HIGH-EFFICIENCY LOADING AND CONTROLLED RELEASE OF CYCLOPHOSPHAMIDE ON GRAPHENE OXIDE AND ADSORPTION ISOTHERM STUDIES

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ABSTRACT

In this research, Graphene oxide (GO) used as a platform, is synthesized for the purpose of nanoparticles for loading of cyclophosphamide (CYP) and the identification of the structure of this new nano drug (CYP/GO) is made using several analytical devices such as XRD, FTIR, SEM, and UV. The morphology, functionalization, stability, loading and controlled release behaviors of cyclophosphamide on GO at different pH, temperatures, contact time and concentrations were investigated. The loading and controlled release of CYP indicated strong pH dependence which is implied hydrogen-bonding interaction between GO and CYP. The equilibrium adsorption data were analyzed by Langmuir and Freundlich models. The results showed that the adsorption behavior could be fitted better to the Freundlich model. Nearly 80 % of CYP was released in simulated gastric fluid, pH 1.2, in 4 h and 68 % in simulated intestinal fluid, pH 7.4, in 30 h.

Keywords: cyclophosphamide, graphene oxide, loading drug, nanoparticle, adsorption isotherm study.

INTRODUCTION

Targeted delivery of drugs to cancer cells has been new and fast developing domain of researches in chemotherapeutic modalities for the purpose of circumventing the side effect of the latter. Carriers of nanoscale drugs appear as communication bridge nanotechnology and delivery of advanced drugs, including nanostructured materials such as graphene, hydrogel polymers, carbon nanotubes and graphene oxide [1 - 5]. Target drugs can be supported in nano materials by different types of mechanisms such as embedding, surface adsorption, hydrogen bonding, and so on; while the loading capacity of the current nanoscale drug-carriers toward the drugs has been poorly developed [6]. The amount of the nano materials' capacity is essential to improve the efficiency of the loading and releasing reaction. Carrier research recently described the extraordinary load capacity of highly aromatic molecules relative to carbon nanotubes through strong π-stack interactions [7]. Graphene, as a growing star in the field of two-dimensional material science has the structure, consists of carbon sp^2 hybridization (like carbon nanotubes) and significant electronics mechanical properties. Its thickness is an atom and large the two-dimensional plane provides a certain level of the specific surface [8]. Graphene oxides for delivery of water-insoluble cancer drugs were reported recently and found nano graphene oxide sheets were applied biologically non-toxic thus it can be used for loading the anticancer drugs with high efficiency [9]. 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. Graphene oxide can be very dispersed in aqueous solutions; this fact makes it a promising substance as a drug carrier material [10].

It can also be compared to fullerenes and CNTs, which differ in the number of walls, diameter, length, and surface chemistry [5]. GO is used in numerous applications, however, is higher than CNTs, taking medication delivery into account. This may be a result of minimal cost, simple customization and manufacture, and a larger surface area. It is also hydrophilic and gathers in physiological due to its extremely negative charge, which buffers when there are salts present, because of the presence of a lot of phenol hydroxyl on its surface, carboxylic groups, and oxygen groups [2 - 5]. As a result, making the necessary changes appears to be a top priority for greater efficiency of GO in the medication delivery field. It is reported doxorubicin hydrochloride that contains chemically reduced graphene oxide modified with chitosan [4]. The drug nanocarrier (CYP/GO) has potential applications in tumor therapy due to its good pH-sensitive behavior and colloidal stability in biological media. Two aspects of this nanocarrier are important. One is about the core and the other is about the wrapped skin. Cyclophosphamide decorated on graphene oxide nanoparticles facilitates handling and compound separation during synthesis. In addition, the carrier property can be delivered to the target site in the cancer therapy program to prevent the spread of the drug to unwanted sites. Also, coating the graphene oxide surface with cyclophosphamide leads to improved interfacial properties of graphene oxide and to the integration of the advantages of both materials, increase the surface area, improves hydrophobicity and dispersion, promotes covalent functionalization, and achieves a high efficiency of drug loading and pore adsorption, allowing both hydrophobic and hydrophilic anticancer drugs to be loaded [4, 10]. Herein, we report a novel noncovalent nanohybrid formed by GO with CYP and we investigate the binding and release of CYP by GO.

The amount of CYP loaded onto GO is significantly high and loading and release of CYP depends on pH value. Furthermore, UV-spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FT-IR), and experimental techniques were used to evaluate the interaction between CYP and graphene oxide. In this study, the equilibrium adsorption data were analyzed by Langmuir and Freundlich models.

EXPERIMENTAL

Instruments

Fourier transform infrared spectra were recorded on a Perkin Elmer (RXI). The scanning electron microscope (SEM) micrographs were obtained on an SEM-S4700, Hitachi (XL30). UV-spectroscopy was carried out on an ultraviolet-visible near IR spectrophotometer (UV-vis-NIR) HACH (DR5000).

Reagents and solutions

Graphite was purchased from Sigma-Aldrich, Germany (99 % purity). Cyclophosphamide (CYP) was purchased from Baxter Oncology GmbH, D-33790 Halle, 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 (PBS) was used to adjust the pH of the solutions.

Preparation of GO dispersion

The GO is prepared according to the modified Hummers method that has been reported and characterized. [3, 11, 12].

Loading of graphene oxide (GO) with cyclophosphamide (CYP)

CYP (50 mg) and GO (25 mg) added to 50 mL phosphate-buffered solution (pH 7.4) and stirred for 24 hrs at room temperature in darkness. The product (GO-CYP) was collected by repeated centrifugation and washing with PBS until the supernatant became CYP free by measuring the absorbance at 720 nm. The resulted GO-CYP dried under the vacuum [6, 13 - 18]. The amount of unbound CYP was determined by measuring the absorbance at 720 nm (the characteristic absorbance of CYP) based on the calibration curve recorded under identical conditions [6] to estimate the cyclophosphamide loading efficiency. To quantify free CYP, the centrifuged solution has to be prepared and diluted with deionized water in a flask to 150 mL. The amount of free CYP was determined by a UV-Vis spectrometer (HACH-DR5000 spectrometer) at the wavelength of 720 nm (measured in the range of 400 nm to 800 nm to optimize). Standard CYP water solution (100 μg mL^{-1}) was prepared for quantitative analysis.

RESULTS AND DISCUSSION

Infrared spectra

Cyclophosphamide (CYP) is an anticancer drug and is commonly used to treat tumors, GO has a two-dimensional nano-structure consisting of sp^2-hybridized carbon containing carboxyl, hydroxyl, and epoxide functional groups [11, 12]. The sp^2 hybridized π conjugated structure of graphene sheet can form π–π stacking interaction with nitrogen gropes of CYP [19, 20], while amine (NH) and oxygen groups of CYP can also form a strong hydrogen-bonding interaction with hydroxyl (OH) and carboxyl (COOH) groups in GO (Fig. 1) that allows controlled release of CYP [13, 21 - 23].

The FTIR spectra of Cyclophosphamide (CYP) and CYP-loaded GO (GO-CYP) are shown in Fig. 2(a) and Fig. 2(b). Fig. 2(a) represents the FTIR spectrum of CYP, in which the characteristic phospho ketone and phospho carbonyl peaks of CYP appeared at 3434 cm^{-1}, 2960 cm^{-1}, 1654 cm^{-1}, 1457 cm^{-1} and 1047 cm^{-1}. The peak at 1550 cm^{-1} is due to the stretching bands of the N-H group, while the peak at 817 cm^{-1} is due to the stretching bands of C-O-P. The peaks at 878 cm^{-1} and 774 cm^{-1} are due to the secondary amine (NH) wagging and N-H deformation bonds, respectively. Fig. 2(b) is the FTIR spectra of CYP-loaded GO. Compared to Fig. 2(a), some additional absorption bands are observed at ~1110 cm^{-1} and 806 cm^{-1} (corresponding to the stretching bands of C-O-P from CYP), and at ~848 cm^{-1} and 746.9 cm^{-1} (corresponding to the secondary amine NH group and N-H deformation bonds from CYP, respectively). These observations confirm the loading of CYP to GO [22, 24].

UV-VIS spectrum

The UV-Vis spectrum of the CYP-GO nano-hybrid solution not only confirms the stacking of CYP onto GO but also shows the change in the absorbance (redshift) due to the interaction. For example, the peaks of GO at 230 nm shifted to 236 nm and the peak of CPY at 720 nm shifted to 722 nm after interaction with GO, which is generally believed due to the ground-state electron donor-acceptor interaction between the two components [25, 26].

Adsorption studies

The effects of experimental parameters such as initial concentration, pH, temperature and contact time on the adsorption of CYP on graphene oxide were investigated.

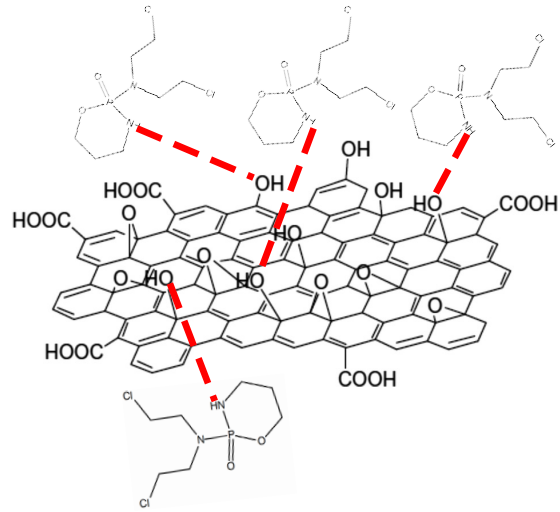


Fig. 1. Hydrogen bond interaction between CP and GO.

Experiments were executed with different amounts of CYP (adsorbent). Isotherm studies were performed by adding 0.05 g of graphene oxide to a series of test tubes filled with 5 mL diluted solutions of CYP (10 to 50 mg L^{-1}) in proper buffers, at different pH (1.2, 5, 7.4 and 9) and different temperatures (25, 37, 40 and 45°C). The adsorption percentage was calculated as follows:

$$\text{Adsorptivity (\%)} = \frac{C_i - C_f}{C_i}\left(\frac{C_0 - C_f}{C_0}\right)\left(\frac{C_0 - C_f}{C_0}\right) \times 100 \quad (1)$$

where, C_0 and C_f are the initial and final CYP concentrations (after contact to the adsorbent), respectively. The amount of CYP adsorbed in (mg g^{-1}) at equilibrium (q_e) was calculated by applying the following equation:

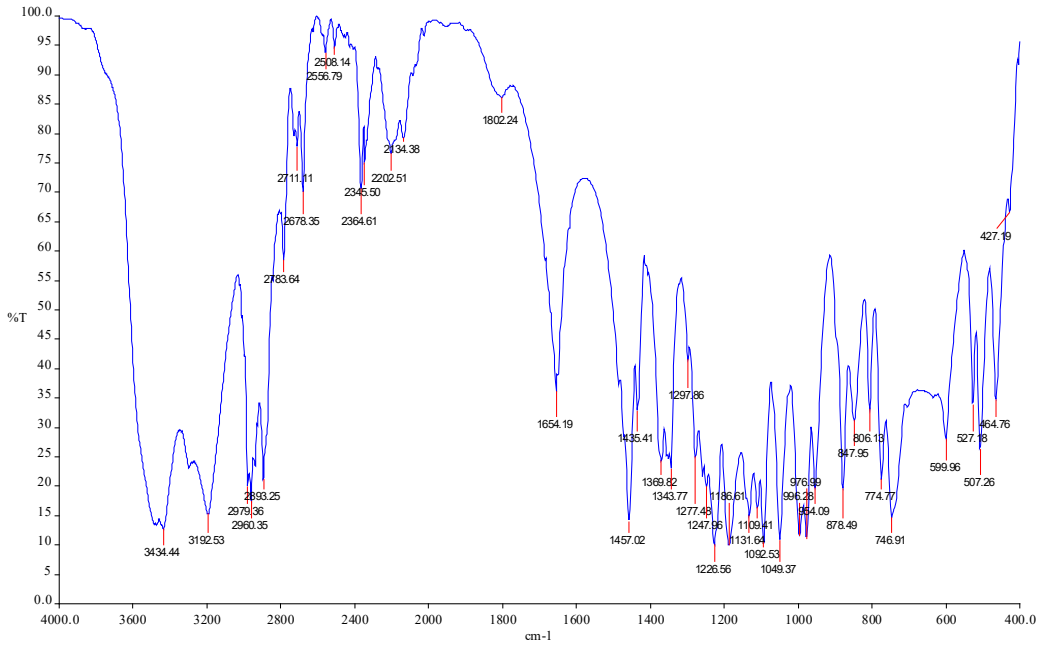
$$q_e = \frac{(C_0 - C_e)V}{W} \quad (2)$$

where C_0 is the initial CYP concentration and *Ce* is CYP concentration (mg L^{-1}) 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.

Effective parameters on adsorption

For studying the effect of concentration, the values were varied from 10 to 50 mg L^{-1}. The other parameters

a)



b)

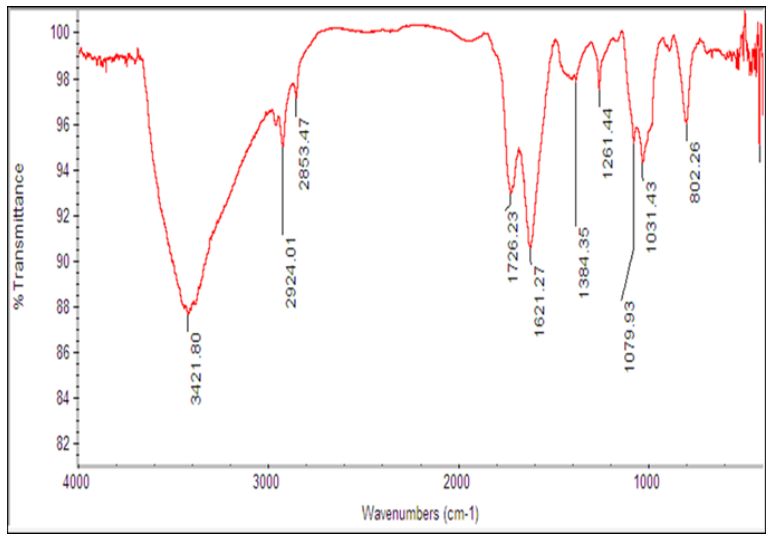


Fig. 2. (a) FTIR spectra of CYP; (b) FTIR spectra of GO-CYP.

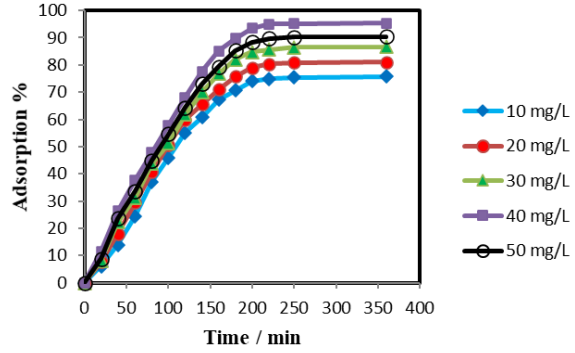


Fig. 3. The effect of initial concentration on adsorption of CYP onto GO at 40°C and pH 7.4.

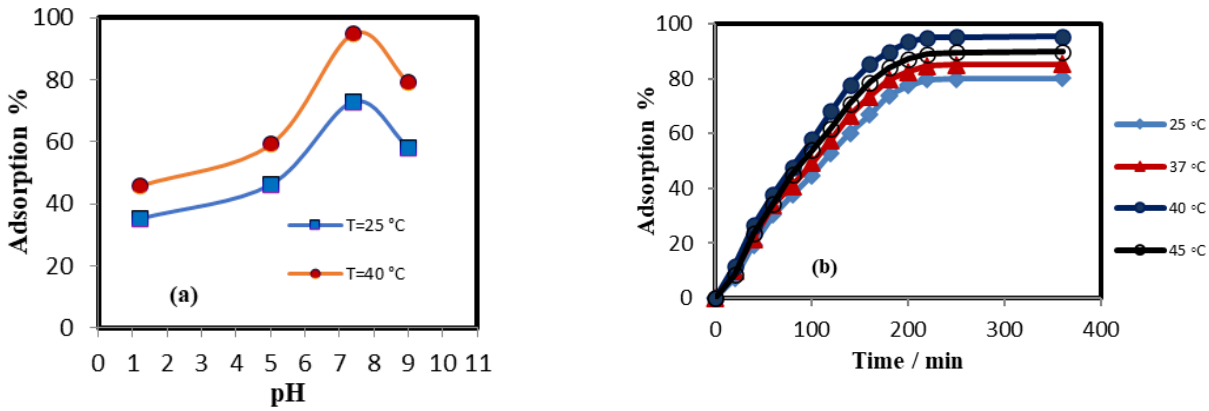


Fig. 4. At initial concentration 40 mg L^{-1}: **(a)** The effect of pH on adsorption of CYP onto GO at 25°C, 40°C; **(b)** The effect of temperature on adsorption of CYP onto GO at pH 7.4.

were 250 min contact time, pH (1.2, 5, 7.4 and 9) and temperatures (25°C, 37°C, 40°C and 45°C). One of the outcomes of these operations is shown in Fig. 3. This figure illustrates that, at temperature 40°C and pH equal to 7.4, increase of initial concentration up to 40 mg L^{-1} could enhance the adsorption but further increase of concentration up to 50 mg L^{-1} could not increase the adsorption, therefore, 40 mg L^{-1} concentration is better to be chosen in these conditions. Also, at this temperature and pH 1.2, 5 and 9, the results are similar. This discipline is obeyed also at temperatures 25°C, 37°C and 45°C and above mentioned pH.

For investigating the effect of contact time, the values were varied from 0 to 360 minutes for all initial concentrations, different pH and temperatures. The results for all of them were the same. As can be seen in Fig. 3, 250 minutes is the proper time. Further increase in contact time could not increase adsorption significantly.

For investigating the effect of pH, the values were varied from 1.2 to 9. The other parameters used were 250 minutes of contact time and 40 mg L^{-1} concentration. The outcome of this operation at temperatures 25°C and 40°C is shown in Fig. 4(a). The data show that in both cases, the best adsorption occurs at pH 7.4.

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°C, 37°C, 40°C and 45°C to investigate the effect of temperature. At initial concentration of 40 mg L^{-1} and pH 7.4, adsorption increases with increasing temperature. Fig. 4(b) illustrates that at this condition the best temperature is 40°C.

Adsorption isotherms

It is known that adsorption isotherms are significant for the description of how an adsorbate interacts with an adsorbent and they are important in scaling and optimizing for practical applications. Here adsorption isotherms are the presentation of the amount of CYP 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 [27] is:

$$\frac{C_e}{q_e} = \left(\frac{1}{q_{max} K_L}\right) + \left(\frac{C_e}{q_{max}}\right) \quad (3)$$

where C_e is CYP concentration (mg L^{-1}) at equilibrium, q_{max} is the maximum CYP adsorption capacity corresponding to complete monolayer coverage on the GO surface (mg g^{-1}), and K_L is the Langmuir adsorption constant (L mg^{-1}). The essential characteristics of the Langmuir equation is expressed in terms of a dimensionless separation factor (R_L). This factor can be defined as:

$$R_L = \frac{1}{(1 + K_L C_0)} \quad (4)$$

It is known that R_L value expresses the adsorption nature to be either irreversible ($R_L = 0$), favorable ($0 < R_L < 1$), linear ($R_L = 1$), or unfavorable ($R_L > 1$) [28] and C_0 is the initial CYP concentration (mg L^{-1}).

The Freundlich isotherm is an empirical isotherm to define heterogeneous systems with the heterogeneity factor of 1/n:

$$\ln q_e = \ln K_F + \left(\frac{1}{n}\right) \ln C_e \quad (5)$$

where n also is the intensity of the adsorbents and K_F is the Freundlich constant [29]. It is defined as the adsorption or distribution related to the bond energy. Langmuir and Freundlich parameters, which are calculated from Eq. (3) to Eq. (5), are listed in Table 1. As can be seen in Table 1, the values of parameters confirm the favorable adsorption of CYP, also by comparing the correlation coefficient (R^2), Freundlich model could fit the data better than Langmuir model.

Table 1. Isotherm parameters obtained by using linear method at 40°C and pH 7.4.

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

DRUG RELEASE

Drug (CYP) release from CYP-GO nano composites

CYP-GO nano composite specimens (appropriate amounts) were placed in a buffer solution (30 ml) at 37°C and gently agitated. A known quantity (2 ml) of solution from the container was removed after every time step, making sure to replace it with the same amount of fresh buffer solution. UV-Vis spectroscopy determined the release rates of the CYP drug from the CYP-GO nanocomposite in appropriate pH. The controlled releasing of CYP by GO in simulated gastric fluid (pH 1.2), in simulated intestinal fluid (pH 7.4), and in pH 10 are illustrated in Fig. 5.

It is obtained that about 68 % of CYP was released in the simulated intestinal fluid (pH 7.4) at 37°C over a period of 30 h, while at this temperature and in the simulated gastric fluid (pH 1.2) CYP was released approximately 80 % in 4 h, 90 % in 15 h and 92 % over a period of 30 h. At 37°C and the period of 30 h, the maximum amount of CYP release was 76 % at pH 10. Obviously, in the simulated gastric fluid (which is harsh acetic media.) CYP was better released. Also, the effect of temperature on CYP, which was released at pH 7.4, was investigated during 10 h and the results are shown in Fig. 6. As can be observed as the temperature is raised up to 45°C, the release of CYP is increased.

Drug release response

The loading capacity of CYP was found to be around 1 mg mg^{-1} of GO nano-sheets. Such a high value of drug loading is far beyond the common drug carrier materials [19, 24, 27] which are generally below 1 mg mg^{-1} at saturated carrying concentration. The above-mentioned observations indicate that GO is indeed a promising candidate for drug carrier herein, the CYP release from CYP-GO at the temperature of 37°C in the phosphate buffer solution. The experimental parameters like the temperature of 37°C and pH (1.2 and 7.4) were selected because of the resemblance with physiological conditions.

The fate of a drug from a drug carrier depends on various experimental factors such as pH, degradation rate, particle size, the interaction between the drug and the surface and behavior of GO in the solvent. In the current study, we have chosen CYP as a model drug to demonstrate the loading and release from a graphene oxide-based nanocarrier [30, 31]. We found

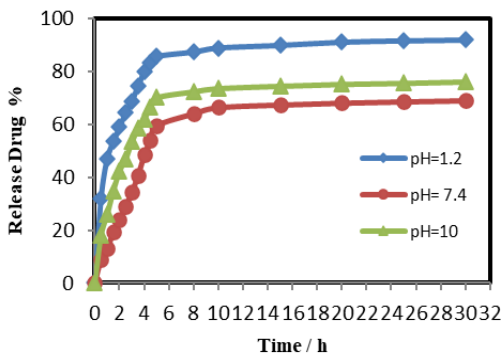


Fig. 5. The release of CYP on GO at different pH and at 37°C.

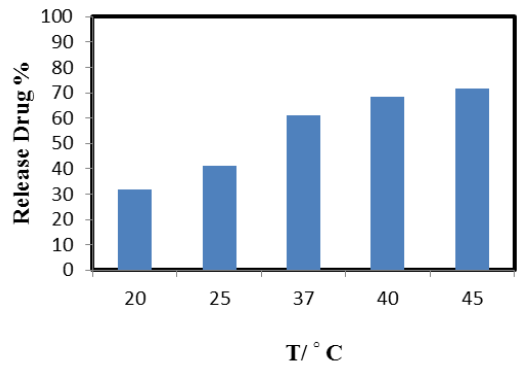
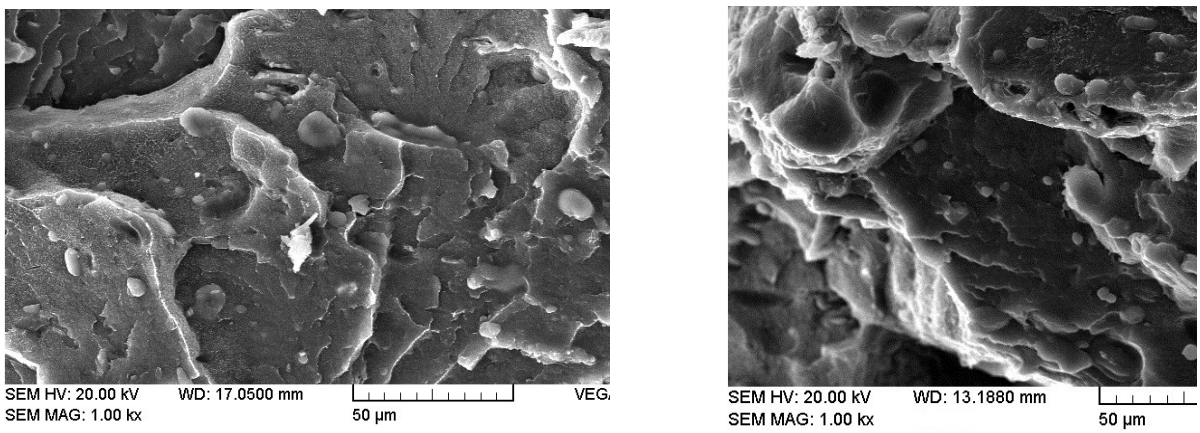


Fig. 6. The effect of temperature on releasing of CYP at pH 7.4, during 10 h.



a) b)

Fig. 7. SEM micrograph of the nanodrug: (a) before drug release; (b) after drug release.

that at pH 7.4, CYP released at a slower and controlled manner from CYP-GO system. The controlled release pattern under acidic media indicates that the amount of CYP released in the first 4 h, is much higher than at neutral conditions. Under acidic conditions, the amine groups of CYP get protonated resulting in the partial dissociation of hydrogen-bonding interaction, hence the amount of released CYP from GO is much higher. The loading and release of CYP depend upon the hydrogen bonding interaction with GO and is a function of pH. Hydrogen bonding can be formed between -OH of GO and -NH group of CYP. Furthermore, at low pH, the proton in solution would compete with the hydrogen bond-forming group and weaken the hydrogen bonding interaction, which may lead to a greater release of CYP [24, 26, 27, 32].

The SEM micrograph suggests the diffusion of the drug from the carrier. The surface of the carrier sample was observed to be nearly non-porous before drug release while after drug release the sample shows enough evidence of surface segregation and development of significant porosity upon release (Fig. 7(a) and Fig. 7(b)) [21, 30 - 35].

CONCLUSIONS

In this study, the adsorption of cyclophosphamide (CYP) on Graphene oxide (GO) was investigated as a function of temperature, pH, contact time and initial concentration. The interaction of CPY to GO is ascribed to the hydrogen bonding between CPY 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 to the Freundlich adsorption isotherm than the Langmuir model. Nearly 80 % of CYP was released in simulated gastric fluid, pH 1.2, in 4 h and 68 % in simulated intestinal fluid, pH 7.4, in 30 h.

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