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The Toxicity of Doxorubicin with Nanotransporters

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***Abstract*—**Doxorubicin (DOX) is an anthracycline drug used to treat many cancer diseases. Similarly to other cytostatic drugs, DOX has serious side effects; the biggest obstacle is the cardiotoxicity. With the aim of lowering the negative side effects and to target the DOX into the tumor tissue, the different nanoparticles (NPs) are studied. The aim of this work was to synthetized different NPs and conjugated them with DOX and determine the binding capacity of the NPs. For this experiment, carbon nanotubes (CNTs), graphene oxide (GO), fullerene (FUL) and liposomes (LIP) were used. The highest binding capacity was observed in GO (85%). Subsequently the toxicity of NPs and NPs-DOX conjugates was analyzed in *in vivo* system (chicken embryos). Some NPs (GO) can increase the toxicity of DOX, whereas other NPs (LIP, CNTs) decrease DOX toxicity.

***Keywords*—**Chicken embryos, Doxorubicin, Nanotransporters, Toxicity

I. Introduction

DOXORUBICIN (DOX) is an anthracycline cytostatic drug used to treat wide spectrum of cancer diseases. DOX is a drug isolated from the bacterial culture *Streptomyces peuceticus* and its application is limited by DOX cardiotoxicity [1]. Therefore the different ways for the toxicity decrease are looked for [2]. One of the possibilities is the usage of nanoparticles (NPs) [3]. NPs can increase the water solubility, bioavailability, can prolong the circulation time of the drug and increase the accumulation of drug in the tumour tissue [4]. The release of drug from NPs can be pH dependent, because there is lower pH in the tumour than in normal tissue [5]. NPs can decrease the toxicity of the drug, by the rapid targeting of the drug to the tumour tissue [6]. The encapsulation of DOX in NPs can increase the concentration of DOX in the tumour and simultaneously decrease the cardiotoxicity [7].

Among numerous types of nanomaterials developed in recent years, research on carbon nanostructures such CNTs, GO and FUL represents a booming area, especially in the development of delivery vehicles for cancer drugs and imaging probes [8]. Carbon nanostructures possess sufficient surface-to-volume ratio, rigid structural properties capable of post chemical modification and excellent biocompatibility [9]. CNTs are allotropes of carbon with a cylindrical nanostructure that can significantly increase the toxicity of DOX to the cancer cells [10]. GO is one-atom-thick sheet of carbon [11]. The scaled-up and the production of graphene derivatives offer a wide range of possibilities to synthesize graphene-based functional materials for various applications [12]. FUL can be exploited in theranostics and are important antioxidants [13]. Their anti-tumour effect may be associated with the anti-angiogenesis and immunostimulatory activity [14].

Liposomes are phospholipid membrane vesicles that can act as carriers for targeted drug therapy [15]. Liposomes are the only NPs approved for the use of DOX therapy in human medicine. Significant advantage is the biocompatibility and the similarity with biological membranes [16], the extended circulation half-life [17] and decreased in cardiotoxicity while keeping its antiproliferative effect [18], [19].The aim of this work was the preparation of CNTs, GO, FUL and LIP conjugates with DOX and their binding capacity determination. Next step was the characterization of the NPs-DOX conjugates toxicity and study of their distribution in the body.

II. Materials and Methods

A. Synthesis of LIP-DOX

LIP (LIP-10) was prepared from 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (100 mg) and phosphatidylcholine (100 mg) which were dissolved in chloroform (4 ml). This solution was divided each of 1 or 10 mg into the microtubes. A lipid film was obtained by rotary evaporation of solvent and residual chloroform was blown out by nitrogen. Subsequently the microtube with LIP was filled with 1 mL DOX (Sigma-Aldrich®, 0.5, 1 or 2 mg/mL) and homogenized in ultrasonic bath Sonorex Digital 10P (Bandelin, Berlin, Germany) for 15 min. The homogenized mixtures were then heated and shaken for 15 min at 60 ºC in Thermomixer Comfort (Eppendorf). The samples were then washed several times with pure water (pH = 10) on Amicon 3k (Millipore). The final solution was filled up to the 1 ml by phosphate buffer.

B. Synthesis of FUL-DOX

The FUL (2 or 10 mg; Sigma-Aldrich®) were purified with concentrated HNO_3 (70 % in ACS water, 1 mL) for 15 min in ultrasonic bath and shaken (1400 rpm, 90°C, 15 min, Thermomixer® comfort, Eppendorf, Germany). The solution was centrifuged (25 000 g, 20°C, 15 min; Centrifuge 5417R, Eppendorf) and the acid was removed. The fullerenes were washed with 1 mL of pure water (7-times) until the neutral pH was reached. To the washed fullerene, 1 mL of DOX was added (0.5; 1; 2 mg/mL). The solution was sonicated in the ultrasonic bath for 15 minutes. The solution was vortexed for 24 hours and subsequently centrifuged using Amicon 3k (4500

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g, 20 °C, 15 min; centrifugal filters for sample purification and concentration; EMD Millipore Corporation, Billerica, USA). The solution was subsequently washed with pure water and centrifugation was repeated (6- time). Finally the solution of fullerenes and doxorubicin was filled up to 1 mL by phosphate buffer.

C. Synthesis of CNTs-DOX

Multiwalled carbon nanotubes (Sigma-Aldrich®) were prepared by the same procedure such as FUL.

D. Synthesis of GO

The first step of graphene oxide preparation was according to the standard method of Hummers [20]. The graphite (2 g) was added to 46 mL of concentrated sulfuric acid and mixed by stirring and cooled with ice, followed by an addition of 1 g $NaNO_3$ and 6 g of $KMnO_4$. The mixture was left 24 hours at laboratory temperature, in order to thicken it. All the black graphene oxide was stirred in pure water (500 µL). 2 mg of GO in water were mixed with 500 µL of DOX solution (1, 2, 4 mg/mL). The solution was vortexed for 24 hours and subsequently centrifuged (4500 g, 20°C, 15 min). The solution was thereafter washed with pure water (6-time). Finally the solution was filled up to 1 mL by phosphate buffer.

E. Microscopy of NPs

Microscopic studies were performed using an inverted Olympus IX 71S8F-3 fluorescence microscope (Olympus, Tokyo, Japan). Images were acquired with a Camera Olympus DP73 (Olympus, Tokyo, Japan) and processed by Stream Basic 1.7 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) with the software resolution of 1,600 × 1,200 pixels and magnification 100x.

F. In vivo Experiment

The fertilized eggs of of Lankenfeld roosters and ISA Brown hens (Integra, a.s., Czech Republic) were incubated in the incubator RCom 50 MAX (Gyeongman, Korea) with temperature (37.5°C) and humidity control (55% rH). After the seven days of the incubation the vitality of embryos was checked and then DOX (0.05, 0.1 mg, 0.2 mg per embryo), NPs (250 mg) or DOX+NPs (0.05 mg DOX, 250 mg NPs) was applied by the injection throw small hole into the air cell. After that the hole was covered by the plaster. The vitality of embryos was checked every day by digital egg monitoring system Avitronics (Vetronic services, England). The incubation was finished after 10 days of incubation with DOX or NPs.

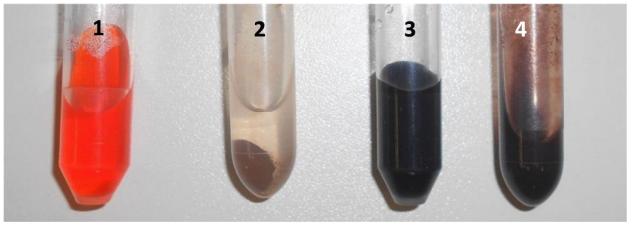
G. Fluorescence Imaging

Fluorescence monitoring was performed using an *in vivo* Xtreme system by Carestream (Rochester, NY, USA). Parameters were set as follows: excitation wavelength - 480 nm, emission wavelength - 600 nm, exposure time - 2 s, binning - 1x1, fStop - 1.1, field of view – 17 x 17 cm. The images were processed and the intensity of the fluorescence analyzed by Carestream Molecular Imaging software (Xtreme Edition, Carestream).

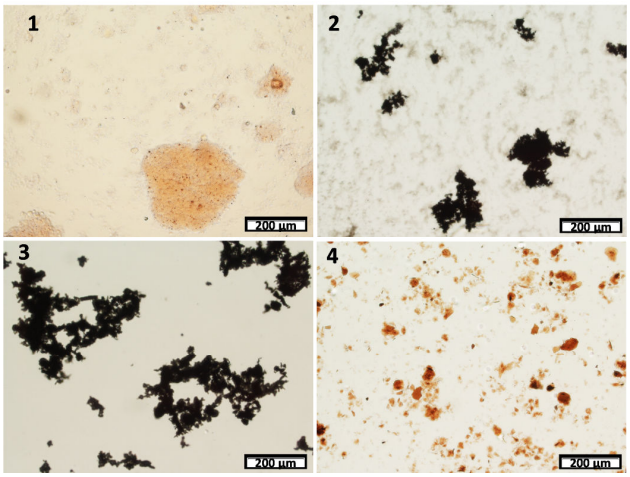
III. Results and Discussion

A. Binding Capacity of NPs

The binding capacity of NPs was determined after the incubation of NPs with DOX and washing out of unbound DOX by Amicon 3k using pure water (Fig. 1 (a)).



(a)



(b)

Fig. 1 NPs with DOX conjugates after washing of unbound DOX: (a) Solution of NPs+DOX conjugates (1 mL) in microtube; 1) LIP; 2) FUL; 3) CNTs; 4) GO. (b) Microscopy images of conjugates; 1) LIP; 2) FUL; 3) CNTs; 4) GO

The microphotographs of prepared NPs are in Fig. 1 (b). The concentration of bound DOX to the NPs can be determined according to the detection of DOX fluorescence intensity. Fluorescence enables sensitive DOX detection, but is limited by DOX concentration quenching of fluorescence [21]. From this reason, the amount of washed out DOX was established by measurement of DOX absorbance ($y = 0.0019x + 0.0812$, $R^2 = 0.9925$). The incubation of DOX and NPs in the ratio 1:1 has showed 85% binding capacity of GO and 10% binding capacity of CNTs. Low binding capacity was observed in fullerene (0 %), FUL incubated with DOX in the ratio of 1:20 showed 40% binding capacity. This low capacity led to the exclusion of this nanotransporter from the next experiments. The LIP with DOX in the 1:1 caused the precipitation of DOX, 10- times higher concentration of LIP enabled 100% binding of DOX. 100% binding enabled incubation of DOX with NPs in the ratio 2:1 (GO) or 10:1 (CNTs).

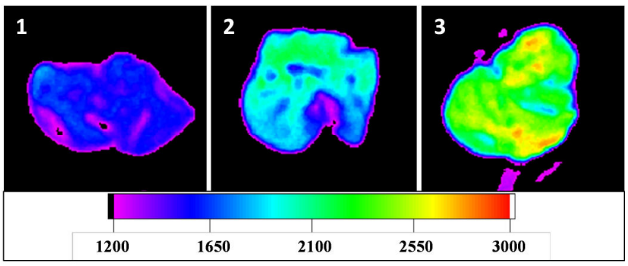
B. Toxicity of the NPs

The toxicity of DOX, NPs and synthetized NPs-DOX conjugates was analyzed in chicken embryos during ten days of their incubation (7^{th} – 17^{th} developmental day). The small hole was made on the bottom end of the egg and through this hole, the analytes were applied by the injection. DOX applied in high concentration (0.1 mg, 0.2 mg per egg) exhibited significant toxicity, after the application of 0.05 mg DOX the mortality was 22% (detected 24 hours after the application) or 66% (detected 10 days after the application).

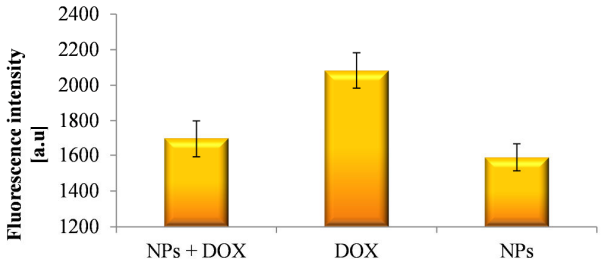
The toxicity of liposomes (200 µg) was not detected; CNTs and GO exhibited similar toxicity to chicken embryos such as DOX (60-70%). It was observed, that analytes soluble in water, such as cadmium ions can successfully penetrate the chorioallantoic membrane [22]. But the stuck of the NPs on the chorioallantoic membrane was observed. The reason could be the formation of clusters, which was confirmed by optical microscopy. We observed that that NPs retained on the membrane and probably small amount was received by the embryo. Therefore for the subsequent experiments, we applied the NPs-DOX conjugates into the egg yolk. Probably higher amount of conjugates could get to the embryo, but we also observed the retention of NPs-DOX clusters in the yolk. LIP and CNTs decreased the toxicity of DOX to chicken embryos (50% mortality) whereas GO-DOX exhibited higher toxicity, than free DOX. We presume that the GO improved the transport of DOX to the embryo due to the presence of smaller particles and therefore increased dying was observed (75%).

C. Detection of NPs in the Organism

Doxorubicin is a drug with very good fluorescence properties [23]. Fluorescence of DOX enable its detection in different nanotransporters [24]-[26]. Doxorubicin fluorescence can be also detected in the tissue [27]. The obstacle in the usage of fluorescence in the medicine is limited by light scattering and quenching in the tissue. There is need for the highly fluorescence probes and sensitive fluorescence detectors [28]. In this work, the fluorescence of DOX was analyzed by fluorescence detection system with the aim to analyze the distribution of DOX in the embryo body. The fluorescence of DOX was possible to detect very well, when 0.2 mg or 0.1 mg of DOX per embryo was applied. These concentrations were very toxic and the most of the embryos did not survive more than a few days. From this reason the applied amount of DOX was decreased to the 0.05 mg DOX per embryo. But this DOX amount was difficult to detect by fluorescence detector. After the scarification of embryos the fluorescence of DOX in the body was analyzed by fluorescence imaging camera, but DOX was not detected. Therefore the abdominal cavity was opened by the scalpel and the individual organs were taken out and fluorescence of DOX detected. The fluorescence of DOX was possible to detect especially in the liver tissue. High accumulation of DOX was observed in the liver. The fluorescence intensity of DOX was 2080 a.u. The autofluorescence of the tissue was 1590 a.u. Low fluorescence intensity of DOX was detected in the liver of embryos exposed to the NPs+DOX (1700 a.u.). The conjugation of DOX with NPs decreased its transport in the body a therefore lower amount of DOX was detected in the liver.



(a)



(b)

Fig. 2 (a) Detection of the fluorescence of DOX in the liver tissue by fluorescence imaging camera. 1) Liver of the embryo incubated with LIP (control); 2) chicken embryo incubated with DOX; 3) chicken embryo incubated with LIP-DOX conjugate; (b) Quantification of the fluorescence intensity (average values from all measurements)

IV. Conclusion

In this work, the NPs-DOX conjugates were prepared and their toxicity analyzed. The highest binding capacity was detected in GO, contrary the FUL are not suitable for DOX transport because of low binding capacity. NPs can significantly influence the toxicity of DOX and decrease the accumulation of DOX in the liver tissue.

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