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RESEARCH ARTICLE

A new theranostic complex based on bismuth-iron oxide nanoparticle for myocardial ischemia: in vitro study

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ABSTRACT

Objective(s): Magnetic nanoparticles of bismuth oxide and iron oxide (BOIO NPs) are extensively used for more accurate medical diagnosis in MRI and CT imaging due to their improved X-ray absorbance and magnetic properties. The nanoparticles (NPs) utilized in the diagnosis of damaged tissue can also be considered an improvement factor in treatment. In this study, an anti-inflammatory drug, indomethacin, was loaded on BOIO NPs.

Methods: The BOIO NPs were synthesized using the hydrothermal precipitation method. To ensure stable dispersion, the NPs were coated with silica-polyethylene glycol (PEG). Indomethacin was also used to alleviate the initial inflammation. The drug was loaded on the PEG shell with a solvent.

Results: Cytotoxicity and anti-inflammatory tests were carried out on H9C2 cells and H2O2 treated cells. MRI and CT signal amplification was evaluated in the phantom. In MRI and CT, the signal surge of these NPs investigated at four concentrations (0, 0.85, 1.7, 3.4 mg/ml) by significant differences ($P< 0.05$). The best drug loading efficiency (DLE) and drug loading content (DLC) were measured as 58.5 ±8 % and 10.5 ±1.3 % respectively. Invitro drug release evaluated after 72 h was about 44.08±0.01%.

Conclusions: According to the results of MRI and CT techniques and signal amplification relative to water for PEGsi-BOIO NPs, it is concluded that these NPs could be employed to track any inflammated tissue. The cytotoxicity results demonstrated the improvement of the induced reactive oxygen species (ROS) in H9C2 tissue cells and its capability through the delivery of indomethacin.

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INTRODUCTION

Experimental evidence suggests that Myocardial Infarction (MI) is associated with the activation of inflammatory reactions. Inflammatory mediators are directly involved in the pathogenesis of vulnerable plaque, leading to coronary artery occlusion and necrosis in the myocardial territory. Cardiomyocyte necrosis also stimulates the systemic inflammatory response, leading to the infiltration of circulating inflammatory cells, which helps filter out dead cells and matrix residuals. Although leukocyte subsets play an important role in repairing myocardial infarction, the long-term activation of inflammatory pathways is also involved in chronic adverse left ventricular remodeling and heart failure [1, 2].

Indomethacin is one of the non-steroidal anti-inflammatory drugs used in MI to prevent its

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spread. However, caution should be practiced in its application [3]. The use of this drug is highly dependent on its dose and MI size [4]. Therefore, it seems essential to adopt a method for targeted delivery of this drug and alleviation of its side effects.

There is ample evidence that ischemic heart can be passively targeted by drug-containing NPs [5]. Passive targeting mainly draws on high permeability and high retention effects (EPR) [6], which is also characteristic of ischemic areas. Hence, in this study, NPs have been used to deliver indomethacin to ischemia.

The diagnosis of myocardial ischemia by two non-invasive MRI and CT techniques has received growing attention. Since ischemic heart disease is fairly prevalent, improving the diagnosis and function of the disease with non-invasive imaging techniques could have a direct impact on the development of a more cost-effective diagnosis and therapeutic method [7]. The CT technique, which is based on late iodine enhancement, is similar to MRI-based late gadolinium enhancement [8]. The CT technique offers fast scanning and high spatial resolution, but it has poor soft-tissue contrast and a considerably high dose of radiation. Heavy metal NPs (including high Z elements such as Au, Pt, Bi, Ta, Gd, and Lu17, 18) are promising as contrast agents (CA) in computed tomography (CT) due to their high X-ray photon capture cross section and Compton scattering effect. The NPs based on Bismuth have been employed as the CT-CA which are commonly used in clinical imaging [9]. MRI, on the other hand, offers higher soft-tissue contrast and functional and molecular imaging capabilities but suffers from long scanning time and poor geometric accuracy. It is also difficult to measure MRI in absolute units. Therefore, a hybrid of these two methods can be highly efficient in diagnosis [10]. The coordination complexes of iron (III) and organic molecules showed excellent T1 MRI signals [11]. Magnetic iron oxide-based NPs with T1 contrast and bismuth have been the subject of growing attention as signal intensifiers in MRI [12, 13] and CT techniques [14]. Fe2O3 iron oxide NPs have magnetic properties in Ms (0.9 emu/g) [15] [16] which can be used in MRI imaging.

A combination of these two in the form of nanocrystals and core-shell bismuth-oxide iron can be used in both MRI and CT techniques [17, 18].

In this research, BOIO NPs with silane-polyethylene glycol (silane-mPEG) coating have been utilized. PEGsi-BOIO NPs have been designed in an attempt to improve image contrast in the MRI/CT hybrid. Indomethacin was utilized to alleviate inflammation in MI and prevent ischemia. The drug was inserted in the mPEG coating of these NPs to contribute to the diagnosis and improvement of ischemia. The efficacy of the drug loaded on these NPs was assessed on H9C2 cells and the enhanced MRI and CT signal by these NPs was investigated.

Experimental section

Synthesis of BOIO NPs

Iron II sulfate hydrate (2.5 mM, 97% Merck), bismuth nitrate pentahydrate (0.5 M, 98% Sigma) and sodium nitrate (50 mM, 98% Merck), as well as 20 ml of sulfuric acid (0.2 M, 98% Merck), were completely dissolved in a 160 ml volume of 50% ethanol (98%, IndiaMART) and placed in a three-necked flask at 90 °C on a magnetic stirrer for 0.5 h. Finally, sodium hydroxide (97-98% Merck) was added dropwise at a concentration of 66 mM, which formed a reddish-brown deposit. The reaction vessel was placed in an oven for 24 h at 90 °C. The NPs were centrifuged three times by deionized water (DI) for 3 min at 2000 rpm. In this step, the core–shell bismuth- iron oxide NPs were fabricated [17].

Silane-PEGylation of BOIO NPs

BOIO NPs containing 2.5 ml of DI water in 150 ml of 2-propanol containing 75 µl of tetraethoxy

Silane (TEOS, 98% Sigma) were dispersed in the reaction vessel. Also, 4 ml of ammonia solution (25%, Merck) was added dropwise to the reaction vessel containing the NPs at room temperature and then placed on a magnetic stirrer under argon gas for 10 min. The NPs were then washed by centrifugation at 10,000 rpm for 0.5 h with 2-propanol (95%, Sigma) solvent three times. The NPs were sintered at this stage and then centrifuged three times at 10,000 rpm with acetonitrile solvent for 0.5 h. After that, 10 ml of acetonitrile containing 0.25 g mPEG-silane (M_w=5000, Sigma) already dissolved in 2 ml of DI water was added to the dispersed NPs in 20 ml of acetonitrile and placed on a magnetic stirrer for 3h at ambient temperature. Finally, the PEGsi-BOIO NPs were separated by centrifugation at 3000 rpm for 15 min and at 5000 rpm, which was then repeated with 50% acetonitrile before being centrifuged 3 times with DI water at 10,000 rpm [17].

Steps of drug loading in PEGsi-BOIO coating

Indomethacin (99%, Supplied by Jalinous pharmaceutical company, Iran) was detected in chloroform solvent at 318-320 nm by the spectrophotometer (CECIL UV-VIS model CE 7250). First, 20 mg of the drug is dissolved in 2 ml of methanol solvent as the initial stock. The NPs (100 mg) were washed several times and their supernatant was removed, and then divided in four 25 mg of NPs in four falcons to which 1000, 500, 200 and 100 µl (10, 5, 2 and 1 mg) of drug stock was added to them. The final volume of methanol was completed to 1 ml in each falcon. Next, all three samples were placed in an ultrasonic bath for approximately 30 min and then placed in a freeze dryer to dry the methanol. Finally, 2 ml chloroform was added to each sample to remove the unabsorbed drug and the product was placed in an ultrasonic bath for 10 min. The chloroform solvent is easily separated from the NPs at 4 °C after centrifugation. A UV-VIS spectrophotometer in the absorbance spectrum of 318-320 nm was used to determine the concentration (c) of unabsorbed and chloroform-soluble drugs. Drug concentration calibration was performed using a linear model regression and R^2 = 0.98 in the chloroform solvent. The drug loading efficiency and drug loading content (%) were calculated from the following formula:

$$Drug\ loading\ \ efficiency\,(\%) = \frac{(M - c \times v)}{M} \times 100 \qquad (1)$$

$$Drug\ loading\ \ content\,(\%) = \frac{(M - c \times v)}{25 + (\mathrm{M} - \mathrm{cv})} \times 100 \qquad (2)$$

Where M is the mass of the initial drug (10, 5, 2 and 1 mg), c is the concentration of the drug in the supernatant (chloroform) and v is the volume of the chloroform solvent.

Assessment of drug release from PEGsi-BOIO coating

To evaluate drug release from the desired NPs at a concentration of 4.5 mg/ml in 8 ml, a stock containing 25 mg of NPs along with 2.5 mg of the loaded drug was used. The release was carried out in a dialysis tubing of 12 kDa in phosphate buffer at pH = 7.4, containing 0.05% tween 80 (due to hydrophobicity of the drug) and at a temperature of about 37 °C. Sink conditions with a buffer volume of 3 ml were considered in each case of sample removal.

Cell toxicity method

By performing the MTT test, the viability of H9C2 cardiac cells on NPs before and after drug loading in the maximum drug loading state, i.e. for 5 mg of the initial drug, is determined based on three different concentrations of NPs. To do so, 6,000/well H9C2 cells (pasture institute) were placed in a 96-well plate with 100 µl of DMEM-HG medium (Bio Idea). They were then incubated for 24 h. In the next step, it was incubated for 24 h by medium change at three concentrations of 100, 300 and 500 µg/ml of NPs in the culture medium with four replications in the plate. The drug concentration was determined based on the amount of drug loaded on the NPs (10, 30 and 50 µg/ml). After 24 h of incubation, the culture medium was removed. 40 µl of MTT solution (Sigma) at a concentration of 0.5 mg/ml was added to each well. The container of the specimens is placed at 37 °C for 4 h. After incubation, 160 µl of DMSO (99%, Sigma) was added to each well. The container was then wrapped in foil and shaken by a shaker for 15 min. Finally, the absorbance at OD=595 nm within 1 h was read.

Methods to evaluate the anti-inflammatory properties of NPs

MTT assay evaluates the viability of H9C2 cardiac cells after exposure to the inflammatory H_2O_2 (30%, Merck) agent for ROS generation at three different concentrations of NPs (100, 300 and 500 µg/ml). Then, 6,000/well H9C2 cells are placed in a 96-well plate with 100 µl of DMEM-HG medium and then incubated for 24 h. In the next step, the medium is removed from the cell and 100 µl of H_2O_2 is added to the culture medium at a concentration of 500 µM [19] in each well and placed in an incubator under humid air conditions at 37 °C and 5% CO_2 for 4 h. In the next step, after removing the culture medium, three concentrations of 100, 300 and 500 µg/ml NPs before and after drug loading and three concentrations of the target drug (10, 30 and 50 µg/ml) are cured with four repetitions in each well. The positive control group already incubated with H_2O_2 and the negative control group without H_2O_2 are considered. After 24 h of incubation, the culture medium is removed and 40 µl of MTT solution at a concentration of 0.5 mg/ml is added to each well. The container of specimens is placed at 37 °C for 4 h. Following incubation under humid conditions at 37 °C and

5% CO_2, 160 µl of DMSO is added to each well. The container is then wrapped with foil and shaken on a shaker for 15 min. Finally, absorbance at OD=595 nm is read within 1 h.

RESULTS AND DISCUSSION

Image analysis of transmission electron microscopy (TEM)

PEGsi-BOIO NPs revealed in the TEM (EM 208S) are displayed in Fig. 1. As can be seen, the dimensions of the NPs are about 17.2±3.6 nm. The boundary between the NPs by PEG creates a cluster-like bond during drying, as depicted in Fig. 1b.

Analysis of drug loading on PEGsi-BOIO coating

Indomethacin has two maximum wavelength at about 290 and 320 nm [20] but in this research the peak at about of 318 nm was used [21] because in the detectable range of drug concentration the peak at 290 nm has high optical density. Therefore, Indomethacin was detected at a wavelength of 318-320 nm by a spectrophotometer in the chloroform solvent (Fig. 2a). As shown in Fig. 2b, as the drug concentration increases, drug loading content (%) in the PEG coating of the NPs is augmented. This surge is due to the greater difference in concentration and higher penetration of the drug from the solvent into the mPEG.

The best condition of drug loading is 5 mg of the initial drug at an amount of 58.5 ±8 % and 10.5±1.3 % (mean ± SD, n = 3) for DLE and DLC respectively. These conditions are used in cellular inflammation analysis.

Analysis of drug release

The release amount on the first day and after 4 h is about 38.60± 0.35%, indicating that a burst release in the early hours. Release is then observed with a gentle slope, and after 72 h, an amount of 44.08± 0.01% (mean ± SD, n = 3) is released from the initial dose (Fig. 3). The indomethacin drug is hydrophobic and the PEG coating in the aqueous solution swelled and absorbed water causing the hydrophobic drug to leak out of the coating.

Analysis of crystal structure of NPs

After being dried at 100 °C for 48 h, NPs were investigated by X-ray diffraction analysis (2.2KW amp X-ray with Cu LFF anode and 60KV voltage). X-ray diffraction analysis for PEGsi-BOIO NPs in Fig. 5 shows that the two elements of iron oxide Fe_2O_3 with respect to the peak at position 41.45° [22] and bismuth oxide Bi_2O_3 at position 30.65° [23] are in the form of nanoscale crystals in the structure of NPs.

Infrared spectrum analysis

In Fig. 6 according to the absorbance diagram in four different modes, i.e. drug, polyethylene glycol polymer (PEG), PEG-coated NPs, PEG-coated NPs with drug and drug the variation in the chemical structure of the NP and its interaction with the drug can be observed. In the infrared absorbance spectrum of FTIR (TENSOR 27) related to PEG, the absorption peak at 1110 cm-1 related to ether COC stretch of PEG structure [24] is evident in Fig. 6d, but this peak disappears after drug loading in Fig. 6b. One probable scenario is that it is combined in

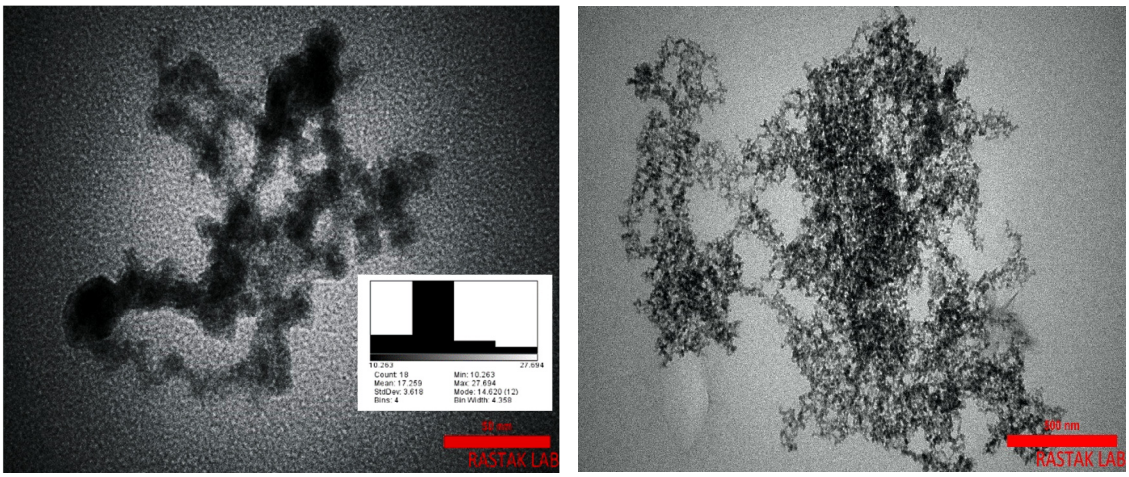

Fig. 1. TEM image of PEGsi-BOIO NPs (a) TEM image of BOIO pegylated (scale bar=50 nm) (b) scale bar=500 nm

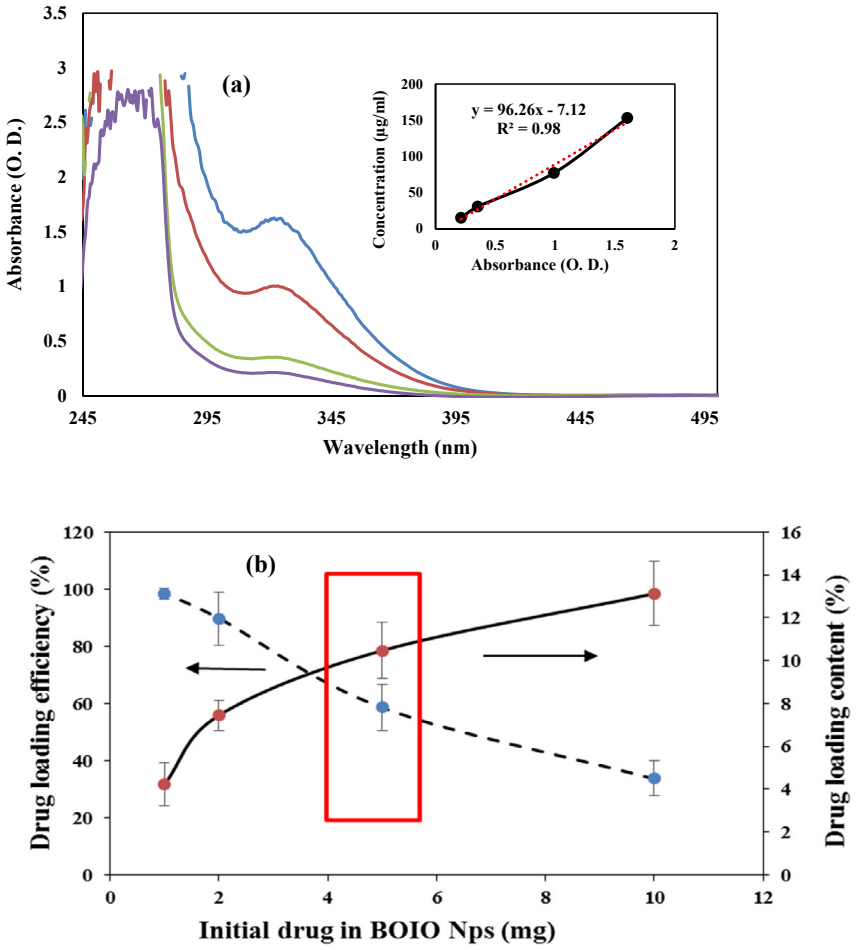

Fig. 2. UV absorbance of indomethacin drug in chloroform (λ_{max}=318-320 nm) (a), Diagram of drug loading efficiency (%) and drug loading content (%) on PEGsi-BOIO NPs per 25 mg NPs (b)

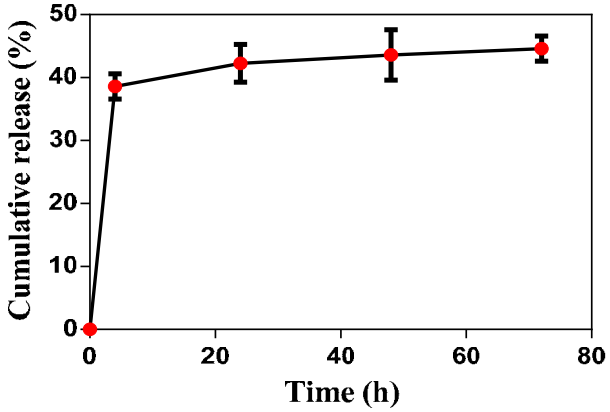

Fig. 3. Diagram of the indomethacin release from PEGsi-BOIO NPs

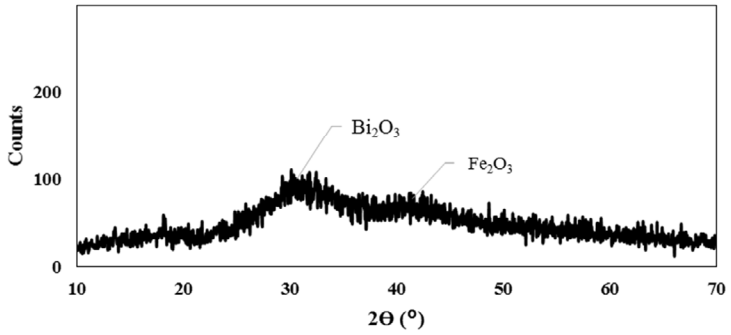

Fig. 5. X-ray diffraction analysis (XRD) for peg-coated NPs

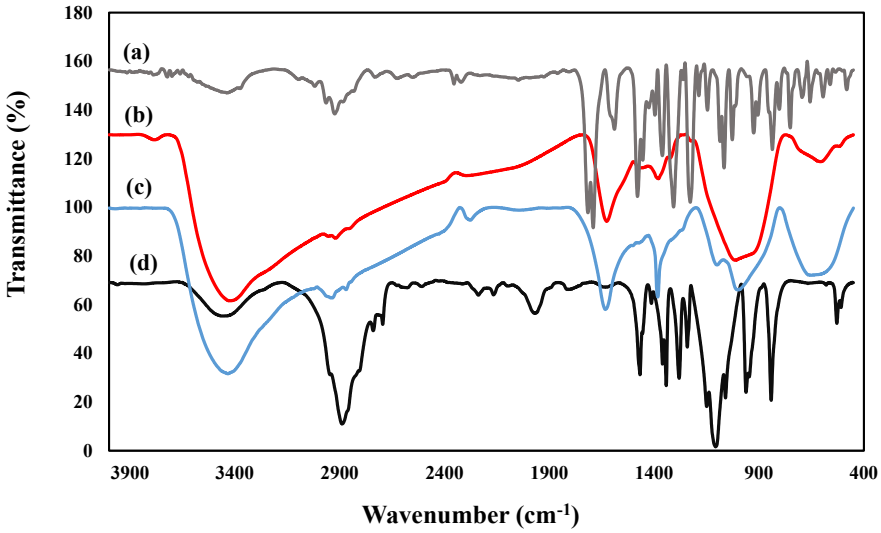

Fig. 6. Diagram of the infrared spectrum in three states, indomethacin (a) PEGylated NPs with drug (b), PEGsi-BOIO NPs without drug (c) and polyethylene glycol polymer (silane-PEG) (d)

the drug structure. According to the drug-related form and the PEGylated NPs along with the drug, it can be indicated that specific regions of the drug-related absorbance peak in Carboxylic acids (O – H bend) 925 cm-1 [25], 2918 cm-1 are related to stretching vibration CH_3 and CH_2 [26] and 3780 cm-1 are present in the NP structure, suggesting that the drug has been successfully incorporated into the PEG structure.

Investigation of toxicity and anti-inflammatory properties of NPs

The viability of H9C2 cardiac cells with NPs before and after drug loading (N and ND, respectively) at the best drug loading condition (i.e. per 5 mg of the initial drug) and based on three different concentrations of NPs (100, 300 and 500 µg/ml) is shown in Fig. 7a. The drug concentrations in each case are equal to 10, 30 and 50 µg/ml, respectively. Normally (without adding H_2O_2 or inflammatory agent), no cytotoxicity was observed in N, ND and drug alone (D). It can be concluded that PEG coating reduces the cytotoxicity of NPs, however, different mechanisms may be important for NPs [27].

MTT assay evaluated the viability of H9C2 cardiac cells after exposure to the H_2O_2 inflammatory agent at three different concentrations of NPs (100, 300 and 500 µg/ml). The results suggest its growing effect on cell viability compared to control (±) of N and ND (Pvalue<0.001). The high improving effect by more proliferation in N group demonstrated the N itself has potentially anti-inflammatory effect because of bismuth (high Z atom) and iron ion by

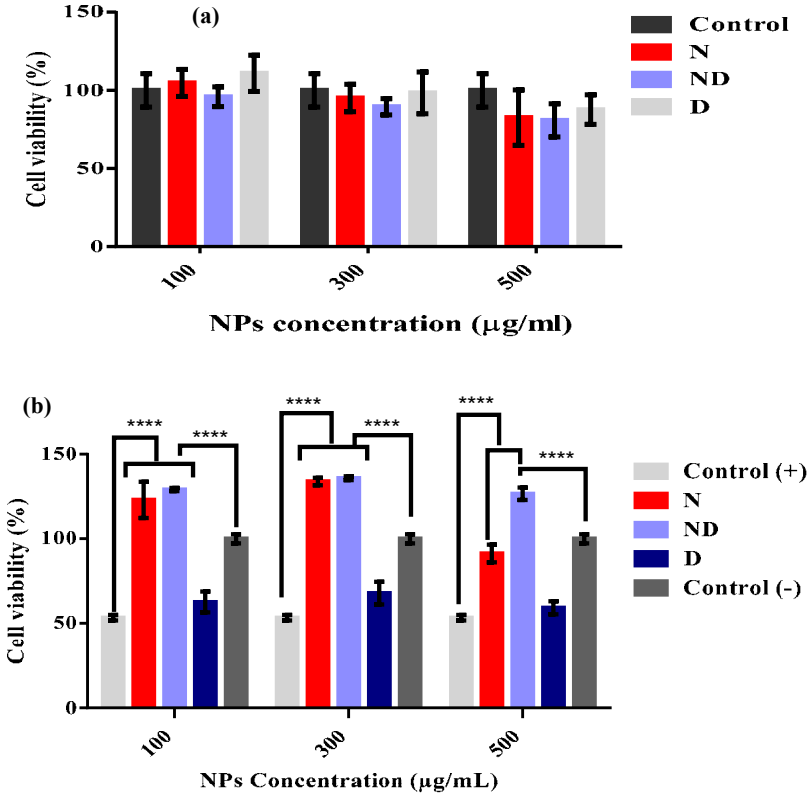

Fig. 7. MTT analysis diagram, NPs in the normal state (in three concentrations of 100, 300 and 500 µg/ml based on PEGsi-BOIO NPs in four groups: control, PEGsi-BOIO NPs (N), PEGsi-BOIO NPs with drug (ND) and indomethacin (10, 30 and 50 µg/ml), which is equal to the amount loaded in NPs (D)) (a), in the inflammatory state with the inflammatory agent H2O2 (in three concentrations of 100, 300 and 500 g/ml based on PEGsi-BOIO NPs for four groups of positive and negative control (control ±), PEGsi-BOIO NPs (N), PEGsi-BOIO NPs with drug (ND) and indomethacin in amounts of 10, 30, and 50 µg/ml) equivalent to the amount loaded on the NPs (D)) (b).

chelating of electrons and radicals. But in more concentration of N, the cytotoxicity of NPs appeared [28] due to inflamated cells were more sensitive than normal cells. In D this growing effect is low in related to control (±) (Pvalue<0.05) because of hydrophobicity of drug and its aggregation. In ND by comparison to N group (Pvalue<0.001) the cell survival increases at 500 µg/ml of NPs. However, at higher concentrations for N it has a slight effect on cell survival. As can be seen, at concentrations of 500 µg/ml, the effect of indomethacin on NPs is higher because of more drug exposure (50 µg/ml) to cells. This may be due to the drug prevented the cytotoxicity of NPs (Fig.7b). The results analyzed by 2wayAnova (Tukey, P_{value}< 0.0001) test and multiple comparison.

RESULTS RELATED TO MRI

For MRI evaluation of NPs, a phantom was used in a chamber contained some wells with water in bottom of chamber. Each wells of phantom contained micro tubes with 1 ml of NPs solution. Enhanced contrast of BOIO and PEGsi-BOIO at three concentrations of 0.85, 1.7, 3.4 mg/ml in MRI imaging (SIEMENS 1.5 Tesla) is depicted in Figs. 9a and b in terms of average transvers relaxation time (R1) of T1-weighted scan (in TRs=800, 1000, 1500, 2000, 2500). As it is evident, increased concentration is associated with visible changes in the amount of signal surge. This growth is also observed in all concentrations of NPs. In general, the signal difference between NPs and water is remarkable (Fig. 8b). For the statistical investigation, the correlation analysis was used and the Pearson r for BOIO NPs and PEGsi-BOIO obtained r=0.9236 and r =0.8221 respectively. The results showed the significant behavior of correlation analysis for BOIO NPs. But the correlation results were not significant for PEGsi-BOIO NPs. T-test (paired) analysis of R1 between

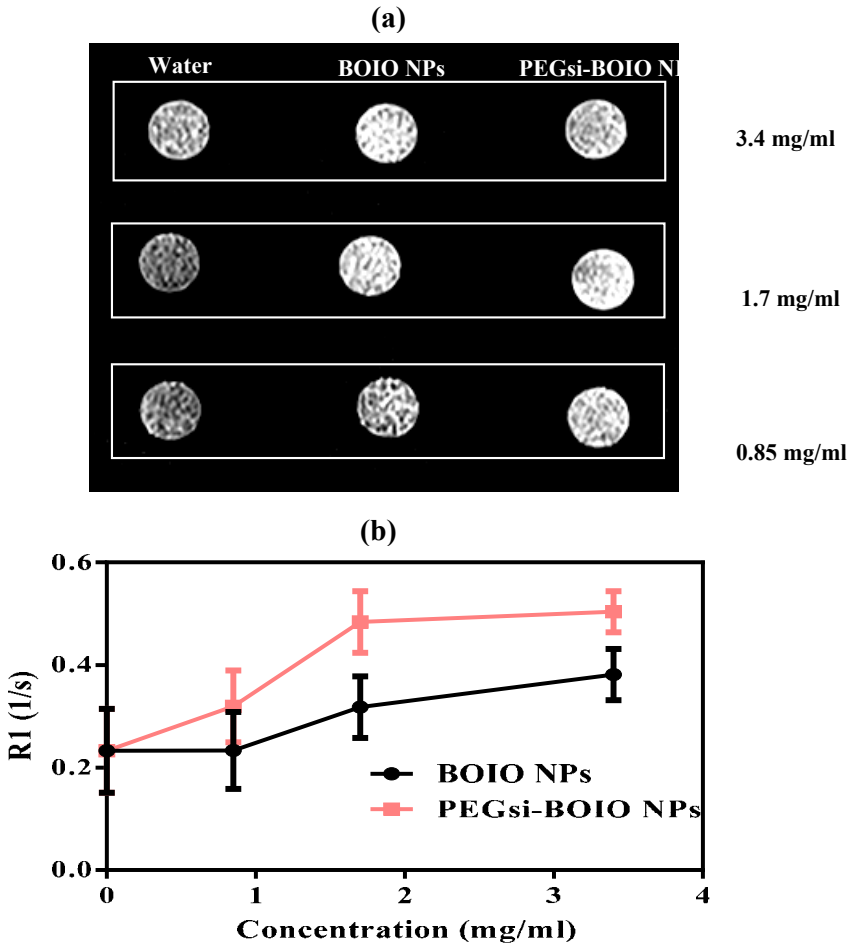

Fig. 8. Investigation of the increased contrast of BOIO and PEGsi-BOIO NPs at four concentrations of 0, 0.85, 1.7, 3.4 mg/ml in MRI imaging technique (a), average relaxation transvers time investigation vs concentration (b)

BOIO and PEGsi-BOIO NPs obtained significant differences with Pvalue=0.0401 (Pvalue<0.05).

Result of CT scan imaging

CT evaluation of NPs was performed in the phantom in Fig. 9. The increased contrast of BOIO and PEGsi-BOIO NP images at four concentrations of 0, 0.85, 1.7, 3.4 mg/ml in CT imaging (SIEMENS 16 slice, slice thickness=2 mm, pitc=1.5, 80-160 kv, 150 mA) in Figs. 10a and b is shown in terms of Hounsfield unit (HU). As can be seen, HU increases as the concentration rises. This surge is still observed in 1.7 and 3.4 mg/ml. In addition, the augmented contrast in PEGsi-BOIO NPs is greater than that of BOIO, which may be due to the improved uniformity and dispersion in the solution by pegilation in NP structure. The distribution of PEGsi-BOIO at each scan improved the HU values in comparison with BOIO NPs. Using linear regression for better comparison, the R^2 of BOIO NPs and pegsi-BOIO were obtained to be 0.91 and 0.97, respectively. The slope of the linear equation for BOIO NPs is estimated to be approximately 4.1 ± 0.9 and for pegsi-BOIO is calculated to be 11.4 ± 1.3. The increasing slope in pegylated structure was investigated in a previous study [29]. T-test (paired) analysis of HU values between BOIO and PEGsi-BOIO NPs obtained significant differences with P_{value}=0.0184 (P_{value}<0.05). In a study for dextran coated bismuth-iron oxide NPs at a concentration of 10.75-15.75 mg/ml, the HU value of the NPs was about 120-300, which by comparing it with the present study, at the concentration between 0.85-3.4 mg/ml, the HU value was obtained about 90-130 HU that the improvement can be related to the percentage of materials in the NPs [30].

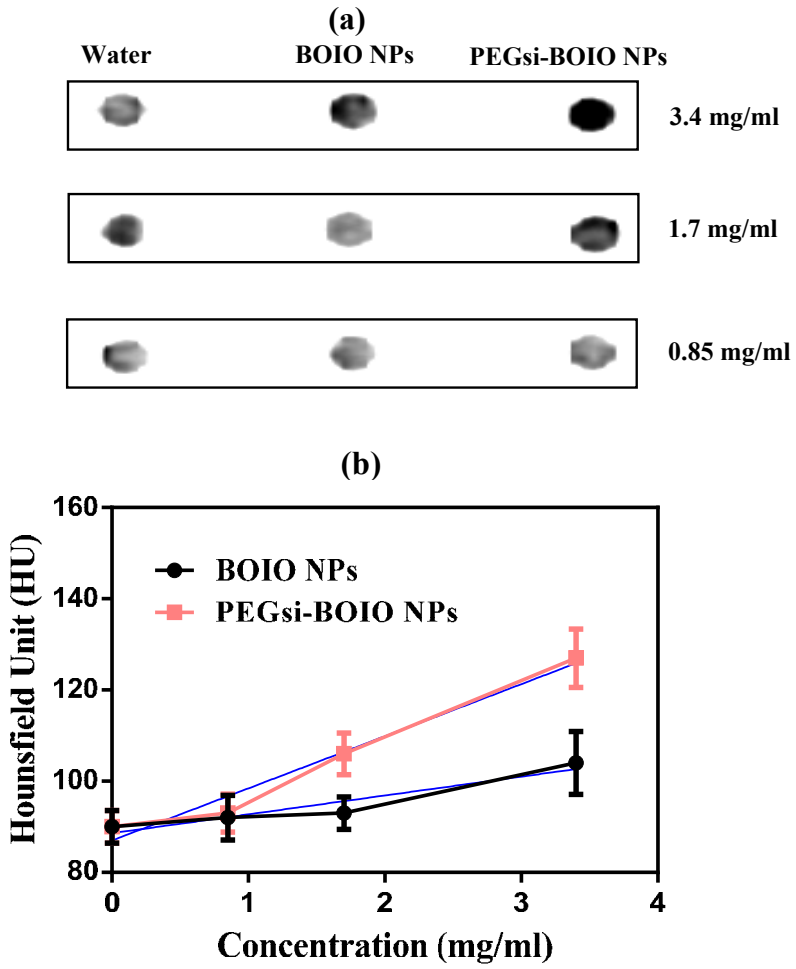

Fig. 9. Investigation of the increased contrast of BOIO and PEGsi-BOIO NPs at four concentrations of 0, 0.85, 1.7, 3.4 mg/ml in imaging technique (a), Hounsfield unit investigation vs concentration in CT scan (b)

CONCLUSION

The analysis of PEGsi-BOIO particle size and morphology by TEM and XRD analysis suggested they are about 17.2±3.6 nm in size and have a crystalline structure. NPs of this size can conveniently penetrate the cell and contribute to the drug delivery into the cell. In addition, NPs together with and without drugs (ND and N groups) at a concentration of 100 and 300 µg/ml prompts cell growth and proliferation. We concluded that N group at the concentration of 100 and 300 µg/ml has potentially growing and proliferation effect on H_2O_2 treated cells because of chelating of electrons. This growth and proliferation in cells treated with H_2O_2 and at concentrations of ND at 500 µg/ml could be due to the high drug concentration of 50 µg/ml delivering in comparison to N group. As a result, the N group in low concentration has potentially improving effect and at high concentration decreased cell viability by more toxicity in inflamated cells. Indomethacin drug by hydrophobicity structure was improved in nano scale and for more investigation the in vivo study must be done in future. Light of the results of MRI and CT signal, a signal increase for PEGsi-BOIO NPs indicated that these NPs, by targeting the damaged tissue and its improvement by targeted delivery of indomethacin, can be easily tracked by these two techniques and demonstrated an improvement process.

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COMPETING INTERESTS

"The authors have no relevant financial or non-financial interests to disclose."

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