Multifunctional magnetic ZnFe$_2$O$_4$-hydroxyapatite nanocomposite particles for local anti-cancer drug delivery and bacterial infection inhibition: An in vitro study

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In this study, a co-precipitation method was applied to synthesize the ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$–Hydroxyapatite (HAp) nanostructures. The microstructure and morphological characteristics of the nanoparticles were studied and well discussed. A dose-dependent biological evaluation comprising of their minimum inhibitory concentration (MIC) antibacterial features as well as their magnetic characteristics were analyzed. The results of vibrating-sample magnetometer (VSM) showed nanoscaled ZnFe$_2$O$_4$–HAp had lower saturation magnetization as well as higher coercive field than ZnFe$_2$O$_4$, which enables the ZnFe$_2$O$_4$–HAp nanoparticles to stimulate cell proliferation, differentiation and adhesion. A remarkable inhibitory effect of the nanoscaled ZnFe$_2$O$_4$–HAp was recognized on bacterial proliferation and growth in the optimal dose, 0.078 mg/L. Besides, a dose-dependent cytocompatibility tests of the nanoparticles on the HEK normal cell and G292 cancer cell was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide assay (MTT). All nanoparticles were cytocompatible and no cytotoxicity effect on normal and cancer cells was observed in the dose-dependent test. In addition, an in vitro test of the drug release from drug-loaded ZnFe$_2$O$_4$–HAp nanocarrier were investigated and well described. The Inhibitory effect of the drug-loaded ZnFe$_2$O$_4$–HAp nanoparticles was investigated in-vitro so that the nanoparticle possessed the ability for inhibiting cancer cell proliferation and growth. By the increment of the nanoparticles concentrations, G292 cancer cell proliferation was inhibited, while, HEK normal cell proliferation was stimulated. Conclusively for the first time, a robust composite based nanostructure as a promising material was developed for multiple applications of bone filler, drug delivery and cancer treatment.

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1. Introduction

Magnetic nanostructures are typically applied for different biomedical conditions due to their excellent properties [1,2]. The applied characteristics of magnetic nanostructures are specifically improving that they could be utilized in various biomedical applications, including drug delivery [3–5], hyperthermia [6,7], cell labeling [8,9], cell separation [10,11], immunoassay [12], biosensors [13,14] and MRI contrasting [15]. Inert surface of magnetic nanostructures is utilized to enhance the cytocompatibility of the nanoparticles because they should be presented into the blood stream [16]. ZnFe$_2$O$_4$ nanoparticles have been received an exceptional deal of attention in medicine nanotechnology because of the low toxicity properties of Zn$^{2+}$ [17]. Zn$^{2+}$ is identified to present a key role in the metabolism of hard tissue; its impact to stimulate the formation of bone and its capability in enhancing the proliferation of the bone-like cells, increasing the expression and promoting the viability of osteoblast and fibroblast cells has been already exhibited in vitro [18,19].

Recently, several developments in synthesis and surface modification of iron oxide nanostructures have been established amongst some magnetic materials [2]. Enhancing the biocompatibility of the particles via surface engineering is one of the best approaches that could qualify the particles for biomedical applications. One of them is coating of bioglass, ceramics and hydroxyapatite (HAp) on magnetic particles [20–22]. Several researches have been established on various magnetic nanostructures of ferromagnetic ceramics which prepare magnetic features for hyperthermia and MRI targets [2]. An important mineral material in the hard tissues is HAp,

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with a formula of Ca₁₀(PO₄)₆(OH)₂. Nanostructured HAp demonstrates the superior characteristics varying from its bulk in physicochemical and biological behaviors [23]. HAp contains appropriate cytocompatibility features and sufficient bio-degradation rate that has been broadly utilized in bone replacements and also in drug delivery systems for targeted and controlled release [2,24]. Moreover, the physical, chemical and biological properties of the synthesized HAp can be tailored by changing its compositions and crystal structures [25]. In the other hand, HAp has also shown an interesting inhibitory effect on the some cancer cell growth in vitro and in vivo including MGC-803, Os-732, Bel-7402 and breast cancer cells [23].

Zoledronic acid (ZA) is a potent nitrogen-containing bisphosphonate that inhibits osteoclastic cells while can have an effect on the bone remodeling simultaneously as well as bone metastasis treatment [26]. The ZA antitumor features is originated from inhibiting the apoptosis stimulation, decreasing the cancer cell proliferation, avoiding angiogenesis and decreasing the cancer cell adhesion, invasion, and migration. The verification of clinical trials demonstrates that ZA amends long-time survival of cancer patients [27]. Moreover, ZA is known as bone-targeted agent for bone drug delivery systems due to its affinity to bone minerals [28]. Amongst the different materials and bioceramics [29] utilized for bone tissue replacement and bone target site reconstruction, it is clear that hydroxyapatite (HAp) is adjusted as the best drug carrier with good cytocompatibility and osteointegrative features for bone clinical drug delivery [30–32]. Furthermore, since the chemical, physical and structural composition of HAp bioceramic is closely similar to hard tissues, these bioactive materials can be resorbed again in the remodeling process in vivo by bone osteoclast cells and also could enhance the bone formation eventually [33]. Inflammatory of joint and bone defects or tumors involves a lot of people in the world. The massive increment of joint replacements and bone graft surgeries demonstrates that they maybe involve the infection associated medical devices [19,34–36]. Bacterial infections related to implants or tissue graft contaminations are a crucial obstacle that often leads to the graft failure with considerable influence on public health concerns [37]. Additionally, managing the infections associated implants often relies on surgical intervention or proscribed use of oral or intravenous antibiotic administrations that cause bone loss [38]. Therefore, an urgent need exists in clinical setting of the bone defect or bone tumor treatments to improve new biomaterials or materials surfaces that present the multi-functions: promoting the bone regeneration and inhibiting the pathogenic microorganisms growth after bone grafting as well as inhibiting the tumor recurrence after the safe margin ressection [34].

Hereby, in this study a novel targeted multi-drug delivery carrier was synthesized and developed to inhibit the bone tumor cell growth through ZA loaded ZnFe₂O₄–HAp nanocomposite particles while bacterial infections inhibition is also fulfilled through loading the anti-bacterial drug to the nano-carrier. ZA was conjugated to the HAp coated surface, and its drug release profile was investigated by UV–vis spectroscopy. MTT assay was used to validate the drug released from the nanocomposite system. Moreover, the antibacterial properties of the nanoscaled carrier and its was quantified using MIC experiment.

2. Materials and methods

Hydroxyapatite and ZnFe₂O₄ were prepared using chemical co-precipitation method using Ca(NO₃)₂·4H₂O and (NH₄)₂HPO₄ precursors for HAp as well as ZnCl₂ and FeCl₃·6H₂O precursors for ZnFe₂O₄. Moreover, ZnFe₂O₄–HAp nanocomposite particles were synthesized by surface treatments of magnetic nanoparticles with citric acid followed by HAp precursor’s precipitation to form a nanocomposite particle. The synthesis procedures were fully detailed in the supporting information.

The viability and proliferation rate of the G292 osteosarcoma cell lines and human embryonic kidney (HEK) cell line as normal cell were assessed using MTT assay in the presence of the ZnFe₂O₄ and ZnFe₂O₄–HAp nanoparticles. Moreover, Zoledronic acid drug was loaded with 3 different concentrations of 0.01, 0.02 and 0.04 mg/L at 4 °C overnight and the effect of released supernatant at 37 °C on the G-292 and HEK cells viability was assessed by MTT protocol. ZA loading efficiency for each of the nanoparticles was assessed through the following equation:

\[
\text{ZA loading efficiency} = \left( \frac{\text{Total amount of ZA} - \text{amount of free ZA}}{\text{Total weight of nanoparticle}} \right) \times 100
\]

To investigate the antibacterial capability of the ZnFe₂O₄ and ZnFe₂O₄–HAp nanoparticles, their minimum inhibitory concentration of the particles was analyzed using broth micro-dilution technique.

The whole experimental section was fully elaborated in the supporting information.

3. Results and discussion

The functional groups and chemical structure of ZnFe₂O₄/HAp nanocomposites were investigated by Fourier-transform infrared (FT-IR) spectroscopy and shown in Fig. 1. The peaks at 1289 cm⁻¹ and 1046 cm⁻¹ was related to C–H in-plane vibration, whereas C–C out-of-plane ring deformation vibration and C–N stretching vibration were located at 931 cm⁻¹ and 1217 cm⁻¹, respectively. C–H ring out-of-plane bending mode was shown at 631 cm⁻¹ and 789 cm⁻¹. Moreover, a strong band at 572 cm⁻¹ exists in the profile of ZnFe₂O₄ proved the Fe–O stretching vibration mode.

Based on Fig. 1, no ZnFe₂O₄ band could be seen in the nanocomposite spectrum. This may be due to the overlapping of the ZnFe₂O₄ vibrational bands with HAp bands. The intense variations were observed in HAp vibrational bands demonstrated that there is an interaction between HAp and ZnFe₂O₄. In HAp spectrum, the broad bands of –OH groups were displayed at almost 630 cm⁻¹. The broadband was presented at around 960 cm⁻¹ related to ν₁ symmetric P–O stretching vibration of the PO₄³⁻. The band range from 570 cm⁻¹ to 600 cm⁻¹ were corresponded to ν₄
vibration mode of $\text{PO}_4^{3-}$ group that showed there was two sites occupation in the crystal lattice. The peak at 1092 cm$^{-1}$ of $\text{PO}_4^{3-}$ was recognized as the $\nu_3$ vibration band, which was the most strengthened peak among the phosphate vibration modes. The bands in the range of 1366–1370 cm$^{-1}$ were referred to $\nu_2$ asymmetric stretching of the C–O bond of $\text{CO}_2^{3-}$ in both A- and B-type substitutions in HAp lattice. $\text{P}_2\text{O}_7^{2-}$ peak was seen at 1216 cm$^{-1}$. The peaks observed in the range of 1732–1736 cm$^{-1}$ were referred to the combination of $\nu_1$ asymmetric stretching and $\nu_4$ bending of $\text{CO}_2^{3-}$ group. Addition of HAp in the nanocomposite may prevent the substitutions of ions in $\text{ZnFe}_2\text{O}_4$ lattice leading to the reduced strength of some group bands.

X-ray diffraction (XRD) patterns of the synthesized HAp, $\text{ZnFe}_2\text{O}_4$ and $\text{ZnFe}_2\text{O}_4$/HAp nanocomposite particles were shown in Fig. 2. The main diffraction peaks of HAp related to the (002), (102), (201), (112), (300), (202), (130), (222), (312), (213), (321), (140), (402) and (004) planes of hexagonal symmetry in the JCPDS file number 74–0566. The main diffraction peaks of $\text{ZnFe}_2\text{O}_4$ phase were corresponded to the crystal planes of cubic spinel $\text{ZnFe}_2\text{O}_4$ based on the JCPDS file number 82–1049. The main phase of $\text{ZnFe}_2\text{O}_4$ was only seen at (311) plane. The X-ray patterns of the nanocomposite particles exhibited well-fitting of peak intensities and locations with the standard HAp phase. Some research reported similar results in literature which exhibited the functionalization of magnetite particle was well carried out in the HAp hexagonal structure. To more confirmation of HAp structure stability in nanocomposite, the crystallite size and the lattice parameter of $\text{ZnFe}_2\text{O}_4$ and HAp phases in nanocomposite were extracted. HAp lattice parameters in the nanocomposite were in the limit of $a = 0.9350–0.9407 \text{nm}$ and $c = 0.6837–0.6896 \text{nm}$, near to the standard HAp phase ($a = 0.9418 \text{nm}$ and $c = 0.6884 \text{nm}$). The average crystallite sizes of $\text{ZnFe}_2\text{O}_4$ and HAp in the nanocomposite estimated from the $\text{ZnFe}_2\text{O}_4$ (311) plane and the HAp (002) plane using Scherrer’s equation were obtained to be in the limit of 60.02–63.05 nm and 45.76–81.38 nm, respectively.

Fig. 3 exposed morphological microstructure of $\text{ZnFe}_2\text{O}_4$ nanoparticles and $\text{ZnFe}_2\text{O}_4$/HAp nanocomposite particles was captured by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). It seems the nanoscaled particles are poly-dispersed and, they were not spherical entirely in shape. The average particle size of $\text{ZnFe}_2\text{O}_4$, $\text{ZnFe}_2\text{O}_4$/HAp nanocomposite particles was evaluated to the range from 20 nm to 80 nm and 40 nm to 130 nm, which were properly consistent with the $\varepsilon_b$ values estimated by XRD pattern.

The hysteresis loops of the $\text{ZnFe}_2\text{O}_4$ nanoparticles and $\text{ZnFe}_2\text{O}_4$/HAp nanocomposite particles was shown in Fig. 4. The Ms amount of $\text{ZnFe}_2\text{O}_4$ and $\text{ZnFe}_2\text{O}_4$/HAp is around 31 and 7 emu/g. Moreover, coercive field of around 100 and 200 Oe was obtained for $\text{ZnFe}_2\text{O}_4$ and $\text{ZnFe}_2\text{O}_4$/HAp respectively in which the effect of surface bonded atoms to the hydroxyapatite structure is responsible for in increasing of the coercivity. It has also been reported that magnetic HAp-based scaffolds might stimulate cell proliferation, differentiation and adhesion in comparison with the pure HAp scaffold because of the inherent coercivity supplied by the mixed magnetic materials [39]. Thus, $\text{ZnFe}_2\text{O}_4$/HAp nanocomposite particles with higher coercivity taken from this research is

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estimated to be effectual in the future biomedical application specially in biosensors [40] and bone repair and replacement.

The G-292 and HEK cells were treated with various concentrations of ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$-HAp nanocomposite particles for 24 h. Then, cell viability was investigated by an MTT test. As shown in Fig. 5, there was no toxicity effect on cell viability in the dose-dependent evaluation compared to the control sample which are G-292 and HEK cells without any intervention. Therefore, the both nanoparticles were cytocompatible for further evaluations. Moreover, the MTT results showed that the both nanoparticles had dose-dependent proliferation on the bone-like osteoblast cells. With increasing the concentration, the G-292 cells proliferation was enhanced. This behavior seems to be due to the release of Zn$^{2+}$ as a promising ion that promotes DNA synthesis for bone formation and cell proliferation [41]. In contrast, with the increment of ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$-HAp nanoparticles concentration, the HEK cells proliferation was decreased and unchanged respectively.

MIC assay was conducted on the ZnFe$_2$O$_4$–HAp nanocomposite particles to evaluate their antibacterial features. The MIC assay of the nanoscaled particles was studied against S. aureus, a Gram-positive bacterial pathogen, depicted in Fig. 6. In concentrations lower than 0.078 mg/l, the inhibitory effects of the loaded nanoparticles are constant, although, in high concentrations of the samples, S. aureus bacteria were stimulated to proliferate. By concentration decrement from 5 to 0.078 mg/l, the bacterial inhibitory effect promoted that the optimal concentration of 0.078 mg/l in which significant inhibition of bacterial growth was observed in comparison with the positive control.

Antibacterial characteristics of materials and nanostructures are associated to bacterial cell membrane that its structure is altered [42,43]. Therefore, the nanostructures could penetrate to the bacterial cells with enhanced permeability through endocytosis process [43,44]. Iron oxide nanostructures can penetrate to bacteria cells due to higher surface area and smaller size than its membrane pores. It seems the nanoparticles could establish a remarkable influence on the synthesis process of DNA and protein, so could change their functions [43]. Reactive oxygen stress (ROS) is produced from hydrogen peroxides (H$_2$O$_2$), hydroxyl radicals (OH$^-$) and superoxide radicals (O$_2^-$) through reaction of the nanoparticles in biological environment. ROS is a key factor to inhibit the DNA and protein synthesis, so the cells proliferation is blocked [45]. It is known that at high concentrations of nanoparticles, the particles agglomeration is probably occurred, so there is not any fortune for penetrating the nanoparticles to the pores of bacterial membranes. At high nanoparticle concentrations, stimulating the bacterial proliferation in comparison with the positive control could also be related to the use of iron-based component as a functional source for upregulating the metabolism pathways of the bacteria and their following growth. With the decrease of the concentration, the bacterial inhibition properties reached to the point in which there was the best significant inhibitory effect compared to the positive control. For lower concentrations than 0.078 mg/ml, it seems that there is no inhibitory effect in comparison with the positive control, demonstrating the lack of sufficient nanoparticles, stimulated bacterial growth.

Based on loading efficiency equation and UV spectroscopy analysis at 215 nm, ZA loading was reported to be around 43.2%, 52%, 59% and 56.3%, 59.9%, 66.78% for primary ZA concentrations of 0.01, 0.02 and 0.04 mg/L in ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$–HAp samples, respectively. The in vitro profiles of the drug release from drug-loaded nanoscaled carrier were depicted in Fig. 7. Drug-loaded nanocomposite particle exhibited an early burst release throughout the first 20 h followed by a fairly slow drug release until 170 h. The burst release of the drug from the nanoscaled carrier is probably because of the quick release of surface-bound drug molecules [46]. In contrast, the second period (slow release) is obtained by diffusion process and ZA chemical binding [46,47].

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The drug-loaded nanocomposite particles had a robust bond with the drug rooted in covalent bindings. For bonding to the mineral phase of the hard tissues and cell-mediated antiresorptive activity, the two phosphate groups are both important. Amino (NH$_{3}$) or hydroxyl (OH) functional groups presented in HAP are established a robust chemical bond with the mineral materials of bone phases, most similarly through tridentate bonding to calcium-based component [45]. Moreover, other groups devoted to predominantly determine the antiresorptive potency of the bisphosphonates are existed. The existence of nitrogen atoms in the drug, ZA, is corroborated to the capability of a special bisphosphonate for inhibiting the cell growth. Likewise, this feature could influence on the bone affinity, as an activity of the nitrogen moiety ability, in producing a strong bond to the bone mineral surface. It seems that the chemically established bonds between the ZnFe$_2$O$_4$–HAp nanoparticles and ZA are included three main steps detailed as follows: (1) A crucial complicated reaction between Ca$^{2+}$ ions of the HAP-based nanocarrier and the OH group of the drug. (2) The complicated of Ca$^{2+}$ ions and ZA molecules accumulated with PO$_4^{3-}$ ions. (3) O–H and P–O groups of the nanoscaled carrier form chemical bonds with the -NH$_2$ and -OH groups in the drug, ZA, molecule, following in ZA drug layers that were sturdily connected to the carrier surface [26].

An MTT assay was applied to assess the cytotoxicity of ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$–HAp loaded with ZA on the cancer cell, G-292, and the normal cell, HEK. Based on the MTT results, by increasing the drug concentration from 0.01 to 0.04, the inhibitory effect of ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$–HAp on G-292 cells was significantly enhanced to about 25% compared to control sample without drug loaded nanoparticles. Moreover, higher dosage increment was impractical (Fig. 8 (a, b)). Fig. 8 also depicted that the inhibitory effects of ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$–HAp loaded with ZA were dose dependent. According to the MTT results of ZnFe$_2$O$_4$–HAp nanocomposite particles which are cytocompatible with the cell proliferation of more than 120% compared to control sample, the ZA loaded composite is cytotoxic by which cell proliferation is suppressed compared to control sample without ZA loaded nanoparticle. Therefore, with comparing cytotoxicity results of the bare nanocomposite particles and drug loaded ones, it is concluded that ZA release is the main cause of cancer cell proliferation suppression. Conversely, with the increment of ZA concentration from 0.01 to 0.04, the cell proliferation of ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$–HAp loaded with the drug ZA on HEK normal cells was significantly stimulated which was more than control sample. It is demonstrating that ZA-loaded nanoparticles in the presence of Ca- and P-based components could stimulate the normal cell proliferation.

4. Conclusion

The HAp, ZnFe$_2$O$_4$ and ZnFe$_2$O$_4$–HAp nanostructures were synthesized by co-precipitation approach. Their microstructures were investigated via SEM, TEM, FTIR, and XRD. Then, their biomedical characteristics including MIC antibacterial properties were surveyed. Higher coercivity was observed for nanoscaled ZnFe$_2$O$_4$–HAp rather than ZnFe$_2$O$_4$ which might stimulate cell proliferation, differentiation and adhesion. Furthermore, the dose-dependent HEK normal cell and G292 osteosarcoma cell line viability of the nanoscaled materials was evaluated by MTT assay. There was no toxicity effect on normal and osteosarcoma cells viability in the dose-dependent evaluation, so both nanoparticles were cytocompatible. A significant inhibitory effect of the nanoparticles was observed on bacterial growth in an optimal dose. Likewise, the in vitro profiles of the drug release from drug-loaded ZnFe$_2$O$_4$–HAp carrier were explored. Eventually, by increasing of the
nanoparticles concentrations, G292 cell proliferation was inhibited, although, HEK normal cell proliferation was stimulated.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jtice.2018.10.018.

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