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Functionalized SPION immobilized on graphene-oxide: Anticancer and antiviral study

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ABSTRACT

The progressive and fatal outbreak of some diseases such as cancer and coronavirus necessitates using advanced materials to bring such devastating illnesses under control. In this study, graphene oxide (GO) is decorated by superparamagnetic iron oxide nanoparticles (SPION) (GO/SPION) as well as polyethylene glycol functionalized SPION (GO/SPION@PEG), and chitosan functionalized SPION (GO/SPION@CS). Field emission scanning electron microscopic (FESEM) images show the formation of high density uniformly distributed SPION nanoparticles on the surface of GO sheets. The structural and chemical composition of nanostructures is confirmed by X-ray diffraction and Fourier transform infrared spectroscopy. The saturation magnetization of GO/SPION, GO/SPION@PEG and GO-SPION@CS are found to be 20, 19 and 8 emu/g using vibrating sample magnetometer. Specific absorption rate (SAR) values of 305, 283, and 199 W/g and corresponding intrinsic loss power (ILP) values of 9.4, 8.7, and 6.2 nHm kg$^{-1}$ are achieved for GO/SPION, GO/SPION@PEG and GO/SPION@CS, respectively. The In vitro cytotoxicity assay indicates higher than 70% cell viability for all nanostructures at 100, 300, and 500 ppm after 24 and 72 h. Additionally, cancerous cell (EJ138 human bladder carcinoma) ablation is observed using functionalized GO/SPION under applied magnetic field. More than 50% cancerous cell death has been achieved for GO/SPION@PEG at 300 ppm concentration. Furthermore, Surrogate virus neutralization test is applied to investigate neutralizing property of the synthesized nanostructures through analysis of SARS-CoV-2 receptor-binding domain and human angiotensin-converting enzyme 2 binding. The highest level of SARS-CoV-2 virus inhibition is related to GO/SPION@CS (86%) due to the synergistic exploitation of GO and chitosan. Thus, GO/SPION and GO/SPION@PEG with higher SAR and ILP values could be beneficial for cancer treatment, while GO/SPION@CS with higher virus suppression has potential to use against coronaviruses. Thus, the developed nanocomposites have a potential in the efficient treatment of cancer and coronavirus.

1. Introduction

Cancer as one of the life-threatening diseases on human health, causes almost 10.0 million deaths annually (2020), reported by world health organization (WHO) in February 2020 factsheets [1]. Beyond different reported treatments for cancer, including chemotherapy, radiotherapy, and surgery, magnetic hyperthermia is a non-invasive cancer therapy with minimal detrimental side effects [2]. In such treatment, magnetic materials act as a heating source, generating heat under the applied external magnetic field [3]. Cancer cells are typically more susceptible than the healthy cells to mild heat (~ 42 °C) due to their less blood flow which consequently limits the heat dissipation to the surrounding area [2,3]. Since tumor cells are more heat sensitive, exploiting effective and specific targeting therapeutic agent (i.e., magnetic nanomaterials) would be a promising tool to combat cancer cells [4].

The incidence (251 million confirmed cases) and mortality (5 million deaths) of the newly emerged coronavirus SARS-CoV-2 require urgent development of materials that can neutralize the virus function and prevent the infection [5]. Several recent evidences have highlighted that nanomaterials (e.g., graphene oxide, GO) have a high potential to neutralize cell-protein interactions and prevent viral endocytosis due to their large surface area or having various functional groups [6–8]. Moreover, it has been reported that chitosan shows inhibitory activity against covid-19 [9,10]. Positively charged chitosan can interact with the receptor-binding domain (RBD) of the virus and assemble protein-
polymer complexes, which could inactivate the virus and obstruct its connection with human cells [11].

Recently, superparamagnetic iron oxide nanoparticle (SPION) has received immense attention in biomedical applications such as targeted delivery of drug [12], magnetic resonance imaging (MRI) [13], tissue engineering [14] and hyperthermia for cancer therapy [15] owing to its magnetic property, low toxicity, resilient mechanical structure and no remnant magnetization [16,17]. However, using this outstanding material in vivo faces longstanding obstacles. Among them, particle agglomeration, less colloidal stability in physiological media and short circulation time in the blood are major challenges. To overcome particle agglomeration and preserve narrow size distribution of SPION, exploiting favorable support such as 2D materials (e. g. GO) could be a good choice [18]. The high specific surface area of GO makes it a suitable candidate to be decorated with nanoparticles with rather good distribution [19]. Moreover, GO has numerous functional groups such as hydroxyl, carboxyl, and epoxy existing on its surface that makes possibility for connection between GO sheets and many other molecules. Many researchers have focused on using GO/iron oxide nanoparticles composite in medical applications. For instance, Wang et al. [20] controlled drug release of temozolomide from GO/iron oxide nanoparticles. Kumar et al. [21] synthesized GO/iron oxide nanoparticles for radio-frequency hyperthermia therapy. Borras et al. [22] prepared GO/SPION nanocomposite as contrast agents in MRL. Recently, the attractive ability of GO to interact, degrade membrane and inhibit enveloped viruses like SARS-CoV-2 encourages researchers to use this material to combat coronaviruses [23,24].

Functionalization is a versatile strategy to tackle shortcomings or donate salient features to materials. For instance, attempt has been made to improve colloidal stability and blood circulation time of SPION via polymeric surface coating. Bach et al. [25] prepared SPION nanoparticles capped with polyethylene glycol (PEG) to reduce the agglomeration. Kazemzadeh et al. [26] enhanced SPION stability in water/oil emulsion with the aid of chitosan. Dolatkhah et al. [27] developed nanocomposites of GO loaded with PEGylated SPION nanoparticles and grafted with methotrexate and stimuli-responsive linkers for photothermal and chemotherapy of breast cancer. Ma et al. [28] synthesized a multifunctional PEG functionalized GO/SPION composite to acquire high stability in physiological solutions. This nanostructure also exhibited strong optical absorbance which is beneficial for applications in cancer therapy.

The novelty of current study involves following aspects: (i) since biocompatibility is one of main issues of using materials in medical field [29–35], cell behavior of PEG functionalized SPION decorated on GO sheets (GO/SPION@PEG) and chitosan functionalized SPION decorated on GO sheets (GO/SPION@CS) has been studied and the morphology of cells with and without applying magnetic field has been observed, (ii) the magneto thermal ability of GO/SPION@PEG and GO/SPION@CS to kill cancerous cells is investigated through applying an external magnetic field, (iii) the ability of GO/SPION@PEG and GO/SPION@CS to inhibit covid-19 activity is addressed.

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG, 6000 molecular weight), chitosan medium molecular weight, iron (II) chloride tetrahydrate (FeCl₂·4H₂O, 99%), iron (III) chloride hexahydrate (FeCl₃·6H₂O, 99%), sodium hydroxide (NaOH, 99%), glutaraldehyde (C₅H₈O₂, 99%), and acetic acid (CH₃COOH, 2% w/v) were purchased from Sigma-Aldrich. Graphene Oxide was prepared using graphite powder, sodium nitrate (NaNO₃, 99%), sulfuric acid (H₂SO₄, 98%), potassium permanganate (KМnO₄, 99%), hydrochloric acid (HCl, 10%) and hydrogen peroxide (H₂O₂, 30%) which all were provided from Merck. Ammonia (NH₄OH, 30%) and trisodium citrate (Na₃C₆H₅O₇, 99%) were also purchased from Merck and used for the synthesis of GO/SPION.

2.2. Synthesis of graphene oxide

Graphene oxide was prepared by chemical oxidation and exfoliation of natural graphite powder via the modified hummer’s method [36]. Graphite powder (5 g) and NaNO₃ (2.5 g) were mixed in 115 mL concentrated H₂SO₄ solution in a beaker, kept under an ice bath with continuous stirring. Then, KMnO₄ (15 g) was added very slowly while maintaining the temperature below 15 °C for 2 h. Further, the suspension was transferred into a water bath and stirred for 3 h at 35 °C until it became pasty brownish. Then, 230 mL of deionized water was added slowly to the above suspension and consequently, temperature rose to 98 °C due to the exothermic reaction. After 30 min, the solution is finally treated with 50 mL H₂O₂ to terminate the reaction by appearance of yellow color. Further, the mixture was washed with HCl and deionized water several times for purification followed by freeze drying.

2.3. Synthesis of SPION

FeCl₃ and FeCl₂ with molar ratios of 1:2 were dispersed in distilled water and stirred for 30 min. Next, 300 mL of 0.5 M NaOH solution was prepared and heated up to 70 °C. Then, the mixture of FeCl₃ and FeCl₂ solution was dropped gradually during 1 h into the NaOH solution and stirred for 4 h. The solution color changed from brown to black and iron oxide nanoparticles precipitated at the bottom of the beaker. Finally, nanoparticles were washed with ethanol and distilled water several times, separated by a magnet and freeze-dried.

2.4. Synthesis of GO/SPION

0.1 g GO was ultrasonicated in 100 mL of deionized water to disperse completely. Then, as prepared iron oxide nanoparticles were added to the GO suspension under nitrogen atmosphere and the mixture was heated up to 80 °C and stirred for 1 h. To adjust the PH of the mixture, 5 mL of ammonia solution (30 %) was added. Finally, 1.0 g trisodium citrate was added and the temperature was kept at 80 °C for 24 h to form black precipitate as GO/SPION. The final product was washed with deionized water by centrifugation and dried in an oven at 60 °C overnight.

2.5. Synthesis of GO/SPION@CS

0.4 g chitosan was ultrasonicated in 200 mL acetic acid (2% w/v) for 2 h to dissolve completely. Then, 0.1 g SPION nanoparticles and 2 mL of glutaraldehyde were added to the chitosan solution and the mixture was stirred for 1 h to cover the surface of nanoparticles. Then, the precipitated SPION@CS were washed and dried. In the next step, 0.3 g of GO were dispersed in water, SPION@CS was added to the suspension and the suspension was transferred to a water bath at 50 °C and stirred for 90 min. To adjust the pH, 10 mL of 1 M NaOH was added. The final product was obtained as black powder, washed with deionized water and dried in an oven overnight.

2.6. Synthesis of GO/SPION@PEG

0.2 g of as prepared SPION nanoparticles were dispersed in 50 mL deionized water by ultrasonication. Then 0.6 g of polyethylene glycol (PEG) was added to the solution and stirred for 24 h. After stirring, SPION@PEG nanoparticles were washed with deionized water and dried at room temperature. In the next step, 0.04 g of GO was dispersed in 40 mL of deionized water. 0.04 g of SPION@PEG was added to the suspension and stirred overnight at room temperature. Finally, GO/SPION@PEG was washed with distilled water by centrifugation and dried in an oven at 30 °C.
2.7. Characterization

The size, morphology and distribution of SPION on the GO sheet were studied using field emission scanning electron microscopy (FESEM, TESCAN MIRA3). The hydrodynamic diameter of functionalized GO/SPION nanocomposites was measured by dynamic light scattering (DLS) (Nano S, Malvern, UK). The crystalline phase was evaluated by X-ray diffraction (XRD, XPert PRO MDP, with Cu kα radiation λ = 0.15418 nm, 40 mA, 40 kV). Scanning was carried out within the range of 20 angle from 10° to 80° with a 0.02° step at room temperature. The crystallite size of SPIONs was calculated from the XRD pattern using Scherrer’s equation: \( D = \frac{k\lambda}{\beta \cos \theta} \), where \( k \) is the shape factor that can be assigned a value of 0.89 if the shape is unknown, \( \lambda \) is X-ray wavelength of Cu Kα radiation 1.54 Å. \( \beta \) is the full width at half maximum in radians and \( \theta \) is the Bragg angle [37].

Fourier transforms infrared spectroscopy (FTIR) (NicoletMAGNA-IR 560) was performed in the range of 400–4000 cm\(^{-1}\) to identify functional groups and chemical interactions between GO and bare/functi- nalized SPION nanoparticles.

Assessment of magnetic properties of the samples was performed by vibratory sample magnetometer (VSM) (Daghigh Meghnatis Kashan Co., Kashan, Iran) at room temperature, over the range of ±10 kOe.

2.8. Magnetic fluid hyperthermia

To assess the efficiency of SPION nanoparticles as well as bare and functionalized GO/SPION for magnetic hyperthermia, the magnetic heating was done using an AC magnetic induction system (Hyperthermia, nastypec, 1000 W output power, \( f = 400 \) KHz, HAC = 120 Oe). To measure the specific absorption rate (SAR) and intrinsic loss of power (ILP) of all magnetic colloids, 5 ml of each sample with the concentration of 3 mg/ml were placed into the center of the heating coil and the fiber optic probe was placed in the center of the samples to record the temperature rise at interval of 60 s. To calculate the SAR and ILP values, the initial linear slope of \( \Delta T/\Delta t \) curve was taken. SAR was determined by calorimetric measurements using following equation:

\[
\text{SAR} = \frac{\Delta T}{\Delta t} \frac{C_p}{\rho} (1)
\]

where \( C_p \) is the heat capacity of liquid solvent (4.186 J/g K for water) and \( \rho = 3 \times 10^3 \) is the mass of magnetic samples per unit mass of deionized water.

The intrinsic loss power (ILP) is a heating transformation ability which normalized SAR with respect to field strength and frequency and is measured using following formula:

\[
\text{ILP} = \text{SAR}/fH^2 (2)
\]

where, \( H \) is the applied AC magnetic field and \( f \) is frequency of the coil.

2.9. In vitro studies

2.9.1. In vitro cytotoxicity assay

The cytotoxicity was assessed for samples using MTT assay [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) bromide].

Fibroblast cell line (L-929) was cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic penicillin-streptomycin solution followed by incubation (37 °C, 5% CO\(_2\)). The cells were trypsinized and passaged up to 80–90% confluence, and third passage cells were utilized for further investigations.

Sample sterilization was performed by exposure to UV light for 20 min. L-929 cells were seeded in 200 μl of culture medium at a density of \( 10^4 \) cells/well in a 96-well plate and incubated for 24 h at 37 °C. Then, culture media was aspirated and the cells were treated for another 24 and 48 h with 200 μl of media containing different concentrations of the following formulations: cells without nanoparticles (control), and five synthesized samples including, SPION nanoparticles, GO, GO/SPION, GO/SPION@PEG, and GO/SPION@CS at concentrations of 100, 300, and 500 ppm. After completion of incubation time, cells were further incubated for 3 h with 20 μl of MTT (5 mg/ml). After treatment, media was discarded from each well, and subsequently, 200 μl of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Readings were taken from a multi-plate reader (Thermo Fisher) at 540 nm. Percentage cell viability was calculated using following equation.

\[ \text{Relative cell viability} \% = \left( \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right) \times 100 \]

where OD\(_{\text{d}}\) and OD\(_{\text{c}}\) represent the optical density of the sample and control, respectively.

2.9.2. Flow cytometric analysis of apoptosis/necrosis

L-929 cells were incubated with GO/SPION@PEG and GO/SPION@CS at concentration of 100 ppm for 24 h. The cells were washed with PBS and then centrifuged at 1300 rpm for 5 min. Next, 5 μl of Annexin V-fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide (PI) were added to the cell suspension and they were incubated at ambient temperature for 15 min in the dark. Finally, the samples were analyzed using a flow cytometer instrument (BD Biosciences, USA).

2.9.3. Magnetic in vitro cytotoxicity study

To investigate the effect of applying magnetic field on fibroblast cells, L-929 cells were seeded in two cell culture plates at a density of \( 10^4 \) cells/well and incubated for 24 h at 37 °C. Then the cells were exposed to 100 ppm sterilized nanocomposites for 24 h. Subsequently, static magnetic field (350 mT) was placed under one of the cell culture plates and both plates were incubated separately for 90 min (37 °C, 5% CO\(_2\)).

After completion of incubation time, cells were further incubated for 3 h with 20 μl of MTT (5 mg/ml). After treatment, media was discarded from each well, and subsequently, 200 μl of DMSO was added to solubilize the formazan crystals. Metabolic activity was determined for magnetic field treated and non-treated L-929 cells.

To estimate the magnetic cytotoxicity effect of GO/SPION@PEG and GO/SPION@CS on human bladder carcinoma cancer cell, EJ138 cells were seeded in cell culture at a density of \( 10^4 \) cells/well and incubated for 24 h at 37 °C. Then the cells were exposed to 100, 300, and 500 ppm sterilized nanocomposites for 24 h. Subsequently, static magnetic field (350 mT) was placed under the cell culture plate and both plates were incubated separately for 90 min (37 °C, 5% CO\(_2\)). After completion of incubation time, cells were further incubated for 3 h with 20 μl of MTT (5 mg/ml). After treatment, media was discarded from each well, and subsequently, 200 μl of DMSO was added to solubilize the formazan crystals.

2.9.4. Cell morphology

L-929 cells were seeded on two cell culture plates at a density of \( 10^4 \) cells/well and incubated for 24 h at 37 °C. After incubation period, the cells were exposed to 100 ppm sterilized samples for 24 h. Subsequently, static magnetic field (350 mT) was placed under the cell culture plate and was incubated for 90 min (37 °C, 5% CO\(_2\)). After completion of incubation time, cells were further incubated for 3 h with 20 μl of MTT (5 mg/ml). After treatment, media was discarded from each well, and subsequently, 200 μl of DMSO was added to solubilize the formazan crystals.

2.10. SARS-CoV-2 surrogate neutralization antibody ELISA

Neutralizing antibodies against SARS-CoV-2 was measured using functional surrogate neutralization antibody ELISA designed based on SARS-CoV-2 RBD and human angiotensin-converting enzyme 2 (ACE2) interaction in the 96-well ELISA plate (Pishptaz Teb Diagnostics; cat.
numbers: PT-CoV2NT-96). In this assay, 50 μl of nanostructures dispersed in PBS and 50 μl horseradish peroxidase conjugated ACE2 were mixed in the appropriate wells coated with SARS-CoV-2 RBD protein and then incubated for 30 min at 37 °C. After adding chromogenic substrate 3,3’,5,5’-Tetramethylbenzidine (TMB) into the wells, the absorbance of the developed colors was measured. The quantity of neutralization was calculated based on a standard curve drawn against humanized SARS-CoV-2 neutralizing monoclonal antibody. Percentage of inhibition was calculated using following equation.

Relative virus inhibition (%) = (OD\textsubscript{standard} − OD\textsubscript{sample} / OD\textsubscript{standard}) × 100

where OD\textsubscript{sample} and OD\textsubscript{standard} represent the optical density of sample and standard with highest concentration, respectively.

2.11. Statistical analysis

Student t-test with a significance of P values <0.05 was utilized to analyze the data. The SPSS software was used for statistical analysis (V 25, New York, US). The obtained data were reported as mean and standard deviation values.

3. Results and discussion

3.1. Characterization

The morphology of the pristine GO as well as synthesized nanostructure and decoration of SPION on the GO sheet are presented in Fig. 1a. The FESEM images of GO display exfoliated sheet like morphology which is consistent with 2D hexagonal lattice structure of GO. The sheets have smooth surface and wrinkled edges with approximately 45 nm thick. FESEM images of GO/SPION and GO/SPION@CS reveal that the GO sheet is decorated by a large quantity of isolated SPION nanoparticles and is almost uniformly distributed on the surface of GO without agglomeration. Moreover, the sizes of decorated SPION nanoparticles are almost uniform and spherical in shape with diameter of 20 ± 5.1 nm and 25 ± 7.4 nm for GO/SPION and GO/SPION@CS, respectively. The FESEM results of PEG functionalized SPION-GO (particle size ~25 ± 12.2 nm) show that the SPION@PEG is fully covered the GO sheets.

These results suggest that the SPION nanoparticles are decorated on GO sheets which enable a good dispersion of these nanoparticles. Some reports suffered from agglomeration and formation of clusters of SPION on the surface of GO sheets [38,39]. Good distribution of SPION on GO sheets is a crucial aspect to obtain uniform properties in the nanostructures.

Fig. 1b shows the FT-IR spectra of GO, SPION, GO/SPION, GO/SPION@CS and GO/SPION@PEG. The FTIR spectrum of GO has a peak at 1029 cm\(^{-1}\) which is ascribed to the C−O bond, confirming the presence of oxide functional groups after the oxidation process [40]. The peak at 1635 cm\(^{-1}\) shows that the C=C bond still remains before and after the oxidation process [41,42]. The peak at 1245 cm\(^{-1}\) is ascribed to C=OH stretching [43,44]. The absorbed water in GO is shown by a broad peak at 3434 cm\(^{-1}\), corresponding to the O−H stretch of H\(_2\)O molecules [41,44]. In the spectrum of SPION nanoparticles, the peak at 578 cm\(^{-1}\) is characteristic of Fe−O vibrations. Moreover, the peaks at 869 cm\(^{-1}\) and the broad region absorption band around 3345 cm\(^{-1}\) are both attributed to the hydroxyl stretching vibrations of OH groups [45,46]. The FTIR of GO/SPION reveals a peak centered at 559 cm\(^{-1}\) corresponding to the stretching vibration of the Fe−O bonds in the tetrahedral crystal lattice of SPION [47,48]. The peaks at 1572 cm\(^{-1}\), 2921 cm\(^{-1}\) and 3395 cm\(^{-1}\) are assigned to the skeletal vibration of graphitic chain, C−H stretching of (CH\(_2\)) and hydroxyl (OH) functional group, respectively [48]. In addition, the peaks at 1110 cm\(^{-1}\) can be attributed to ether (C−O−C) group [45,48]. For GO/SPION@CS, the peaks at 571 cm\(^{-1}\), 1647 cm\(^{-1}\), 2926 cm\(^{-1}\) and 3447 cm\(^{-1}\) are ascribed to Fe−O stretching, N−H vibration of NH\(_2\) in chitosan [49], stretching vibration of C−H in chitosan and O−H stretching of hydroxyl group [50], respectively. Additional, the peaks at 1044 cm\(^{-1}\) and 1442 cm\(^{-1}\) are corresponded to the stretching vibration of O=C-O in the glucose circle of chitosan as well as CH\(_2\) and CH\(_2\) symmetrical bending, respectively [49,50]. For GO/SPION@PEG, the characteristic peaks appear at 585 cm\(^{-1}\), 1052 cm\(^{-1}\), 1427 cm\(^{-1}\), 1625 cm\(^{-1}\), 2924 cm\(^{-1}\), and 3421 cm\(^{-1}\) corresponded to Fe−O stretching, C−O stretching, C−O−C bending, C=C, C−H and O−H stretching, respectively [44,47,51,52]. This result confirms the successful coating of PEG on the iron oxide nanoparticles.

The particle size of functionalized GO/SPION nanocomposites was also measured using DLS. The particle size of GO/SPION@CS was found to be ~396.5 with proper polydispersity index (PDI) (0.347), as shown in Fig. 1c. The mean particle size of 730.9 nm was obtained for GO/SPION@PEG, with PDI (0.214) (Fig. 1c). Since DLS method measures the hydrodynamic diameter of the particle including the layer solvation, the obtained values were larger than the diameters measured by FESEM images. Moreover, The PDI values of both samples indicated that the nanocomposites had a narrow size distribution.

Fig. 1d displays magnetization hysteresis curves of the synthesized samples. According to the sigmoid shape of magnetization curve, all the synthesized nanostructures have superparamagnetic character. The saturation magnetization (M\(_S\)) of SPION, GO/SPION, GO/SPION@PEG and GO/SPION@CS were estimated around 41, 20, 19 and 8 emu/g, respectively. The highest saturation magnetization is attributed to SPION nanoparticles which is in harmony with reported results in literatures [48,53]. GO/SPION hysteresis curve presents higher magnification among nanostructures. The decrement in the magnetization of functionalized GO/SPION compared with GO/SPION could be attributed to the presence of non-magnetic polymer species surrounding iron oxide nanoparticles which plays an adverse effect on the performance of magnetization expression [54].

The crystalline nature and average crystallite size of synthesized SPION as well as GO/SPION were evaluated using XRD. As can be seen in Fig. 1e, the absence of peak at 26° corresponding to graphite and presence of the peak at 11.16° in the XRD pattern of GO show that the product is oxidized after the chemical oxidation and exfoliation, indicating an increase in d-spacing [52]. XRD spectrum of SPION nanoparticles indicates several diffraction peaks at 2θ = 30.56°, 35.71°, 43.26°, 53.81°, 57.21°, and 62.28° which indexed to (220), (311), (400), (422), (511), and (440) planes of SPION respectively, according to JCPDS card # 019–0629 identifying a cubic spinel structure [48]. The crystallite size of SPION was estimated to be 11.8 nm using the most intense peak (311) in XRD pattern. No impurities were found in the sample. Moreover, XRD pattern of GO/SPION also confirms the presence of SPION nanoparticles on the surface of GO sheets.

3.2. Magnetic hyperthermia

In order to evaluate the magnetic-heating ability of synthesized samples, superparamagnetic samples (SPION, GO/SPION, GO/SPION@PEG, and GO/SPION@CS) at the concentration of 3 mg/ml in deionized water were subjected to applied magnetic field (AMF) (400 KH, 120 Oe) for 900 s with 60 s intervals. Fig. 2a demonstrates a time dependent gradual increase in temperature of all samples under AC magnetic field generated by hyperthermia system. It has been reported that the heating efficiency of superparamagnetic nanomaterial is originated from Neel and/or Brownian relaxation which refers to the rotation of magnetic moment and physical rotation of magnetic nanoparticles, respectively [55,56]. The comparison of the ΔT for different samples suggests that SPION has the highest amount of ΔT, and as such, the amount of ΔT follows the order SPION > GO/SPION@PEG > GO/SPION@CS. No significant temperature rising is observed after 660 s for GO/SPION@PEG and GO/SPION@CS.

SAR values of superparamagnetic samples were calculated from the
Fig. 1. (a) The FESEM images of synthesized GO, GO/SPION, GO/SPION@CS, GO/SPION@PEG and the measured particle size distribution. (b) FTIR spectra of synthesized SPION nanoparticles, GO, GO/SPION, GO/SPION@CS, and GO/SPION@PEG. (c) DLS analyses of the size distribution of GO/SPION@CS and GO/SPION@PEG. (d) Magnetization curves of synthesized SPION, GO/SPION, GO/SPION@PEG and GO/SPION@CS. (e) XRD pattern of synthesized SPION nanoparticles, GO and GO/SPION.
ΔT versus time graph using the initial linear slope method at fixed frequency of 400 kHz and with an AMF of 120 Oe (~9.55 kAm$^{-1}$). The SAR values are found to be 594, 305, 283 and 199 W/g for SPION, GO/SPION, GO/SPION@PEG, and GO/SPION@CS, respectively (Fig. 2b). This result coincides with the $M_s$ value of these compositions obtained from VSM technique (Fig. 2c). The higher the $M_s$ value, the heating efficiency is more pronounced. It has been reported that the following phenomena may affect SAR value: (1) fluctuation in the flip direction of magnetic moments of SPION during Neel relaxation due to the alteration in barrier heights of magnetic anisotropy and/or inter-particle and inter-aggregate dipolar interactions; (2) deflection from log-normal distribution of magnetic dipole moments and twinning defects [55]. Among hybrid nanostructures, GO/SPION shows a higher SAR value, which indicates its superior heating profile. Kumar et al. reported that decoration of SPION on GO sheets increases the colloidal stability which in turn, enhances the heating properties of the synthesized compositions [21]. The decrement of SAR values of GO/SPION@PEG and GO/SPION@CS than GO/SPION could be ascribed to the polymeric coating, which consequently influences the inter-particle dipolar interactions [56]. The estimated SAR values of GO/SPION and functionalized GO/SPION nanocomposite are in accordance with the data reported by Sugumaran et al. [57] for GO/SPION and PVP-functionalized SPION nanoparticles.

Since many parameters such as particle shape, size, distribution, nanoparticles concentration, frequency and magnitude of external AMF affect SAR value, the ILP is considered as an alternative value to evaluate the heating transformation ability [21,55–60]. ILP is independent of frequency and field strength of AMF, giving the opportunity of comparing the heating efficiency of different nanomaterials synthesized in other laboratories [57]. System-independent ILP values are found to be 18.3, 9.4, 8.7, and 6.2 nHm$^2$kg$^{-1}$ for SPION, GO/SPION, GO/SPION@PEG, and GO/SPION@CS, respectively (Fig. 2a). Similar to the SAR value, there is a direct relationship between the $M_s$ and ILP value of compositions (Fig. 2d). It is recommended that the ILP values of higher than 3 nHm$^2$kg$^{-1}$ are desirable for magnetic hyperthermia therapy [21]. It is worth mentioning that our samples have ILP values even higher than the best available commercial materials [57]. Although both GO/SPION@PEG and GO/SPION@CS have the potential to use in cancer therapy, GO/SPION@PEG with higher SAR and ILP values is preferred.

3.3. In vitro studies

3.3.1. In vitro cytotoxicity assay

It is necessary to investigate the safety level of the synthesized nanomaterials for in vivo application. The first step of this kind of investigation is in vitro analyses, e.g., the mitochondrial activity. Therefore, the cytotoxicity of the synthesized GO, SPION as well as hybrid GO/SPION was evaluated on L929 cells using MTT assay after 24 and 72 h (Fig. 3). The biocompatibility results show the differences in mitochondrial activity of L929 cells that depend on the type of
As can be seen in Fig. 3a and b, SPION nanoparticles exhibit no cytotoxicity in time periods of 24 and 72 h ($p > 0.05$). Shundo et al. demonstrated that even higher concentration of iron oxide nanoparticles up to 1000 $\mu$g/mL does not reduce the cell viability [58]. This can be associated with low uptake of SPION by the cells [38]. Low toxic effect of SPION on Cos-7 monkey kidney cells and GH3 pituitary tumor cells was also reported [59,60]. Merely a slight decrease in cell viability was noted at a dose of 100 and 300 ppm in GO sample after 72 h. The low toxicity of GO can be assigned to the presence of oxygen containing functional groups at the edges of the graphene sheet, which folds the sharp edges [45]. The effects of GO on mouse fibroblast cells depend on GO dose as well as the culture time [38]. No significant cytotoxicity is detected for all three hybrid GO/SPION samples after 24 and 72 h, even at high concentrations but all samples show decreases in viabilities in a dose dependent manner. The cell viability of hybrid composites at 24 and 72 h incubation at 500 ppm concentration was calculated and found to be $92 \pm 0.3\%$ and $87 \pm 1.8\%$ respectively for GO/SPION, $77.6 \pm 1\%$ and $77 \pm 0.66\%$ respectively for GO/SPION@PEG, $85 \pm 1.8\%$ and $71 \pm 1.5\%$ respectively for GO/SPION@CS. The cell viability of GO/SPION@PEG does not change considerably even with increasing incubation time from 24 to 72 h. However, the cell viability of GO/SPION and GO/SPION@CS shows descending trend with time increment. Khafaji et al. [61] reported more than 90% cell viability of L929 cell line against PEGylated silica coated iron oxide nanoparticles after 24, 48, and 72 h. Liu et al. [62] reported that PEG molecular weight could influence the cell viability. Gul et al. research revealed that less chitosan de-acetylation increases its degradability and degradation products which have a harmful effect on cell viability [63].

The in vitro studies are a valuable method to evaluate the interaction of nanomaterials with the living cells and the possibility of their usage in biological environment. L929 fibroblasts are recommended by ISO 10993-5 for cytotoxicity tests. Actually, the cytotoxicity of the nanomaterials is usually inconclusive between researchers as it is highly dependent on various factors such as particle size, shape, chemical composition, functional groups, synthesis techniques, cell lines, incubation time and concentration [64]. Size of nanomaterial plays a key role in interactions with the biological system and many biological-
nanomaterial related mechanisms such as cellular uptake depending on it [65]. Nanomaterial sizes larger than 1 μm (e. g. GO) cannot easily enter the cell, but they interact with proteins absorbed in the cells [65]. Furthermore, the optimal size for internalization inside the cells is strongly related to the nanomaterial's surface chemistry [66,67]. In general, Van der Waals or electrostatic forces are critical in the nano-material interactions. Surface modification is a well-known strategy to decrease toxicity, increase stability, and to control and modulate cellular internalization in biomedical applications. Surface functionalization is predominantly comprised by PEG, carboxyl group, hydroxyl group and amine groups [67,68]. Moreover, interaction of nanomaterial with biomolecules such as proteins upon contact with the biological environment, known as protein corona, may also affect the nanomaterial-cell interactions due to alteration of nanomaterial size or surface chemistry [65–71]. Regarding the effects of GO and hybrid GO/SPION on cell viability, the mechanism is not well explained and still requires further analysis. Many in vivo studies of GO-based systems have been also conducted in recent years. Rodrigues et al. demonstrated that lateral dimensions play a fundamental role in the pulmonary response to GO after intranasal instillation in mice [72]. Yang et al. reported that PEGylated GO did not induce appreciable toxicity in mice after intravenous administration in a period of 3 months [73]. Karthika et al. performed in vivo toxicity assay on zebrafish and the result showed the high biocompatibility of the rGO/Fe₃O₄/CS without inducing significant abnormalities [74]. Zan et al. indicated that graphene/Fe₃O₄ nanocomposite can be cleared from the body through the metabolic processes of macrophages and therefore it is harmless to the living body [75].

Here, we clearly demonstrate that bare, PEG and chitosan functionalized GO/SPION shows more than 70% cell viability in the time period of 72 h even at 500 ppm concentration. Therefore, we believe that such hybrid nanomaterials with high cytocompatibility would be desirable for the application in biomedicine, e.g., as a drug carrier and/or in hyperthermia.

3.3.2. Flow cytometric analysis

The flow cytometry was performed to quantitatively evaluate live, necrotic, early, and late apoptotic cells. The type of cell death (apoptotic or necrotic) was analyzed by double staining with FITC and PI. Cells which were PI and Annexin V negative were considered healthy, the cells which were PI negative and Annexin V positive were considered early apoptotic, cells which were positive for both PI and Annexin V were considered late apoptotic and those which were PI positive and Annexin V negative were considered necrotic. The cells with no treatment were considered as the control group.

According to the flow cytometry results, a lower number of early, late apoptotic as well as necrotic cells are found in GO/SPION@PEG and GO/SPION@CS. This result reveals that the developed nanomaterials in this study do not have a harmful effect on cell division. These findings are in concurrence with the MTT assay and demonstrate that functionalized GO/SPION nanocomposites have very low cytotoxicity to the tested L929 cells at the concentration of 100 ppm. Kooti et al. reported that the magnetic GO inlaid with silver nanoparticles (10 ppm) had not led to cytotoxicity and cellular apoptosis of eukaryotic cells, and the cells were being continuously proliferated [76].

3.3.3. In vitro magnetic cytotoxicity analysis

To determine the effect of magnetic field on the cytocompatibility of normal cells, the samples (concentration of 100 ppm) were exposed to L-929 cells under the applied constant magnetic field (350 mT) for 90 min.

Fig. 4a shows that the cell viability of SPION nanoparticles treated L929 cells is almost 100%. However, a substantial decrease in cell viability (~53.36%) is seen in the presence of a constant magnet. The cell viability shows descending order from 97 ± 0.5% to 72 ± 3% for GO/SPION, 90 ± 2% to 75 ± 0.6% for GO/SPION@PEG and 83 ± 2.1% to 81 ± 0.5% for GO/SPION@CS under the applied magnetic field (Fig. 4a).

According to the flow cytometry results, a lower number of early, late apoptotic as well as necrotic cells are found in GO/SPION@PEG and GO/SPION@CS. This result reveals that the developed nanomaterials in this study do not have a harmful effect on cell division. These findings are in concurrence with the MTT assay and demonstrate that functionalized GO/SPION nanocomposites have very low cytotoxicity to the tested L929 cells at the concentration of 100 ppm. Kooti et al. reported that the magnetic GO inlaid with silver nanoparticles (10 ppm) had not led to cytotoxicity and cellular apoptosis of eukaryotic cells, and the cells were being continuously proliferated [76].

Fig. 4. (a) Relative cell viability of SPION nanoparticles as well as GO/SPION hybrid composites against L-929 cells at the concentration of 100 ppm after 24 h of treatment in the presence of constant magnet, * = p < 0.05. (b) Relative cell viability of GO/SPION@PEG and GO/SPION@CS against EJ138 cells at the concentrations of 100, 300 and 500 ppm after 24 h of treatment in the presence of constant magnet, * = p < 0.05. (c) Relation between relative cytotoxicity of GO/SPION@PEG and GO/SPION@CS (concentration ~ 300 ppm) and their saturation magnetization.
their magnetic lethality subsided. Because polymer coating reduces the magnetic impact of nanoparticles on cell killing under the applied magnetic field and therefore intercepts cell destruction [54]. Following the recommendation of the ISO-10993-5 in which viabilities higher than 70% are not considered cytotoxic, we proposed the in vitro use of functionalized GO/SPION in biomedical application which is necessary to utilize the external magnetic field.

To investigate the magneto thermal effect of functionalized GO/SPION on EU138 human bladder cancer cells, GO/SPION@PEG and GO/SPION@CS were exposed to a static magnetic field (350 mT) for 90 min at the concentrations of 100, 300 and 500 ppm. Fig. 4b shows the cell viability of cancer cells treated with functionalized nanocomposites. As can be seen, the cell viability shows a descending trend under the applied magnetic field in both samples. The presence of magnetic SPION nanoparticles leads to the focus of the static magnetic field on the cells and provides vulnerable conditions for cell viability [55]. The relation between magnetic property and relative magnetic cytotoxicity of samples is shown in Fig. 4c. The results demonstrate that with decreasing sample magnetization, the relative magnetic cytotoxicity percentage shows a downward trending. As it is expected, the ability to kill cancer cells is more pronounced for the GO/SPION@PEG than GO/SPION@CS due to higher magnetization. Moreover, at higher concentrations (300 and 500 ppm), GO/SPION@PEG shows the ability of killing even more than 50% of cancer cells which could be effective for cancer therapy. To conclude, when targeted magnetic cytotoxicity is considered as a matter of necessity, such as magnetic cancer therapy, GO/SPION@PEG could be beneficial. Due to the relative biocompatibility of GO/SPION@CS, it could play an operative role in biological targeting without the need for tangible cytotoxicity such as magnetic drug delivery carriers.

3.3.4. Cell morphology

Optical microscopy has been used to evaluate changes in cell morphology induced by magnetic compositions with and without a constant magnet. Black and red arrows represent normal and abnormal cells, respectively (Fig. 5). As can be seen, the cells retain their normal shape when exposed to SPION nanoparticles and merely a few numbers of cells have changed their morphology, exposing nanostructures without magnetic field. Furthermore, alive cells exhibit spindle and elongated morphology, while dead cells are more likely to exhibit round morphology. It has been confirmed that cells usually tend to show elongated shapes in favorable environments, which could be a sign of biocompatibility of specific surfaces toward cell attachment [14]. By applying an external magnet, in accordance with MTT assay results, cells undergo deformation and change their morphology and such cellular deformation is more prevalent for SPION and GO/SPION with higher magnetization. It is obvious that magnetic SPION nanoparticles can effectively deform cell appearance and consequently influence cell survival and proliferation under strong magnets. This could be attributed to DNA damage which caused by magnetic nanomaterials under the applied magnetic field [77].

The cellular morphology and cytoskeleton structure of L-929 cells induced by magnetic samples in the presence and absence of a magnet were analyzed by SEM micrographs. As shown in Fig. 6, control cells display their characteristic spindle-shaped morphology (filopodia) and cover the glass substrate. It seems that they reflect mostly the healthy phenotype of fibroblast cells [78]. No significant changes in morphology occurred in the absence and presence of a magnetic field.

L-929 fibroblasts in the vicinity of SPION and GO/SPION retain their original shape, and their membranes are not ruptured in the absence of a magnet (Fig. 6). The cells adhere well to the substrate and flatten [79]. Round shape cells are rarely observed. Based on the MTT results, we would not speculate that the cells undergo severe damage at these samples. Although flattened cells can be distinguished in exposure to GO/SPION@PEG, and GO/SPION@CS, cell attachment had been severed in some areas and cells became more rounded due to loss of their polar orientation [80]. The cell morphology has changed specifically for GO/SPION@CS.

On the contrary under a magnet, the L-929 morphology in the vicinity of SPION nanoparticles as well as GO/SPION and GO/SPION@PEG has changed. The cells lost their original, elongated shape, and the flattened cells had no interconnectedness. Such observation is compatible with the MTT results in the presence of a magnet. However, no noteworthy differences in cell morphology were noticed between magnetic and non-magnetic conditions for GO/SPION@CS, and this nanostructure was not able to cause notable magnetic morphological changes, which could be originated from its lower magnetization compared with other nanostructures.

3.3.5. Antiviral analysis

The inhibitory effect of GO, SPION, GO/SPION, GO/SPION@PEG and GO/SPION@CS on SARS-CoV 2 RBD and its receptor ACE2 interaction at the concentration of 100 ppm was evaluated using SARS-CoV-2 surrogate neutralization antibody ELISA method (Table 1). According to the results, GO neutralizes 43% of RBD-ACE2 binding, which nearly equals to inhibition observed at standard B. The strong interaction of GO with spike, ACE2 receptor and spike-ACE2 complex was reported by Unal et al. [23]. Maio et al. stated the complete SARS-CoV-2 inhibition in GO functionalized face mask materials [81]. No sign of antiviral activity is observed for SPION nanoparticles. GO/SPION reveals merely 23% viral inhibition, which lessens than non-decorated GO. This could be ascribed to the decoration of GO sheets with SPION nanoparticles which hampers the connection of SARS-Cov-2 RBDs to GO surfaces, confirming by FESEM images (Fig. 1a). GO/SPION@PEG shows no neutralizing effect of SARS-CoV-2 virus specific anti-spike
monoclonal antibodies. As shown in Fig. 1a, the surface of GO sheets is fully covered by SPION@PEG, which does not allow any access to SARS-CoV-2 RBDs. The highest level of inhibition is related to GO/SPION@CS with more than 86% viral inhibition.

The SARS-CoV-2 virus, the causative agent of coronavirus-19, is a single-stranded RNA with an envelope [82]. The virus contains several structural proteins, including envelope protein (E), spike protein (S), nucleocapsid protein (N) and membrane protein (M) (Fig. 7a) [83,84]. S protein of coronaviruses subdivided to the N-terminal S1 subunit, which forms the bulbous head of the S protein, and the C-terminal S2 subunit that forms the stalk region of the protein and is directly embedded into the viral envelope [85]. Upon interaction with a potential host cell, the S1 and S2 subunits are responsible for receptor recognition/binding and

![Fig. 6. FESEM images of cell morphology of L929 cells exposed to the SPION nanoparticles as well as GO/SPION nanocomposites in the presence and absence of constant magnet.](image)

| Standard (μg/ml) | Virus inhibition (%) | Samples     | Virus inhibition (%) |
|-----------------|----------------------|-------------|----------------------|
| A (0)           | 0                    | GO          | 43                   |
| B (1)           | 46                   | SPION       | 0                    |
| C (2.5)         | 62                   | GO/SPION    | 26                   |
| D (5)           | 80                   | GO/SPION@CS | 86.5                 |
| E (10)          | 87                   | GO/SPION@PEG| 0                    |
| F (40)          | 100                  | SPION@PEG   |                      |

Table 1
The inhibitory effect of GO, SPION, as well as bare and functionalized GO/SPION on SARS-CoV-2 virus.
membrane fusion, respectively [85]. S1 subunit contains a RBD that identifies and binds to the receptor at the cellular level of ACE2, which leads to endocytosis into lung cells and virus replication [86]. Infection with SARS-CoV-2 virus induces acquired immune responses and as a result, specific antibodies against viral antigens are found in patients' circulatory system [84]. Therefore, specific antibodies against RBD can play a protective and neutralizing role by inhibiting RBD-ACE2 binding.

Understanding the mechanism of nanomaterial-virus interaction is somehow difficult due to the low size of viruses (2–300 nm) [85]. Several mechanisms include the blockade of viral proteins (polymerases or enzymes) responsible for entry into human cells, or inhibition of important viral enzymes responsible for genome replication or viral assembly, and some target host cellular proteins that are involved in virus activity [23,87]. Some research endeavors suggested that graphene and its derivatives (i.e. GO) exhibited the ability to counter a variety of human viral pathogens [85,88,89]. They proposed that GO could directly interact with viruses via electrostatic interactions, hydrogen bonding and redox reactions [90]. For instance, simulations conducted by Raval et al. [91] revealed a strong binding efficiency between pristine multilayer exfoliated graphene and RBD of SARS-CoV-2 virus. They also found that the surface reactivity of the graphene material was enhanced with increasing the number of carbon layers [90]. Unal et al. revealed that GO had affinity toward the ACE2 receptors, spike protein and spike-ACE2 complex [23]. Furthermore, GO can absorb charged lipids and destroy membranes, suggesting possibility of interaction with enveloped viruses like SARS-CoV-2 virus [81].

The high surface area of GO provides an outstanding opportunity to be functionalized or to be used as a substrate for loading other antiviral agents [92]. It has been reported that chitin and chitosan can fight against viral infection [87]. The proposed mechanisms of chitosan antiviral activity are as follows: (i) destruction of virus structure through electrostatic interactions between positively charged chitosan and negatively charged capsid protein S; (ii) inactivation of viruses by attaching to its tail fiber and dissociation of a virus; (iii) interfering with the viral replication process; (iv) binding to the viral ligands and preventing their interaction with the host cell receptors [87]. Herein, GO/SPION@CS shows superb antiviral performance due to the synergistic effect of GO and chitosan to fight against corona viruses (Fig. 7b). The well distribution of SPION@CS on GO surfaces makes it a promising candidate to be used as an antiviral platform nanomaterial.

4. Conclusions

In summary, bare, PEG and chitosan functionalized SPION nanoparticles were uniformly decorated on graphene oxide sheets. GO sheets decorated with SPION nanoparticles as well as GO/SPION@PEG and GO/SPION@CS possessed a super-paramagnetic behavior. Due to polymer coating, $M_r$ values of functionalized GO/SPION were lower than GO/SPION but not pronounced for GO/SPION@PEG. The developed GO/SPION demonstrated low toxicity on L929 cells even up to 500 ppm of concentration. High SAR value of 305 W/g and ILP value of 9.4 nHm²kg⁻¹ for GO/SPION@PEG make this composition a good candidate for cancer hyperthermia treatment. This issue was further supported by MTT assay by killing of cancer cells exposed to the GO/SPION@PEG under the applied magnetic field. Meanwhile, GO/SPION@CS was capable of neutralizing SARS-CoV-2 virus effectively and could be considered as a promising material against Covid-19.

CRediT authorship contribution statement

Shaghayegh Kohzadi: Investigation, Methodology, Writing – original draft. Najmeh Najmoddin: Supervision, Conceptualization, Writing – review & editing. Hadi Baharifar: Supervision, Methodology, Writing – review & editing. Mahdi Shabani: Methodology, Writing – review & editing.

Fig. 7. (a) Schematic representation of the structural proteins of SARS-CoV-2 virus and its interaction with host cell receptor ACE2 through spike protein. Receptor binding domain (RBD) plays a key role during this binding, (b) GO/SPION@CS and its interaction with RBD inhibits RBD-ACE2 binding.
Declaration of competing interest

The authors declare no potential conflicts of interest concerning the research, authorship, and publication of this article.

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