# Enhanced Ehrlich tumor inhibition using DOX-NP and gold nanoparticles loaded liposomes

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**Abstract**: Treatment with doxorubicin (DOX) is a common treatment for different types of cancer. DOX-NP is one of a well established marketed liposomal formulation for DOX. It has advantages over free DOX in reducing the cardiac toxicity and increasing the efficacy. Gold nanoparticles (GNPs), have been widely used in biomedical applications such as medical imaging and biosensors. Mice bearing Ehrlich tumor were injected with saline, free doxorubicin (DOX) in solution, gold nanoparticles loaded liposomes and commercial liposomal encapsulated doxorubicin (DOX-NP). The results showed that GNPs loaded liposomes could enhance the antitumor activity of commercial liposomal formulation (DOX-NP) and displayed significantly decreased systemic toxicity compared with free DOX and commercial liposomal formulation (DOX-NP) at the equivalent dose. So the injection of GNPs and DOX-NP is expected to increase the cell killing and make it a promising approach to cancer treatment.

**Keywords**: Liposomes, doxorubicin, Ehrlich carcinoma, cytotoxicity, gold nanoparticles.

## INTRODUCTION

Doxorubicin (DOX) is one of the anticancer drugs prescribed alone or with other agents. It is used for the solid tumors and hematological malignancies treatment (Carvalho *et al*., 2009). However, the use of free DOX in biomedical application is still limited since it has severe side effects associated with its use. To overcome these side effects, DOX has been encapsulated in liposomes with polyethylineglycol, preventing the reticuloendothelial system (RES) to recognize the drug (Gabizon, 2001). This formulation will be passive accumulated in the tumor leaky vasculature, known as the enhanced permeability and retention (EPR) effect (Maeda *et al* 2000; 2003; 2009, Barenholz, 2012). DOXIL, a clinically approved liposomal DOX formulation, is currently used for the cancers treatment such as Kaposi's sarcoma and ovarian cancer (Gabizon, 2001, Bennett and Calhoun, 2004).

Gold nanoparticles (GNPs) have scientific and technological significance because of their easy preparation, chemically stable, and unique optical properties. GNP scan bebiomolecules linked and have many applications such as delivery of drug, transfer gene, bioprobes in tissue and cell analysis, and biological processes studies at the nanoscale (Chen *et al*., 2008, Wangoo *et al*., 2008, Byrne *et al*., 2008, You *et al*., 2007). However, the uptake of GNPs by tumor *in vivo* has compromised by the nanoparticles opsonization with plasma proteins and their subsequent clearance by RES components such as macrophages and monocytes. So, injected GNPs are eventually accumulated in the spleen and liver (Kah *et al*., 2009).

Our approach to improve the GNPs circulation lifetime, cellular uptake and the *in vivo* stability is the particles incorporation on or into the liposomal surface (Hainfeld *et al*., 2004; 2006, Zheng *et al*., 2006, Kim *et al*., 2008). Liposomes can also provide possibility of targeted delivery due to easy linkage of ligands to surface of liposomes containing nanoparticles (Mady *et al*., 2012).

In the present work, we prepared and characterized GNPs loaded liposomes. We further investigated those GNPs loaded liposomes could enhance the antitumor activity and reduce the cytotoxicity of commercial liposomal encapsulated DOX (DOX-NP).

## MATERIALS AND METHODS

### *Materials*

The GNPs used in this study were 5nm in diameter coated with polyethyleneglycol PEG (product MKN-Au-05, MK Impex Corp, Canada). The GNPs were in an aqueous solution to give concentration of 0.01%. Liposomal encapsulated doxorubicin (DOX-NP), phosphatidylcholine hydrogenated Soy (Hydro Soy PC) lipid, cholesterol and polyethylineglycol 2000 (PEG-PE) were purchased from Avanti Polar Lipids, Alabaster, Al, USA). Adriblastina consisting of DOX hydrochloride was obtained from Pharmacia Italia, SPA, Italy. Chloroform ($CHCl_3$) was purchased from Sigma Aldrich (St. Louis, MO, USA). All other reagent and solvents were of

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analytical grade and were used without further purification.

***Preparation of GNPs loaded liposomes***

We prepared multilamellar liposomes (MLVs) from 9.5 mg/ml Hydro Soy PC, 3.19mg/ml cholesterol and3.19 mg/ml PEG-PE. The lipids were first dissolved $CHCl_3$, in around-bottom flask to make a homogeneous lipids mixture. A thin lipid film on the flask sides are formed after removing the solvent by rotary evaporator. The lipid film was dried thoroughly by placing the flask on a vacuum pump overnight. An aqueous medium (containing GNPs) was added to the dry lipid to hydrate of the dry lipid film and agitating. The hydrating medium temperature was above the lipid phase transition temperature (50°C). The resulting lipid suspension was extruded through 200-nm polycarbonate membranes (Nucleopore GmbH, Germany), using a commercially available extruder Liposo Fast, Avestin Inc., Canada). The non-encapsulated GNPs were removed by centrifugation at 13,000 rpm. This was repeated twice for 15 min each.

Dynamic laser light scattering (Zetasizer 3000 HS, Malvern Instruments, Germany) was used for size measurement and the size of the GNPs loaded liposomes was in the range of 105±10 nm. Transmission electron microscopy (TEM) (JEOL-JEM-1011, Tokyo, Japan) was used to characterize the GNPs loaded liposome samples.

***Inoculation of mice with tumor cells***

Ehrlich ascites tumors are a rapidly growing experimental tumor model in which various experimental designs for anticancer agents can be applied (Elbialy *et al*., 2010). Ehrlich ascites carcinomas cells ($1\times10^6$ cells) were obtained from the National Cancer Institute, Cairo University. After injection ascites fluid was collected on the seventh day. The Ehrlich cells were washed and resuspended in 5ml saline. Female balb mice, around 22-25g in body weight and 6-8 weeks old (obtained from the animal house of Pharmacy College, King Saud University), were subcutaneously injected in their right flanks where the tumors were developed in a single and solid form. Tumor growth was monitored post-inoculation until the desired volume was about 0.3-0.6$cm^3$.

***Tumor size measurements***

The change of Ehrlich tumor volume (ΔV) was monitored over 9days for four groups. Ellipsoidal tumor volume (V) was calculated using the formula:

$$V = (\pi/6)\,(d)^2\,(D)$$

(Montgomery *et al*., 2000, Ogawara *et al*., 2009, Elbialy *et al*., 2010, Elbialy and Mady, 2015), where d and D are the short and long axes, respectively, measured with a digital caliper (accuracy 0.01mm). Fisher's least significance difference (LSD) multiple-comparison test was conducted to check the significance between group pairs.

***Cell culture***

MCF-12A non-carcinogenic breast cells were used in this study. Cells will be maintained in DMEM/F12, supplemented with 100µg/ml streptomycin, 100U/ml penicillin, 10% fetal bovine serum, and 0.25µg/ml amphotericin B in a humidified 5% $CO_2$ atmosphere at 37°C.

***Apoptosis analysis by annexin V staining***

The Annexin V staining technique was employed in this study (Harata *et al*., 2004). The current study used the Vybrant Apoptosis Assay kit 2 (Molecular Probes, Eugene, WI, USA). Briefly, cells ($5\times10^5$) were cultured in a 60-mm plate to 50-60% confluence and then treated with different liposomal formulations. The DOX and GOLDIL concentration were 10µg/ml. Cells were collected at 24h, centrifuged, and resuspended in 1ml phosphate-buffered saline. Then cells were stained by red-fluorescent propidium iodide (PI) and Alexa Fluor 488 annexin V. Stained cells were analyzed by flow cytometry using the FACS caliber apparatus and the Cell Quest Pro software from Becton Dickinson. Apoptotic cells show a significant degree of surface green fluorescent labeling, dead cells show both green and red fluorescence (because of membrane staining by annexin V and nuclear staining by PI, respectively) and normal cells show little or no fluorescence. Experiments were performed in triplicate. Similarly, non-medicated vesicles were treated to study the cytotoxic effect of liposome components.

***In vivo anti- tumor activity***

Treatment was initiated when tumors reached the desired volume (0.3-0.6$cm^3$). Forty mice were used and randomly divided into four groups. Group A (control), group B (DOX), group C (DOX-NP) and group D (DOX-NP/GOLDIL). Mice of group A were injected with saline solution. The treatment groups (B, C and D) were injected intravenously with a single dose of DOX, DOX-NP and DOX-NP/GOLDIL respectively.

## RESULTS

Size measurements of the Au NPs were taken using dynamic laser light scattering and were around 105±10 nm (fig. 1). The morphologies of GNP-loaded liposomes were investigated by TEM (fig. 1). In the TEM image, GNPs were observed at the liposomal assembly boundary surface.

The cytotoxicity of the formulations used in this study was assessed *in vitro* using the annexin V staining technique. The cytotoxicity of free DOX, DOX-NP and DOX-NP/GOLDIL formulations on an MCF-12A non

cancer breast cell line are shown in Fig. 2. The free DOX resulted in death (apoptosis) for most cells after 24h. The DOX-NP/GOLDIL formulation exhibited a reduced cytotoxicity compared with free drug. Two days after drug application, the apoptosis % values of free DOX, DOX-NP and DOX-NP/GOLDIL formulations were 96.72, 23.07, and 12.49, respectively (fig. 2).

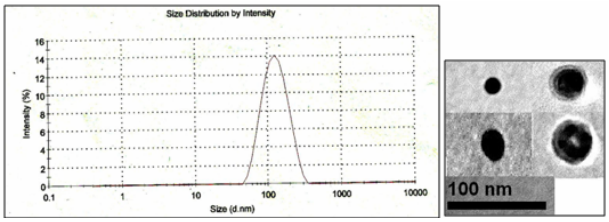


**Fig. 1**: Size distribution diagram and transmission electron micrograph of GNPs loaded liposomes.

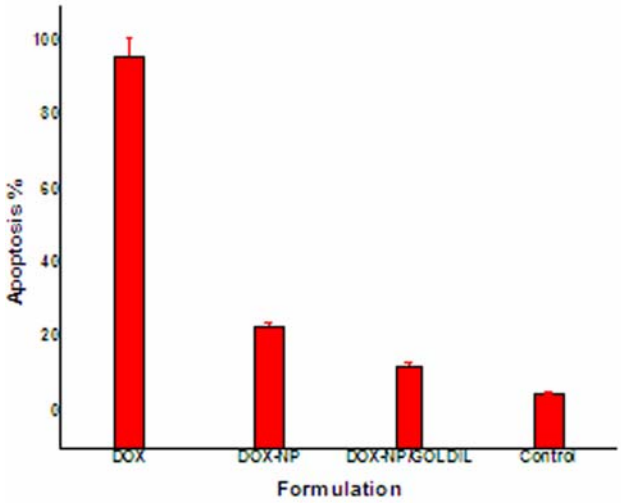


**Fig. 2**: Recorded cytotoxic effects of different formulations. Apoptosis was measured using the annexin V staining assay kit # 2 (Molecular Probes). The final drug concentration was 10µg/ml and the cells collected after 48 hours (n=3).

The efficacy of the drug delivery system used in this study was assessed *in vivo* by following up the change in Ehrlich tumor volume over 9 days for the tested groups (fig. 3). Under our experimental conditions, a marked inhibition in tumor growth was demonstrated in the DOX-NP-treated group (Group C). The control group (A) showed a marked increase in tumor volume (growth) with time (fig. 3).

## DISCUSSION

The GNPs-loaded liposomes were spherical in shape and are less aggregated. The GNPs may be physically associated with the liposomes at the surface without disturbing the membrane packing. It is suggested that GNPs are entrapped in the bilayer hydrophobic part. The darker color of the liposomes is due to the GNPs presence on the surface.

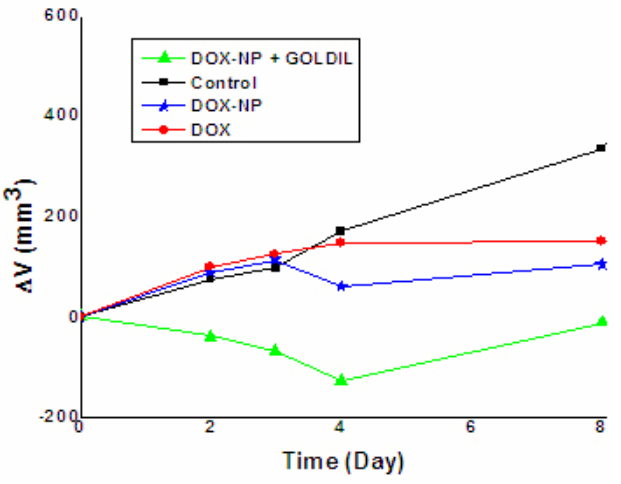


**Fig. 3**: The change of Ehrlich tumor volume as a function of time for the different formulations.

All liposomal formulations demonstrated lower toxicity than free drug, probably because of the delayed release of DOX from liposomes. For DOX-NP-treated cells, the marked decrease in the apoptosis % value may be attributed to the sustained release of DOX from liposomes. For DOX-NP and GOLDIL formulations, there was a marked reduction of the apoptosis % compared with the free drug, i.e., the combination of DOX-NP and GOLDIL had a lower cytotoxicity compared with that of free drug alone.

The nucleus is an important site of cytotoxic action by the anticancer drug, where DOX intercalates DNA, forming DNA adducts and inhibiting topoisomerase II (Gewirtz 1999). Doxorubicin that is released from liposomes into the tumor interstitial space is diffused widely within the tumor. Doxorubicin can diffuse into protein associated or surrounding cell membranes, or diffuses into mitochondria and nuclei.

The marked decrease in Ehrlich tumor volume upon treatment with both DOX-NP and GOLDIL was attributed to antitum or activity of the encapsulated DOX and GNPs and can be explained by the EPR effect of tumor vasculature. Moreover, the liposomal formulation destabilized in the slightly acidic environment of tumor and triggered the release of DOX and GNPs. For the group administered free DOX, a slight inhibition in tumor volume was observed. This result can be attributed to the rapid elimination of free DOX solution from plasma, being excreted into bile and urine. As a consequence, the amount of DOX delivered to tumor tissues was quite low.

The following mechanisms may be behind the anti-tumor effects of DOX loaded liposomes recognized in tumor

bearing mice. The liposomes in the blood circulation gradually extravasated into the interstitial space of the tumor tissue due to EPR effect. Therefore, DOX encapsulated in the liposome must be first released into the interstitial space of the tumor to be taken up by the cells via passive diffusion.

GNPs can also easily permeate tumor vasculature and remain in tumors owing to the EPR effect, as gaps in tumor vasculature and are larger than the gaps in the endothelial lining of normal (healthy) capillaries. GNPs easily pass through these gaps, are able to remain in the tumor tissue because tumors lack lymphatic clearance and have a disordered extra cellular matrix (Kah *et al*., 2009, Lee *et al*., 2014). GNPs loaded liposomes are transported via the endo-lysosomal pathway and internalized by endocytosis.

GNPs may bind to a receptor and cause some cellular/tissue responses (e.g., change in the electrical activity of the cell), blocking the growth and spread of disease by interfering with cancer cells involved in tumor growth and progression (Hashmi *et al*., 2014).

It is clear that the combination of such DOX-NP and GOLDIL formulations facilitate the passive targeting of drug and GNPs to tumors. Such formulations used synergistically are showcased in this study as an efficient oncological modality over the other traditional chemotherapeutic agents.

## CONCLUSION

A liposome-based system was developed for enhanced intracellular delivery of GNPs and DOX. The internal aqueous volume of the liposomes is able to carry chemotherapeutic drugs and nanoparticles as a combination therapy (nanoparticles and chemotherapy).

We investigated those GNPs loaded liposomes could enhance the antitumor activity and reduce the cytotoxicity of commercial liposomal encapsulated DOX (DOX-NP).

Future studies will focus on determining the stability and tumor-targeting efficacy of these DOX-NP and GNPs-incorporated liposomes *in vivo* for developing multiplex systems towards improved diagnosis and therapeutics in cancer treatment.

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