells were evaluated for each group. Cells were prepared on chamber slides. After 24 hours, mediums of cells were changed with mediums, which contain samples in IC\textsubscript{50} concentrations. After 24 hours, medium of chamber slides was removed and washed with PBS 2 times. Slides with cells were incubated in PBS with paraformaldehyde (4%) (pH 7.4) solution at 4°C, for 25 minutes. Then the slides were incubated in PBS with Triton-X (0.2%) for 5 minutes to increase the cell permeability. Then the slides were washed with PBS 3 times for 5 minutes. One hundred µL of equilibration tampon was added to the slides and incubated for 10 minutes. During this time, rTdT incubation tampon was prepared: 90 µL equilibration tampon, 10 µL nucleotide mixture and 2 µL rTdT enzyme. After 5-10 minutes, liquid in the slides were removed and nucleotide mixture was added instead of this, then lamella was used. Samples were incubated at 37°C for 60 minutes. At room temperature, samples were incubated in 2XSSC for 15 minutes to stop the reaction. The slides were washed with PBS for 5 minutes and this process was repeated 3 times. The slides were incubated in propidium iodid solution (1 µg/mL) for 15 minutes. After the slides were dried, the cells were imaged by fluorescence microscope by using green (520 nm) and red (620 nm) filters.

**Time Dependent Incorporation Study of \textsuperscript{125}I Labeled Samples on Cells**

Cells were grown to determine the optimum time parameter for incorporation of \textsuperscript{125}I labeled TQ, TQG, TQ-Fe\textsubscript{3}O\textsubscript{4}, TQG-Fe\textsubscript{3}O\textsubscript{4}, TQ-Fe\textsubscript{3}O\textsubscript{4} (MF) and TQG-Fe\textsubscript{3}O\textsubscript{4} (MF) on cells. 24-well plates were prepared and time parameters specified as 30., 60., 120. and 240. minutes.

0.1 µg/mL of sample and \textsuperscript{125}I 1 µCi/mL were used for per well. 0.5 mL of \textsuperscript{125}I labeled ligand was added to the wells. 1 µCi/mL of \textsuperscript{125}I was used to determine the incorporation of unbound \textsuperscript{125}I. Only medium was added to cells as a control. Cells were incubated at 37 °C. When incubation time was over, cells were washed with PBS two times and suspended by treating with 200 µL RIPA lyse buffer solution. After 30, 60, 120 and 240 minutes, the protein concentration of the lysed cell suspension (25µL) was determined by the Bicinchoninic acid (BCA) kit. Also, 100 µL of lysed cell suspension was used to count the radioactivity by a liquid
scintillation counter (Packard TriCARB-1200). Radioactivity corresponding to per µg of protein was used in the calculation of incorporation values.

**Statistical analysis**
The statistical significance of cell culture studies was assessed by one-way ANOVA and linear regression using the Graph Pad (prism 2.01V) program. Probability values $P < 0.05$ were considered statistically significant for studies.

**RESULTS AND DISCUSSION**

**Structural Analysis Studies of TQG and $^{127}$I-TQG Compounds**

Table 1 shows LC-MS/MS m/z values of the TQG and $^{127}$TQG compounds and some other fragments. The identification of the TQG was performed using LC-MS/MS with positive mode \([M+H]\) allowed the detection of corresponding molecular ion at m/z 488.48. $^{127}$TQG and some fragments were 454, 334.22, 274.51, 260.56, 246 respectively. It is seen that the glucuronidation occurred on hydroxyl groups by transforming the carbonyl group in quinone ring of molecule to quinol.

**Characterization of Silane Conjugated Fe$_3$O$_4$ Nanoparticles**

*Dynamic Light Scattering (DLS)*: Dynamic Light Scattering analysis of silane conjugated Fe$_3$O$_4$ nanoparticles in ethanol was performed by Malvern Nano ZS equipment. The average dynamic size of the silanated nanoparticles is 200 nm (Figure 1).

*TEM*: When Figure 1 is examined, it is seen that there is an aggregation between nanoparticles and size of nanoparticles is ranging between 7.63-26.95 nm. Also, the resulting nanoparticles were found to be spherical in shape.

*EDX*: As seen in Figure 1, the amount of Fe, O and Si are compatible with the elemental structure according to EDX results of silane conjugated Fe$_3$O$_4$ nanoparticles. The results are appropriate with expected structure.

**Quality Control Studies by Chromatographic Methods**

*High Performance Liquid Chromatography (HPLC)*: Retention time (R$_t$) values of some compounds are given in Figure 2, R$_t$ value of TQ is determined as 2.65 min. while TQG’s is 33.35 min R$_t$ value.

This result shows that the reaction of glucuronidation was performed with yield of 69.16±3.28 % (n=3). R$_t$ values of radiolabeled compounds are given in Table 3.
Table 3. \( R_t \) values of \( ^{125}\text{I}, \text{Oxi.}^{125}\text{I}, ^{125}\text{I-TQ}, ^{125}\text{I-TQG}, ^{125}\text{I-TQ-Fe}_3\text{O}_4 \) and \( ^{125}\text{I-TQG-Fe}_3\text{O}_4 \).

<table>
<thead>
<tr>
<th></th>
<th>( ^{125}\text{I} )</th>
<th>( ^{125}\text{I-TQ} )</th>
<th>( ^{125}\text{I-TQG} )</th>
<th>( ^{125}\text{I-TQ-Fe}_3\text{O}_4 )</th>
<th>( ^{125}\text{I-TQG-Fe}_3\text{O}_4 )</th>
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<tbody>
<tr>
<td>( R_t )</td>
<td>2.23</td>
<td>5.12</td>
<td>5.26</td>
<td>5.77</td>
<td>6.03</td>
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</table>

The thin layer radio chromatography (TLRC): \( R_t \) values of the radiolabeled components (\( ^{125}\text{I} \) and Oxi. \( ^{125}\text{I}, ^{125}\text{I-TQ}, ^{125}\text{I-TQG}, ^{125}\text{I-TQ-Fe}_3\text{O}_4 \) and \( ^{125}\text{I-TQG-Fe}_3\text{O}_4 \)) for TLRC1 developing bath are given in Table 3. The separation between compound peaks was satisfactorily achieved and radiolabeling yields of all compounds (TQ, TQG, TQ-\( \text{Fe}_3\text{O}_4 \) and TQG-\( \text{Fe}_3\text{O}_4 \)) were determined over 95%.

Cytotoxicity Studies

The cytotoxic effects of the samples were investigated on A549 cell lines via WST-8 cytotoxicity assay kit. Five different concentrations were tested after 24, 48 and 72h of incubation at 37 °C. The obtained results are summarized in Figure 3. \( \text{Fe}_3\text{O}_4 \) nanoparticle conjugated samples have a negligible cytotoxic effect on the cell line for 24h. \( \text{IC}_{50} \) values of samples were calculated and given Table 4. The results show that neither \( \text{Fe}_3\text{O}_4 \) nor \( \text{Fe}_3\text{O}_4 \) nanoparticle conjugated samples are cytotoxic at the concentration studied until 12.5 µg/mL for 24h. The cell viability of A549 is more than 80% for concentrations < 6.25µg/mL until 48h. However, in all concentrations the cytotoxic effect of \( \text{Fe}_3\text{O}_4 \) nanoparticle conjugated samples was less than \( \text{Fe}_3\text{O}_4 \).
Figure 3. Viabilities of A549 cells after a) 24h b) 48h c) 72h incubation.
Table 4. IC\textsubscript{50} values of samples on A549 cells.

<table>
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<th>A549</th>
<th>TQ</th>
<th>TQG</th>
<th>TQ-Fe\textsubscript{3}O\textsubscript{4}</th>
<th>TQG-Fe\textsubscript{3}O\textsubscript{4} (\text{MF})</th>
<th>TQ-G-Fe\textsubscript{3}O\textsubscript{4} (\text{MF})</th>
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<td>IC\textsubscript{50} ((\mu g/mL))</td>
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<tr>
<td>24 hours</td>
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<td>72 hours</td>
<td>6.59</td>
<td>15.62</td>
<td>12.40</td>
<td>11.88</td>
<td>7.04</td>
<td>9.579</td>
</tr>
</tbody>
</table>

Comparison of Incorporation Efficiency between A-549 and BEAS-2B cells

Comparison of incorporation efficiency of \(^{125}\text{I}\) labeled samples on BEAS-2B and A549 cells is shown in Figure 4. After 2 hours, \(^{125}\text{I}-\text{TQ}\) and \(^{125}\text{I}-\text{TQG}\) were incorporated by healthy cells higher than carcinoma cells, and after conjugation of Fe\textsubscript{3}O\textsubscript{4} affinity to carcinoma cells increased 2 times for \(^{125}\text{I}-\text{TQ-Fe}_3\text{O}_4\) and increased 3 times for \(^{125}\text{I}-\text{TQG-Fe}_3\text{O}_4\). This ratio increases 5-fold for \(^{125}\text{I}-\text{TQ-Fe}_3\text{O}_4\) and 6-fold for \(^{125}\text{I}-\text{TQG-Fe}_3\text{O}_4\) after magnetic field.

![Figure 4](image.png)

Figure 4. Comparison of time dependent incorporation ratios of the \(^{125}\text{I}\) labeled samples on BEAS-2B and A549 cell lines (Dose: 1 \mu g ligand/1 \mu Ci \(^{125}\text{I}\), Incubation time: 2h).
Apoptosis Study
According to results (Figure 5), there is no apoptotic effect of the samples on BEAS-2B cells was found. It is seen that magnetic nanoparticle conjugated TQG (TQG-Fe₃O₄), could inhibit the apoptosis on A549 cells. With magnetic field TQG-Fe₃O₄ could increase the apoptosis of the cells more than TQG-Fe₃O₄ and there is no inhibition of apoptosis for other samples.

**Figure 5.** Apoptosis assay images of the TQG, TQG-Fe₃O and TQG-Fe₃O₄(MF) on A549 and BEAS-2B cells (left images are for A-549 cells, right images are for BEAS-2B cells).
**Time Dependent Incorporation Study**

Results of time dependent incorporation study of $^{125}$I labeled samples on A549 cells are given in Figure 6. Incorporation ratios of all samples increased by the time and the highest amount is determined for Fe$_3$O$_4$ conjugated TQG. The magnetic field has a dramatic effect on the incorporation ratios with the compounds loaded onto magnetic nanoparticles.

![Figure 6](image_url)

**Figure 6.** Time dependent incorporation ratios of the $^{125}$I labeled samples on A549 cells.

Results show that magnetic nanoparticles could be used as efficient carriers for the bioactive molecules along with drugs, and these nanoparticles might be easily accommodated to the targeted for diagnosis and therapy applications$^{16}$.

**CONCLUSION**

Preparation of TQ glucuronide derivatives, labeling of them with $^{131}$I or with other radionuclides ($^{125}$I, $^{124}$I, $^{123}$I, other radiohalogenated isotopes and $^{211}$At), conjugation with Fe$_3$O$_4$ nanoparticles and preparing radionuclide labeled TQG conjugated Fe$_3$O$_4$ nanoparticles of solid, semi-solid and liquid formulations, in which TQG's prepared using enzyme preparations prepared from human cancer cells in the synthesis, obtaining radionuclide labeled of them by attaching with radioiodine, preparing of them solid, semi-solid and liquid dosage forms.

TQ glucuronide preparation of derivatives $^{131}$I or with other radionuclides ($^{125}$I, $^{124}$I, $^{123}$I, other radiohalogenated isotopes and $^{211}$At) labeling, conjugating with Fe$_3$O$_4$ nanoparticles prepared TQG conjugated Fe$_3$O$_4$ nanoparticles of solid,
semi-solid and liquid form is the preparation of formulations, in which by molecules inserted radionuclide feature cancer Single Photon Emission Tomography (SPECT) and Positron Emission Tomography (PET) radionuclide labeled TQ of which enables the use of imaging TQ or TQG which is conjugated to Fe\textsubscript{3}O\textsubscript{4} nanoparticles.

TQ glucuronide derivatives preparation of labeling with \textsuperscript{131}I or with other radionuclides \textsuperscript{120}I, \textsuperscript{124}I, \textsuperscript{125}I, other radiohalogenated isotopes and \textsuperscript{211}At, conjugated with Fe\textsubscript{3}O\textsubscript{4} nanoparticles prepared thymoquinone radionuclide labeled glucuronide (TQG) conjugated Fe\textsubscript{3}O\textsubscript{4} nanoparticles of solid, semi-solid and liquid form is the preparation of formulations, in which MR which enables the use in imaging lymph node or tumor contains radionuclides or radionuclide transport marked the thymoquinone or thymoquinone glucuronide bound Fe\textsubscript{3}O\textsubscript{4} nanoparticles.

TQ glucuronide derivatives of labeled with \textsuperscript{131}I or with other radionuclides \textsuperscript{125}I, \textsuperscript{124}I, \textsuperscript{123}I, other radiohalogenated isotopes and \textsuperscript{211}At labeled, conjugated with Fe\textsubscript{3}O\textsubscript{4} nanoparticles, prepared thymoquinone radionuclide labeled glucuronide (TQG) conjugated Fe\textsubscript{3}O\textsubscript{4} nanoparticles of solid, semi-solid and liquid form is the preparation of formulations, in which according to the different cancer molecule attached radionuclide feature that can be used in diagnostic and therapeutic radionuclides or contains TQ marked TQG connected Fe\textsubscript{3}O\textsubscript{4} nanoparticles.

Thymoquinone glucuronide preparation of derivatives \textsuperscript{131}I or with other radionuclides \textsuperscript{125}I, \textsuperscript{124}I, \textsuperscript{123}I, other radiohalogenated isotopes and \textsuperscript{211}At labeled, conjugated with Fe\textsubscript{3}O\textsubscript{4} nanoparticles, prepared thymoquinone radionuclide labeled glucuronideconjugated Fe\textsubscript{3}O\textsubscript{4} nanoparticles of solid, semi-solid and liquid form is the preparation of formulations, in which In the treatment of different cancer with hyperthermia and providing thymoquinone (TQ) be used to monitor therapy or thymoquinone glucuronide (TQG) which is connected Fe\textsubscript{3}O\textsubscript{4} nanoparticles.

TQ glucuronide preparation of derivatives \textsuperscript{131}I or with other radionuclides \textsuperscript{125}I, \textsuperscript{124}I, \textsuperscript{123}I, other radiohalogenated isotopes and \textsuperscript{211}At) marking, conjugated to Fe\textsubscript{3}O\textsubscript{4} nanoparticles, prepared thymoquinone radionuclide labeled glucuronide (TQG) conjugated Fe\textsubscript{3}O\textsubscript{4} nanoparticles of solid, semi-solid and liquid form is the preparation of formulations, in which beta-glucuronidase rich in the enzymes that allow the use of different cancers in vitro diagnostic kit for the diagnosis of cancer TQ or TQG bound Fe\textsubscript{3}O\textsubscript{4} nanoparticles it contains.

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