Doxorubicin Loaded Liposomal Nanoparticles Containing Quantum Dot for Treatment of Breast Cancer

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ABSTRACT: *In addition to increasing the efficacy of various drugs, Nanoparticles reduce their side effects. In this study, different nanoparticle formulations of Doxorubicin anticancer drugs were prepared. The efficacy of the formulations produced in the cell culture medium was studied compared with the free drug. Reverse phase evaporation was used to form the liposome containing doxorubicin. The graphite nanoparticles were prepared. These nanoparticles were mixed with the liposome containing doxorubicin, and the related Nano-complex was conjugated. Spectroscopy methods for visible light-ultraviolet, light and light dynamics differentiation were used to describe nanoparticles. For the toxicity of different formulation, MTT and MCF-7 cells were used. The amount of drug loading in the liposomes was 72%. The largest amount was related to the Nano-conjugated complex and the smallest size was related to the graphene-oxide nanoparticle with a nanometer size. The controlled release in 96 hours and the amount of drug release was 95.43%. Doxorubicin-containing liposome toxicity was 75% and Nano- conjugated complex was 85%, at the lowest drug concentration (10μM). The free drug created 35% cell toxicity in 10μM and 89% in 2500μM. The results of the study showed Liposomes act as a suitable nanoparticle for doxorubicin. It was found that the effect of nanoparticles of graphene oxide is very important. In the presence of this nanoparticle in the complex, toxicity increased significantly.*

KEYWORDS: *Graphene oxide nanoparticles; Liposomes; Doxorubicin.*

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INTRODUCTION

The use of multifunctional nanostructures in medicine is increasing. Recent advances in chemical engineering and technology have led to the production of various types of nanostructures. These include quantum dots, nanoparticles, graphene oxide nanoparticles, paramagnetic nanoparticles, carbon nanotubes and lipid-based systems [8]. These compounds reduce the side effects and increase the efficacy of chemotherapy agents [10]. They are also used to cross biological barriers, protect the drug and releasing optimal dosage [6]. The use of nanoparticles as carriers of the drug comes from two characteristics of these substances: 1- Due to their small size, these nanoparticles can penetrate through the very small capillaries within the cells. Resulting in an effective accumulation of the drug at target sites in the body. 2- The use of biodegradable materials for the preparation of nanoparticles results in the sustained and uniform distribution of the drug at the target site for several days or several weeks [1].

Liposomes are used as commonly used drug carriers. Some have been approved by the FDA like Doxil [12]. Liposomes are a phospholipid structure made up of a hydrophobic head and a hydrophilic head. Due to their amphipathic nature, they can encapsulate water-soluble drug molecules in the internal phase and fat-soluble molecules in their hydrophobic-membrane [2]. Improved future generations of liposomal drug carriers include controlled release and detection by adding operational molecules. The purpose of the operating molecules is molecules that are sensitive to temperature or PH or have a detectable ability [17]. In the medical field of nanoparticles of graphene oxide, due to their physical-chemical properties of size and shape, operational molecules are noteworthy [30]. They are used in a variety of applications such as adjusting the intracellular gene expression, chemotherapy, and drug delivery. Liposomes are an effective method for intracellular delivery of graphene oxide nanoparticles. The cellular absorption of graphene oxide nanoparticles is enhanced by the use of liposomes as carriers. Also, the easy attachment of ligands to the surface of liposomes containing graphene oxide nanoparticles has enabled the activated targeting of these carriers [20].

There are three types of liposome-graphene oxide nanoparticle complexes. The first type of graphene oxide nanoparticle is placed inside the liposome. This type of complex is made through the recovery of graphene oxide ions in the presence of a resuscitator [31]. This type is used for evaluating the distribution of liposome in vivo [12]. Resuscitation may hurt the activity of the drug. In the second type of this complex, graphene oxide nanoparticles are present in the lipid membrane [22]. But since the thickness of the two-layer lipid is only 5 nm, for this reason, only a limited number of graphene oxide nanoparticles can be integrated into the membrane. The third complex is a modified liposome with graphene oxide nanoparticles at the surface. This kind of complex is easily obtained by combining liposomal suspensions with graphene oxide nanoparticles [28].

Breast cancer is currently common in human societies, and cancer has become the second cause of death in developing countries [21, 20]. Doxorubicin is an anticancer drug. The use of this drug is common in the treatment of myeloproliferative disorders and breast cancer [15, 25]. This drug has side-effects with anti-cancer properties. Drowsiness, nausea, vomiting, and diarrhea are part of its side effects. Also joined, mucositis, anorexia, stomatitis, bone marrow toxicity, hair loss, skin changes, change of liver enzymes, creatinine and blood urea are other side effects related to this drug [15].

Based on these observations, it was decided to formulate this drug using nanotechnology to increase drug efficacy. The complex was made through a liposome suspension containing doxorubicin with nanoconjugates and pure graphene oxide nanoparticles. As expected, the drug's degree of toxicity in nanoparticle compared to free drugs showed a significant increase. But the interesting thing was the role of graphene oxide nanoparticles. So, if this nanoparticle is present in the formulation, toxicity significantly increased, especially at low concentrations.

EXPERIMENTAL SECTION

Isopropanol and ethanol were purchased from Merck Inc, and Cholesterol, Doxorubicin, Phosphatidylcholine, Graphite, and MTT from the Sigma Company. Polyethylene glycol (3500) was obtained from the Kimiagaran company. The 1640RPMI medium was purchased from Invitrogen Inc. The MCF-7 cell was prepared from the Cellular Bank of Pasteur Institute of Iran. The water used throughout the study was distilled water.

Construction of PEGylated liposomal nanoparticles containing Doxorubicin

For Constructing the liposomal nanoparticles containing Doxorubicin, the compounds of lecithin/cholesterol/polyethylene glycol (25/50/500 mg) were poured into 50 ml of 98% ethanol (40°C hot bath). Then, 10 mL of phosphate-buffered saline (7.2 PH) and doxorubicin (20 mg) were added to the suspension.

Determination of the drug loading

For this reason, Spectrometer device (Shimadzu UV-1601PC) and Optical Density of the supernatant was measured at a wavelength of 215 nm. Subsequently, the drug loading was calculated using the following formula

$$\text{Loading}\% = \frac{\text{The amount of enclosed doxorubicin}}{\text{Th amount of initial doxorubicin}} \times 100 \quad (1)$$

Different concentrations of doxorubicin were prepared to draw the standard curve and the absorbance was measured by spectrophotometry at 215 nm wavelength. Figs. 1 and 2 show conjugation of the nanoparticles of graphene oxide and complex.

Description of prepared nanoparticles in terms of size, size distribution, and zeta potentials

To characterize the nanoparticles in terms of size, size distribution, zeta potential Zeta Sizer was utilized (3600 Zs Zen-nano UK instruments Malvern).

Study the cytotoxicity of Different formulations of the drug and free drug

To evaluate the cytotoxic effects of doxorubicin and comparing their effects with the relevant formulations, MCF-7 cells and MTT were used (30).

Statistical data analysis

The T-student test was used for data analysis. The values of P-Value≤0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Results

Control liposomal nanoparticles and containing the drug were made successfully. Combining the liposome containing conjugated drug with graphene oxide nanoparticle Led to the complex. Among the different formulations, the smallest size and size distribution of

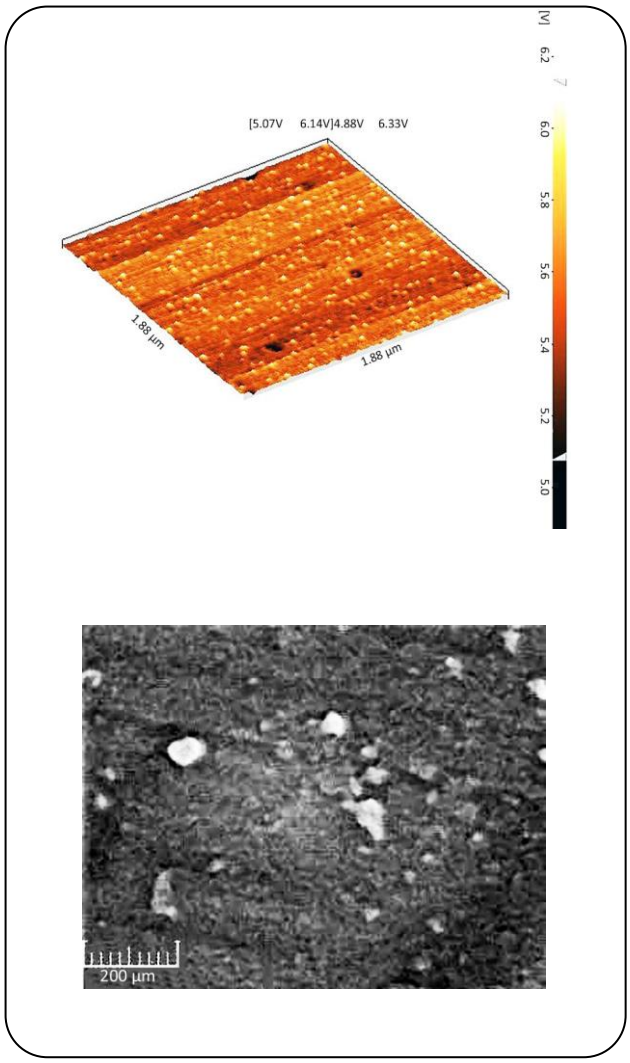

Fig. 1: Graphene nanoparticle AFM schematics.

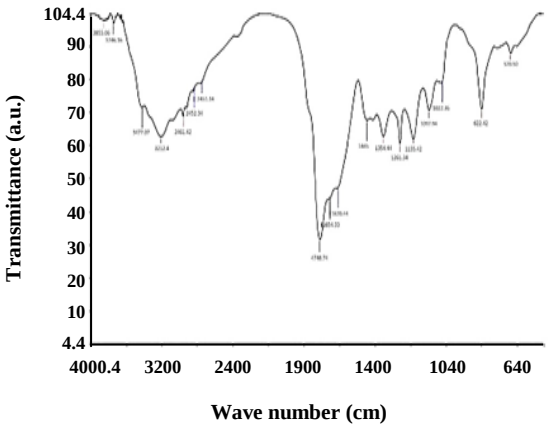

Fig. 2: Graphene nanoparticle FTIR schematics.

Table 1: the properties of nanoparticles based on the type of formulation.

Properties / Formulation	Size (nm)	Size distribution	Zeta potential (mv)
Controlled liposomal	473	0.46	-21
Liposome containing doxorubicin	230.5	0.38	-41.8
Graphene oxide nanoparticles	25	0.92	-9.9
Liposomal complex containing the drug + graphene nanoparticle 1.5 µM	320	0.43	-28
Liposomal complex containing the drug + graphene nanoparticle 3 µM	502	0.41	-32

the graphene oxide nanoparticle was calculated as a nanometer. However, the conjugation of this nanoparticle with a drug-containing liposome resulted in a dramatic increase in size(nm). The zeta potential of all formulas was calculated minus. Liposomes containing doxorubicin showed the lowest zeta potential equal to mv. The highest zeta potential was related to the liposome complex with the graphene oxide nanoparticle, which was calculated in mv. Also, based on the results, the size distribution of all formulations were appropriate. Graphene oxide nanoparticles showed the smallest size distribution and the highest was for controlled liposomal nanoparticles. Table1 shows, the properties of nanoparticles have been remarkably modified based on the type of formulation.

Verification of the conjugation of graphene nanoparticles with drug-containing liposomes

The results of Spectroscopy by spectrophotometry of visible-ultraviolet light confirmed the conjugation (Fig. 3)

Fig. 3: The spectroscopy of graphene oxide nanoparticles (green) and liposomes containing the drug(blue). Two different spectra have been obtained: the green color refers to the golden nanoparticles and the blue color of the liposome containing the drug.

Drug loading efficiency

The loading efficiency was calculated according to the standard curve of doxorubicin. The percentage of the drug-loaded was estimated at 72%.

Evaluation of drug release profile of doxorubicin

The study of the release of Doxorubicin drug from the liposomal system was performed by dialysis bag, in the vicinity of the PBS buffer at 37°C and PH=7.4, during 96 Hours.

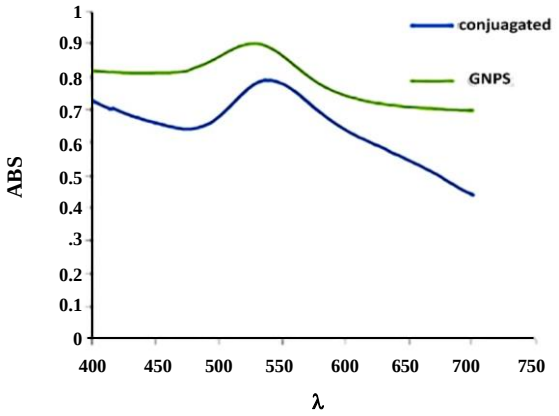

Fig. 3: The spectroscopy of graphene oxide nanoparticles (green) and liposomes containing the drug (blue). Two different spectra have been obtained: the green color refers to the graphene nanoparticles and the blue color to the liposome containing the drug.

The predictive model of drug release kinetics

The release of the drug is obtained from the liposomal system formulation (2).

$$\text{The release} = \frac{\text{The cumulative amount of drug released at any time}}{\text{The initial amount of the drug}} \quad (2)$$

$$\text{The release} = Kt^2 \quad (3)$$

The amount of drug release can be estimated by the mathematical model of quasi-experimental, The Peppa model, which is introduced in the formula. Nonlinear regression analysis conducted by MATLAB software. To verify the quality of fitting data, Root Mean Square Error R^2 (RMSE) was used. Although the rate of R^2 is higher and *RMSE* is lower, The data is better suited to the model, Formulas(4) and(5):

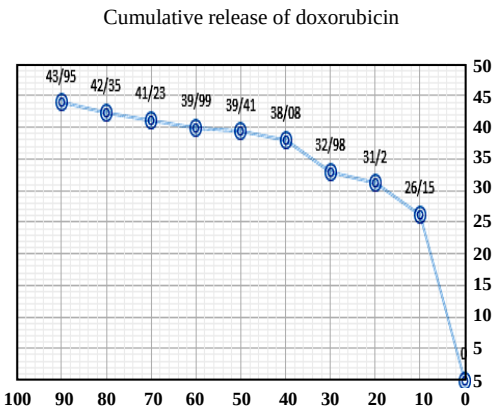

Fig. 4: The release profile of liposomal doxorubicin in PBS at 37°C and PH=7.4

$$\mathrm{RMSE} = \left[\frac{1}{N}\sum_{i=1}^{N}\left(\mathrm{Release}(\%)\mathrm{per,i} - \mathrm{Release}(\%)\mathrm{exp,i}\right)\right]\frac{1}{2} \quad (4)$$

$$R^2 = 1 - \frac{\sum_{i=1}^{N}\left(\mathrm{Release}(\%)\mathrm{exp,i} - \mathrm{Release}(\%)\mathrm{per,i}\right)2}{\sum_{i=1}^{N}\left(\mathrm{Release}(\%)\mathrm{exp} - \mathrm{Release}(\%)\mathrm{exp,i}\right)2} \quad (5)$$

In the above Formulas, *N* is the number of observations, *Z* is the number of constants of the model and i, i is the first data.

Evaluation of the release of doxorubicin from liposomes

After examining the release of the drug at different times, the dose of Doxorubicin is slow-release, after 96 hours 43.95% of doxorubicin is released from the system. The Fig. 3 shows the release profile of doxorubicin in the water at 37°C (Fig. 4).

The fit analysis of this model, based on experimental data shows RMSE and R^2 are 0.87 and 0.9966, respectively, which shows good agreement with experimental data. Also, Coefficients and functions of the model for n and k are 0.1983 and 17.25. Since the coefficient n is less than 0.5, therefore, the release of Doxorubicin from the liposomal system follows the Fick's law

The cytotoxicity of various products

Initially, it was found that the drug-free liposomal nanoparticle at concentrations containing the drug did not apply any toxicity on the cell and is completely healthy. It was also found that the drug toxicity was higher in all formulations than in the free drug (Fig. 5). However, at maximum concentrations (2500 µM), the toxicity of all three nanoparticle formulations containing the drug and the two Nano-drug complexes were estimated to be approximately the same, but at a minimum concentration, this formulation was a conjugated complex which increased the toxicity significantly (Fig. 6). An interesting point about both Nano-complexes was observed: the toxicity was reduced proportionally with the reduction in drug concentration. But at concentrations less than 80 µM for the graphene oxide nanoparticle complex 3µM, and 40µM for the graphene oxide nanoparticle complex 1.5 µM, the toxicity showed a rising trend again in contrast with the reduction of the concentration (Fig. 7). It should be noted that in these concentrations in the graphene nanoparticle complex of 1.5µM the graphene nanoparticle concentration was 1.5µM, and in the graphene nanoparticle complex of 3µM this number was 3µM (Fig. 8).

The results were presented as an average of ± 5% error from at least three independent tests. As the figure shows, the efficiency of the drug in the loaded state on nanoparticles significantly increased compared to the free drug. This means that in the presence of nanoparticles and the same concentrations of the drug, more survival reduction was observed in comparison with free drugs. The survival rate was inversely related to the concentration of the drug. This may be due to the phenomenon of delayed-release drug in the loaded state on nanoparticles.

The results were presented as an average of ± 5% error from at least three independent tests. In this figure, the increase in drug efficiency in nanoparticulate states is observed in comparison to free status. Cell survival increased proportionally with the reduction of drug concentration. This situation occurred to a concentration of about 80 µM. Interestingly, at concentrations below 80µM of the drug, the survival rate was significantly reduced. Based on the results of Fig. 1, this phenomenon cannot be related to the effect of liposome in drug release. This can be attributed to the effect of graphene nanoparticles. This means that in the low concentrations of graphene nanoparticles (about 3µM), the Nano-carriers complex works better and the drug's entry into the cell is probably increases.

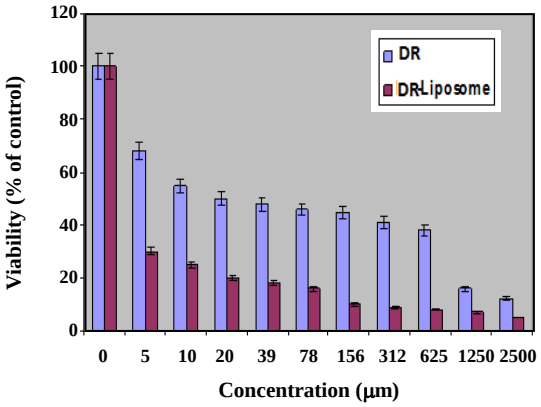

Fig. 5: The effects of cytotoxicity of doxorubicin and loaded doxorubicin on the liposomal nanoparticles on MCF-7 cells after 48 h incubation.

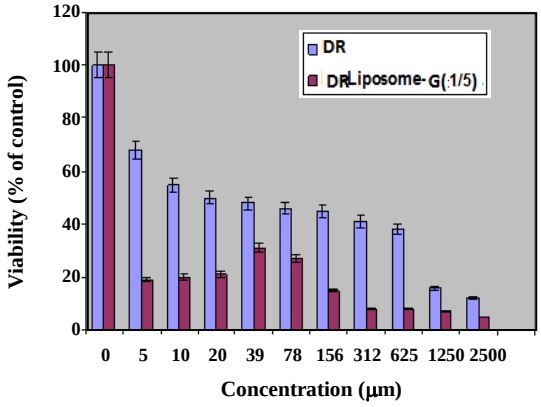

Fig. 7: The effects of cytotoxicity of doxorubicin and loaded doxorubicin on liposomal nanoparticles complex- graphene nanoparticles 1.5 µM on MCF-7 cells after 48 h incubation.

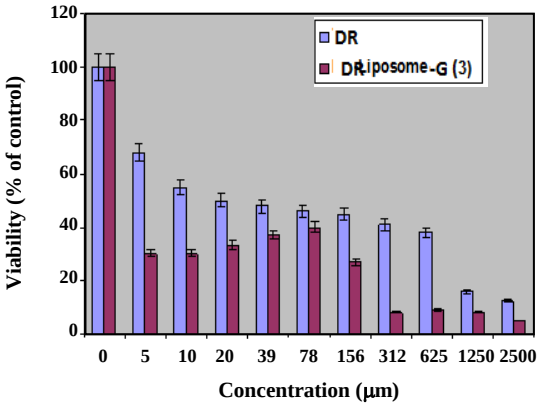

Fig. 6: The effects of cytotoxicity of doxorubicin and loaded doxorubicin on liposomal nanoparticles complex-graphene nanoparticles 3 µM on MCF-7 cells after 48 h incubation.

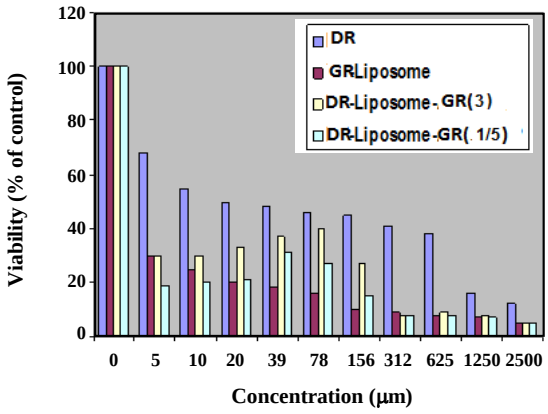

Fig. 8: The comparison of the cytotoxicity effects of doxorubicin and loaded doxorubicin on liposome, and loaded on liposomal nanoparticles complex- graphene nanoparticles 3 µM, and loaded on liposomal nanoparticles complex- graphene nanoparticles 1.5 µM on MCF-7 cells after 48 h incubation.

The results were presented as an average of ± 5% error from at least three independent tests. Like the liposomal nanoparticle complex-graphene nanoparticles, the increase in drug efficacy in nanoparticle states was observed compared to free status. The cell survival pattern was repeated here as well as the liposomal nanoparticle complex- graphene nanoparticles. However, instead of 80µM concentration in the liposomal nanoparticle complex-graphene nanoparticles, concentrations below 40µM led to a gradual reduction in survival or increase in cytotoxicity. This can be attributed to the presence of the graphene 1.5 µM in this complex.

The results were presented as an average of ± 5% error from at least three independent tests. The results show the comparative superiority of the formulations containing graphene nanoparticles 1.5µM in low drug concentrations, and simple drug formulation with liposome in High concentrations.

Discussion

Cancer is a disease of abnormal cell development [3]. Which has become the second cause of death in developing countries [20, 21]. Meanwhile, breast cancer is one of the most common causes of death among

cancers [15]. Targeted drug delivery is one of the main challenges of breast cancer treatment. The discovery and the development of new anticancer drugs is needed due to problems with currently available medicines, like toxicities and drug resistance [11].

The problems of most common drug delivery systems are included poor bioavailability of the drug, low in-vivo stability, poor drug solubility, and low intestinal absorption. Also, the weakness in drug targeting and its continuous delivery to the target site is part of the challenges of drug delivery systems. The use of nanotechnology in drug delivery is a good way to overcome these challenges.

Nanostructures are used in this technology. Generally, nanostructures are capable to preserve their encapsulated drugs from the Hydrolytic and enzymatic experience in the digestive system. Also, they can deliver a large number of drugs to different areas of the body for continuous release [13, 23, 27]. Liposomes are one of the nanoparticles studied in the field of drug delivery [8].

In this study, reverse phase evaporation used for making liposomes containing optimal doxorubicin. The resulting liposomes had the proper properties. Size, size distribution, and zeta potential were in the desirable range. Also, these liposomes investigated in terms of their sustainably. In this way, after 2 months, the resulting suspension was investigated again by Zeta Sizer. It was found that the size did not change significantly compared to the day it was made. It was also found that the effect of sonication and homogeneity on the size of the liposomes is fundamental. So that their size decreases by about a quarter after sonication and homogeneity. Doxorubicin drug loading on the liposomes was estimated in the optimal range of 72%.

In another part of this study, graphene nanoparticles were made. The resulting graphene nanoparticles were relatively small. In comparison with the free drug, all formulations applied more toxicity.

This could indicate delayed drug release of these formulations. Cytotoxicity decreased in parallel with decreasing drug concentration in different formulations. This was true for the concentration of 80µM for 1.5µM graphene nanoparticle complex, and 40µM for 3µM graphene nanoparticle complex at lower concentrations, the toxicity level increased again in these two complexes, in contrast with decreasing drug concentrations. The difference between these two complexes is the presence of graphene nanoparticle in comparison with the drug-containing liposome. Therefore, this toxicity increase

can be attributed to the presence of this nanoparticle in the complex, in contrast to the decrease in drug concentration. It was found that a specific concentration of gold nanoparticles in the complex is required to maximize the efficiency of the complex. Since this concentration was very low (1.5µM), Therefore, it provides the advantage to decrease in accordance with the required drug concentration. It is possible that the graphene nanoparticle entry into the cell or its interaction with the cell increases in this concentration, which causes the subsequent consequences. This increases the drug's entry into the cell. This effect occurred at low concentrations. Graphene nanoparticles may be distributed individually in these concentrations and have better activity. Another point about the formulation was the presence of polyethylene glycol in various formulations. Polyethylene glycol increases its stability in the blood by increasing its efficacy [32].

Finally, the results of the study demonstrated liposomal nanoparticles act as a suitable Nano-carrier for doxorubicin. The placement of graphene nanoparticles to the surface of the liposome containing doxorubicin increased the efficacy of the drug. Interestingly, the toxicity of the complex containing graphene nanoparticle in the low concentrations of this nanoparticle has increased dramatically. As a result, the use of graphene nanoparticles in the form of a liposomal complex for various pharmaceutical formulations can be considered. Another conclusion from this study is that liposomes, in addition to doxorubicin, can act as an essential way to deliver graphene nanoparticles for therapeutic and diagnostic applications. Also, due to the increased efficacy of the complex formulation, it is possible to debut the related in-vivo studies.

CONCLUSIONS

The successful results of this study showed that the presence of graphene nanoparticles in the formulation of liposomal doxorubicin increases the efficacy of the drug. Especially this effect becomes more important when this effect is observed at low concentrations of graphene nanoparticles. The use of graphene nanoparticles is recommended in various liposomal formulations

containing the drug. Also, based on the results of the study, especially this effect is more important when it is recommended in low concentrations of graphene nanoparticles in various formulations of the liposomal drug. additionally, based on the results of the study, intracellular tests can be initiated with the resultant formulation.

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