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Synthesis and Characterization of Cisplatin-Loaded BSA (Bovine Serum Albumin) Nanoparticles as Drug Delivery System against Pancreatic Cancer Cells

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Abstract: Cisplatin as chemotherapeutic agents is consideredone of important drug for treatment of many malignant disorders. Prolonged and Conventional administration methods for cisplatin reduce its effectiveness to the target organ beside side-effects, as nephrotoxicity and ototoxicity. Nanotechnology holds several promises in cancer therapeutics. It can increase the ability of the drug toward the target site of the tumor cells, reach otherwise lesser-accessible sites and reduce the frequency of administration. The present study explored the efficacy of cisplatin coated albumin nanoparticle as a sustained delivery system.To attain this goal, cisplatin was loaded onto albumin nanoparticles (NPs), characterization of the formed albumin nanoparticles was done using surface plasmon spectra using UV-spectroscopy.The morphology of nanoparticles (NPs) were determined by transmission electron microscope (TEM) and Fourier transform infrared spectroscopy (FTIR) was done. The effect of particles on RBCs was assayed.Cellular uptake of cisplatin loaded nano-albumin was qualitatively visualized by Confocal laser scanning microscopy.The anti-cancer efficacy of the cisplatin coated albumin nanoparticles were tested on pancreatic cancer cell lines by Dimethylthiazol Diphenyl Tetrazolium Bromide (MTT) assay. The result showed that nanoparticles have the ability for releasing the drug for more than 50 h,the release kinetics is diphasic.Transmission electron microscopy (TEM) investigated particle sizes in the range 55nm with encapsulation efficiency of 98%.Cisplatin-conjugated albumin nanoparticlesdid not exhibit any toxicity or hemolysis to the RBC"s in hemolysis test assay.A significant decreasingin survival rate of the tumor cells withincreasing concentration of cisplatin loaded albumin nanoparticles. CLSM results showed cisplatin loaded albumin NPs could be absorbed easier and sooner into the nucleus than albuminNPs.Finally, it was found that cisplatin was an appropriate anticancer drug has the ability to be encapsulated in polymeric nanoparticle and released from the carriers with favor rate and no burst effect.

Key words: Cisplatin, albumin nanoparticle, drug release, pancreatic cancer cells.

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I. INTRODUCTION

Almost many years after the cisplatin discovery, it is certainly still one of the most successful available anticancer drugs[1].Cisplatinis used as anticancer drug of many tumors (i.e., ovarian cancer, cell lung cancer, osteosarcomas, germ cell tumors, etc.), with high rate of a cure in testicular cancer 90% [2].Cisplatin, cis-diamminedichloride platinum(II), is apromising metal drug widely used in the systemic treatment of many solid tumors, withthe dose of administration by radiotherapy is 40 mg/m^2.Unfortunately, cisplatin is limited for clinical use due to its severe toxic side effect and poor patient compliance as renal toxicity, asthenia, ototoxicity ,gastrointestinal toxicity and nausea/vomiting [3,4].The exact mechanism by which cisplatin kills cells may still uncertain. In cancer cells, the organic cation transporter "OCT2" and the copper transporter "Ctr1" have been played an important role in cisplatin uptake [5, 6]. Cisplatin bind to DNA bases and forming adducts that eventually lead to cell apoptosis and death [7]. Cisplatin has been shown to covalently bond with the nucleophillic N7 atom of purine bases of DNA to form intra-strand and inter-strand crosslinks, these DNA adducts affect DNA replication[8]. Many signaling proteins bind and recognize the structural distortions in the DNA and initiate different signaling sequences leads to apoptosis of the cells [9]. Cisplatin has been accumulated in both cancerous and normal tissue, and also have non-specific interactions with extra- and

intracellular proteins. The effect of many drug conventional formulations is lasting for short time;therefore, frequent doses of administration are required to maintain their effect on the therapeutic window. Now, thedrug development is directed todelivery vehicles that can decrease these disadvantages and directly target cancer cells also intrinsic and acquired drug resistance .A clinically viable formulation based drug delivery system throughnanoparticle are now applicable by prolonged in the plasma adequate concentration of the drug fort prevent frequent repeated doses of administration. Particles of volume less than 100 nm can escape phagocytosis to other lesser-accessible sites [9]. Nanotechnology now is consider a promising technology with great empowering applications in the drug delivery system through nanoparticles that have great surface to volume ratio enable it to attract a larger amount of particle and molecules as drug. The release of the drug from polymeric nanoparticles are related to the degradation of the matrix so it can gain sustained drug release and achieve higher concentrations due to their small size. It has also been reported that nanoparticles actively absorbed on the surface of malignant cells[10].Many polymeric nanoparticles have been demonstrated and evaluated in many literatures [11–16].

Drug carriers of different materials are frequently used, such as albumin,chitosan [15],poly (lactideco-glycolide) [17], poly-butylcyanoacrylate[18].Albumin is a major soluble protein of the circulating system, involved in transport of nutrients andfreely soluble protein that dissolve in diluted salt and water to the cells[17]. Albumin has several advantages; high solubility at pH 7.4,stable at pH 4 to 9 and could be heated at 60C° for more than 10 hours without denaturation [18]. It is obtained from different sources, including human serum albumin (HSA), egg white (ovalbumin) and bovine serum albumin (BSA) which is recently used for drug delivery because of low cost, abundance with unusual ligand-binding properties and easily purification [19, 20]. Because of many endogenous molecules and drugs are attached with the albumin, it could be considered an effective macromolecular carrier has the ability of accommodating many drug, andnow albumin is widely used in the preparation of many nanocapsules and nanospheres [21].

In this article cisplatin as an effective anticancer drug was loaded into albumin nanoparticles (NPs) and were tested for effectiveness onpancreatic cancer cell lines, besideof drug kinetic, RBCs toxicity, and cell uptake as a trial to overcome its disadvantages.

II. MATERIAL AND METHODS

2.1. Preparation of Albumin Nanoparticles loaded by Cisplatin

Albumin nanoparticles were prepared by the coacervation method [13, 14].Eight ml of the 2% w/v of aqueous solution of bovine serum albumin was added to 16 ml of ethanol with stirring. Glutaraldehyde (1.56 µg/mg protein) was added to obtain a coacervate. Three batches of nanoparticles were prepared using different speeds of centrifugation 45000, 30000 and 20000xg forces.The pellets were incubated 4 h in phosphate buffer saline (PBS; pH 7.4) containing cisplatin, with drug protein ratioswere 1:8 and 1:16. Cisplatin-loaded nanoparticles were separated by centrifugation at (10000 rcf for 45 min and the samples were lyophilized with mannitol (2% w/v) at -48°C. Encapsulation efficiency was calculated as
(Weight of the feeding drug / Weight of the drug in nanoparticle)×100.

2.2. Drug release kinetics

The release of the cisplatin from the albumin nanoparticleswas determined by dialysis at pH 7.4 at room temperature with stirring for about 80 hours. At certain timeintervals,equal volumes of PBS were withdrawn and replaced by the same volume of PBS, the concentration of drug released was determined by calculating the absorbance of the drug at 265 nm by UV visible spectrometer [22].

2.3. Scanning Electron Microscopy.

Cisplatin –albumin nanoparticles were visualized using a Tescan Mira3 SEM operating at an accelerating voltage of 20.0 kV. Samples were prepared by pipetting 1ml droplets of the Cisplatin –albuminNPs solutions onto copper tape and leaving them to dry in the air for at least two hours. The SEM images of the Cisplatin –albuminNPs were used for determining the size distribution and shape of the particles. For each sample analysis, a representative image was selected and the size of 100 particles was measured using ImageJ, which allowed for the calculation of the average size and standard distribution of the Cisplatin –albumin NPs [23].

2.4. Ultraviolet-visible spectroscopy.

UV-visible spectra of albumin nanoparticles were determined using a Varian Cary 50 Bio spectrophotometer running Cary WinUV scan software [13].

2.5. FT-IR spectroscopy

FT-IR spectroscopy was done to albumin nanoparticles,cisplatin and the changes on albumin nanoparticles interaction with the cisplatin . Samples were mixed with dry potassium bromide powder. The spectra were recorded on FT-IR spectrometer in a range of about 400– 4000 cm21[14].

2.6. Hemolysis test

The hemolytic effect of cisplatin-albumin nanoparticleswas evaluated using sheep blood. Fourml of physiological saline was added to 8 ml of heparinized sheep whole blood and centrifuged for 12 minutes at 2400 rpm. The sediment of erythrocyte was washed then suspended with physiological saline to obtain a 2% erythrocyte standard suspension [24].Cisplatin-albumin NPs (5 mg/ml)were dissolved in physiological saline. Several solutionconcentrations of (0.25, 0.5, 1.0, 1.5, 2and 2.5 ml) were prepared and added to six tubes, each containing 2.5 ml of the erythrocyte suspension. All the tubes were incubated at 37°C for 24 hours. Physiological saline with erythrocyte suspension was used as negative control and water with erythrocyte suspension was used as positive control. After blending the agglutination of red cells was observed.

2.7. Assaying of nanoparticles cytotoxic effecton pancreatic cancer cells

Pancreatic cancer cells were used as cancerous cell lines kindly, obtained from King Fahed hospital at KSA.The cells were cultured in RPMI 1640 medium mixed with 10% heat-inactivated fetal bovine serum, streptomycin (100 µg/ml) and penicillin (100 U/ml) and incubated in a humidified atmosphere containing CO_2 (5%) at 37°C.To demonstrate cisplatin –albumin nanoparticles cytotoxicity, different concentrations were prepared and plated over the cancerous cells. The samples were placed into 96 well cultured plates which contained 50,000 cells and a medium supplemented with serum which was subsequently put into the incubator at 37 °C and 5 % CO2 for 1 week. The proliferation rate of pancreatic cancer cells was measured using dimethylthiazol diphenyl tetrazolium bromide (MTT, Sigma) assay. Briefly pancreatic cancer cells were added to microtiterplates of 96-well (10,000 cells/well) and incubated overnight, leaving wells with no sample and used as negative controls. The cells were then treated with various doses of cisplatin –albumin nanoparticles solution in the culture medium for 24 h. At the end of the treatment period, the medium was removed and 100 µm of a 0.5-mg/ml solution of MTT was added. The formed formazan crystals were then dissolved through the addition of 100 µl dimethyl sulfoxide DMSO (Sigma) to each well and incubated at 37 °C for 4 h prior to absorbance measurements. The optical density was recorded on microplate reader at 570 nm.

2.8. Assaying the cellular uptake in vitro

Cellular uptake of cisplatin loaded nano-albumin was qualitatively demonstrated by Confocal laser scanning microscopy (CLSM). The cells treated with albumin and cisplatin loaded albumin NPs was treated with cold PBS and fixed with 4% paraformaldehyde.The CLSM images of pancreatic cancer cells after incubation with albumin nanoparticles alone and cisplatin loaded nano-albumin particle for 5, 10, 20 and 30 min.Finally, by confocal laser scanning microscopy, cells were observed. Images were examined using differential interference contrast with excitation at 488 nm.

III. RESULTS

3.1. Encapsulation efficiency

Encapsulation efficiency was analyzed to evaluate the amount of free drug.Bovine serum albumin combination with the best encapsulation efficiency as shown in Table 1;the drug concentration was from 10 to 25mg, albumin gave the best encapsulation efficiency of 98%.

Table 1:Encapsulation efficiency of different ratios of cisplatin to albumin.

	Drug concentration (mg)	Encapsulation efficiency
1	10	79
2	15	98
3	20	92
4	25	80

3.2. Kinetics release of cisplatin

Total concentration of the drug release of cisplatin at pH 7.4 and predefined time intervals was plotted as seen in “Fig. 1”. It is also noticed from the plot that the release kinetics is diphasic and nanoparticles have the ability for releasing the drug for more than 50 h. The first early phase which is corresponded to the release of bounded drug into the surface of the protein and the another delayed phase which was related to the release of covalently bound drug from the disintegrated nanoparticles.

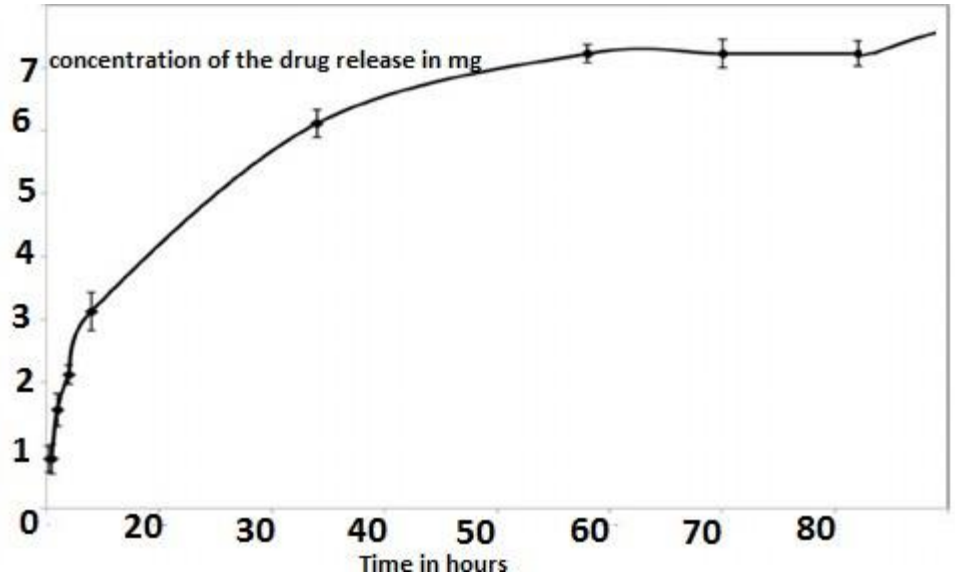


Figure1. Cisplatin release kinetics.

UV-Spectrophotometryof saturated solutions of albumin nanoparticle was monitored by UV-Vis Spectrophotometer at range (300-800) for the metal ions stability. The peak was obtained at 421nm as showed in "Fig. 2".

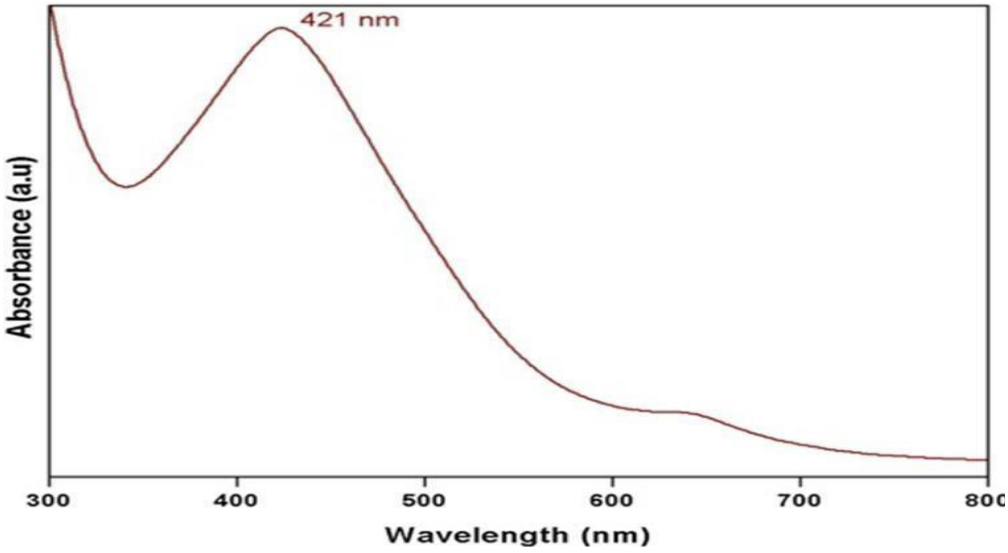


Figure 2.UV-vis absorption spectra of saturated solutions of albumin nanoparticle

3.3. Transmission Electron Microscopy (TEM)

Albumin nanoparticles and cisplatin-albumin nanoparticles was spherical. Most of the particles showed identical size distribution without any precipitation of crystals.The morphology of the Cisplatin –albuminNPs-nanostructures and that of drug delivery was investigated by transmission electron microscopy (TEM) as seen in "Fig. 3".

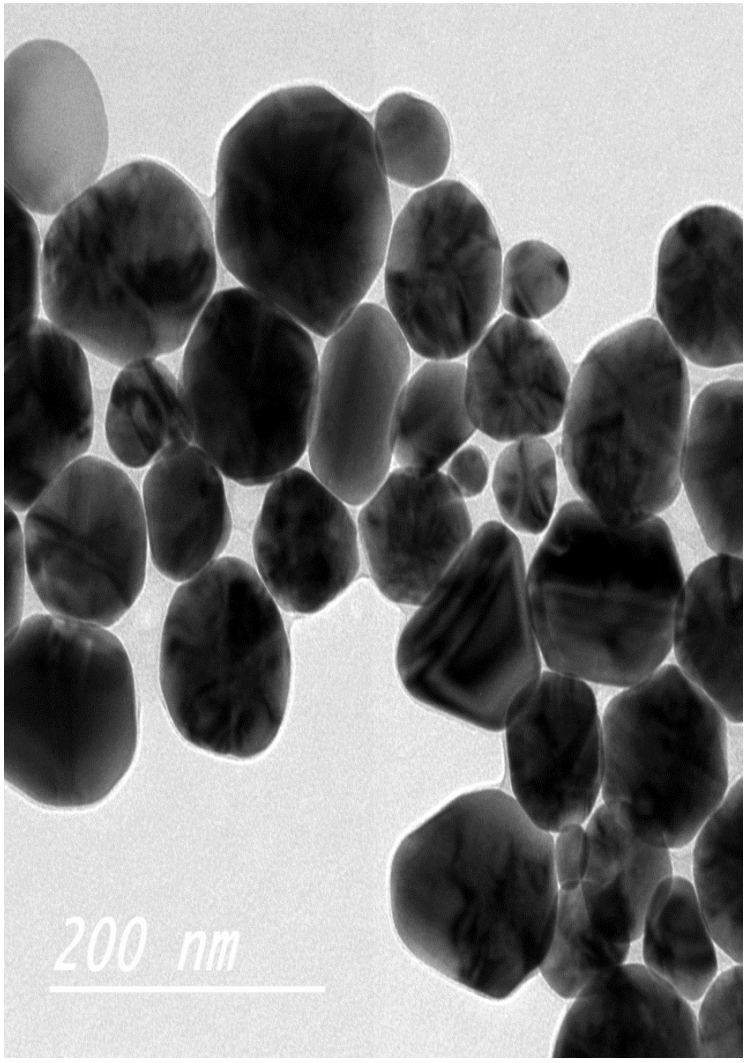


Figure3: Transmission electron microscopy imaging of the nanoparticles. Cisplatin- albumin nanoparticles of size 200 nm and magnification 30X.

3.4. FTIR spectroscopy

FTIR spectra of albumin nanoparticles, cisplatin-albumin nanoparticles and cisplatinis shown in**"**Fig. 4". The characteristic peaks of cisplatin were demonstrated at1649.81 cm^{-1} and 3364.21 cm^{-1} indicating aromatic N-H and C=O stretching respectively, while the characteristic bands of albumin nanoparticles were

three Amides at 1640 cm^{-1}, 1532 cm^{-1} and 1233 cm^{-1} respectively. The drug interaction between cisplatin and albumin nanoparticles interaction spectra were evaluated by the broadening of the peaks of N-H that indicating the possible interaction of the drug with the aromatic residues of albumin(tyrosine ,tryptophan).

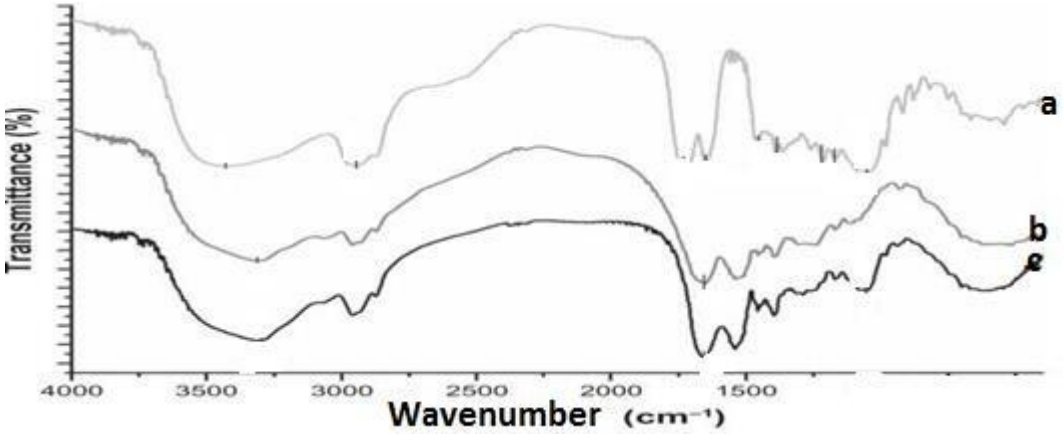


Figure 4: Fouriertransform infrared spectra of samples: (a) drug (cisplatin), (b) bovine serum albumin (c) cisplatin-conjugated albumin nanoparticles. Notes: The peaks correspond to: (1) 3430.36 cm−1, Free OH; (2) 1051.92 cm−1, secondary cyclic alcohols;; (3) 1730.37 cm−1, C=O; (4) 1646.20 cm−1, C=O; (5); 1655.78 cm−1, amides (6) 1387.74 cm−1, CH3; (7) 1213.16 cm−1, acetates; (8) 1173.75 cm−1, formats; (9) 2954.43 cm−1, C-CH3 ;(10) 3306.93 cm−1, free NH; (11) 1455.32 cm−1, CH2.

3.5. RBC's hemolysis assay

`The Hemolyticactivity of cisplatin loaded albumin nanoparticles in RBC's from left to right was shown in ”Positive control(distilled water) showed complete lysis of (RBC‟s) while negative control and other tubes with concentration of cisplatin loaded albumin nanoparticles 10,15 & 20 µg/ml did not show any toxicity or hemolysis to the RBC‟s .

3.6. The cytotoxicity assay

The cytotoxic effect induced by cisplatin loaded albumin nanoparticles on pancreatic cell line was measured using the MTT assay, a common method have been used for evaluating the effects of substances in cell cultures by measuring cell mitochondrial function in the culture plate. The concentrations of the cisplatin loaded albumin nanoparticles used in this study were chosen in such a way that they would be safely and readily achievable .The results show a significant decrease survival rate of the pancreatic cells when concentration of cisplatin loaded albumin nanoparticles increased as seen in „Fig. 5”.

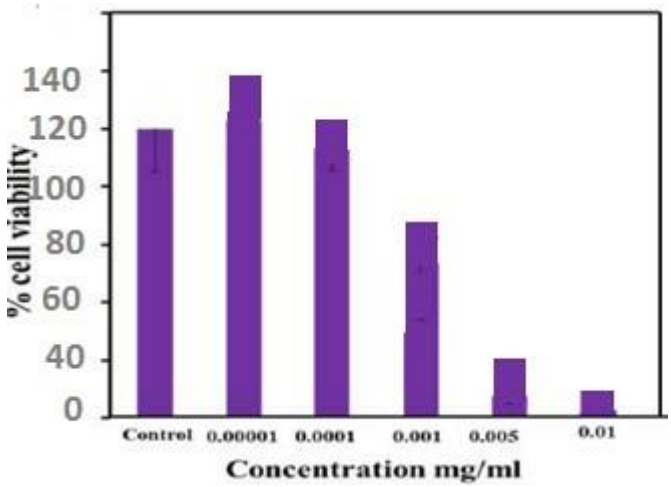
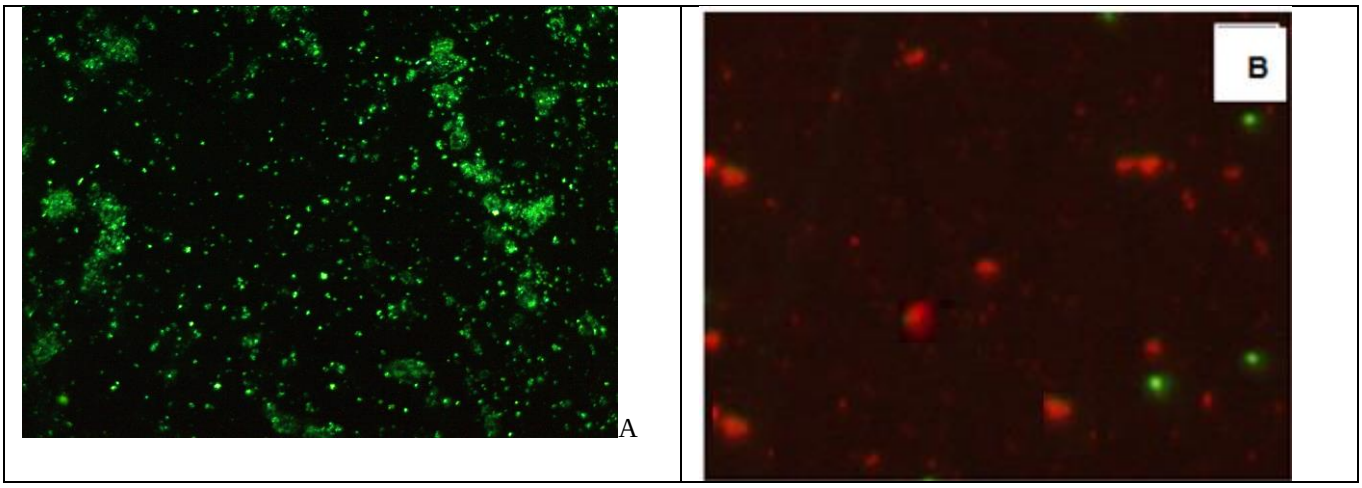


Fig.5: MTT assay of pancreatic cancer cells lines with serial concentration (0.00001, 0.0001, 0.001, 0.005, 0.01 mg/ml) of the cisplatin loaded nano-albumin.

3.7. Estimation ofcellular uptake in vitro.

Cellular uptake of cisplatin loaded nano-albuminwas qualitatively demonstrated by Confocal laser scanning microscopy (CLSM). The CLSM images ofpancreatic cancer cellsafter incubation with albumin nanoparticles alone and cisplatin loaded nano-albumin particle for 30 min were shown in "Fig. 6- A". CLSM image showed the closely appearance cisplatin fluorescence (green) around the cell membrane. However, at 30 min of incubation, cisplatin was successfully internalized into pancreatic cancer cells. "Fig. 6-B"indicated that cisplatin loaded albumin NPs could be absorbed easier and sooner into the nucleus than albuminNPs.



A

Figure 6.Confocal laser scanning microscopy (CLSM) images of pancreatic cancer cells treated with albumin nanoparticles (A) and cisplatin loaded albuminNPs (B) for 30 min.

IV. DISCUSSION

The particle size of albumin nanoparticlescan be controlled during preparation by many different factors. Drugs loadedin albumin nanoparticles were digested by proteases and the entrapping of the drug can be quantified. Protein-based nanoparticle preparation is a viable option for sustained drug deliveryand different polymers have been used for preparing albumin nanoparticles[25-32].Albumin is main component integral plasma protein, as it is biodegradable and biocompatible.Preparation of cisplatin loaded albumin nanoparticles

<70 nm diameter of high entrapment efficiency drug delivery with 50% release at 16 h when compared with the free drug that showed 85% release at first 5 h as drug-release study efficiency of cisplatin loaded albumin – NPswas measured to study the release of cisplatin drug in vitro as seen in curve Fig 1. Drug release from bovine serum albumin nanoparticles have been reported in the literatures, it has been found that bovine serum albumin is stable in the first hours [33]. In our study at the first 10 hours, the release was nearly constant .After 12 hours, the speed rate of cisplatin loaded albumin -NPs increased rapidly and reached to the maximum at 60-80 hours.

MTT assay was used as a classical procedure to investigate the cell proliferation of many cancer lines in many investigations[34].Sowe used it in studying the proliferation of on pancreatic cancer cells. The inhibitory activity of cisplatin loaded nano- albumin. The concentrations of cisplatin loaded albumin -NPs tested were 0.00001, 0.0001, 0.001,0.005 and 0.01 mg/ml.Cell proliferation was inhibited after 48 hours of incubation and increased by increasing concentration. Previously nanoparticles have been demonstrated with the entrapped drug in the cytoplasm by endocytic mechanism [35].

Fourier transform infrared spectra of samples: (a) drug (cisplatin), (b) bovine serum albumin (c) cisplatin-loaded albumin nanoparticles are presented in Fig 5 which shows the peaks 10 and 11 of bovine serum albumin and 1–9 peak for cisplatin . The FTIR spectrum of cisplatin-conjugated albumin nanoparticles (c) showed the characteristic peak of cisplatin (peak 9) at wavenumber 1051.92 cm−1 and the characteristic peak of bovine serum albumin (peak 10) at 3306.93cm^{-1}. As bovine serum albumin has a large number of amide bonds, at 1655.78 cm−1 wave number there is an absorption peak. Peak 11 was more clearly seen in curve c than curve b, as the combination of cisplatin to bovine serum albumin depends mainly on the formation of amide bonds. Thus, the Fourier transform infrared spectra show that cisplatin was successfully combined with bovine serum albumin.

The results of the hemolysis assay can be analyzed. Our results demonstrated that all the tested concentrations didn‟t showany hemolytic activity or thrombus formation making it safe and suitable for circulation. The highest drug concentration was 20 µg/ml wasn‟t showed any agglutination and hemolysis at 37°C. Also there are no any adverse reactions recorded between the surface of the nano-carrier and the serum proteins. Thus, cisplatin loaded nano albumin -NPs is a safe drug carrier can be used as drug delivery targeting tumor cells. The results were consistent with the other results the hemocompatibility action of docetaxel - loaded albumin nanoparticles on blood cells[36] also with who studied chitosan/vitamin E succinate copolymer for paclitaxel selective delivery [24].

Cellular uptake is very important for the drug delivery system as few cellular uptake leads to decrease in the levels of intracellular cisplatin and undesired therapeutic effects. The result in Fig 8 B showed internalization of cisplatin into pancreatic cancer cells. Such drug loaded protein-based nanoparticles forms an important vehicles as anticancer drug delivery as the ultra-small size of the particles enable it for reaching otherwise lesser-accessible sites and the bioavailability of the drug is likely to be more in tumor cells than normal one as a result of higher surface to volume ratio and sustained release , this would translate into benefits in the drug delivery system by decrease frequent doses of administration of the drug and reduced toxicity of cisplatin like nephrotoxicity and ototoxicity.

V. CONCLUSION

Loading of anti- cancer cisplatin on albumin nanoparticles could be more effective against cancer cell line, more cancerous cell uptake with no toxic effect on normal cells as RBC‟s. Also the obtained data demonstrated that coating of cisplatin with mentioned nanoparticles lead to increase the drug performance and also enhance the release period.

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