Fabrication of a magnetic alginate-silk fibroin hydrogel, containing halloysite nanotubes as a novel nanocomposite for biological and hyperthermia applications

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In this study, the main focus was on designing and synthesizing a novel magnetic nanobiocomposite and its application in hyperthermia cancer treatment. Regarding this aim, sodium alginate (SA) hydrogel with CaCl2 cross-linker formed and modified by silk fibroin (SF) natural polymer and halloysite nanotubes (HNTs) followed by in situ Fe3O4 magnetic nanoparticles preparation. No important differences were detected in red blood cells (RBCs) hemolysis, confirming the high blood compatibility of the treated erythrocytes with this nanobiocomposite. Moreover, the synthesized SA hydrogel/SF/HNTs/Fe3O4 nanobiocomposite does not demonstrate toxicity toward HEK293T normal cell line after 48 and 72 h. The anticancer property of SA hydrogel/SF/HNTs/Fe3O4 nanobiocomposites against breast cancer cell lines was corroborated. The magnetic saturation of the mentioned magnetic nanobiocomposite was 15.96 emu g⁻¹. The specific absorption rate (SAR) was measured to be 22.3 W g⁻¹ by applying an alternating magnetic field (AMF). This novel nanobiocomposite could perform efficiently in the magnetic fluid hyperthermia process, according to the obtained results.

As mentioned in scientific definitions, hydrogels with a three-dimensional structure, flexible frameworks can be prepared with natural- or synthetic-based materials1-3. Typically, the flexible hydrogel networks are prepared through gelation, cross-linkage, or self-assembly routes. Hydrogels emerge expanded characteristics including hydrophilicity, elastic nature, reversibility, favorable functionality, and biocompatibility, making these structures idealistic in multifunctional biomedical applications. Due to the physical or chemical cross-linkage in hydrogel structures, these materials have the capability to keep high water or biological liquids ratios1,4,5. Recently, these flexible frameworks have been utilized because of their ability to carry chemical moieties, such as molecules, active pharmaceutical ingredients, proteins, etc., to proliferate and differentiate cells6-7. Generally, polysaccharides and protein chains are the two main participants in natural hydrogels. Hence, an extensive range of polymeric carbohydrate molecules including cellulose, chitin, dextran, chitosan, hyaluronic acid, dextran, chitosan, pectin, starch, and xanthan gum have been applied to form hydrogel lattice6-10.

Alginate is a linear polysaccharide derived from mannuronic and guluronic acid residues that have been used in the biomedicine field according to its biocompatible, low toxic, high hydrophilic structure11,12. The water

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swelling ability of sodium alginate (SA) has been introduced as a leading component in various biological fields, such as wound dressing and drug delivery. According to the structural sameness of the SA scaffold with the extracellular matrix, this biopolymer functions as a support to enhance cellular adhesivity and growth. The SA biodegradable polymer has been proven to be a stabilizer and an agent to upgrade the nanocomposite’s hemocompatibility. In this study, calcium chloride acts as SA natural polymer’s cross-linking agent, which is highly soluble in aqueous solutions, and hence this cross-linking procedure is not controlled. Some influential factors in the cross-linking process include the SA and CaCl₂ concentration and polymers web topology alteration due to dispersion hindrance caused through cross-linkage.

Besides, natural proteins with effective biological characteristics have been widely contemplated. The foremost example of natural proteins, silk fibroin (SF), possess enhanced mechanical strength and no cytotoxic and carcinogenic nature. The SF polymer has attracted scientists’ interest due to its biocompatibility and adhesion increment and the multiplication of fibroblasts and keratinocytes cells. As presented in many reports, the combination of SF natural polymer and metal nanoparticles, other natural polymers, and GQDs would improve antimicrobial activity related to wound healing requirements. Microporous aluminosilicate-based halloysites nanotubes (HNTs) consisting of oxygen silicon tetrahedron at external surface and alumina oxygen octahedrons at internal lumen. The hydroxyl groups in the Si–OH and Al–OH components exist at the inner and outer areas of the nanotubes. Since the HNTs own a multilayer structure, most OH groups concentrate in the lumen compared to the surface. On the whole, the OH groups inside and outside the HNTs aid nanotube’s convenient modification. The outside of HNTs is negatively charged, while its lumen is positively charged. This property causes various chemical activities. Moreover, the HNTs possess remarkable properties, including enhanced thermal and mechanical resistance, environmentally-friendliness, and eco-based material with improved aspect ratio. The HNTs incorporation in a polymeric matrix results in enhancing the physicochemical characteristics of the polymer, which causes thermal and mechanical efficiency improvement.

On the other hand, because of an empty limen inside of HNTs, the loading of chemically and biologically active species on HNTs would be possible. Thus, they are applicable in specific chemical facets, such as antimicrobial, anticorrosion, flame-retardant, drugs, and microfluidics’ modeling. Also, the morphology and physicochemical features of HNTs, rendering a large volume of cavities, remain intact in different experiments, and the structure of HNTs would not be damaged. Besides, the interaction between HNTs with a large aspect ratio and guest molecules through hydrogen bonding and electrostatic interactions miserates the HNTs’ features by loading various species like pharmaceutical compounds on its internal and external surfaces. Furthermore, as HNTs have the possibility to immobilize diverse magnetic metal oxides, they can act as nanocomposite’s substrate. The immobilized magnetic metal oxides are chemically stable, they have convenient separation from the reaction flask and reusability. Nanoparticles, and more specifically, magnetic nanoparticles (MNPs) are very reactive due to their high aspect ratio. Further, these can be applied in biological functions, namely hyperthermia, cancer therapy and drug delivery. It is noted that MNPs that were exposed to an alternative magnetic field excited and produced heat. In continuation with other MNPs features, Fe₃O₄ MNPs also exhibit superparamagnetic behavior, magnetic sensitivity, coercivity, low Curie temperature, facile workup and separation.

MNPs hyperthermia is a complex approach with both advantageous aims and challenges. The cancer cells could be straightly destructed employing MNPs hyperthermia which caused easy mass transmission in drug delivery application leading to the thermal-responsive drug release or enhanced connection with other therapies to upgrade the general outcome. There are many impressive parameters in hyperthermia procedure progression. On this subject, selecting appropriate NPs due to their features, cementation, and related function have been widely reported. The injection strategy is another effective parameter in MNPs hyperthermia process. MNPs reach the tumor tissue either by injection or intravenously. The delivery procedure was determined for the in vivo assessment. In this case, the systematic injecting of MNPs into the tumors does not encounter similar difficulties as the direct injection procedure.

The prepared biocomposite can be introduced as a suitable candidate for drug delivery because the hydrogel composition renders a pH-sensitive or thermo-sensitive structure. In the case of considering wound dressing or tissue engineering applications, the hydrogel was used to control wound secretions and, at the same time, provide moisture to the damaged area. Also, silk fibroin was utilized, which has many applications in the cell cycle and can be applied in wound dressings and tissue engineering. Also, incorporating HNTs into the composite matrix increases biocompatibility and reduces toxicity, which is considered a vital issue in the field of tissue engineering. Another noteworthy point is the selection of natural materials for preparing this nanocomposite; even the mineral HNTs used are natural. As a result, both toxicity and hemolysis were reduced by this procedure.

Herein, considering each of the components’ structural and prominence properties in the composition, viz. cross-linked SA biopolymer with CaCl₂ cross-linking agent, extracted SF, HNTs, and Fe₃O₄ MNPs demonstrate enhanced synergistic properties. This biocompatible scaffold with high dispersity was utilized in hyperthermia cancer therapy. Indeed, the utilization of HNTs improves the structure strength and correlation of the SA/SF/HNTs/Fe₃O₄ biocomposite scaffold (Scheme 1).

**Materials and methods**

**Materials.** Sodium alginate (CAS No. 9005-38-30), CaCl₂·2H₂O (CAS No. 10035-04-8, Mw: 147.01), LiBr (CAS No. 7550-35-8), Na₂CO₃ (CAS No. 497-19-8), Tris base (CAS No. 77-86-1), EDTA (CAS No. 6381-92-6), FeCl₃·6 H₂O (CAS No. 10025-77-1), FeCl₂·4 H₂O (CAS No. 13478-10-9), ammonia (CAS No. 7664-41-7), solvents, and reagents were provided from Merck and Sigma-Aldrich company. Also, the Sigma-Aldrich chemical company was the supplier of halloysite nanotubes and dialysis tubing cellulose membrane (14000 Dalton). Milli-Q ultra-pure water was utilized in this study. Apart from other materials, silkworm cocoons were provided by the locals.
Preparation of cross-linked SA hydrogel. Here, the cross-linked SA hydrogel with CaCl₂ cross-linking agent was prepared with respect to previous approaches. Concisely, 0.7 g SA powder was dissolved in distilled water and stirred for 10 min at 50 °C until the appearance of a clear solution. After that, 0.15 g CaCl₂ as the cross-linker was added to the as-prepared suspension and manually blended for complete cross-linking between polymer chains. In the next step, 1 mL of the mixture was shed into the microplates and then frozen at −70 °C for 24 h in the refrigerator. Eventually, the sample was freeze-dried and further kept in a dry environment.

Preparation of cross-linked SA hydrogel/SF. Regarding previous studies on silk fiber’s processing approaches and extraction, silkworm cocoons were cut into small pieces at the first step. Then, in 500 mL of the specified aqueous solution of 1.06 g Na₂CO₃ (0.21% w/v), the cocoon pieces were boiled for 2 h. After the mentioned time (2 h), the non-woven silk fibers were washed several times with distilled water and then dried overnight at room temperature. Simultaneously, in another beaker, 0.242 g tris and 0.058 g EDTA were added in 200 mL of boiled distilled water. At this stage, the membrane was placed in this solution for 2 h. In the next step, the dried silk fibers were dissolved in a suitable concentration of lithium bromide (9.3 M), and the solution was kept for 2 h under stirring at 60 °C. The resulting solution was poured into the dialysis membrane to prepare the dialysis procedure condition in the presence of distilled water. The dialysis procedure lasted for three days at room temperature. When the SF extraction was completed, 10 mL of SF were added to the cross-linked SA hydrogel with 1:1 ratio at 25 °C for 5 min. Hence, the resulting cross-linked SA hydrogel/SF was provided and cast in microplates to be exposed to freezing at −70 °C for 24 h. Then, cross-linked SA hydrogel/SF was freeze-dried and stored in a dry place.
Preparation of cross-linked SA hydrogel/SF/HNT. Since the HNTs strengthen and cohesion the structure, at this stage, 0.6 g HNT was added to the cross-linked SA hydrogel/SF network structure. Then, the mixture was sonicated to have homogeneity and a uniform HNT dispersity in the whole structure. The effect of changing the amount of HNTs or even other components involved in the nanocomposite can be investigated in terms of various properties, such as mechanical properties, biological properties such as toxicity and hemolysis, and also some chemical properties.

Synthesis of cross-linked SA hydrogel/SF/HNTs/Fe3O4 biocomposite scaffold. In the final stage of cross-linked SA hydrogel/SF/HNTs/Fe3O4 biocomposite scaffold preparation, 1.94 g FeCl3·6H2O and 0.88 g FeCl2·4H2O were added to the as-prepared mixture at 70 °C for 2 h in the reaction flask of the previous step. The reaction mixture was then subjected to mechanical agitation under N2 atmosphere followed by continuous heating while adding ammonia to the mixture solution for 30 min. By ammonia solution addition, the reaction mixture was stirred under constant conditions (70 °C) for 2 h. After this time, the cold mixture solution and black sediment separation was performed by an external magnet. Also, the purification procedure of magnetic nanocomposite was performed several times to remove non-reactive compounds and achieve neutral pH (pH 7).

Fourier-transform infrared spectroscopy. Due to the significance of functional group characterization in each synthesis level, the Fourier-transform infrared (FT-IR) spectrometer (Shimadzu FT-8400 s model, Japan) was applied based on pellets containing 0.1–1.0% of the samples, which were further mixed with 200–250 mg of KBr powder. Moreover, the resolution of the spectra was 4 cm−1 in the 400–4000 cm−1 frequency range. All spectra were captured at ambient temperature and the mean number of scans varied between 6 and 18.

Field-emission scanning microscopy. The morphology and shape of the structure were depicted by field-emission scanning microscope (FE-SEM) (ZEISS-Sigma VP model, Germany), functioning at a 15 kV. A double-sided carbon tape fixed the sample on a stainless-steel stub, and they were further subject to gold sputter coating (Agar Sputter Coater model, Agar Scientific, England).

Energy-dispersive X-ray spectroscopy. The authentication of elements that existed in the composite was performed with an energy-dispersive X-ray (EDS) detector (Oxford instrument, England) coupled with the ZEISS-Sigma VP model, Germany.

Thermogravimetric analysis. An analytical device which conducted the thermogravimetric (TGA) analysis was Bahr-STA 504 (Germany). 0.5 g of sample was placed in the alumina pans of the instrument at argon atmosphere exposure. The argon flow rate was 1 L h−1; each thermal cycle was performed from 50 to 800 °C with a constant 10 °C min−1 heating rate.

X-ray diffraction. In this X-ray diffractogram (XRD) pattern was applied to determine the crystalline phase and structure utilizing the Bruker X-ray diffractometer instrument (D8 Advanced Model, Germany), owning a Lynxeye detector (0D mode), and Cu-Kα radiation (λ = 0.154 nm, 40 kV, 40 mA). Besides, the scanning angle of 5° ≤ 2θ ≤ 90° and 0.2°/s rate were used.

Hemolysis assay. Red blood cells (RBCs) hemolytic assay was used to determine the hemocompatibility of the synthesized SA hydrogel/SF/HNTs/Fe3O4 nanobiocomposite on human erythrocytes. Primarily, RBCs were transferred to the 96-well microplate; v-shaped bottom (Citotest, China). 100 µL of dispersed nanobiocomposite mixture was then subjected to mechanical agitation under N2 atmosphere followed by continuous heating while adding ammonia to the mixture solution for 30 min. By ammonia solution addition, the reaction mixture was stirred under constant conditions (70 °C) for 2 h. After this time, the cold mixture solution and black sediment separation was performed by an external magnet. Also, the purification procedure of magnetic nanocomposite was performed several times to remove non-reactive compounds and achieve neutral pH (pH 7).

MTT assay. Cytotoxicity of SA hydrogel/SF/HNTs/Fe3O4 nanobiocomposite were measured by using of BT549 cells (breast cancer cell line) and HEK293T cells (human embryonic kidney cell line) which was prepared by the Pasteur Institute of Iran, in comparison with Cisplatin anti-cancer drug as positive control (Sigma-Aldrich, MO, United States). Briefly cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. Then, 5 × 103 cells/well were seeded in 96-well plates and serially dilutions (0.0156, 0.312, 0.625, 0.125, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 mg mL−1) of nanobiocomposites were added to the wells and incubated for 48 h and 72 h. Culture medium alone was served as negative control. The plates were incubated for 2 h at 37 °C and then centrifuged at 2000 rpm for 10 min. The supernatant of each well was transferred to the flat bottom plate and OD was quantified by the ELISA reader (Biohit, Finland) at 405 nm.
Results and discussion

characterization of cross-linked SA/SF/HNTs/Fe₃O₄ biocomposite scaffold. FT-IR analysis. Figure 1a(i,ii) represents the FT-IR spectra of synthesized SA hydrogel/SF and SA/SF/HNTs/Fe₃O₄ biocomposite, respectively. As could be seen in Fig. 1a(i), four absorption bands at 1234 cm⁻¹, 1516 cm⁻¹, 1628 cm⁻¹, and 588 cm⁻¹ were assigned to amide (III), amide (II), amide (I), and amide (V), respectively, and also the broadband at 3414 cm⁻¹ was related to the N–H bond. In addition, three absorption peaks at 1402 cm⁻¹, 1032 cm⁻¹, and 2366 cm⁻¹ were corresponded to stretching vibration modes of CH₃, C–O and CH. Also, a polyalanine IR peak was observed at 914 cm⁻¹ in the SF IR spectrum. The vibration band at ca. 2300 cm⁻¹ is due to the CO₂ in the atmosphere. As illustrated in the FT-IR spectrum of synthesized biocomposite in Fig. 1a(ii), the two distinctive peaks of HNTs that arose at 3717 and 3618 cm⁻¹, correlating with the surface OH groups in the HNTs’ lumen and the inner OH groups located between the tetrahedral and octahedral sheets, respectively. Furthermore, SF peaks that belong to amid (I), amid (II), and amid (III) were observed in 1226 cm⁻¹, 1516 cm⁻¹, and 1268 cm⁻¹. Appearing absorption bands at 1628 cm⁻¹ and 1432 cm⁻¹ can be related to carbonyl groups’ resonance stretching in SA. Besides, a peak at 2360 cm⁻¹ and 674 cm⁻¹ could determine CH and Fe–O bonds, respectively.
X-ray diffraction. As depicted in Fig. 1b(i), the famous secondary structures of SF, i.e., the Silk I and Silk II, have been analyzed by XRD. A metastable structure of Silk I correlates with the α-helix conformation, while Silk II corresponds to the β-sheet structure, which is a conformation with alternate directions of molecular chains, resulting in stable antiparallel chain pleated sheets. One of the most significant β-sheet crystallization indicators is Silk I to Silk II transformation. Silk I demonstrates three distinctive diffraction peaks at ca. $2\theta = 12.2, 19.7$, and $24.7^\circ$, and Silk II diffraction peaks are arose at ca. $2\theta = 9.1^\circ$ and $20.7^\circ$ (pattern (i))50,51. Also, the main diffraction peaks of SA appears at $13.76$ and $21.5^\circ$ 52. As shown in the Fig. 1b(ii), the XRD pattern of synthesized SA/SF/HNTs/Fe$_3$O$_4$ biocomposite was determined. In this pattern, characteristic peaks at $2\theta = 30.18^\circ$, $35.65^\circ$, $43.09^\circ$, $62.47$ confirmed the presence of Fe$_3$O$_4$, which corresponded to (1 1 2), (2 0 0), (1 0 3) and (0 4 0) plane in its crystalline structure (JCPDS card No. 01-075-1709). Also, a weak peak at $2\theta = 9.1, 12.2, 19.7, 26.72^\circ$ could be ascribed to SF, which proved its low crystallinity. In addition to this, HNT peaks in this sample were assigned at $2\theta = 12.50^\circ$, $20.17^\circ$, $24.97^\circ$, and these peaks were related to (0 0 1), (0 2 0), and (0 0 2) plane.

Field-emission scanning microscopy and elemental composition. The morphology and structure of SA/SF/HNTs/Fe$_3$O$_4$ biocomposite were acquired by FE-SEM imaging, shown in Fig. 2a. The SEM image shows spherical nanoparticles with an average diameter of 58.62 nm, representing Fe$_3$O$_4$ magnetic NPs in synthesized biocomposite. Also, adding SF biopolymer to the cross-linked SA/SF hydrogel has changed its mesoporous structure and made it incorporated. On the other hand, the presence of tubular shaped nanoparticles on the surface with an average diameter of 51.99 nm is evidence of successful halloysite nanotubes’ synthesis5. The elemental composition of SA/SF/HNTs/Fe$_3$O$_4$ biocomposite was demonstrated in Fig. 2b. The sharp peaks of Al, Si, O authenticated the Al$_2$Si$_2$O$_5$(OH)$_4$ structure of HNTs. The existence of the Ca element is related to the CaCl$_2$ cross-linking agent of alginate polymeric chains. Besides, the alginate has emerged with C and O element peaks in the EDX spectrum. Fe$_3$O$_4$ magnetic NPs are presented with Fe and O sharp peaks. Also, the comprising elements of silk fibroin, i.e., C, N, and O, are verified.
Thermogravimetric analysis. To investigate the thermal stability of synthesized SA/SF/HNTs/Fe$_3$O$_4$ biocomposite, TGA analysis was applied, as shown in Fig. 3a(i). The first mass loss of biocomposite was about 10% between 53 and 208 °C, related to the loss of absorbed moisture. Besides, by incorporating the organic species with the HNTs, the moisture loss diminishes, while the hydrophobization of the HNTs’ cavity rises$^{53,54}$. The second weight loss was associated with the decomposition of amino acid side chains as well as cleavage of peptide bonds in SF, which was obtained at the temperature between 238 and 607 °C$^{5,11}$. As could be seen in the TGA curve, there is a mass loss in the temperature range of 208 to approximately 300 °C, which was also confirmed by DTG Fig. 3a(ii), that can be associated with the degradation of the SA polymeric chain, and complete decomposition of the SA backbone occurs at the temperatures between 300 and 576 °C$^{11,55}$. The following Table shows the components' mass loss (%) during various temperature ranges. It should be noted that based on the hyperthermia application, a high amount of the Fe$_3$O$_4$ magnetic NPs were applied.

The table of thermogravimetric parameters of SA/SF/HNTs/Fe$_3$O$_4$ biocomposite.

Figure 3. (a) The thermogravimetric analysis (i) and DTG curve (ii) of SA/SF/HNTs/Fe$_3$O$_4$ biocomposite, and (b) the vibrating-sample magnetometer of SA/SF/HNTs/Fe$_3$O$_4$ biocomposite.
Temperature ($^\circ$C) Mass loss (%) Component
53–208  ~ 10.0 Absorbed moisture
208–300  ~ 18.0 Degradation of the SA polymeric chain
238–607  ~ 23.0 Decomposition of amino acid side chains as well as cleavage of peptide bonds in SF
300–576  ~ 12.0 Complete decomposition of the SA backbone
600  ~ 60.0  s Residual mass

Vibrating-sample magnetometer. To investigate the magnetic properties of synthesized SA/SF/HNTs/Fe$_3$O$_4$ biocomposite vibrating-sample magnetometer (VSM) was used, shown in Fig. 3b. Fe$_3$O$_4$ nanoparticles magnetic saturation was measured about 44.2 emu g$^{-1}$ in a previous study which A.S. Sakhthi Athitan and others reported. On the other hand, the magnetization of synthesized biocomposite was measured at approximately 15.96 emu g$^{-1}$. This reduction in magnetic behavior was due to the core–shell shape of synthesized biocomposite and the presence of HNT, SF, and SA layers as shells.

Biological characterization

RBCs lysis inhibition assay. The deionized water that was used as positive control, showed the highest rate of hemolysis, lysing almost all RBCs. Our nanobiocomposite-treated erythrocytes did not show significant differences in RBCs hemolysis compared with physiological serum as a negative control. In some concentrations, this amount was even less than the negative control (Fig. 4). Therefore, SA hydrogel/SF/HNTs/Fe$_3$O$_4$ nanobiocomposite is fully compatible with blood.

Cell proliferation assay. The results showed that the viability percentage of HEK293T normal cells did not change significantly after 48 h (Fig. 5a) and 72 h (Fig. 5b), and therefore the synthesized SA hydrogel/SF/HNTs/Fe$_3$O$_4$ nanobiocomposite are not toxic to this cell line.

At the same time, the proliferation rate and viability percentage of BT549 cancer cells exposed to SA hydrogel/SF/HNTs/Fe$_3$O$_4$ nanobiocomposites was decreased (Fig. 5a,b). Therefore, it can be said that this nanobiocomposite has anti-cancer property against breast cancer cell line.

The survival rate of both cell lines after treatment with cisplatin (as a positive control) can also be seen in Fig. 5c,d.

EC50 values for HEK293T and BT549 cells after 48 h and 72 h also can be seen in the Table 1.

Application of synthetic magnetic nanocomposite in hyperthermia procedure. MNPs generate heat when they are exposed to an alternating magnetic field. This is why they are widely used in a cancer therapy method called hyperthermia, in which the temperature of the tumor is elevated to 41–45 $^\circ$C for a predefined period of time. According to the purpose of this article and biological applications and hyperthermia, we did not seek to increase the nanocomposite's thermal resistance or thermal stability. The composite did not undergo structural changes at 41–45 $^\circ$C temperature, even during the hyperthermia test. Besides, the TGA analysis aims

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**Figure 4.** Hemolysis histogram of SA hydrogel/SF/HNTs/Fe$_3$O$_4$ nanobiocomposite, comes with 96-well plate image.
to prove the nanocomposite structural components based on their mass loss, proving the existence of Alg, SF, and HNTs. However, the amount of the released heat depends on various factors such as the size, shape, and

![Figure 5](image)

This illustration shows the viability percentage of HEK293T and BT549 cells after treatment with SA hydrogel/SF/HNTs/Fe₃O₄ nanobiocomposite after 48 h (a) and 72 h (b), and the survival rate of HEK293T and BT549 cells after treatment with cisplatin (positive control) at days 2 (c) and 3 (d); comes with 96-well plate images.

|                  | EC50 of HEK293T normal cells | EC50 of BT549 cancer cells |
|------------------|------------------------------|---------------------------|
| 48 h             | 11.28                        | 0.2253                    |
| 72 h             | 11.28                        | 0.2253                    |

Table 1. EC50 values of HEK293T and BT549 cells after treatment with SA hydrogel/SF/HNTs/Fe₃O₄ nanobiocomposite after 48 h and 72 h.
magnetic properties of the MNPs and also frequency and the strength of the magnetic field. In order to measure the thermal power of MNPs, SAR is expressed as Eq. (1):  

$$\text{SAR} = \frac{C m}{\Delta T_{\text{max}} / \Delta t}$$  



where $C$ is specific heat capacity of the sample, $m$ is the concentration of the nanoparticles and $\Delta T_{\text{max}} / \Delta t$ shows maximum changes of the temperature of the sample with time. In the present research, heating efficiency of three different concentrations of 1, 2, and 5 mg mL$^{-1}$ of an aqueous solution of MNPs at various frequencies of 100, 200, 300, and 400 kHz were studied for 10 min and the temperatures of the samples were measured every 5 min. Looking at Fig. 6a, it is obvious that SAR decreased with increasing the concentration of MNPs in the samples. Maximum value of SAR (22.3 W g$^{-1}$) was achieved at the highest concentration and under the highest frequency of the magnetic field, which was 8 times as high as the minimum value (2.8 W g$^{-1}$). Although samples with lower concentrations exhibited higher values of SAR, higher temperature rises were observed in the case of samples with higher concentrations in a given period of time, meaning that temperature increases more rapidly as the concentration of MNPs in the nanofluid increases, while higher values of SAR show that a given amount of the sample is more capable of transforming the electromagnetic energy into heat.

The rate at which temperature was increasing rose more when the concentration was increased from 2 to 5 mg mL$^{-1}$ (in comparison with doubling it from 1 to 2 mg mL$^{-1}$) under the frequencies of 100, 200, and 300 kHz, whereas under the highest frequency, increasing the concentration from 1 to 2 mg mL$^{-1}$ was more effective on changing the temperature of the sample (Fig. 7).

As can be seen from Fig. 6b, in all samples, increasing the frequency of the magnetic field from 100 to 200 and 200 to 300 kHz led to a decline in SAR. However, SAR started rising when the frequency increased from 300 to 400 kHz. In fact, not only did all the samples showed the highest magnitude of SAR at 400 kHz, but in a particular time interval, temperature of all the samples also changed the most at this frequency.

Taken together, the present results are of high importance in at least two aspects. Firstly, in the determination of the proper frequency of the magnetic field according to the location of the tumor as deep tumors should be destroyed by lower frequencies and the superficial ones need higher frequencies. Secondly, the amount of the generated heat and temperature of the tumor must be precisely controlled since high temperatures are likely to damage the surrounding healthy tissue.

**Conclusions**

Herein, SA hydrogel with CaCl$_2$ cross-linker was modified with SF and HNTs with a further in situ Fe$_3$O$_4$ magnetic nanoparticles preparation for employing the magnetic fluid hyperthermia procedure. This magnetic nanobiocomposite was prepared for the first time, highlighting the structural stability and homogeneity in aqueous media, which suits hyperthermia application. The structural properties were characterized by various spectroscopic and microscopic analyses, such as FT-IR, EDX, FE-SEM, XRD, TGA, and VSM. The newly emerged functional groups of each synthesis step were characterized by FT-IR spectroscopy. FE-SEM images confirmed the spherical Fe$_3$O$_4$ magnetic nanoparticles and HNTs presence with 58.62 nm and 51.99 nm average diameter, respectively. The nanobiocomposite showed high blood compatibility and non-toxicity toward HEK293T normal cell line considering hemolysis and MTT assays. Further, the anticancer feature of this nanobiocomposite was confirmed against breast cancer cell lines. Comparing the magnetic saturation of bare Fe$_3$O$_4$ (44.2 emu g$^{-1}$) with SA hydrogel/SF/HNTs/Fe$_3$O$_4$ nanobiocomposite (15.96 emu g$^{-1}$), the reduction can be related to the non-magnetic materials involved in the core–shell nanobiocomposite. The maximum value of SAR (22.3 W g$^{-1}$) was
obtained at 1 mg mL\(^{-1}\) concentration and under the highest frequency (400 kHz) of the alternating magnetic field (AMF). However, the sample generated the highest temperature by 5 mg mL\(^{-1}\). This novel nanobiocomposite could perform efficiently in the magnetic fluid hyperthermia process, according to the obtained results.

**Data availability**

All data generated or analysed during this study are included in this published article.

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R.E.-K.: substantial contributions to the conception, design of the work, have drafted the work, writing—review and editing, analysis and interpretation of data and wrote the main manuscript. Z.S.: have drafted the work, analysis and interpretation of data, substantively revised it. Wrote the main manuscript and prepared figures. F.G.: analysis and interpretation of data, substantively revised it, wrote the main manuscript and prepared figures. H.A.M.A.: analysis and interpretation of data, substantively revised it, wrote the main manuscript and prepared figures. S.K.: analysis and interpretation of data, substantively revised it, wrote the main manuscript. N.S.: analysis and interpretation of data, substantively revised it, wrote the main manuscript. M.M.A.: analysis and interpretation of data, wrote the main manuscript. A.K.: analysis and interpretation of data, substantively revised it. M.S.B.: analysis and interpretation of data, substantively revised it. R.A.C.: analysis and interpretation of data, substantively revised it. A.M.: the corresponding (submitting) author of current study, substantial contributions to the conception, design of the work, have drafted the work, writing—review and editing, substantively revised it.

Competing interests
The authors declare no competing interests.

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