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Synthesis and Characterization of Chitosan Hydrogel Reinforced by Graphene in Order to Doxorubicin Delivery

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Abstract: In recent years, considerable advances have been performed in the use of inflammatory biomedical polymers in water as targeted carriers for drug delivery and chitosan has been notable due to its unique physical, chemical and biological characteristics. The purpose of this study was to reinforce the chitosan hydrogel with nano graphene oxide. The graphene-reinforced hydrogel was used to deliver Doxorubicin (DOX) as an antioxidant drug. The structure and morphology of synthesized hydrogels were investigated by various tests including FTIR, XRD and SEM. The drug delivery rate was determined by spectrophotometry device (Vis-UV) at different times. The present study has shown that graphene oxide improves the hydrogel structure and carries out the cancerous drug delivery in a controlled manner. Electrospinning of chitosan containing drugs was also successfully performed. Therefore, chitosan fibers containing drug can be used as wounds and tissue engineering scaffolds for carriers of the drug in cancerous tissues.

Keywords: Chitosan, Doxorubicin, Graphene oxide, Targeted drug delivery.

Introduction

A wide range of synthetic and natural polymers can be used for biological applications. natural polymers have many advantages than synthetic polymers, in particular, these polymers are non-toxic, biodegradable and biocompatible, and their main characteristic is water absorption and therefore, classified in the category of hydrogels (Jayanth & Vinod, 2012; Natarajan & SN, 2012). Chitosan is a naturally deacetylated polyatomic polymer of chitin that it consists of N-acetyl-D-glucosamine units. Chitin is a mucopolysaccharide that is abundantly found in the outer skeleton of arthropods such as shrimp, crabs, and primary plants such as yeasts. Chitosan has various molecular weights, which can have many applications in the industry, especially in drug delivery (Azadi & Hamidi, 2013; Yousefpour et al., 2011; Rampinoa et al., 2013).

Previous researches have indicated that the cationic nature of chitosan is well used for the development of drug delivery systems. The most important processes that make for the chitosan structure are the creation of chemical cross-links between the hydrogel chains. To prepare these structures, glutaraldehyde has been used as the agent for crosslinking between amino groups in chitosan. The addition of glutaraldehyde causes the drug to be attached to the polymer and the immobilization of the drug instead of its encapsulation (Hamidi, 2008).

Doxorubicin is an anticancer drug that conflicted with the growth and spread of cancer cells which interfere with the body. Doxorubicin is used to treat the various types of cancer such as breast, bladder, ovarian, thyroid,

stomach, lung, bone, neural tissues, muscles, joints, and soft tissue. It is also used to treat Hodgkin's disease and certain types of leukemia. Doxorubicin has limited use for several reasons including drug toxicity, low stability of blood circulation, severe infiltration in the heterogeneous tissue of tumors, and drug disappearing by degrading enzymes of inside the body. The most important side effect of doxorubicin is cardiac toxicity, which appear mainly as a chronic congestive heart failure. According to the mentioned constraints and made side effects caused by this drug, the use of a new drug delivery system seems necessary which can reduce these constraints and side effects (Katz wt al., 1997; Lishner et al., 1992).

Drug delivery systems (DDS) are created to improve the medicinal and therapeutic properties of drugs used in patients, and they often contain drugs as a reservoir. These systems release the drug at a given amount and specific place, thus affecting the pharmacokinetics and distribution of the drug in the body. Bio-nanoparticles and hydrogels as drug reservoir, widely used in drug delivery. In recent years, a lot of attention has been paid to the preparation of nanostructures as controlling the release of cancer drugs, because of these structures are considered as a highly effective drug delivery system for reasons such as their control and slow release of drug, the preservation of the drug molecule, the prolongation of the drug in the bloodstream, targeted drug delivery and biocompatibility which increases the therapeutic efficacy of the drug (Benita, 2006). Polymers and lipids are used as drug delivery systems (Kim et al., 2008; Tang & Singh, 2008). Hydrogels are three-dimensional networks of hydrophilic polymers that can absorb a large amount of water and are used for drug delivery. The pores in the hydrogels make an easy to get the drug into them and use it as a drug delivery system (Tamilvanan et al., 2008; Watanabe et al., 2008; Katime et al., 2004; Nguyen & West, 2002).

Graphene is a layer of graphite and as two-dimensional allotropes of carbon with a grid plate structure such as a honeycomb. Graphene has chemical and physical properties including high Young's modulus, high failure strength, and excellent electrical-thermal conductivity, rapid mobility of loads, high surface and high biodiversity compatibility. These characteristics represent graphene as an ideal material in various fields such as quantum physics, nanoelectronics, energy researches, decomposition and nanocomposites engineering and biochemicals (Iijima, 1991). Graphene at the same level as other nanocomposites has four times higher the ability to transfer and drug delivery (approximately 2600 grams per square meter). Another important feature of graphene and graphene oxide in the field of drug delivery is loading ratio (the ratio of loaded drugs to the carrier) which can be up to 200 percent for graphene nanomaterials which is significantly higher than other nanomaterials and other drug delivery systems. According to conducted studies, carbon nanoparticles and nanotubes and their interaction with body cells depend on macrophages and follow several parameters such as size, shape, and surface chemistry. The graphene shape plays a very important role. The unique shape of the two-dimensional, flattened graphene and graphene oxide, and the lack of this shape in the morphology of the human body's biological system are other advantage for the use of this nanocomposite in drug delivery (Liu et al., 2013).

Graphene oxide is a monolayer material that is made from oxidation of graphite powder with very strong oxidants. Graphene oxide sheets act like active surfaces and have amphiphilic properties. By placing graphene oxide in carbonate water, it was observed that graphene sheets penetrated into the water bubbles, they brought themselves to the surface of the water and also graphene oxide could disperse oil droplets in water (Liu et al., 2013).

GO (graphene oxide) can be easily placed in a sheet of monomer plates and dispersed in water due to the large number of oxygenated hydrophilic groups. Along with many sciences, many uses of GOs have been taken into consideration, which reinforced composite materials are one of its applications. Research indicates that graphene oxide can easily improve the mechanical and thermal properties of the polymer, and also the hydrogel composition with these nanoparticles can have particular uses. In this regard, research on graphene composites and hydrogels has been conducted. The hydrogel network poly (N-isopropylacrylamide) reinforced with graphene oxide is obtained by creating a covalent bond between the GO sheets and the PNIPAM gels. This compound is used for drug delivery uses. However, some quantitative investigations have been carried out to produce such structures with good strength and friable properties. The use of the graphene family as a nanoparticle in the base material improves the thermal properties, including the thermal conductivity coefficient. For example, the use of 0.25 percent graphene nanoparticles in the silicon structure increases the conduction heat transfer coefficient up to 6 percent. This increase is also observed for epoxy, polyethylene, polypropylene and paraffin. Therefore, graphene oxide, as a structure with well soluble in water, can be used as an option to improve the thermal and biological properties of hydrogels (Jing et al., 2015; Yoo et al., 2014).

Previous studies have shown that reinforcement of hydrogels with graphene oxide has not been investigated in order to drug delivery. Considering the importance of chitosan as a drug carrier and many special properties of graphene oxide, in this study, synthesis and fabrication of chitosan/graphene oxide hydrogel as a suitable hydrogel for control and delivery of cancerous drugs were investigated. Also, possibility of loading and delivering behavior of doxorubicin drug in the presence of graphene oxide and its chemical structure were studied.

Material and Methods

Material

Chitosan with average molecular weight of 85% was purchased from MP Biomedicals in Netherlands, glycine acetic acid, glutaraldehyde, trifluoroacetic acid from the Merck Company in German, Sodium Hydroxide and Dichloromethane from Sigma Company in Germany. The drug used in this study was doxorubicin from a German company. nano Graphene oxide was synthesized and used based on the modified Hummer method from graphite powder of DAEJUNG company (Wilson et al., 2009).

Devices

Selected samples are attached on a base and are coated with gold medal by Sputter counter made by BAL-TEC Swiss Company, the SCD00S model. Images are provided from each sample by electron microscope (SEM), manufactured by Philips of the Netherlands, XL30 models with different magnifications. A number of selected samples from all of the samples are subjected to X-ray diffraction to measure the change in degree of crystallinity. The samples were subjected to x-ray diffraction for the above experiments. The XRD device is manufactured by Siemens, D5000 model with Cu kα lamp and electrical characteristics of 40 kV/20 Ma. Specific specimens under the IR beam of the Thermo Nicolet device of the NEXUS 870 model are subjected from Canada to identify chemical groups and agents. The spectrophotometer Bio waves 2100 (UV-VIS) of the UK were used to measure the loading and release rate of the drug. To obtain a suitable texture for use in tissue engineering scaffolds or wound structures, chitosan was electros pinned by the ES1000 machine.

Methods

2% chitosan was mixed with 0.1 molar acetic acid for 24 hours in a magnetic stirrer to form a homogeneous solution. 1% weight of synthesized grapheme oxide relative to the polymer weight with water was placed in the ultrasound apparatus. Then, dispersion of grapheme oxide was added to chitosan and a uniform mixture was created using a magnetic stirrer. 1% Glutaraldehyde relative to polymer weight was used to make cross-linking. The different synthesized samples was put in the mold and placed in a 37 °C incubator in order to film preparation and perform the transverse reactions. For the preparation of scaffolds of the chitosan Nano fibers containing grapheme oxide, 2 g of chitosan and 0.1% of grapheme oxide dissolved in TFA (trifluoroacetic acid) and DCM (dichloromethane) solvents for ratio of 30 to 70, and the solution was placed on the ultrasound apparatus for 90 minutes. The scaffold samples were electros pinned with a potential difference of 22 kv and a feeding rate of 3 ml/h.

To evaluate the delivery rate of the drug, 3 mg/ml doxorubicin was added to 10 cc of hydrogels. To determine the drug loaded rate, a ratio of 1:1 solution of sodium hydroxide was removed and centrifuged for 12 minutes at 12,000 rpm to precipitate. The UV absorbance of upper solution was measured by the UV device. The drug loading rate was calculated from Equation 1.

$$\text{Loading}\% = \frac{m_e}{m_p + m_e} \times 100 \quad (1)$$

m_e is the weight of the enclosed drug in the hydrogel, which is obtained using UV .The amount of m_p is the mass of the used hydrogel.

The hydrogel containing the drug was placed in 50 cc phosphate buffer to release the drug into the phosphate buffer, and a certain some amount of solution was removed at certain times and the drug delivery rate was measured.

With a slight buffer increasing, the volume remained constant during the test. 1:1 sodium hydroxide was added to the removed solution and centrifuged at 10,000 rpm for 10 minutes. The absorbance was measured by a UV device at a wavelength of 480 nm and drug delivery rate was obtained from equation 2.

$$Drug\ delivery\% = \frac{Amount\ of\ drug\ released}{Total\ drug\ amount} \times 100 \quad (2)$$

The synthesized hydrogels were weighed and immersed in 100 ml of distilled water at ambient temperature for 3 hours and then re-weighed. Equilibrium swelling (E_S) was calculated by Equation (3).

$$E_S = \frac{W_2 - W_1}{W_1} \quad (3)$$

W_1 and W_2 are the weight of dry and swollen hydrogels, respectively.

Result

Electron Microscope (SEM)

Figure 1 shows the electron microscope images of chitosan and chitosan containing graphene oxide. As shown in Fig. 1a and 1b, the produced chitosan has different particles dispersed on the surface and inside it.

The produced particles are spherical, and adding doxorubicin drug did not make a change in this structure. Figure 1c shows the chitosan hydrogel containing glutaraldehyde and graphene oxide. As it is indicated, glutaraldehyde has created cross-linking in chitosan and has made crystalline networks, and there are no spherical particles of chitosan, but is composed an intricate network structure. As shown in the figures, the presence of porous structure can be a good factor for loading the drug and delivering it appropriately. The presence of graphene oxide with a layered structure is evident in Figures 1c and d, which increases the strength of hydrogel from controlled delivery drug.

Figure 2 illustrates the electrospinned nanofiber of chitosan containing graphene oxide. As it is clear, the presence of graphene oxide has led to the creation of a three-dimensional structure with a nano diameter from chitosan fibers, which created scaffold has uniform distribution. Therefore, it can be used as a wound containing drug or tissue engineering scaffolds of drug carriers.

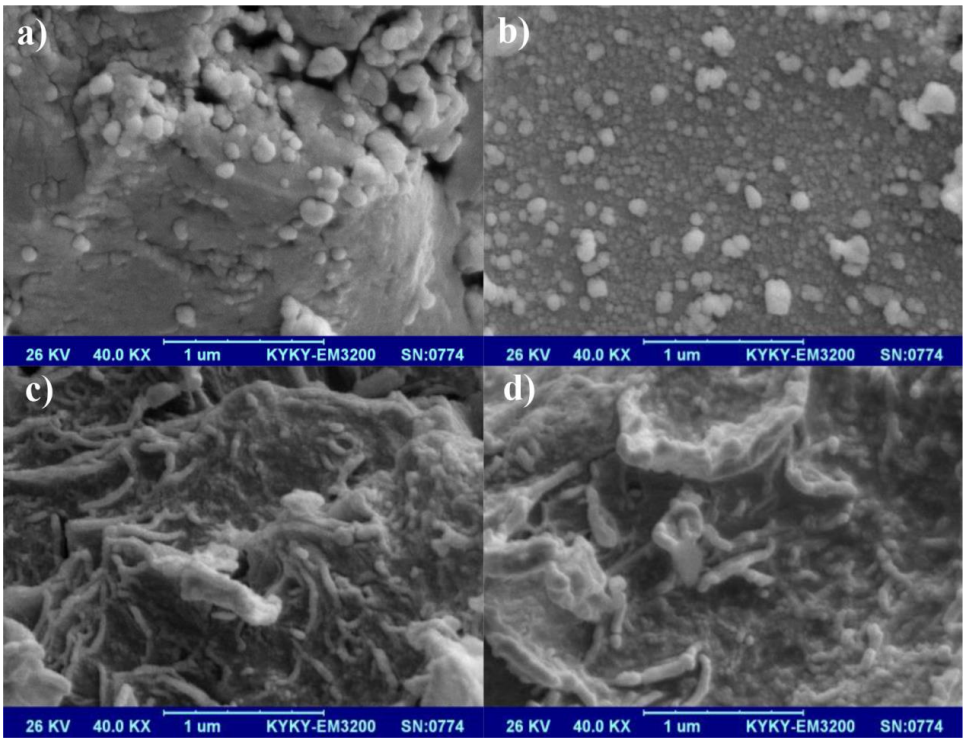

Figure 1. Images of an electron microscope a) Chitosan, b) Chitosan-Doxorubicin drug, c) Chitosan-Glutaraldehyde-Graphene oxide, d) Chitosan-Glutaraldehyde-Graphene oxide-Doxorubicin.

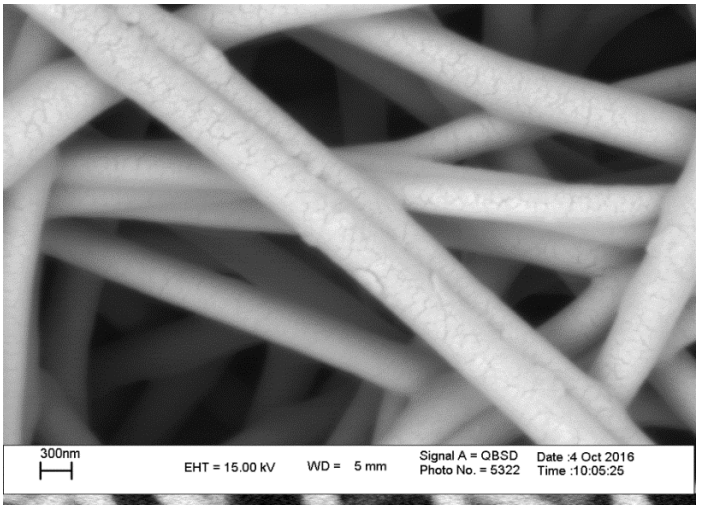

Figure 2. Electron microscope image of chitosan fiber containing graphene oxide

Infrared Spectroscopy (FTIR)

The chitosan infrared spectrum is illustrated in Fig 3a. Chitosan factor groups including 3390 cm^{-1} peak related to the OH and NH functional groups of Amine type one tensile vibrations. The peaks ranging between 3019 cm^{-1} to 3221cm^{-1} are related to N-H tensile vibrations, 2945 cm^{-1} peak is related to tensile vibrations of the C-H group, 1537 and 1578 cm^{-1} peaks are related to the bending vibrations of N-H, 1395 cm^{-1} is related to CH_3, 1336 and 1256 cm^{-1} are related to tensile vibrations of the C-N, 1039, 1090 and 1151 cm^{-1} are related to C-O-C group, and 897 cm^{-1} is related to bending absorption outside the N-H sheet. The b3 spectrum represents the chitosan and doxorubicin agent groups. In this diagram, the peaks related to bending vibration of the N-H and OH are more intense and the tensile vibrations of C-N have been created between 900 and 400 are related to the CH_2 spectra that appear in the form of sweep and flexural vibrational spectra, which is due to the presence of doxorubicin.

Figure 4a indicated the spectrum of chitosan, glutaraldehyde and graphene oxide functional groups. In this diagram, the peaks related to glutaraldehyde also intensify some of the peaks and CH peaks have changed to CH_2. The intensity of this peak can be attributed to the formation of cross-linking between glutaraldehyde and chitosan. By adding graphene oxide to chitosan, new peaks of 3537 and 3347 cm^{-1} related to the OH group and the specific 2819 cm^{-1} related to C-H tensile vibrations was created. The peaks related to N-H appeared again at this stage, and the peak 1018 cm^{-1} can be belong to C-O group of hydroxyl or carboxyl, and the peak related to the tensile vibrations of C-N has been removed.

Figure 4d is the spectrum of chitosan, glutaraldehyde, graphene oxide and doxorubicin. In this diagram, the corresponding peaks that is made by adding the graphene oxide to OH agent group have been removed again. Also, the spectrum related to C-H has been eliminated, and the peak related to the C-N is being weaker, and the peak related to the C-O-hydroxyl or carbonyl is being weaker, indicating the presence of the drug in the chemical structure of the chitosan. Acetic acid causes the protonation of oxygen in carbonyl glutaraldehyde groups. The attack of the nitrogen electron pair to carbon of the protonated carbonyl group creates a bond between nitrogen and carbon, and in the next stage, the group (C=N) is formed by transferring one proton of nitrogen to oxygen, and then losing water. Afterwards, the nitrogen of the imine group can be ready to accept the electron pair of the amino-chitosan group by accepting one proton from surroundings. Consequently, it leads to networking that eventually leads to the formation of a hydrogel.

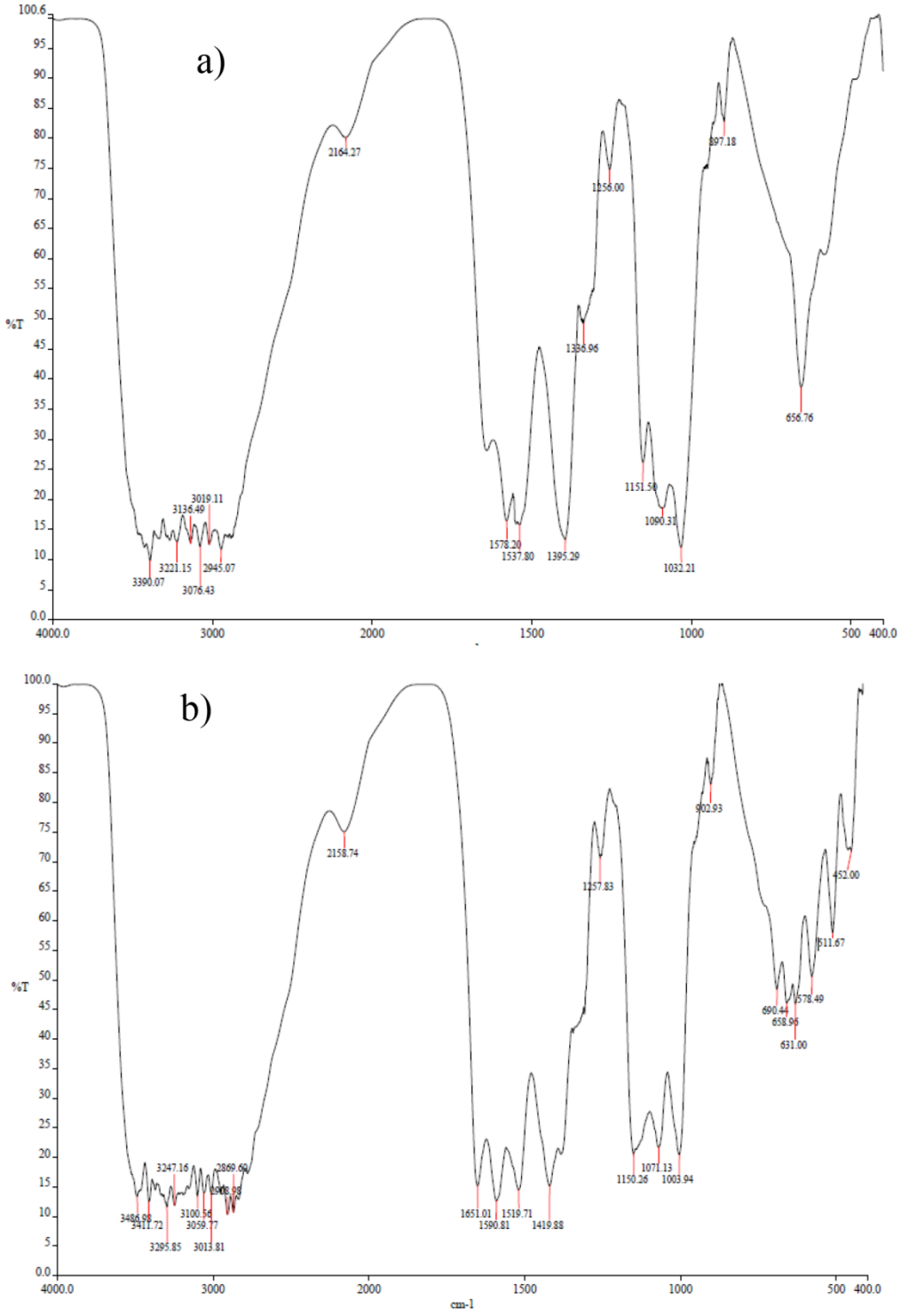

Figure 3. Infrared spectroscopy a) Chitosan, b) Chitosan-Doxorubicin.

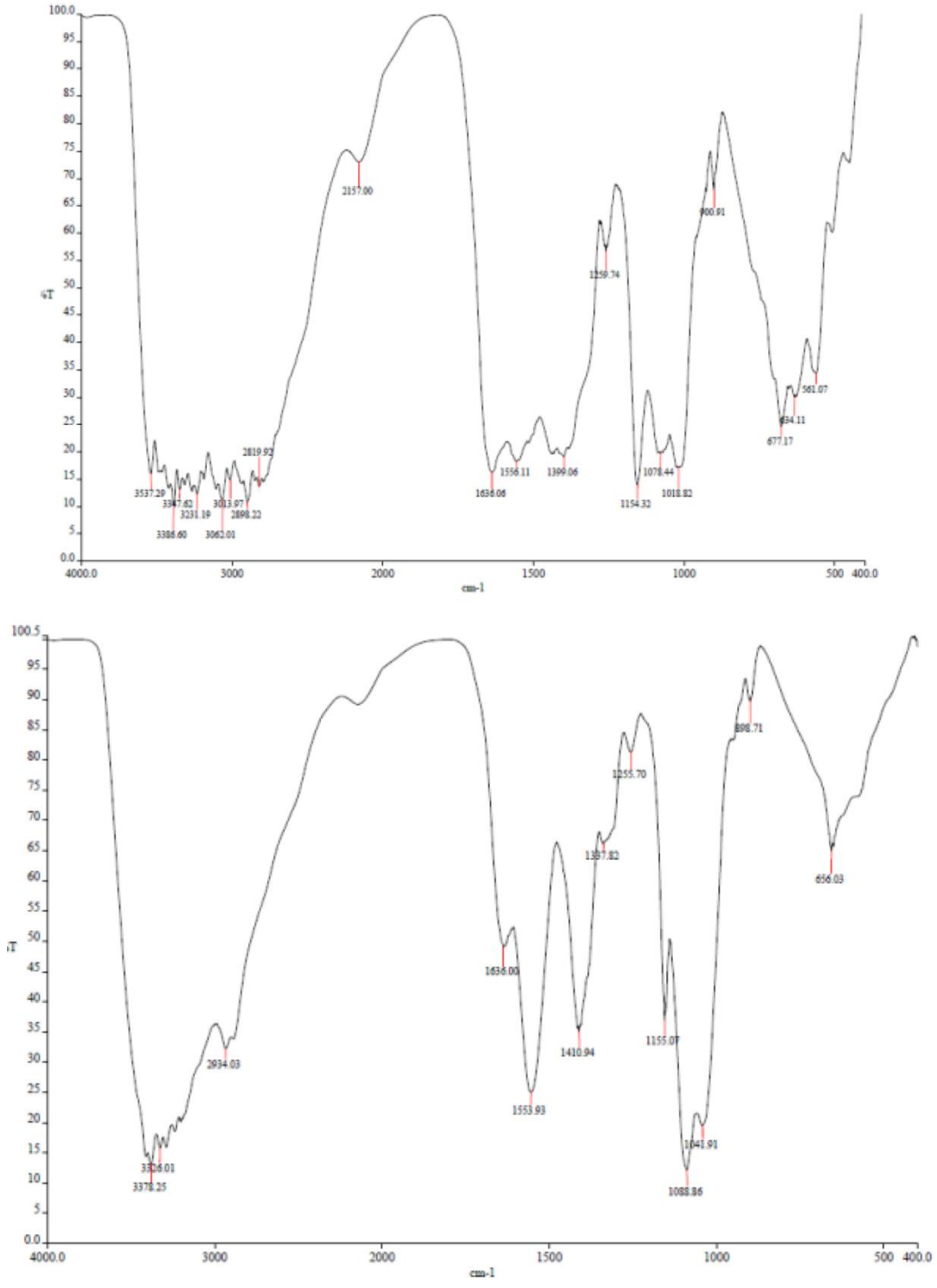

Figure 4. Infrared spectroscopy a) Chitosan-Glutaraldehyde-Graphene oxide, b) Chitosan-Glutaraldehyde-Graphene oxide-Doxorubicin drug.

XRD analysis

Different samples were exposed under x-rays and obtained XRD patterns reported in figures 5 and 6. The spectrum of figure 5a is related to chitosan which has three peaks 2θ=9.3, 11.92 and 21, indicating its crystallinity. High crystallinity of chitosan can be attributed to the presence of hydroxyl and amine groups which have capability for hydrogen bonding and strong intermolecular bonds formation.

Spectrum of b5 resulting from X-ray diffraction on chitosan sample containing doxorubicin drug. Doxorubicin drug has several peaks and has a crystalline characteristic which has created new peaks in chitosan and has intensified the chitosan peaks. Figure 6a shows the X-ray spectra of chitosan samples with glutaraldehyde and graphene oxide. The chitosan crystallinity property decreases after cross-linking between glutaraldehyde and this deformation can be attributed to the strong hydroxyl linkage of the main chitosan due to the substitution of hydroxyl and amino groups which destroyed the main chain of chitosan and led to its amorphous form. The difference in XRD pattern between chitosan and glutaraldehyde can be attributed to the interaction between chitosan and glutaraldehyde. The sharp peak at 11.68 refers to the graphene oxide crystallinity. The addition of graphene oxide to chitosan causes the chitosan crystalline property be stronger. On the other hand, the addition of glutaraldehyde causes to weakness some of the peaks. However, it makes to retain the crystallinity of hydrogel.

The 6d spectrum is related to chitosan samples containing glutaraldehyde, graphene oxide, and doxorubicin. As it is determined, the addition of graphene oxide causes sharpening of the chitosan peak and is a reason for reinforcing the chitosan crystallinity characteristic, and adding doxorubicin causes to exacerbate the chitosan peaks and glutaraldehyde make to weakness the chitosan peaks. In general, it can be stated that by adding graphene oxide, glutaraldehyde and doxorubicin to chitosan, hydrogel retains its crystallinity.

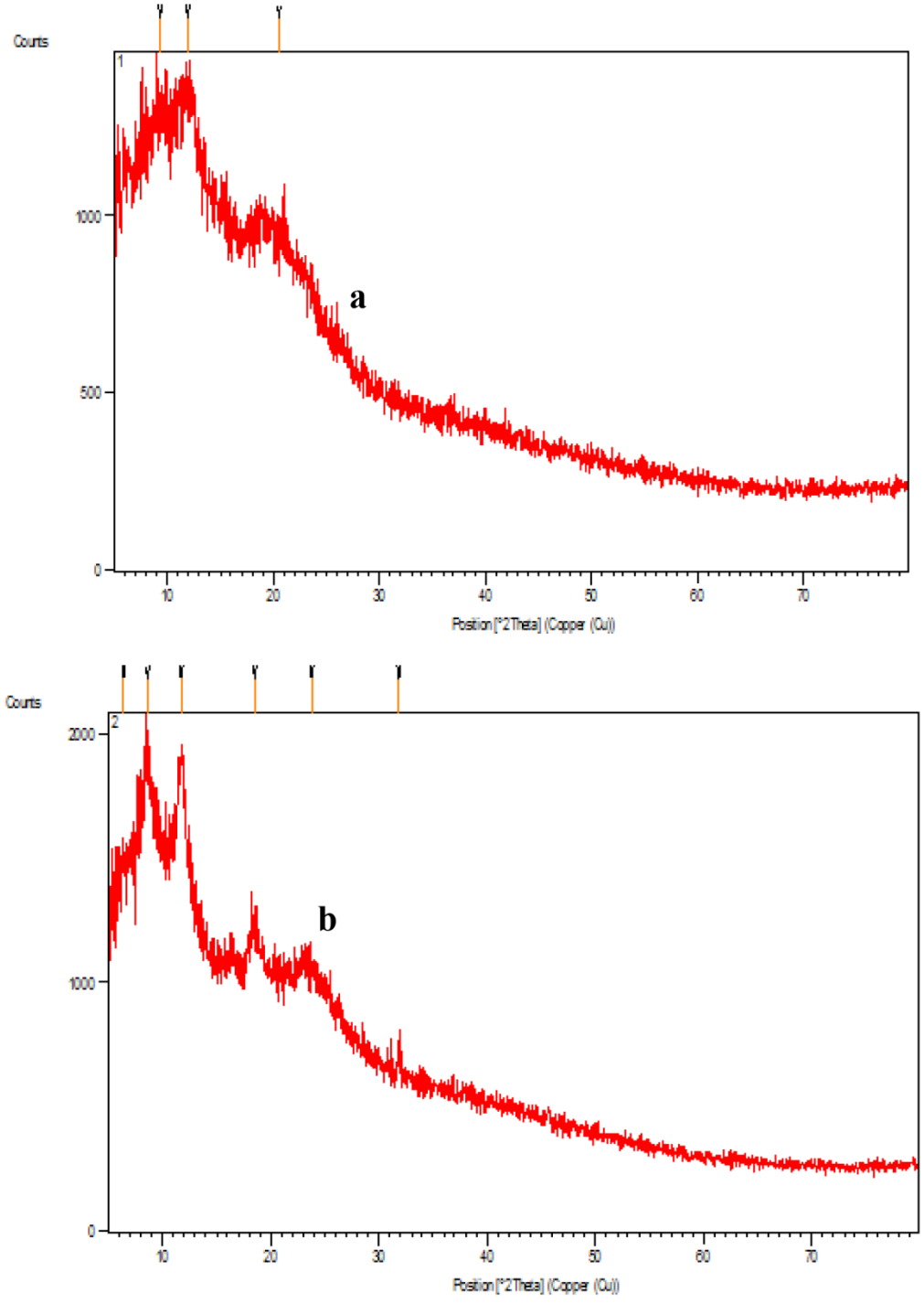

Figure 5. X-ray diffraction spectra of samples a) Chitosan, b) Chitosan-Doxorubicin.

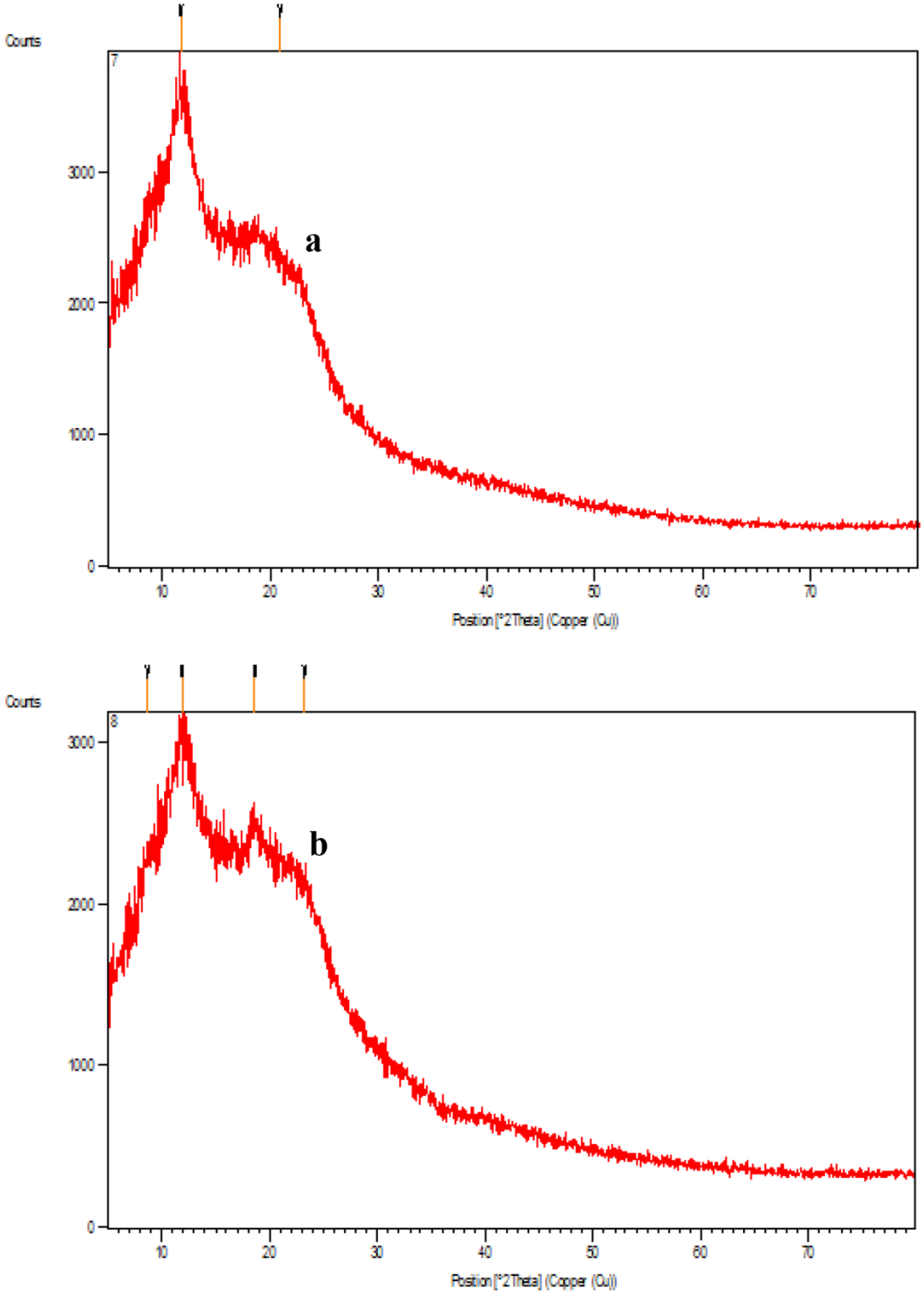

Figure 6. X-ray diffraction spectra Examples a) Chitosan-Glutaraldehyde-Graphene oxide, b) Chitosan-Glutaraldehyde-Graphene oxide-Doxorubicin.

Assessment of hydrogel equilibrium swelling rate

Table 1 indicates the equilibrium swelling of different samples due to water absorption. As it is clear, chitosan samples alone have more water absorption and swelling. By adding glutaraldehyde to chitosan, which causes a strong cross-linking between chitosan and glutaraldehyde, a decrease in water absorption and swelling occurs. Graphene oxide has hydrophilic structure, but due to its inclusion in the chitosan matrix and the occupation of water-absorbent areas, it significantly reduces the amount of water absorption and, consequently, hydrogel swelling decreases. Thus, a hydrogel sample containing graphene oxide can be controlled to absorb water and swelling. Figure 7 also shows swelling variations over several hours. Changes indicate a reduction in the rate of swelling in the presence of graphene oxide and cross-linking by glutaraldehyde.

Table 1. Evaluation of the equilibrium swelling rate of various hydrogels.

Samples	Initial polymer weight	Secondary polymer weight	Water absorption	Hydrogel equilibrium swelling (E_S)
Chitosan	1.175	9.04	7.86	6.69
Chitosan - Graphene oxide	4.85	10.02	5.32	1.09
Chitosan - Graphene oxide - Glutaraldehyde	4.81	9.10	4.28	0.88

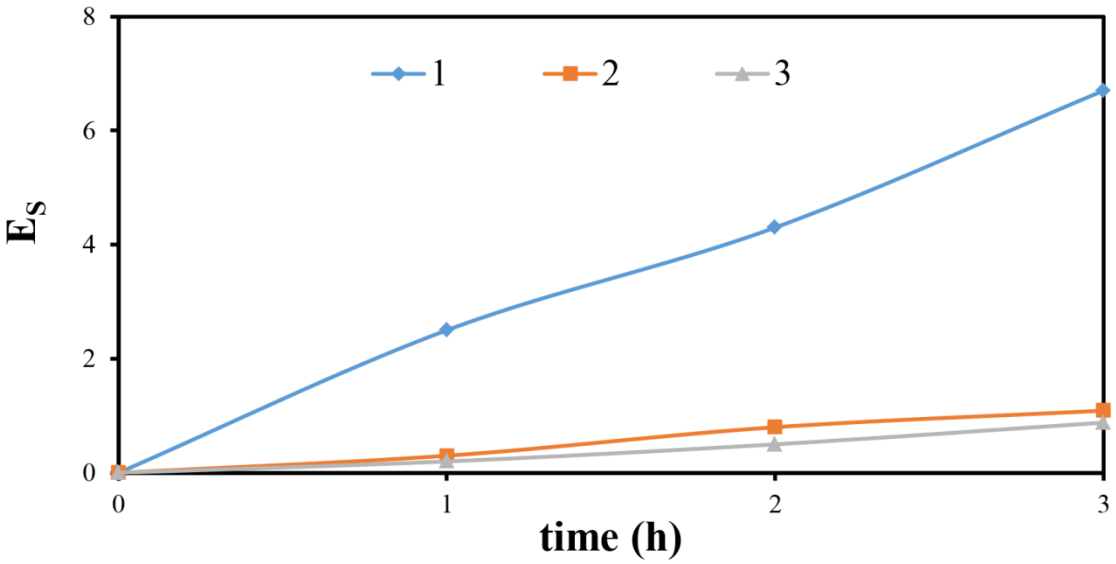

Figure 7. Hydrogel equilibrium swelling rate 1) Chitosan, 2) Chitosan and Glutaraldehyde, and 3) Chitosan containing Glutaraldehyde and Graphene oxide.

Percent of doxorubicin loading

Table 2 shows the loading rate for different samples.

As it is determined, drug loading the in the sample containing glutaraldehyde has increased, and its reason can be attributed to the hydrogel networking. Hydrogel networks increase the absorption and maintenance capacity of the drug. In other words, the creation of a porous structure by hydrogel networks has increased the absorption of the drug. The presence of graphene oxide also improves the drug loading.

The biological and pharmacological properties of graphene oxide have been studied and documented in many studies. Due to the hydrophilicity and frequency of the hydroxyl group in the surface of graphene oxide, the absorption capacity of the drug increases in the structure of the hydrogels containing the graphene oxide.

Table 2. Percent of drug loading in different samples.

Samples	Drug loading (percent)
Chitosan- Doxorubicin	2
Chitosan – Glutaraldehyde- Doxorubicin	3.5
Chitosan - Graphene oxide – Glutaraldehyde- Doxorubicin	5

Drug delivery test

Absorption rate of delivered doxorubicin was obtained at various times using spectrophotometric device. The drug delivery rate graph was prepared in various samples using equation 2 and shown in Figure 8. Based on equation 1, the drug loading rate in each carrier is calculated, and the maximum rate of drug delivery for each carrier was calculated by the ratio of delivery rate at infinite times relative to the percentage of loading. Table 3 shows the maximum drug delivery rate in terms of loading percentages. In the single dose of chitosan, doxorubicin has been delivered as descending, but graphene oxide acts in the hydrogel structure and slows down the delivery, resulting in gradual drug delivery. The reason for this slowdown can be attributed to the layered structure of the graphene oxide, which, by placing the layers in the structure of the hydrogel, slows down the drug delivery. Also, cross-linking and networking make to slow down drug delivery. As stated, the control of the anticancer drugs delivery has great importance. The sudden presence of an anticancer drug in the target tissue, while destroying cancer cells, can also damage the tissues and normal cells, causing a high toxicity.

Table 3. Percent of drug delivery in various samples.

Samples	Maximum drug delivery rate in terms of loading
Chitosan- Doxorubicin	33%
Chitosan – Glutaraldehyde- Doxorubicin	20%
Chitosan - Graphene oxide – Glutaraldehyde- Doxorubicin	16%

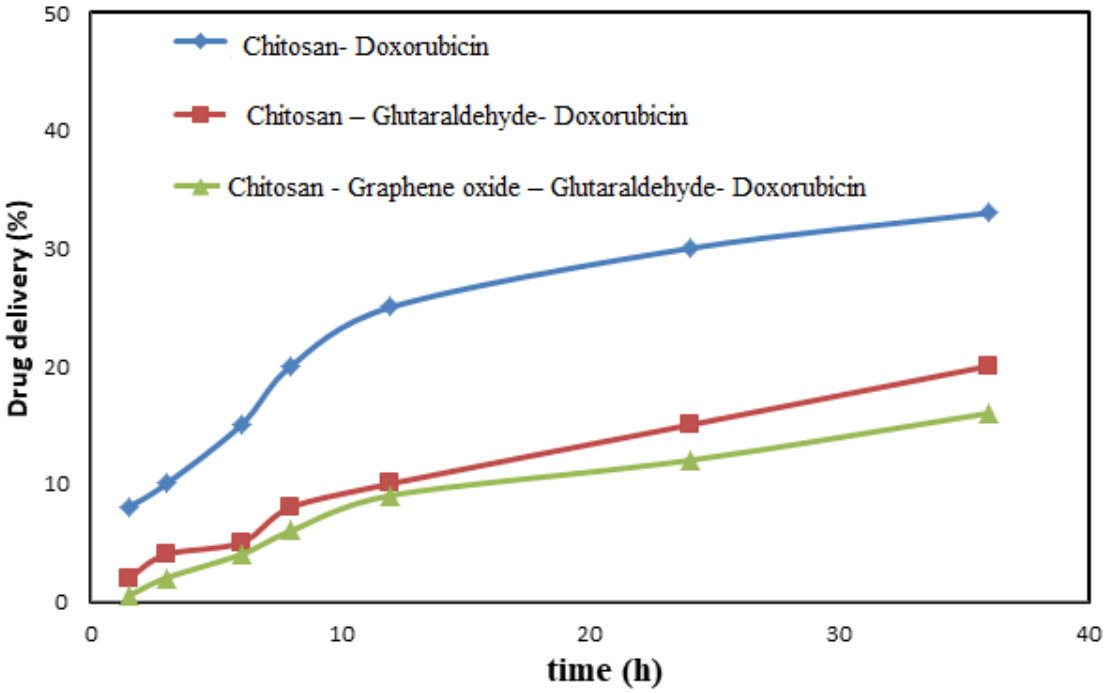

Figure 8. Drug delivery rate in different samples in terms of time.

Conclusion

Drug delivery systems are being developed to improve the pharmaceutical and therapeutic properties of drugs used in patients and they put the drug inside, often in the form of a reservoir.

These systems deliver the drug at a given amount and specific place. As a result, they affect the pharmacokinetics and distribution of the drug in the body. In this research, a new drug delivery system has been used. For this purpose, chitosan polymer was coated with glutaraldehyde and the resulting hydrogel was reinforced as a carrier of the drug using graphene oxide. Doxorubicin anticancer drug was used to evaluate the delivery rate of different carriers.

Adding graphene oxide as a biocompatible and hydrophilic structure increased the drug loading rate, and due to its layered structure, it prevented the drug delivery and, consequently, it causes gradual delivery. The gradual drug

delivery causes the destruction of cancerous cells in the intended tissue and it has the least damage to adjacent tissues and normal cells. In this study, electrospinning of chitosan containing graphene oxide and drug were successfully performed. Based on the results of microscopic images, the chitosan morphology is spherical particles. However, porous hydrogel is obtained by increasing glutaraldehyde. The presence of porosity increases the loading of the drug and then, the absorption of water and hydrogel swelling decreases. Graphene oxide also reduces the amount of swelling and water absorption, which can be attributed to the presence of hydroxyl groups at its surface, which is susceptible to blocking water absorbent positions in the hydrogel.

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