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Magnetic based graphene composites with steroidal diamine dimer as potential drug in hyperthermia cancer therapy

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Abstract

Hyperthermia is a non-invasive process of killing cells through heat, as cells go into apoptosis when heated in the range of 41 °C–47 °C. In this work, the biologically active 4-pregnen-3-one-20β-carboxaldehyde (ketobisnoraldehyde) based steroidal diamine dimer (KPD) was chemically grafted on GO surface (GO-KPD) for the first time through an amidation reaction between amine groups of KPD and activated carboxylic acid sites of GO. Magnetite nanoparticles (Fe3O4) were dispersed on the prepared nanocomposite surface to produce GO-KPD-Fe3O4 nanocomposite with superparamagnetic property. To study the structural effect of KPD, 1,4 diamonobutane (Putrescine) was also grafted chemically on GO via amidation reaction. Successful functionalization of GO surface was confirmed using Fourier transform infrared spectroscopy (FT-IR), Raman spectroscopy, X-ray diffraction (XRD), elemental analysis, and thermogravimetric analysis (TGA). The morphology of the functionalized GO was characterized by scanning electron microscopy (SEM). Furthermore, a cytotoxicity test on Michigan Cancer Foundation-7 (MCF-7) human breast cancer cell line was conducted. The data suggest that the prepared nanocomposite (GO-KPD-Fe3O4) has a cytotoxic potential against the MCF-7 cell line, thus it could be investigated as potential drug in hyperthermia cancer therapy.

1. Introduction

Cancer therapy is an active research area for nanobiotechnological applications because nanotechnology presents novel approaches to major existing problems in the field. A lot of research has been done on magnetite nanoparticles, as they are specifically good candidates for nanomedical applications because of their intrinsic superparamagnetic properties and other desirable qualities [1]. Nonetheless, the field still faces challenges in the clinical translation of nanoparticles such as biocompatibility concerns, toxicity, targeting efficiency, and long-term stability of the magnetite nanoparticles, these issues require an urgent solution before the field can advance [2].

Magnetite nanoparticles can be loaded with drugs and dragged through an extracorporeal magnetic field to the tumorous site [3]. Moreover, they can be heated via hyperthermia in a range of 40 °C–45 °C, contributing to the destruction of the heat-sensitive cancer cells. Also, iron oxide nanoparticles accumulate in tumor sites through passive targeting due to their small size which allows them to remain in the sites for a longer time than blood clearance [4]. However, there are some challenges with using Fe3O4 nanoparticles. Magnetite nanoparticles are highly reactive with oxidizing agents and their hydrophobicity leads to decreased thermodynamic stability. Furthermore, un-coated iron oxide nanoparticles are considered toxic to the body tissues. These issues can be tackled by coating the particles with oxide surfaces such as graphene oxide (GO) [5].

GO is a graphene sheet with randomly distributed aromatic regions (sp2) and oxygenated aliphatic regions (sp3) containing various types of oxygen functionalities distrusted all over the sheets and at the sheet edges [6].
The loading of Fe₃O₄ nanoparticles on GO surface may improve the solubility and stability of the nanoparticles, also provide high surface area for drug loading. Added to that, it can promote the hyperthermia by providing near-infrared optical property to the nanocrystal [5, 7, 8]. Noteworthy, GO itself has many interesting properties such as the distinctive amphiphilic nature that enables access through the cell membrane [9]. However, GO sheets aggregate in physiological saline solutions due to the charge screening effect of the negatively charged oxygen functional groups present on it [10]. Also, the negative charge on GO is disadvantageous in penetrating malignant cell membrane since they exhibit a negative net charge in an electrolyte solution such as saline [11]. Therefore, chemical functionalization of magnetite–GO prior to medical application uses is a necessity.

Various modifying groups have been used to functionalize GO via condensation reaction for medical purposes including polymers, biomolecules, porphyrins, polyamines...etc [12–15]. Pramanik et al investigated the effect of covalently functionalized GO with hyaluronic acid on the bioactivity of Fe₃O₄ nanoparticles. Their results showed great biocompatibility of the nanocomposite and significant potential in hyperthermia and targeted drug delivery [3]. Lemin et al work also indicated that coating nanoparticles such as iron oxide with biocompatible material like chitosan improves the heating efficiency for hyperthermia [1]. Yet, the use of biocompatible steroidal based materials remained unexplored. Although, dimeric steroids—one of the common class of steroids—are well-known for their pharmacological activity, especially polyamine based dimeric steroids [16]. As well, the naturally occurring cephalostatins, cressatinis, and ritterazines are among the most potent natural cytotoxins with extreme cytotoxic activity towards various human tumor cell lines [17, 18]. Polyamine dimeric steroids also found their applications as molecular umbrella for drug delivery [17]. Thus, polyamines dimeric steroids are emerging as a significant chemical resource for biomedical application. Consequently, covalent functionalization of magnetically modified GO with polyamine dimeric steroids will not just prevent the sheets from aggregation but also might render them cytotoxic characteristics toward malignant tumor cells. Furthermore, they might lower the negative charge of GO, contributing to more efficient interaction with the cell membrane.

In this work, the functionalization of GO with 4-pregnen-3-one-20ß-carboxaldehyde (ketobisnoraldehyde) based steroidal dimine dimer (KPD) through condensation reaction to produces GO-KPD nanocomposite were reported. The GO-KPD nanocomposite was loaded with magnetite nanoparticles aimed to produce a dual-targeted delivery system based on the biological activity of the KPD and hyperthermia. To study the structural effect, the dimine itself was also functionalized with GO. The cytotoxicity toward breast cancer cells was reported. Our future goal is to investigate nanotechnology-based hyperthermia methods and their risks/benefits in cancer treatment.

2. Experimental section

2.1. Materials

All reagents and solvents were used without further purification. Ketobisnoraldehyde, 97% sodium triacetoxylborohydride (NaBH₃(OAc)₃), 99% thionyl chloride (SOCl₂), sodium nitrate (NaNO₃), 99% Glacial acetic acid (AcOH), 37% hydrochloric acid (HCL), and 99% ethanol were provided from Sigma-Aldrich (Germany), while 96% sulfuric acid and toluene were bought from Scharlu (Spain). Sodium hydroxide (NaOH), and ≥97% potassium permanganate (KMnO₄) were obtained from Park Scientific (UK). Pure graphite, ≥97% ferrous chloride hexahydrate (FeCl₂·6H₂O), and ≥97% ferrous chloride tetrahydrate (FeCl₂·4H₂O) were purchased from Fischer (UK). While 99% pure Putrescine (1,4 butane diamine) was obtained from Acros (Belgium), and 30% hydrogen peroxide (H₂O₂) was provided from AppliChem (Germany). Dichloromethane and chloroform were purchased from TEDIA company and ~70% magnesium sulfate (MgSO₄) was purchased from CDH company (India).

2.2. Instrumentations

X-Ray diffraction (XRD) was performed on (XRD-6000, Shimadzu) with CuKα radiation λ = 1.5405 Å. The scanning electron microscope (SEM) used was a Quanta FEI 450 SEM. EURO EA elemental analyzer was used for elemental analysis. Ultrasonication was performed on Omni Sonic Ruptor 400 ultrasonic Homogenizer. While Centurion Scientific C2 Series centrifuge was used for centrifugation. Thermal gravimetric analysis (TGA) was performed on Netzsch-Proteus model 209F1-Iris device. The Fourier transform infrared spectroscopy (FT-IR) of the samples was taken in the region between 400–4000 cm⁻¹ with Thermo Scientific Nicolet iS10 FTIR Spectrometer. Raman spectra of nanocomposites were measured with a Bruker Equinox 55 FT-IR spectrometer. BioTek Cytation 5 instrument was used to get automated images of living cells.

2.3. Preparation of Graphene Oxide (GO)

GO was synthesized from natural graphite by the modified Hummer’s method [19]. In brief, 113 ml of 96% H₂SO₄ and 2.5 g of NaNO₃ were mixed and stirred for several minutes. Then, 4.5 g of graphite was added to the mixed solution under stirring condition. Followed by the addition of 15 g KMnO₄ dropwise with vigorous stirring at 0 °C. After the addition, the temperature was raised to 40 °C and the mixture was stirred vigorously for
2.72 g of FeCl₃·6H₂O and 1.06 g of FeCl₂·4H₂O were added to deionized water (DI) and 20 ml of 30% H₂O₂ were added to eliminate the excess KMnO₄. A yellow paste was formed and centrifuged at 6000 rpm for 10 min and washed several times with 10% HCl and DI to remove residual salts and metals [20]. After washing, a dark brown paste of graphite oxide was obtained, then dried in the oven at 70 °C for 24 h.

### 2.4. Preparation of graphene oxide containing acyl chloride groups (GO-COCl)

GO was activated by nucleophilic acyl substitution reaction with SOCl₂ [15]. In brief, 100 mg of GO was dispersed in 25 ml of SOCl₂ in the presence of 2 ml DMF. The dispersion was heated at 70 °C for 24 h under reflux with continuous stirring to generate GO-COCl. Excess SOCl₂ was evaporated in vacuum at 70 °C. Then the prepared sample was washed excessively with toluene and ethanol.

### 2.5. Preparation of putrescine modified graphene oxide (GOP)

GOP was prepared by a condensation reaction between the amine groups of Putrescine and the acyl chloride groups of GO-COCl [15]. 100 mg of GO-COCl was dispersed in a solution of 200 mg Putrescine in ethanol. The mixture was heated to 60 °C under reflux for 2 h with continuous stirring. After that, excess unreacted Putrescine was removed by centrifugation at 6000 rpm for 30 min. The product was washed several times with hot ethanol and hot water then dried at 60 °C under vacuum to generate GOP.

### 2.6. Preparation of Ketobisnoraldehyde-based steroidal diamine dimer (KPD)

KPD were prepared by reductive amination between the aldehyde group of Ketobisnoraldehyde steroid and the amine groups of putrescine by using a selective reducing agent according to literature [21]. Putrescine (0.36 g, 4 mmol) was added to a solution of 1.4 g Ketobisnoraldehyde (4 mmol) in 60 ml DCE in the presence of 2 ml AcOH. The mixture was stirred at room temperature under nitrogen gas for 64 h. Then, 1.7 g of NaH(ACO)₃ (8 mmol) was added to the mixture. The mixture was stirred for another 24 h under the same conditions. The reaction was quenched with 1 M NaOH and the product extracted with chloroform (4 × 50 ml). The combined organic layers were washed with brine (2 × 40 ml), dried over MgSO₄ and filtered. Subsequent evaporation of the solvent in vacuo gave the crude product, which was purified by recrystallization with ethanol to yield KPD.

### 2.7. Preparation of Ketobisnoraldehyde diamine dimer modified graphene oxide (GO-KPD)

GO-KPD was prepared using the same procedure for the preparation of GOP. 100 mg of GO-COCl was dispersed in ethanol in the presence of 200 mg of KPD. The mixture was stirred at 60 °C for 2 h under reflux. Excess KPD was removed by washing several times with ethanol and dried at 60 °C under vacuum to generate GO-KPD powder.

### 2.8. Preparation of magnetite nanoparticles (Fe₃O₄)

Fe₃O₄ nanoparticles were prepared by co-precipitation method as stated in literature with some modifications [22]. First, 2.72 g of FeCl₃·6H₂O and 1.06 g of FeCl₂·4H₂O (2:1 molar ratio) were dissolved in 100 ml of DI water separately. The solutions were stirred for 10 min at room conditions. The temperature then was raised to 40 °C and the two solutions were mixed together for 10 min using overhead stirrer. 20 ml of 3 M NaOH was added to the mixed mixture dropwise in a period of 1 h with continuous stirring. After the addition, the Fe₃O₄ nanoparticles were magnetically separated from the supernatant and washed thoroughly with ethanol and DI until pH was neutralized. The product was dried at 60 °C in the oven.

### 2.9. Preparation of GO-KPD-Fe₃O₄ nanocomposite

50 mg of GO-KPD was dispersed in 100 ml DI by ultrasonication for 15 min 100 mg of Fe₃O₄ was added and the mixture was sonicated for another 15 min. The GO-KPD-Fe₃O₄ nanocomposites were collected by applying an external magnet to remove any floated GO-KPD sheets. Finally, GO-KPD-Fe₃O₄ were alternately washed with ethanol and DI several times [23].

### 3. Results and discussion

#### 3.1. Fourier transform infrared spectroscopy (FT-IR)

Figure 1 (a) illustrates the FT-IR spectrum of KPD. The peak at 3310 cm⁻¹ is attributed to (N–H) stretching of secondary amines. While the peak at 1675 cm⁻¹ is attributed to (C=O) stretching vibrations of the conjugated ketone of ketobisnoraldehyde steroid [21]. The FT-IR spectrum of GO presented in figure 1 (b) shows bands between 3769–3000 cm⁻¹ corresponding to the O–H stretching of adsorbed water molecules and hydroxyl groups [19]. The peak at 1735 cm⁻¹ corresponded to the C=O stretching of carboxylic and carbonyl groups presented in GO, and the peak at 1572 cm⁻¹ is attributed to the C=C stretching band. The peaks at 1232 cm⁻¹...
and 1050 cm\(^{-1}\) contributed to the C–O stretching bands of epoxy and alkoxy groups\(^{[24]}\). In GOP, (figure 1(c)) the functionalization of GO with putrescine was confirmed by the peak presented at about 1719 cm\(^{-1}\) which contributes to C=O stretching vibrations of secondary amide and 1543 cm\(^{-1}\) for C–N in-plane stretching and CHN deformation\(^{[15, 25]}\). Moreover, a reduction in the peak at 1050 cm\(^{-1}\) corresponding to C-O stretching band of alkoxy groups was observed. The GO-KPD spectrum (figure 1(d)) showed two peaks at around 2936 cm\(^{-1}\) and 2847 cm\(^{-1}\) that corresponds to C–H asymmetric and symmetric stretching of methylene groups respectively, demonstrating the presence of KPD on the GO surface\(^{[15]}\). Furthermore, a massive decrease in the peak at 1735 cm\(^{-1}\) was observed compared to pure GO\(^{[24]}\). The appearance of a new peak at 1679 cm\(^{-1}\) along with the peak at 1195 cm\(^{-1}\) could be attributed to the C=O stretching of tertiary amide and C–N stretching of amines respectively, which further proves the successful functionalization of GO with KPD.

The spectra of Fe\(_3\)O\(_4\) nanoparticles illustrated in figure 1(f) shows a characteristic peak at 592 cm\(^{-1}\) which is contributed to the Fe–O stretching vibrations of Fe\(_3\)O\(_4\) nanoparticles\(^{[26]}\).

The FT-IR of GO-KPD/Fe\(_3\)O\(_4\) (figure 1(e)) shows a peak at 594 cm\(^{-1}\) indicating that Fe\(_3\)O\(_4\) nanoparticles have successfully been dispersed on the surface of GO-KPD.

3.2. Elemental analysis

The presence of Putrescine in GOP and KPD in GO-KPD was confirmed by elemental analysis. Table 1 shows the relative percentage (%) of nitrogen (N), hydrogen (H), and carbon (C) in GO, GOP, GO-KPD samples. GOP has higher nitrogen content than GO-KPD, indicating a higher loading of Putrescine in GOP. This is expected due to the steric factor caused by the dimeric steroids attached to the Putrescine in KPD.

| Sample name | N%  | C%  | H%  |
|-------------|-----|-----|-----|
| GO          | 0.000 | 46.95 | 2.195 |
| GOP         | 6.241 | 66.397 | 3.092 |
| GO-KPD      | 2.666 | 61.811 | 4.688 |

![Figure 1. FT-IR spectrum of (a) KPD, (b) GO, (c) GOP, (d) GO-KPD, (e) GO-KPD-Fe\(_3\)O\(_4\), and (f) Fe\(_3\)O\(_4\) samples.](image-url)
3.3. Scanning electron microscopy (SEM)

Figure 2(a) shows the SEM image of GO. The image demonstrates the morphology of GO which resembles a thin curtain. The image also shows a minimal stacking because of the oxygen functional groups and adsorbed water molecules presented between the GO sheets. It also demonstrates the large surface area and the transparency of GO [27]. While the SEM image of GOP (figure 2(b)) shows that GO sheets are layered in a more organized and stacked form compared to GO, which can be attributed to the cross-linking of Putrescine molecules between the GO sheets [28]. The SEM images of GO-KPD (figures 2(c), (d)) illustrate the distribution of the KPD groups on the surface of GO. As shown in the images, the functionalization took place mainly on the carboxylic acid at the edges of GO sheets due to the steric factor. The successful preparation of quasi-spherical Fe₃O₄ nanoparticles is demonstrated in figure 2(e) [29]. The images demonstrate that Fe₃O₄ nanoparticles tend to aggregate. On the
other hand, GO-KPD-Fe₃O₄ image (figure 2(f)) shows the dispersion of Fe₃O₄ nanoparticles on the surface of GO-KPD with a high loading and lower aggregation compared to bare Fe₃O₄ nanoparticles.

3.4. X-ray diffraction (XRD)

Figure 3(a) demonstrates the diffractogram of GO, GOP, and GO-KPD. The diffractogram of GO showed a strong peak at 2θ of 10.38° which is a characteristic peak for GO [15, 30]. XRD of GOP and GO-KPD showed a weak peak at 2θ of 12.1° and 12.6° respectively, and another peak near the 2θ of 23.92° and 23.82° respectively. These results are consistent with the observations of Rani et al [25]. These observations are believed to correspond to the functionalization of GO with amines. The functionalization might remove the intercalated water molecules and reduces the hydrophilic character in GO, allowing closer inter-layer stacking of graphene sheets. Thus, the d-spacing is shifted closer toward pristine graphite [31].

The XRD spectrum of Fe₃O₄ nanoparticles (figure 3(b)) is in good agreement with the results reported in literature [22, 26, 29]. The result showed that the sample has six peaks at 2θ of 30.5, 35.68, 43.46, 54.02, 57.36 and 63.0° representing the corresponding indices of (220), (311), (400), (422), (511), and (440), respectively. The average particle size was calculated using Debye-Scherrer equation [32–34]:

\[ D = \frac{0.89 \lambda}{\beta \cos \theta} \]

Where \( \lambda \) is the wavelength of CuKα radiation (\( \lambda = 1.5405 \) Å), \( \beta \) is the entire width at half maximum, and \( \theta \) is Bragg’s diffraction angle. The average particle size estimated from the equation was 11.35 nm. There was no significant change in the peaks representing Fe₃O₄ nanoparticles after the functionalization with GO-KPD, which indicates that the crystalline structure of Fe₃O₄ nanoparticles remained after functionalization. Another peak at 2θ of 23.04° was observed that contributed to the GO-KPD. The intensity of the peak at 2θ equals 23.04° was low due to its small quantity in the sample.

3.5. Raman spectroscopy

Raman spectroscopy was performed to examine the carbon structure of GO, GOP, and GO-KPD and GO-KPD-Fe₃O₄ (figure 4). In general, the main peaks of GO in Raman spectrum are the D and G bands observed around 1352 cm⁻¹ and 1604 cm⁻¹, respectively [35]. Where the D band refers to the vibration of the sp³ carbons and the defects in GO structure especially at the sheet edges generated by the oxidation process and the G band refers to the vibration of the sp² carbons [24, 36, 37]. After functionalization of GO with Putrescin, there was no significant shift in the G band. However, upon functionalization of GO with KPD the peak shifted to a lower frequency (1600 cm⁻¹) compared to GO. This result is consistent with literature and could be attributed to the de-oxygenation of GO [36, 37]. Another useful parameter that determines the changes in the electronic conjugation of GO during functionalization is the intensity ratio between the D and G bands (I_D/I_G). A higher value of I_D/I_G means more disorder in the sample, which was in good agreement with our results that showed the I_D/I_G ratio for GO was 0.71 indicating, that the G band is predominant in GO and that GO contain a lower number of sp³ bonded carbon and defects compared to sp² bonded carbon. On the other hand, the I_D/I_G ratio of GOP, GO-KPD, and GO-KPD-Fe₃O₄ was 1.4, 0.96 and 1.06, respectively. The increment in the I_D/I_G ratios suggests that more defects in the structure of GO were generated after the modification [24, 35, 38]. The higher intensity ratio in GOP compared to GO-KPD indicates more disorder in the stacked sheets which is correlated to the cross-linking ability and higher reactivity of Putrescine compared to KPD. This is because of the
bulkiness of the dimeric steroids attached to putrescine in KPD which largely restricts its penetration inside the stack layers of GO sheets [31]. Also, the increment in $I_{D}/I_{G}$ value of GO-KPD-Fe$_3$O$_4$ compared to GO-KPD suggests further functionalization [39].

3.6. Thermogravimetric analysis (TGA)
Figure 5 shows the TGA curves of Fe$_3$O$_4$ nanoparticles, GO-KPD, GOP, GO, KPD alone, and GO-KPD-Fe$_3$O$_4$. As illustrated in the figure, GO is thermally unstable. The weight loss of GO started immediately above 40 °C.
with almost 12% loss below 150 °C due to the evaporation of adsorbed water [15]. Followed by a rapid weight loss between 150 °C–160 °C (~79%) which can be attributed to the loss of oxygen functionalities distributed on GO sheets [25]. Above 160 °C GO showed a steady weight loss with almost 9% loss of the total remaining mass below 700 °C. These results clearly indicate a high content of oxygen groups in the prepared GO sample [27]. The weight loss of GOP is lower than that of GO especially between the ranges of 40 °C–200 °C. A small weight loss of about 5% was observed around the 200 °C due to the decomposition of the remaining oxygen groups of GO [25]. A higher weight loss about 23% was observed between 200 °C–445 °C that could be attributed to the degradation of Putrescine molecules attached to GO surface [15]. The overall weight loss of GOP at 700 °C (~49%) was much lower than GO which indicates that the oxygen-containing groups of GO have been converted to amide upon reacting with Putrescine [25]. The TGA curve of KPD alone showed about 5% weight loss under 100 °C which could be attributed to the loss of absorbed water molecules. While the major weight loss of 51% was observed in the temperature range between 400 °C–500 °C. Upon functionalization of GO with KPD the thermal stability of GO greatly enhanced. The residual mass of GO-KPD at 700 °C was about 46%. While the residual mass of GO alone and KPD alone at 700 °C are ~0.15% and ~9.6%, respectively. The enhanced thermal stability of GO-KPD might be ascribed to the chemical reactions between the amine groups in KPD with the epoxy and carboxylic groups in GO. The cross-linking between the two components can promote the thermal resistant property of GO-KPD [13, 40]. The TGA curve of Fe₃O₄ nanoparticles shows a total mass loss of 12.62% at 700 °C. On the other hand, the total mass loss of GO-KPD-Fe₃O₄ is about 15.5% at 700 °C. From the weight loss percentage of GO-KPD, Fe₃O₄, and GO-KPD-Fe₃O₄ at 700 °C which is 54.1%, 12.62%, and 15.5% respectively, we could calculate that the loading of Fe₃O₄ nanoparticles on GO-KPD was about 93% [41].

3.7. Cytotoxicity test

The primary results of the in vitro cytotoxicity test conducted on human breast cancer MCF-7 cell line are shown in figure 6. The cells were cultured in Dulbecco's modified eagle's medium (DMEM) and incubated with 100 μg of GO, GOP, KPD, GO-KPD, and GO-KPD-Fe₃O₄ for 24 h. The negative control in this experiment was untreated MCF-7 cells (UTC) and the positive control was doxorubicin hydrochloride (DOX). As seen in the figure, Fe₃O₄ nanoparticles did not show significant cytotoxicity after 24 h incubation with CMF-7 cells. While the cell viability decreased to about 70% after 24 h of incubation with GO and KPD. On the other hand, cell viability after the incubation of GOP with the cells for 24 h was 80%. Surprisingly, upon functionalization of GO with KPD a synergistic effect was observed and the cytotoxic effect increased tremendously with less than 5% cell viability. The rise in cytotoxicity of the nanocomposite could be explained by the positive charge rendered by the tertiary amide bond, which facilitate transportation through negatively charged cancer cells membrane [42]. Almost complete death of CMF-7 cells was observed upon treatment with GO-KPD-Fe₃O₄ nanocomposites. These results are in good agreement with Pramanik et al reports [3]. Nevertheless, these observations need to be further investigated to understand the anti-cancer effect of GO-KPD and GO-KPD-Fe₃O₄ on MCF-7 cells. Furthermore, the biocompatibility of the nanocomposites on normal cells should also be investigated.
4. Conclusion

In conclusion, KPD molecules were distributed mainly at the edges of GO sheets as proved by the SEM images. The bulkiness of the dimer prevented GO sheets from accumulation without affecting the surface area on which the Fe₃O₄ nanoparticles were distributed. Therefore, allowed a high loading of 93% of Fe₃O₄ nanoparticles on the GO surface as indicated by TGA results. The nanocomposite showed a synergistic effect, with cytotoxicity comparable to that of DOX drug. However, more in vitro, and in vivo studies need to be conducted to determine the cytotoxic effect of the nanocomposite on normal cells. Nanotechnology-based hyperthermia methods and their risks must be thoroughly understood if they are to be correctly developed.

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