Synthesis and Drug Delivery Evaluation of Graphene Oxide Supported 5-Fluorouracil

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ABSTRACT: *A novel 5-fluorouracil and graphene oxide [GO-5-FU] mediated drug delivery system was prepared that involves uniquely combining Graphene Oxide (GO) with anticancer 5-fluorouracil (5-FU) drug for controlled drug release. The nanocarrier system was synthesized by attaching 5-FU to graphene oxide via a strong π–π stacking interaction. The loading and release of 5-FU indicated strong pH dependence and implied hydrogen-bonding interaction between graphene oxide and 5-fluorouracil. The [GO-5-FU] system increased significantly in acidic pH and higher temperature without any burst release. In addition, the equilibrium adsorption data were analyzed by the Langmuir and Freundlich models. The results showed that the adsorption behavior could be fitted better by the Freundlich model. We believe that these materials and pH-dependent properties allow developments in controlled drug release techniques for biological and biomedical applications. It is obtained that about 66% of 5-FU was released in the simulated intestinal fluid (pH 7.4) at 37°C over a period of 30 h, while at this temperature and pH environment of the gastric fluid (pH 1.2), ltngvk.tyn 5 5-FU was released approximately 90% over a period of 30 h. At 37°C and this period, the amount of 5-FU release was 78% at pH 10. As can be observed, as the temperature is raised, the release of 5-FU is increased.*

KEYWORDS: *Anticancer; 5-fluorouracil; Graphene oxide; Controlled drug release; Loading drug; Adsorption isotherm study.*

INTRODUCTION

Cancer disease has been considered a severe threat to humans. The World Health Organization (WHO, 2018) reported that cancer causes one in six deaths. According to a study in 2020, there are 19 million cases related to cancer

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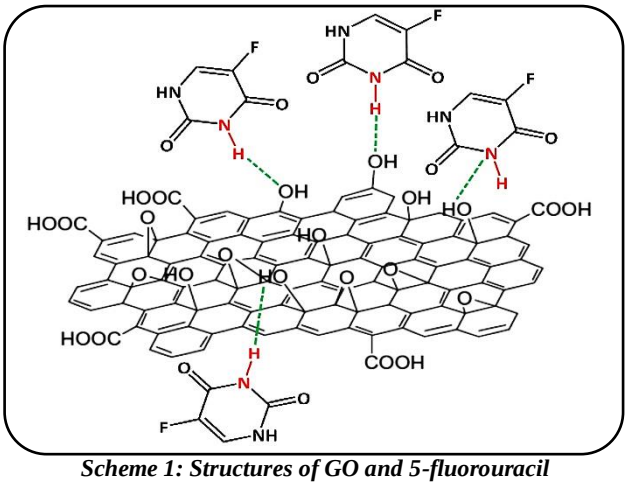

Scheme 1: Structures of GO and 5-fluorouracil

annually, causing 8.8 million deaths (represents 15.7% of deaths annually) [1].

Targeted delivery of drugs to cancer cells has been a new and fast-developing domain of research in chemotherapeutic modalities to circumvent the side effects of the latter. In this field, nano gels have attracted considerable attention as a prominent targeting drug delivery. Usually, drug supports are three-dimensional inorganic materials or polymeric networks either formed chemically (covalent bonds) or physically (hydrogen bonds, Van der Waals, and electrostatic interactions) of cross-linked hydrophilic polymers with small size [1-5]. Target drugs can be supported in these nanomaterials by different types of mechanisms, such as embedding, surface adsorption, hydrogen bonding, and so on types of interactions, while the loading capacity of the currently developed nano-scaled drug carriers toward the drugs has been developed low [6]. Action is important to improve the efficiency of drug loading and release. Carrier research recently described the extraordinary load capacity of highly aromatic molecules relative to carbon nanotubes through strong π-stack interactions[7, 8]. Some compounds reported that are suitable for material absorption have similarity with other applications like drug delivery mechanisms [10-14].

Several drugs have been reported as promising anticancer therapeutics. However, they lack high cell internalization, cell permeation, bioavailability, selectivity, and increased efficiencies [15, 16]. Thus, several carriers, including natural polymers [17], polysaccharides [18], magnetic carriers [19], and Extracellular Vehicles (EVs) [20], were reported for drug delivery. They exhibit high drug-loading capability, controlled release, and increased cell internalization. Nanotechnology has advanced biomedical fields, including cancer treatment [21–23]. Graphene is a new allotrope (two-dimensional, 2D) of carbon nanomaterials with a single layer of sp^2 -hybridized carbon atoms.

Graphene-based nanomaterials such as Graphene Oxide (GO) have advanced biomedicine, including drug delivery [24, 25]. Graphene-based materials offered several advantages, including high drug loading and release abilities [26]. Mixed anticancer drugs such as doxorubicin (DOX) and camptothecin (CPT) can be loaded into GO [27].

Graphene, as a growing star in the field of two-dimensional material science, consists of carbon sp^2 hybridization such as carbon nanotubes, and significant electronic mechanical properties its thickness is an atom, and large the two-dimensional plane provides a certain level of specific surface [9-11]. Graphene oxides for delivery of water-insoluble cancer drugs were reported recently and found that the nano graphene oxide sheets were applied biologically non-toxic thus they can be used for loading the anticancer drugs with high efficiency [12-14]. Graphene oxide and nano-horns mainly load raw materials levels and tips, and when used as drugs, form serious bundles. The carrier material is expected to load the drug graphene oxide sheet through its two faces and its edges. After the reaction, graphene oxide can be introduced with hydrophilic groups such as hydroxyl and carboxylic acids and can be very dispersed in aqueous solutions is a promising substance as a drug carrier material [15-18]. In continuation of our work in medicinal chemistry [38,39] Herein, we report a novel non covalent nanohybrid formed by GO with 5-FU and investigate the in vitro binding and release of 5-FU by GO. The amount of 5-FU loaded onto GO is significantly high and loading and release of 5-FU depends on pH value. Furthermore, the interaction between 5-FU and graphene oxide was investigated by UV-spectroscopy and experimental techniques.

EXPERIMENTAL SECTION

Instruments

Infrared spectra were recorded on a Bruker (EQUINOX55) Fourier transform infrared spectrometer. The scanning electron microscope (SEM) micrographs

were obtained on a SEM-S4700, Hitachi (XL30). UV-spectroscopy was carried out on an ultraviolet-visible near IR spectrophotometer (UV-vis-NIR), Perkin Elmer (RXI).

Reagents and solutions

Graphite was purchased from Sigma–Aldrich, Germany (99% purity). 5-fluorouracil purity 99% (Sigma Aldrich, Germany). Solvents, reagents and all the inorganic acid and salt were products of Merck and Sigma–Aldrich (Germany) or purchased from local suppliers and used as received. Either an acetate buffer or a phosphate buffer was used to adjust the *p*H of the solutions.

Preparation of GO dispersion

The GO is prepared according to the modified Hummers method [19-22]. In detail, concentrated H_2SO_4 (50 mL), $K_2S_2O_8$ (10 g) and P_2O_5 (10 g) are mixed in a 2 L Erlenmeyer flask and heated to 80 °C with a hotplate. 12 g of graphite is added to the mixture under strong magnetic stirring for 4.5 h. After that, 2 L of deionized (DI) water is added to the suspension (initially, water is added very slowly to avoid large amount of heat from the dilution of H_2SO_4). After dilution, the mixture is left overnight and then filtered through a 0.1-micron Teflon Millipore membrane; the filter cake is allowed to dry in air overnight. On the second day, the filter cake is slowly dispersed into 0.46 L concentrated H_2SO_4 in a 4 L Erlenmeyer flask in an ice bath (keep temperature as low as 0 °C) with stirring. 60 grams of $KMnO_4$ was slowly added to the flask with stirring, during which the temperature of the mixture is 5-FUlly controlled not exceeding 10 °C. The Dispel resin is kept at 35 °C for 2 h and then diluted with 900 mL of DI water. (Water should initially be added slowly to avoid rapid heating. During the whole process, the temperature is controlled below 50 °C.) Subsequently 2.8 L of DI water is added over 2 h with continuous stirring, giving a brownish dispersion. Immediately after finishing dilution, 50 mL of 30 % H_2O_2 is slowly added to the dispersion, leading to tremendous bubbling as well as an obvious color change from brown to bright yellow. The mixture is left untouched for at least two days and then filtered through a 0.1-micron Millipore Teflon membrane, and washed with 10 % HCl and 5 L DI water sequentially. The final filter cake is left to dry in air and then kept in desiccators with P_2O_5. The GO product can be easily dispersed in water by mild sonication [23-32].

Preparation of graphene oxide supported 5-fluorouracil

A typical procedure for preparing the GO-5-FU is as follows [5-7]. GO with the final concentration of 10 mg/mL (determined using a standard GO concentration curve at the absorption of 230 nm) was first sonicated with 5-fluorouracil (5-FU) with a certain concentration at a certain pH value (pH=7) for 30 min and then stirred overnight at room temperature in the dark. All samples were adjusted to regarded pH and then ultra-centrifuged at 12000 rpm for 1 h. The resultant mixture was separated and purified by repeating ultracentrifugation (12000 rpm for 10 min) /decantation /resuspension in water for three times. The purified GO- 5-FU were dried overnight under vacuum at 50 °C[2, 3].

Loading of Graphene Oxide (GO) with 5-fluorouracil (5-FU)

5-FU (270/1 mg) and GO (158/1 mg) added to 50 mL phosphate buffered solution (*p*H= 7) and stirred for 24 h at room temperature in darkness. Repeated congregation collected the product (GO-5-FU) and washing with PBS until the supernatant became 5-FU free by measuring the absorbance at 266 nm. The resulted GO-5-FU dried under vacuum [6, 33, 34]. The amount of unbound 5-FU was determined by measuring the absorbance at 266 nm (the characteristic absorbance of 5-FU) relative to a calibration curve recorded under identical conditions[6], allowing the 5-fluorouracil loading efficiency to be estimated. To quantify free 5-FU, the centrifuged 5-FU solution collected and diluted to 150 mL with deionized water in a flask. The amount of free 5-FU was determined by a UV–VIS spectrometer (Perkin Elmer-RXI spectrometer) at wavelength of 266nm (measured in the range of 400 nm to 800 nm to optimize) Standard 5-FU water solution (100 µg/mL) was prepared for quantitative analysis. The 5-fluorouracil loading efficiency was calculated as follows [6, 34]:

5-FU - loading efficiency (%) = 100 (W_{feed} 5-FU − W_{free} 5-FU) / W_{feed} 5-FU.

The 5-FU - loading efficiency estimated was~98%.

Adsorption studies

The effects of experimental parameters, such as initial concentration, *p*H (1.2, 5, 7.4 and 10) and temperature[35, 36] on the adsorption amount of various 5-fluorouracils were investigated in a batch mode of operation for the specific period of contact times (0–400 min). Experiments

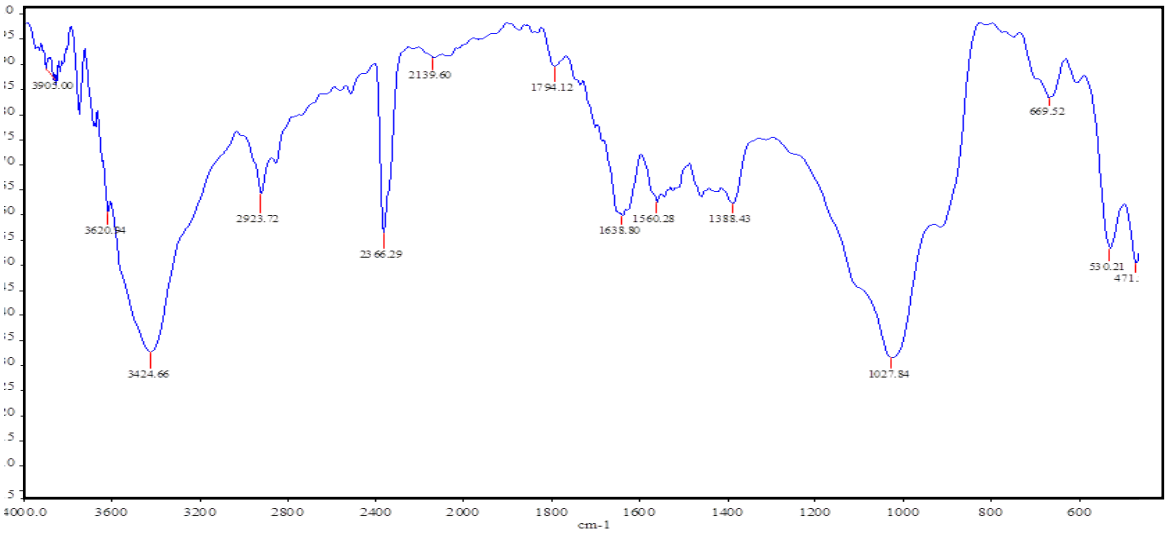

Fig. 1: Infrared spectra of graphite

were executed with different amounts of 5-FU (adsorbent). The range of Initial concentration was between 10 and 300 mg/L by keeping identical the experimental condition. For contact time studies, drug solution of known initial concentration and a certain pH was taken with a stable quantity of adsorbent. The adsorption percentage was calculated as follows:

$$\text{Absorptivity}\ (\%) = \frac{C_i - C_f}{C_i} \times 100 \qquad (1)$$

Here, C_i and C_f are the initial and final 5-FU concentrations (after contact to the adsorbent), respectively. The amount of 5-FU adsorbed in (mg/g) at equilibrium (q_e) was calculated by applying the following equation:

$$q_e = \frac{(C_i - C_e)V}{W} \qquad (2)$$

Which C_i is the initial 5-FU concentration and C_e is 5-FU concentration (mg/L) at equilibrium, V and W are the volume of solution (L) and the mass of the adsorbents (g) respectively. In this study, all the adsorption experiments were done in triplicates and mean cumulative values were represented.

In-vitro drug release response

The drug release profile from the synthesized nano-carrier GO-5-FU was studied at the temperature of 25 °C, 37 °C, 40°C, 45°C and 50°C and pH of 1.2, 10 and pH of 7.4 (the physiological pH). Typically, the GO-5-FU (10 mg) was transferred into dialysis bags with 4 buffer solutions (pH = 1.2, 5, 7.4 and 10) and gently shaken at ambient temperature[15]. The drug release was started when the dialysis bags were placed into the buffer solutions. At predetermined periods, the amounts of 5-FU (W free 5-FU) in the buffer solution were quantified by UV–Vis at 266 nm. The averages of three measurements were reported for all 5-FU drug release [33, 35, 37-40]. The release of 5-fluorouracil from the carrier (GO) could affect the morphology of the nano hybrids drug carrier, so in order to evaluate any morphological changes we studied the GO–5-FU nano hybrid drug carrier samples via Hitachi XL30 SEM-S4700, Scanning Electron Microscopy (SEM).

RESULTS AND DISSCUSSIONS

Synthesis and characterization of graphene oxide (GO)

Infrared spectra

Graphite powder utilized to make graphene oxide was purchased from Sigma–Aldrich, Germany. The after effects of the graphite infrared spectroscopy test are watched, Fig. 1 shows that the graphite contains no carbonyl and epoxide group, and the adsorption in the locale of around 3424 cm^{-1} is identified with the hydroxyl group of the atom water consumed by graphite [20, 26, 29].

Graphene oxide arranged by changed Hummer strategy at that point refined. The FT-IR spectroscopy of graphene oxide demonstrated an absorption frequency of 1740 cm^{-1} relative to the tensile vibrations of the carbonyl group (C = O) and additionally strong absorption in the 3421 cm^{-1} of the hydroxyl group, (OH) 1415 cm^{-1} group of the flexural vibrations of the hydroxyl group (OH) and absorption in the 1040 cm^{-1} locale of the epoxide tensile

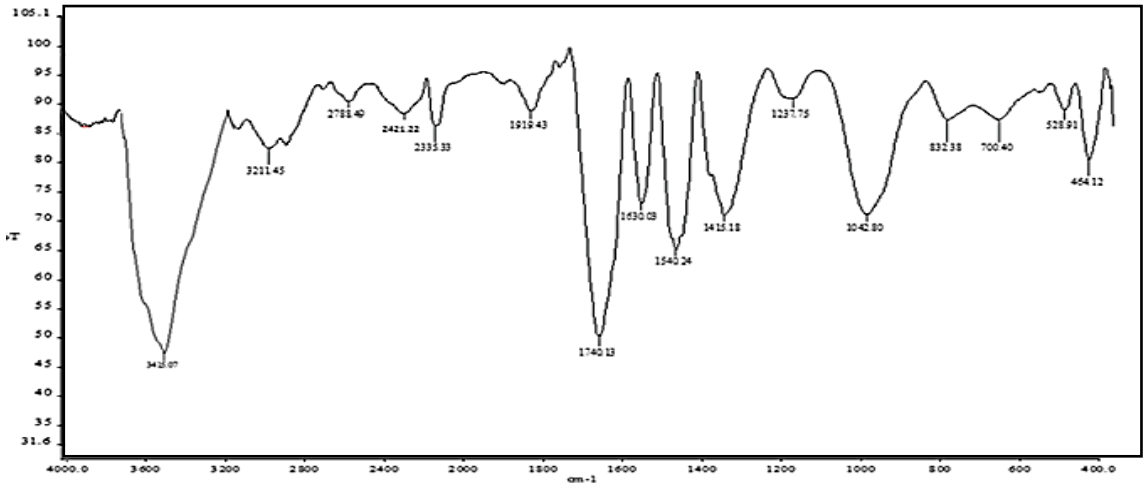

Fig. 2: Infrared spectra of graphene oxide

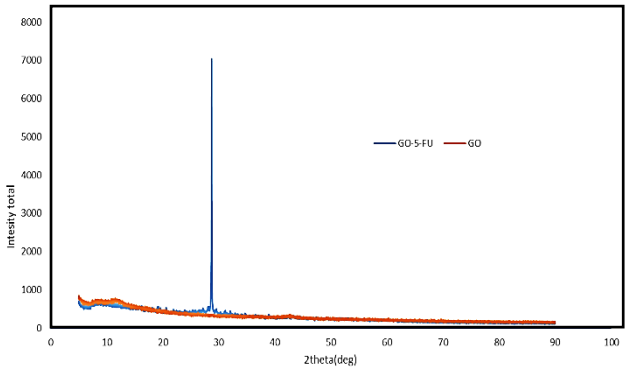

Fig. 3: XRD pattern of graphene oxide/ graphene oxide-5-FU (5-FU encapsulated in graphene oxide)

frequencies (Fig. 2). Likewise, the pinnacle contained in 1630 cm^{-1} is identified with the functional groups (C = C) staying on graphene plates, which have not experienced any progressions amid the oxidation procedure [20, 26, 29].

XRD analysis

The X-ray beam diffraction test utilized to check the structure of graphene oxide. X- Ray beam Diffraction (XRD) for graphene oxide demonstrates a strong peak at 2θ = 10.5, which shows the nearness of oxygen-containing functional groups in void spaces between graphene oxide plates and oxygen bunches from the spaces between the layers of graphene oxide. (Fig. 3)

The XRD pattern of free 5-FU showed a sharp and intense peak at 30◦ and smaller peaks at 16◦ and 33◦, confirming its crystalline nature as also reported in the literature [56]. The peak at 30◦ could also be seen in the diffractogram of GO-5-FU, suggesting that the drug was successfully encapsulated in the carrier.

Raman study

Raman scattering is a valuable device to portray graphite and graphene materials as this dissipating emphatically relies upon the electronic structure (Fig. 4). In the spectra of GO two crucial vibration bands were seen in the scope of 1250–1750 Cm^{-1}. The G vibration mode, inferable from the first-order scattering of E2 g phonons by sp^2 carbon of GO while the D vibration band acquired from a breathing method of j-point photons of A1g symmetry of GO, D band emerges due to sp^2 carbon group.

Graphene oxide supported 5-fluorouracil

Infrared spectra

5-fluorouracil (5-FU) is an anticancer drug and is commonly used to treat tumors[41]. GO has a two-dimensional nano-structure consisting of sp^2-hybridized carbon containing carboxyl, hydroxyl, and epoxide functional groups [30-32, 42-44]. The sp^2 hybridized π conjugated structure of graphene sheet can form π–π stacking interaction with nitrogen groups of 5-FU[33, 35, 37, 39-41], while amine (NH) and oxygen groups of 5-FU can also form a strong hydrogen-bonding interaction with hydroxyl (OH) and carboxyl (COOH) groups in GO, the structure is shown in scheme 1[32, 34, 45-48]. The FT-IR spectra of 5-fluorouracil (5-FU) and 5-FU-loaded GO [GO-5-FU] are shown in Fig. (5a and 5b). A broad band between the 3200 and 3700 cm^{-1}, is attributed to—NH stretching vibrations in the spectrum of 5-FU. This band was seen approximately at 3515 cm^{-1} in the spectrum of drug loaded GO, because the over overlapping of -OH band of GO with -NH band of 5-fluorouracil. In the spectrum of drug loaded GO and 5-FU appeared band of

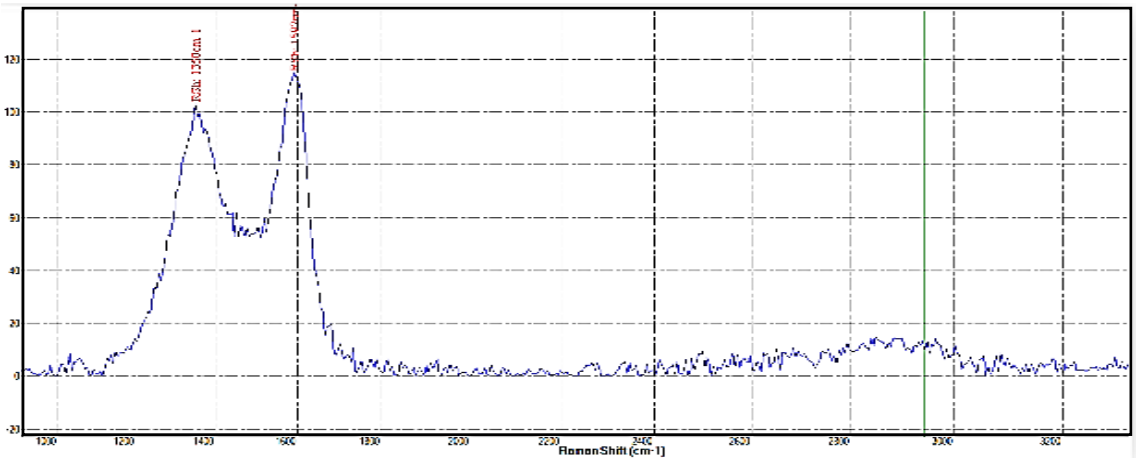

Fig. 4: Raman spectra of GO

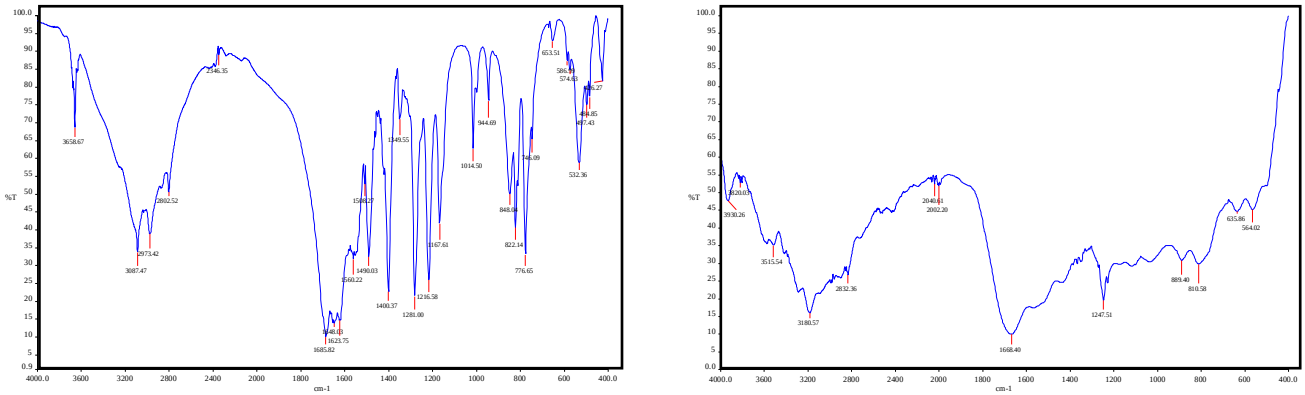

Fig. 5: (a) FTIR spectra of 5-FU, (b) FTIR spectra of GO-5-FU

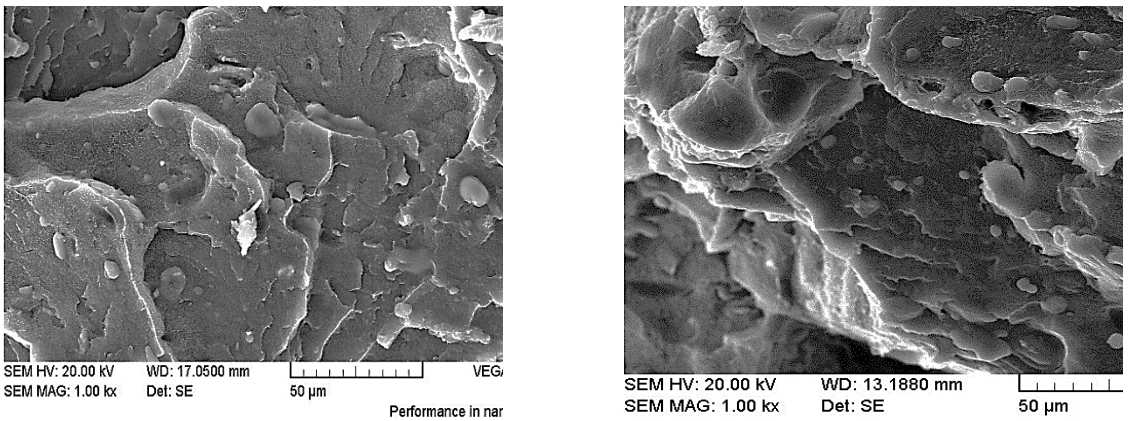

Fig. 6: SEM micrograph of the nano drug right) before drug release and left) after drug release

carbonyl stretching (C=O) at 1620 cm^{-1} - 1660 cm^{-1}. C-H group stretching band of GO and drug loaded GO appeared were seen at 2950 cm^{-1} - 2995cm^{-1}. The peak at 1276 cm^{-1} was belonged to C-F stretching band in the spectrum of 5-FU. This peak was seen at 1247 cm^{-1} in the spectrum of drug loaded GO. Some additional absorption bands are observed at ~848 and 776 cm^{-1} (corresponding to the secondary amine NH group and N-H deformation bonds from 5-FU, respectively). These observations confirm the loading of 5-FU to GO [34, 39, 47]. What is more, in Fig. 6, SEM images of GO before and after drug release nanoparticles can be seen. As can be seen in Fig. 6.

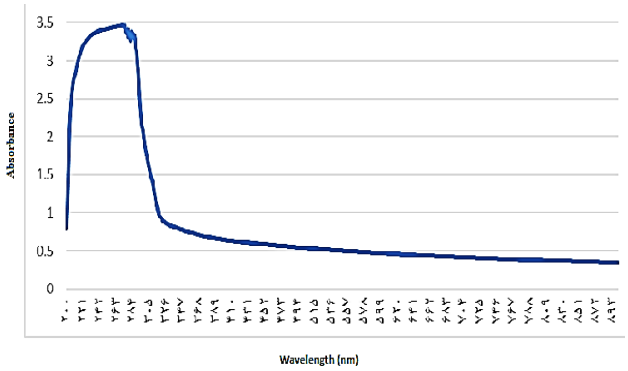

Fig. 7: UV-vis spectra of GO-5-FU

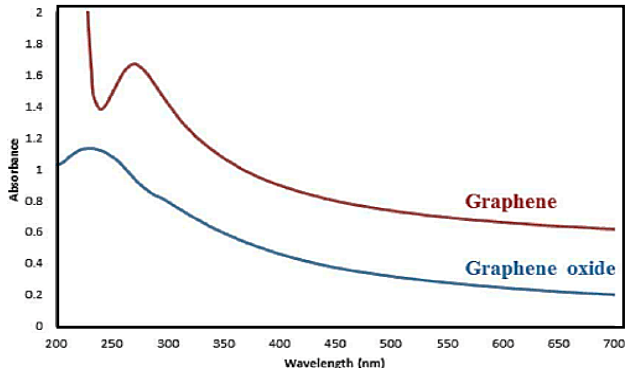

Fig. 8: UV-vis spectra of GO

UV–Vis spectrum

The UV–vis spectrum of the 5-FU- GO nano-hybrid solution not only confirms the stacking of 5-FU onto GO, but also shows the change in the absorbance (blue shift) due to the interaction.

For example, the peaks of 5-FU at 266 nm shifted to 269 nm after interaction with GO, which are generally believed due to the ground-state electron donor–acceptor interaction between the two components [49, 50].

Effective parameters on adsorption /desorption

For studying the effect of concentration, the values were varied from 10 to 50 mg/L. The other parameters were 400 min contact time, pHs (1.2, 5, 7.4 and 10) and temperatures (25, 37, 40 and 45 ˚C). The outcomes of these operations are shown in Fig. 9 to 11. Fig. 6 illustrates that, at temperature 25 ˚C and pH equal to 7.4, increase of initial concentration up to 40 mg/L could enhanced the adsorption, but increase of concentration 5-FU up to 50 mg/L, could not increase the adsorption. Therefore, 40 mg/L concentration is better to be chosen in these conditions.

Fig. 9 shows the same result at temperature 25, 37, 40 and 45 ˚C and pH equal to 7.4. Also at this temperature and

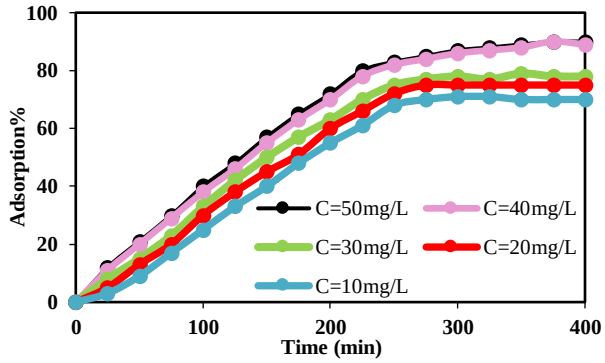

Fig. 9: Effect of concentration of 5-FU on the adsorption in the 25 ˚C and pH equal to 7.4

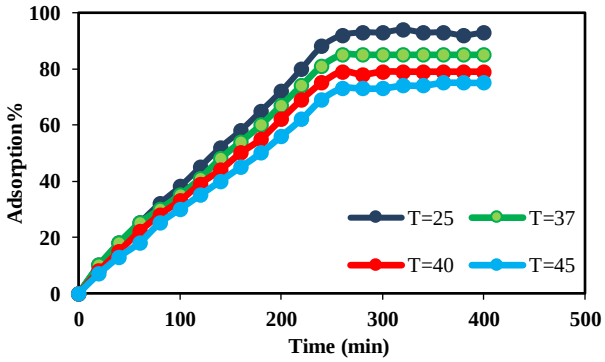

Fig. 10: Effect of temperature on the adsorption of 5-FU in the concentration 40 mg/L and pH equal to 7.4

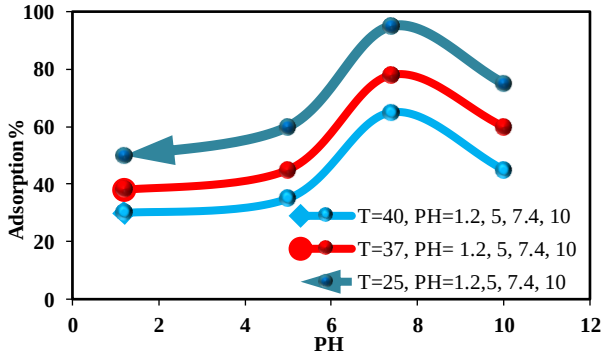

Fig. 11: Effect of pH and temperature on the adsorption of 5-FU

pH 5 and 10, results are similar. This discipline is obeyed also at temperatures 37 and 40˚C and mentioned pH.

The outcome of this operation at temperatures 25 ˚C, 37 ˚C and 40 ˚C is shown in Fig 11. The data show that in all cases, best adsorption is occurred at pH 7.4.

For effect of contact time, the values were varied from 0 to 400 minutes for all initial concentrations, pH and temperatures. The results for all of them were the same. As can be seen in Figs. 9 to 11, 300 minutes is proper time.

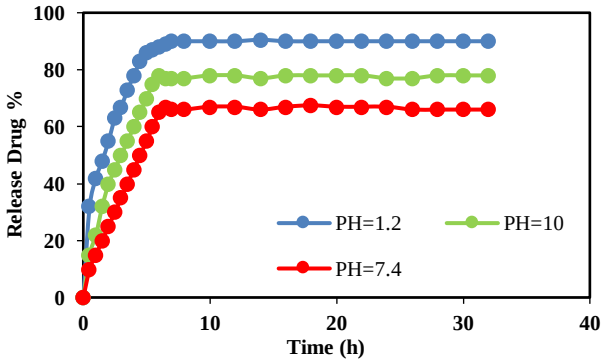

Fig. 12: Effect of pH on the release of 5-FU

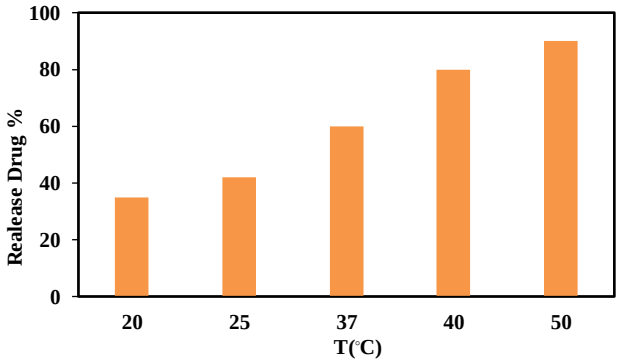

Fig. 13: Effect of temperature on the release of 5-FU in pH equal to 7.4

5-FUrther increase in contact time could not increase adsorption significantly.

Effect of temperature was studied with chosen different temperatures between 25°C to 45°C. The percentages of the adsorption experiments were conducted at 25, 37, 40 and 45 °C to investigate the effect of temperature [50-55].

Drug Release

The releasing of 5-FU by GO in simulated intestinal fluid (pH 7.4), in simulated gastric fluid (pH 1.2) and in pH 10 are indicated in Fig.12. It is obtained that about 66% of 5-FU was released in the simulated intestinal fluid (pH 7.4) at 37°C over a period of 30 h, while at this temperature

and pH environment of the gastric fluid (pH 1.2) 5-FU was released approximately 90% over a period of 30 h. At 37°C and this period of time, the amount of 5-FU release was 78% at pH 10. The effect of temperature on 5-FU which was released at pH 7.4, was also investigated and the results are gathered in fig.13. As can be observed as the temperature is raised, the release of 5-FU is increased.

Adsorption isotherms

It is known that adsorption isotherms are significant for the description of how an adsorbate interacts with an adsorbent and are critical in optimizing the use of adsorbent [54]. Here adsorption isotherms are the presentation of the amount of 5-FU adsorbed per unit of graphene oxide. It is accepted that Langmuir isotherm is a valid model for monolayer sorption on a surface with a number of identical active sites. The Langmuir equation is [56]:

$$C_e/q_e = (1/q_{max} K_L) + (C_e/q_{max}) \quad (1)$$

Where q_{max} is the maximum 5-FU adsorption capacity corresponding to complete monolayer coverage on the

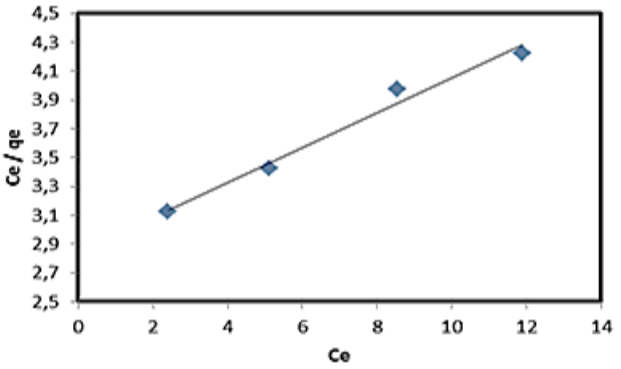

Fig. 14: Langmuir isotherm for adsorption of 5-FU onto GO

FPG-MNP surface ($mg.g^{-1}$), and K_L is the Langmuir adsorption constant (Lmg^{-1}). Fig. 14 shows the Langmuir C_e / q_e vs. Ce plots for adsorption of 5-FU at 25°C and pH 1.2. Langmuir parameters which are calculated from the Eq. (1) for 5-FU adsorption are listed in Table 1.

With compared to many other inorganic nano materials currently explored in nano medicine, nano-graphene oxide can be prepared in large scales with low cost. Also, nano-graphene exhibits ultra-high surface area available for drug loading with high efficiency. Alternatively, both covalent and non-covalent surface coating has been used to improve the biocompatibility and impart specific biological activity to nano-graphene. Moreover, nano-graphene exhibits high NIR absorbance useful in photo thermal therapy, which if combined with other therapeutic agents delivered by nano-graphene would enable more effective cancer killing (e.g., overcome drug resistance). At last, various inorganic nanoparticles could be adsorbed or in situ growth on the surface of nano-graphene to form multifunctional nano-graphene-based nano composite for imaging-guided drug delivery.

Table 1: Isotherm parameters obtained by using linear method at 40 ^{0}C and pH 7.4.

Langmuir isotherm model			
q_{max} (mg/g)	K_L (L.m/g)	R_L	R^2
8.33	0.042	0.373	0.981
Frendlich isotherm model			
K_F (mg/g) (L.m/g)$^{1/n}$	n	R^2	
3.853	1.24	0.997	

One of the most important considerations of these materials is a profound understanding of graphene's interaction with living cells (tissues and organs), especially the cellular uptake mechanism. This knowledge will certainly facilitate future development of graphene-based nano carriers, especially for anti-cancer therapy where these materials showed considerable potential. The most critical issue for the biomedical application of these materials is their biocompatibility and toxicity, which are primary concerns. A review of current literature has revealed the majority of present studies indicate that graphene is biocompatible and a low toxic material, although caution must be taken with conflicting and unclear results obtained by different research groups. However, an agreement has been gradually reached that the functionalization of pristine graphene and GO significantly improves their biocompatibility, and this step is essential for designing stable and safe drug delivery nano carriers. Therefore, more toxicity studies using in vivo animal models are required in future to prove the biocompatibility of graphene and GO. Finally, the recent advances of graphene-based nano carriers for drug delivery applications is a significant development in nano medicine, which opens exciting opportunities for the future and broad use of nano materials in real clinical conditions [73].

CONCLUSIONS

In this study we have demonstrated that the adsorption of 5-fluorouracil on graphene oxide was investigated as a function of temperature, pH, contact time and initial concentration. The interaction of 5-FU to GO is ascribed to the hydrogen bonding between 5-fluorouracil and GO, which is more prominent in the acidic conditions, resulting in a controlled release. The results demonstrate that this work has some advantages with respect to simplicity, lower solvent consumption, and high loading–release efficiency. The equilibrium adsorption data were described as well and fitted better by the Freundlich adsorption isotherm than the Langmuir model. Nearly 92% of 5-FU was released in simulated gastric fluid, pH 1.2 and 62% in simulated intestinal fluid, pH 7.4, in 6 h. This study will provide a deeper understanding of the self-assembly behavior of GO and inspire more novel designs of functional materials based on GO.

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