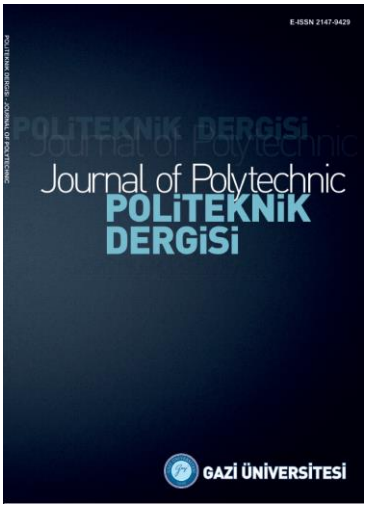

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Preparation of chitosan and glycol chitosan coated magnetic nanoparticles loaded with carboplatin as anticancer drug

Antikanser ilaç olarak karboplatin yüklenmiş kitosan ve glikol kitosan kaplı manyetik nanotaneciklerin hazırlanması

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Preparation of Chitosan and Glycol Chitosan Coated Magnetic Nanoparticles Loaded with Carboplatin as Anticancer Drug

Araştırma Makalesi / Research Article

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ABSTRACT

Surface modified Fe_3O_4 nanoparticles (Fe_3O_4-OA) with an average diameter of 10 nm were synthesized, coated by chitosan (CS) and **glycol chitosan** (GCS), thus magnetic polymer nanocomposites were obtained. The magnetic nanostructures were analyzed by transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), X-Ray diffraction **(**XRD) and vibrating sample magnetometer (VSM). All magnetic structures synthesized in this study exhibited superparamagnetic properties. Loading carboplatin (CPt) as anticancer drug to Fe_3O_4-OA-GCS nanocomposites were carried out with 13.17 % drug content and 38 % encapsulation efficiency. The cytotoxicity studies were occured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on L929 mouse fibroblasts and MCF-7 human breast cancer cells. Fe_3O_4-OA-GCS-CPt nanocomposites showed higher cytotoxicity than free CPt on the MCF-7 cells at 50 µg/ml drug concentrations during 72 h.

Keywords: Fe_3O_4-OA nanoparticles, chitosan / glycol chitosan coating, carboplatin, anticancer drug loading, drug release.

Antikanser İlaç Olarak Karboplatin Yüklenmiş Kitosan ve Glikol Kitosan Kaplı Manyetik Nanotaneciklerin Hazırlanması

ÖZ

Yüzeyi modifiye Fe_3O_4 nanotanecikleri (Fe_3O_4-OA) ortalama 10 nm çapta sentezlenmiş, kitosan (CS) ve glikol kitosan (GCS) ile kaplanmışlardır, böylece manyetik polimer nanokompozitleri elde edilmiştir. Manyetik nanoyapılar geçirimli elektron mikroskobu (TEM), Fourier dönüşümlü infrared spektroskopisi (FTIR), X-ışınları kırınımı (XRD) ve titreşimli örnek magnetometresi (VSM) ile analiz edilmiştir. Bu çalışmada sentezlenmiş tüm manyetik yapılar süperparamanyetik özelik göstermiştir. Fe_3O_4-OA-GCS nanokompozitleri üzerine antikanser ilaç karboplatinin (CPt) yüklenmesi % 13.17 ilaç içeriği ve % 38 enkapsülasyon etkinliği ile gerçekleştirilmiştir. Sitotoksisite çalışmaları MTT (3-(4,5-dimetiltiazol-2-ol)-2,5-difeniltetrazolyum bromit) analiziyle L929 fare fibroblast ve MCF-7 insan meme kanseri hücreleri üzerinde gerçekleştirilmiştir. Fe_3O_4-OA-GCS-CPt nanokompozitleri MCF-7 hücreleri üzerinde 72 saat boyunca 50 µg/ml derişimde serbest CPt'den daha yüksek sitotoksisite göstermiştir.

Anahtar Kelimeler: Fe_3O_4-OA nanotanecikler, kitosan / glikol kitosan kaplama, karboplatin, antikanser ilaç yükleme, ilaç salım.

1. INTRODUCTION

Magnetic nanoparticles with small dimensions and superparamagnetic properties (after eliminating magnetic field there is no permanent magnetization, no coercivity) are important functional structures for hyperthermia, magnetic resonance imaging (MRI), biochemistry, drug and gene delivery [1-4]. They have high surface energy due to high surface to volume ratio and tend to agglomeration to reduce this energy. Fe_3O_4 nanoparticles, which are widely used in bioapplications, are formed composites with different substances such as organic and inorganic materials in order to increase their biocompatibility and stability [5,6]. Chitosan used to coat iron oxides is a natural polymer provided by deacetylation of chitin detected in the skeleton of crustaceans such as shrimp and crab. Deacetylation degree (DD) affects properties of chitosan such as solubility. Chitosan, a cationic polysaccharide can be used in biomedical applications due to its biocompability and nontoxicity [7-10]. Glycol chitosan decorated with ethylene glycol groups is a water soluble derivative of chitosan [11]. As an anticancer drug "carboplatin (CPt)" (cis-diamine (1,1-cyclobutanedicarboxylato)-platinum (II)) is similar to cisplatin with lower side effects. It plays an effective role during treatments of testicular, breast, ovarian, bladder, head, neck and lung cancers [12,13]. In the literature, studies were found about drug loaded chitosan or its derivatives with or without magnetic nanoparticles. Yang et al. (2018) [14] loaded paclitaxel (PTX) to estrone-modified glycol chitosan nanoparticles

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(PTX/GCNP-ES) and realized cell culture studies on MCF-7 cells. They found the highest tumor inhibition ratio as 81.4 % with PTX/GCNP-ES structures. Zamora-Mora et al. (2017) [15] synthesized 5-fluorouracil loaded magnetic chitosan nanoparticles for drug delivery and magnetic hyperthermia (MH) therapy. Encapsulation and loading efficiency of nanoparticles were 80-82 % and 33-35 %, respectively. Folic acid functionalized magnetic (Fe_3O_4) chitosan nanocapsules were prepared by Zhong et al. (2017) [16] for the purpose of targeted delivery and release of coumarin 6. In the size range of 200-350 nm and superparamagnetic nanoparticles were synthesized and used for drug loading studies.

In the present work, synthesis of superparamagnetic Fe_3O_4 nanoparticles and surface modification with oleic acid (OA) were performed to increase coating efficiency and obtain stable nanostructures. Surface modified magnetic nanoparticles were coated with chitosan (CS) and glycol chitosan (GCS) which are natural polymers. Carboplatin used as anticancer drug was loaded to Fe_3O_4-OA-GCS nanocomposites and drug release studies were carried out. To our knowledge, there is no study in the literature loading and release studies of carboplatin to Fe_3O_4-OA-GCS nanocomposites. Cell culture studies were realized to determine cytotoxic properties of Fe_3O_4-OA-GCS-CPt compared to CPt and Fe_3O_4-OA-GCS structures.

2. MATERIALS and METHOD

2.1 Materials

Iron (II) chloride tetrahydrate ($FeCl_2 . 4H_2O$, 99 %, Sigma Aldrich), iron (III) chloride hexahydrate ($FeCl_3 . 6H_2O$, % 97, Sigma Aldrich), sodium hydroxide (NaOH, ≥ 98 %, Sigma Aldrich), hydrochloric acid (HCl, 37 %, Sigma), oleic acid (OA, Alfa Aesar, 90 %), chitosan (Aldrich, medium molecular weight, 75-85 %), glycol chitosan (Sigma Aldrich, > 60 %), tripolyphosphate (TPP, Sigma-Aldrich 90-95 %), tween 80 (Merck), carboplatin (Sigma) and phosphate buffered saline (PBS, Sigma) were used for synthesis of magnetic nanostructures and drug loading studies. The materials of cytotoxicity studies purchased from Sigma-Aldrich were Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), and penicillin–streptomycin and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), respectively.

2.2 Preparation and Surface Modification of Magnetic Nanoparticles (Fe_3O_4 / Fe_3O_4-OA)

Magnetic Fe_3O_4 nanoparticles were synthesized similar to our previous work by co-precipitation of $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ salts [17]. The experiments carried out under nitrogen were realized at constant ratio of iron salts (Fe^{2+}/Fe^{3+}=0.5) and 80 ^oC. In the modification process by OA, oleic acid (30 ml) was added to Fe_3O_4 (0.5 g) nanoparticles and the mixture was stirred at 150 rpm for 90 minutes. The Fe_3O_4-OA nanoparticles were separated by a magnet and washed with acetone (x3) and dried by nitrogen [18].

2.3 Preparation of Chitosan / Glycol Chitosan Coated Magnetic Nanoparticles (Fe_3O_4-OA-CS / Fe_3O_4-OA-GCS)

The polymer coating of the magnetic nanoparticles was carried out by mixing and modifying of the methods in the literature [19-21]. 0.5 % (v/v) Tween 80 was added to the CS (or GCS) solution (2 mg / ml) in 1 % acetic acid and formed solution was mixed. Fe_3O_4 - OA nanostructures were placed in the polymer solution adjusted to pH 4.6 with 10 M NaOH (polymer / Fe_3O_4 - OA w:w 4:1). The TPP solution (crosslinker, 1.5 mg / ml) was dropped (10-15 min) to the magnetic polymer solution, stirring was continued at 300 rpm (15-20 min) as the dropping was completed (polymer / TPP w: w 4: 1). The nanostructures were separated by centrifugation (wash with x 3 DI) and the lyophilized samples were stored at + 4 °C.

2.4 Characterization

The characterization of magnetic nanostructures were realized by transmission electron microscopy (TEM, FEI Tecnai G2), Fourier-transformed infrared spectroscopy (FTIR, 8400 S Shimadzu) with spectra in the range of 4000-500 cm^{-1} and X-Ray diffraction (XRD, Rigaku Ultima-IV) using a scanning rate 2^o/min. The magnetic properties of nanoparticles were determined by vibrating sample magnetometer (VSM, Cryogenic Limited PPMS).

2.5 Anticancer Drug Loading and Release Studies

The drug loaded magnetic polymer nanoparticles were synthesized according to the polymer coating method given in section 2.3. The anticancer drug carboplatin (10 mg) was completely dissolved in the polymer solution. The other stages of the synthesis method were the same. The drug loading and encapsulation efficiency were calculated from following equations given in our previous study [22]. Fe_3O_4-OA-GCS-CPt drug-loaded nanocomposites were weighed after lyophilization and the value was used for drug loading calculations.

$$\text{Drug loading (\%)} = \frac{\text{weight of drug in nanocomposite}}{\text{weight of } Fe_3O_4\text{-OA-GCS-drug nanocomposite}} \text{x100} \quad (1)$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{weight of drug in nanocomposite}}{\text{weight of initial amount of drug}} \text{x100} \quad (2)$$

The drug release studies were realized from Fe_3O_4-OA-GCS magnetic nanoparticles. 10 mg lyophilized drug loaded magnetic nanoparticles were dispersed in PBS solutions (1.5 ml) at different pH values (7.4 and 5.5) containing 1 % (w/v) Tween 80. The suspensions were shaked in an orbital shaker at 150 rpm and 37 ^{o}C. All of the mixtures were centrifuged at specified time intervals and the supernatants were replaced with fresh PBS (1.5

ml). HPLC analyses were used to evaluate drug loading and release efficiency of the Fe_3O_4-OA-GCS magnetic nanoparticles. Analysis using Inertsil ODS-3 column with particle size 5 µm (250 x 4.6 mm) were realized at 233 nm. The flow rate of mobile phase consisted of acetonitrile / water (10:90 v/v) was 1 ml/min. Column temperature and injection volume were adjusted as 25 ºC and 20 µL, respectively [13].

2.6 Cytotoxicity Studies

In vitro cytotoxicity assays with MTT (3-(4,5-dimethylthiazol-2-ol)-2,5-diphenyl tetrazolium bromide) were performed using L929 mouse fibroblasts and MCF-7 human breast cancer cells. The culturing of the cells was carried out in a CO_2 (5 %) incubator at 37 °C in DMEM medium containing 10 % fetal bovine serum (FBS) and 1 % penicillin streptomycin. In the cell culture studies, 4 different groups were used: TCPS (tissue culture polystyrene, control), CPt, Fe_3O_4-OA-GCS and Fe_3O_4-OA-GCS-CPt nanocomposites. TCPS contained only cells and culture medium to determine the effect of the drug and nanoparticles on the cells comparatively. L929 and MCF-7 cells were allowed to reach confluency in 24-well cell culture plates. The culture mediums were replaced with the groups at 50 µg / ml concentration to observe 24, 48 and 72 h cell viability. MTT added cells were incubated for 3 hours and after removing the solution, isopropanol: HCl (0.1 N) solution was placed to dissolve the formed formazan crystals for 1 hour at 37 °C [23]. At the end of this period, the absorbance value of the solutions measured via the ELISA microplate reader (Rayto, RT2100C) at 570 nm.

2.7 Statistical Analysis

Statistical analysis was realized by using the software GraphPad InStat, Version 3.10 according to one-way analysis of variance (ANOVA). P values less than 0.05 indicated statistical significance.

3. RESULTS AND DISCUSSION

3.1 Characterization of The Magnetic Nanoparticles

TEM images of magnetic and magnetic polymeric nanoparticles are given in Figure 1. The average particle size of Fe_3O_4 (Figure 1a) and Fe_3O_4-OA (Figure 1b) nanoparticles were found approximately 8 nm and 10 nm, respectively [24]. As seen from images, surface modification with OA reduced the agglomeration of Fe_3O_4 nanoparticles. It was proven clearly that the modified magnetic nanoparticles were coated with chitosan and glycol chitosan polymers (Figure 1c and 1d).

Figure 2 indicates XRD pattern of Fe_3O_4, Fe_3O_4-OA, Fe_3O_4-OA-CS and Fe_3O_4-OA-GCS nanoparticles. XRD characteristic peaks which belong to (220), (311), (400), (422), (511), (440), (622) crystal planes of synthesized Fe_3O_4 nanoparticles are shown in Figure 2a.

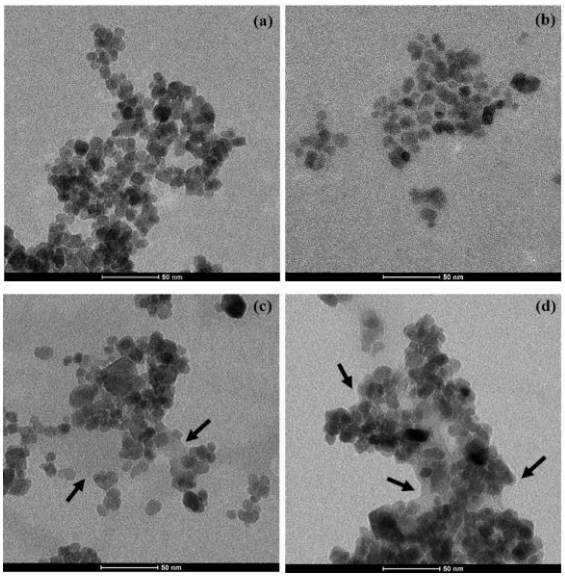

Figure 1. TEM images of (a) Fe_3O_4 (b) Fe_3O_4-OA (c) Fe_3O_4-OA-CS and (d) Fe_3O_4-OA-GCS nanoparticles

The XRD analysis results showed no change in the crystal structure of Fe_3O_4 nanoparticles because of surface modification and polymer coating. The crystal diameters of synthesized nanostructures were calculated by Scherrer equation [25,26]. The results of Fe_3O_4, Fe_3O_4-OA, Fe_3O_4-OA-CS and Fe_3O_4-OA-GCS nanostructures were found as 4.6-19.2, 6.7-10.6, 8.0-19.3 and 6.6-10.6 nm, respectively.

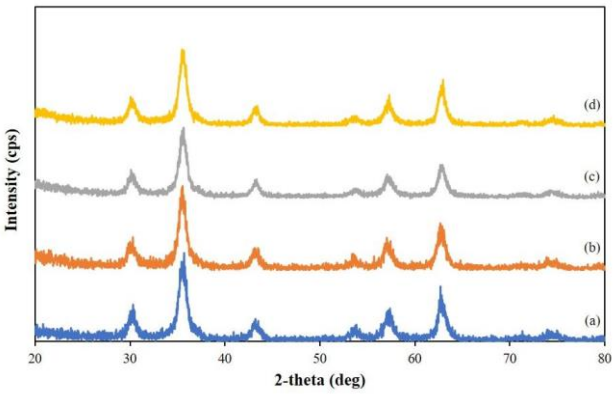

Figure 2. XRD pattern of (a) Fe_3O_4 and (b) Fe_3O_4-OA nanoparticles, (c) Fe_3O_4-OA-CS and (d) Fe_3O_4-OA-GCS nanocomposites

When the FTIR spectrum of Fe_3O_4 nanoparticles was examined, it was found that the characteristic peaks at 578 cm^{-1} and 3441 cm^{-1} which belong to Fe-O and –OH (Figure 3a) [1,6]. The characteristic bands of oleic acid modified Fe_3O_4 nanoparticles were shown at 2924 and 2854 cm^{-1} (CH_3 and CH_2 symmetrical and asymmetric streching), 1635 and 1458 cm^{-1} (COO- symmetric and asymmetric stretching), 1118 cm^{-1} (O-C-O vibration), respectively (Figure 3b) [27]. In the spectrum shown in Figures 3c and 3d, the peaks seen in 3464 cm^{-1} (3441 cm^{-1}, O-H and N-H streching vibration), 1658 cm^{-1} (C=O stretching), 1535 cm^{-1} (1527 cm^{-1}, N-H bending

vibration), 1087 cm^{-1} (C-N vibration) proved that the oleic acid modified magnetic nanostructures were successfully coated with chitosan and glycol chitosan [27,28].

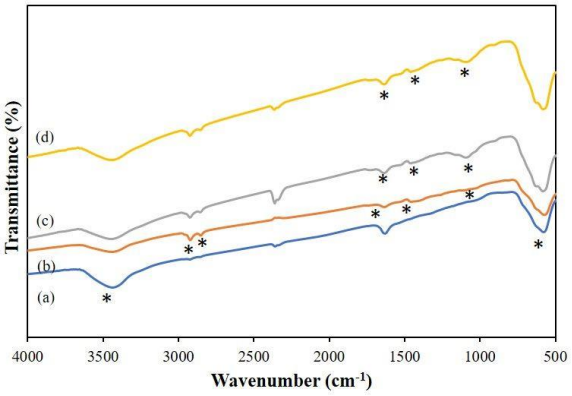

Figure 3. FTIR spectrum of (a) Fe_3O_4 and (b) Fe_3O_4-OA nanoparticles, (c) Fe_3O_4-OA-CS and (d) Fe_3O_4-OA-GCS nanocomposites

VSM results with no coercivity showed that all magnetic structures synthesized in this study had superparamagnetic properties which desired in bioapplications. The saturation magnetization value of the Fe_3O_4 nanoparticles was determined as 54.4 emu/g. Due to surface modification and polymer coating, the saturation magnetizations of Fe_3O_4-OA, Fe_3O_4-OA-CS and Fe_3O_4-OA-GCS decreased to 53, 46 and 48 emu/g, respectively.

3.2 Anticancer Drug Loading and Release Studies

It was concluded that the loading capacity of carboplatin on the Fe_3O_4-OA-GCS nanocomposites was more effective by analyzing the synthesis fluids in drug loading studies. The drug release studies were carried out by using glycol chitosan coated magnetic nanoparticles. The results of TEM, XRD and VSM analyses of Fe_3O_4-OA-GCS-CPt nanocomposites were given in Figure 4. The characteristic crystal structure of nanocomposites was preserved after anticancer drug loading (Figure 4b). The nanocomposites continued to show superparamagnetic properties, but saturation magnetizations (32.6 emu/g) reduced as expected (Figure 4c).

The drug content and encapsulation efficiency of Fe_3O_4-OA-GCS-CPt nanocomposites were found 13.7 % and 38 %, respectively. Similar results were found in the literature [23]. The release of CPt from magnetic structure was examined at pH 7.4 and 5.5 which given in Figure 5.

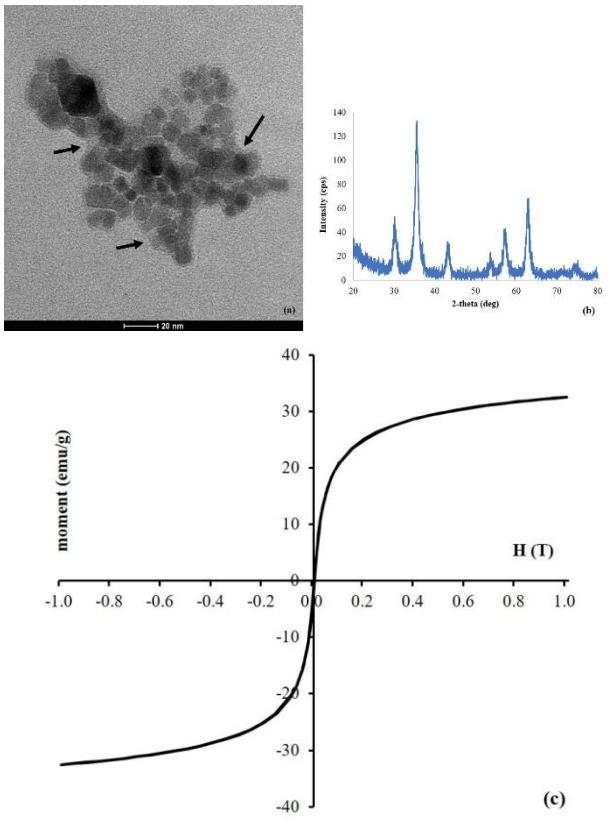

Figure 4. (a) TEM image (b) XRD pattern (c) VSM result of Fe_3O_4-OA-GCS-CPt nanocomposites

The results of the "burst release" in the first 8 hours were about 13 % at pH 7.4 and 5.5. After 48 h, cumulative release values of 15.04 % and 15.63 % were reached at different pH values. A slightly higher release value was reached at pH 5.5, which is important for cancerous areas. Carboplatin loading studies on Fe_3O_4-OA-GCS nanocomposites have not been found in the literature until now.

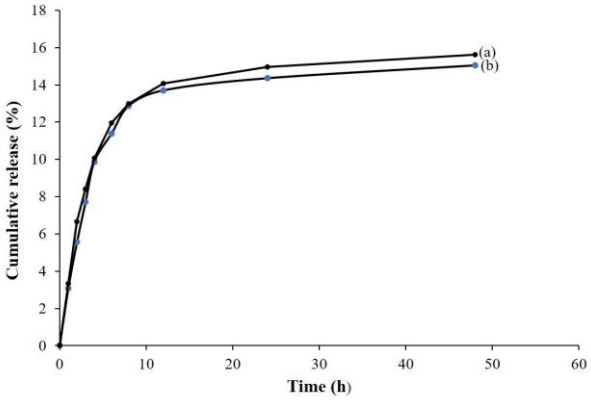

Figure 5. CPt release profiles from Fe_3O_4-OA-GCS nanocomposites (a) pH 5.5, (b) pH 7.4

3.3 Cytotoxicity Studies

The results of the cell culture studies performed with L929 mouse fibroblasts and MCF-7 human breast cancer cells at the end of 24, 48 and 72 h are given in Figure 6. Compared to the TCPS, it was determined that the different groups (CPt, Fe_3O_4-OA-GCS and Fe_3O_4-OA-GCS-CPt) had no remarkable toxic effect on L929 cells (Figure 6a).

Figure 6b indicates that the drug-loaded magnetic nanoparticles (Fe_3O_4-OA-GCS-CPt, $P < 0.05$) showed more toxic effects than free drug on the MCF-7 cancer cells. While the free drug is easily diffused from the cell membrane, the drug loaded nanostructures led to controlled release and high intracellular concentration. Mean values of cytotoxic effects of free CPt and Fe_3O_4-OA-GCS-CPt nanostructures with the same drug amount (50µg / ml) on MCF-7 cells were 6.73 % and 21.18 %. Fe_3O_4-OA-GCS nanostructures had no valid cytotoxic effect on the cancer cells according to the TCPS. When the viability of the cells were examined, it was found that the low concentration (50µg / ml) was not very effective on cell viability [23].

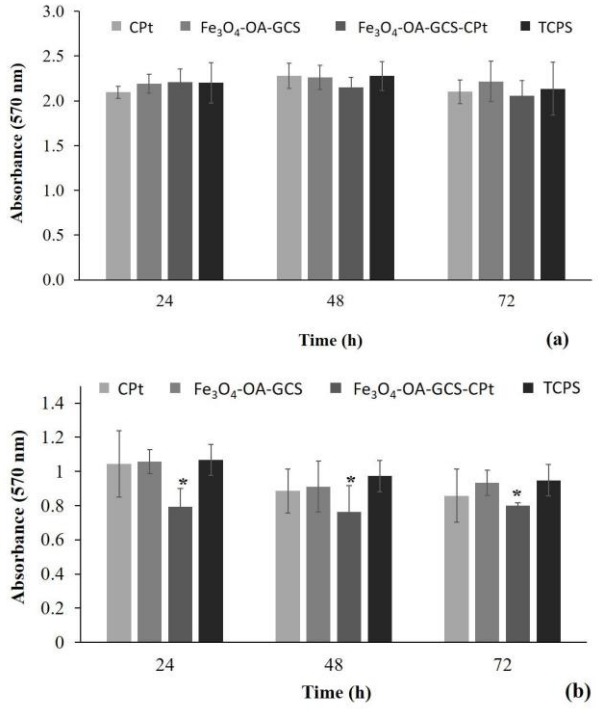

Figure 6. Cytotoxic effect of different groups on (a) L929 (b) MCF-7 cells (* $P < 0.05$)

4. CONCLUSION

Superparamagnetic Fe_3O_4 nanoparticles modified and coated by oleic acid (OA), chitosan (CS) and glycol chitosan (GCS). Fe_3O_4-OA-GCS nanocomposites were selected for carboplatin loading and the loading study was performed successfully with 13.17 % drug content and 38 % encapsulation efficiency. It was determined that the polymer coating and drug loading did not cause any negative properties on the crystal structure and superparamagnetic property. Fe_3O_4-OA-GCS-CPt nanocomposites exhibited higher cytotoxic effect than CPt on the MCF-7 human breast cancer cells, which confirmed controlled release.

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