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RESEARCH PAPER

Converting the Hematotoxicity of Doxorubicin to Antioxidant on Blood by Coating with PEGylated Chitosan Nanoparticles

Tuqa Sami Al-Salmani *, *Hanaa Naji Abdullah, Mayada Noori Iqbal*

Department of Medical Laboratory Science Technology, College of Health and Medical Technology, Middle Technical University, Baghdad, Iraq

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ABSTRACT

Doxorubicin (DOXO) is a potential antineoplastic agent used for treating several tumors. It has severe side effects on the hematological profile. Chitosan is an effective nano-carrier for delivering therapies. This study aims to convert the oxidative effect of DOXO to an antioxidant effect by coating it with chitosan nanoparticles (CsN). Three steps prepared this; (1) the CsN preparation, (2) the CsN coated with polyethylene glycol (CsN-PEG), and (3) the DOXO coating with CsN-PEG to produce DOXO-CsN-PEG polymers. The prepared polymers were characterized using AFM, FE-SEM, and TEM analysis techniques. The cytotoxicity was evaluated on human blood cells. The redox reaction was studied using cyclic voltammetry (CV). The hemolysis percentage of blood treated with DOXO-CsN-PEG was less than 5% for all studied concentrations and no changing in red blood cells (RBCs) forms. The electrochemical study showed that the oxidation peak of blood was elevated when treated with DOXO, and decreased after treated with DOXO-CsN-PEG. The study concluded that the DOXO was converted from an oxidative agent to an antioxidant of blood when coated with CsN-PEG polymers.

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INTRODUCTION

Nanotechnology is a global branch of science that deals with the designing and forming of tiny structures known as, nanoparticles (range from 5-100 nm in size) [1]. Nanotechnology employs various techniques to measure the size and shape of nanoparticles, which are crucial for several applications [2]. Previously, it was primarily used in chemical engineering or material physics. Recently, it has been utilized in various industries, including medicine, pharmacy, healthcare, food, dental industry, and chemical industries [3-5].

Doxorubicin (DOXO) is a potential antineoplastic agent which widely used for the management of several solid tumors such as; breast, lung, uterus, ovary, prostate, stomach, liver, and bile duct tumors and hematological malignancies [6]. The antitumor mechanism depends on its ability to inhibit DNA replication and protein synthesis within tumor cells by covalently binding into the DNA strands and many proteins important in DNA replication and transcription [7]. Doxorubicin has severe side effects on the hematological profile of blood by disrupting the red blood cell production, causing blood clotting disorders, leukopenia, and anemia [8]. Several attempts have been made to reduce the harmful adverse effects of DOXO by reducing the free radicals generation and oxidative

* *Corresponding Author Email: tuqa.sami86@mtu.edu.iq*

damage in normal cells [9,10].

Chitosan is a natural polysaccharide that is produced after the de-N-deacetylation of chitin in the shells of seafood (e.g. shrimp, crab, and crayfish) and insect's chitin [11,12]. Chitosan has ideal characteristics such as biocompatibility, biodegradability, mucoadhesivity, nontoxicity, and hemocompatibility that render it an effective nano-carrier for the delivery of the various drugs [13]. Chitosan structure consists of powerful H-bonds in the hydroxyl (-OH) and amine (-NH2) groups. These bonds facilitate its reaction with hydroxyl radicals [14].

The cyclic voltammetry (CV) is an electrochemical technique employed to determine the redox action [15]. Newly, the CV technique was used to study the electrochemical properties of several pharmaceuticals [16,17].

This study concentrated on the hemotoxicity of DOXO chemotherapy and measured the oxidative effect of DOXO on the blood medium using the CV technique. At the same time, this oxidative effect converted to an antioxidant effect on blood by coating DOXO with chitosan nanoparticles (CsN).

MATERIALS AND METHODS

Doxorubicin Coating with Chitosan Nanoparticles (DOXO-CsN-PEG)

The DOXO-CsN-PEG polymer preparation was achieved depending on three main steps including; (1) the preparation of CsN, (2) the preparation of CsN-PEG, and (3) the preparation of DOXO-CsN-PEG polymers. All these polymers were prepared according to the optimum preparation conditions, as appeared in Table 1. Firstly, the CsNs were prepared according to the ionic-gelation process with few modifications. After mixing 0.4% TPP (Avonchem/UK) solution with 1% acetic acid (Alphachemika/India) Cs (200kD, Avonchem/UK) solution, a white cloud quickly developed as an indicator of the CsN-TPP crosslinking [18]. Secondly, the prepared 5%PEG (Avonchem/UK) aqueous solution was mixed with an equal volume of 1% CsN solution. The linking was shown as clouds developed after dropping the TPP solution. Finally, the DOXO-CsN-PEG polymers were prepared by mixing 5µg/ml of DOX (Pfier/USA) with the 1% CsN-PEG solution (away from light) to avoid light degradation of DOXO molecules.

Characterization of the Prepared Polymers

The prepared polymers (CsN, CsN-PEG, and DOXO-CsN-PEG polymers) were characterized using many techniques. The morphology of the prepared polymers was documented using FE-SEM and TEM analysis techniques. The nano-sizes of the prepared polymers were measured by AFM analysis.

Cytotoxicity on Human Blood Cells

Hemolysis Test

A hemolysis test was carried out for CsN-PEG, DOX, and DOXO-CsN-PEG depending on the previously documented method, with mild alterations [19]. According to the National Institute of Health and Food and Drug Administration and the statement of ethical principles of the declaration and regulation of Helsinki of 1975, a 10ml fresh blood sample was collected by venipuncture from a healthy donor and emptied in the EDTA tubes with ratio1.5mg/1ml. 100µL of whole blood and 700µL of PBS (pH 7.4) was added to empty tubes, and then 100µL of each prepared compound was added with six serial concentrations (from 31.25 to 1000µg/ml). The PBS was utilized as a -ve control (0% hemolysis), and deionized DW was utilized as a +ve control (100% hemolysis). The tubes were incubated for 1 hour at 37°C, and centrifuged for 10 minutes at 2000 rpm. Finally, the supernatants were examined by UV-Vis spectrophotometer to measure the absorbance at wavelength 540nm to measure the liberated hemoglobin value. The samples with lower and higher concentrations were examined microscopically to show the hemolysis effect. The hemolysis activity was measured using the Eq. 1 [20]:

Electrochemical Analysis by Cyclic Voltammetry

The electrochemical behavior of CsN, CsN-PEG, and DOXO-CsN-PEG polymers was studied using the CV device (Pioneering electrochemical technologies/USA) to determine the redox reaction for each prepared compound. This apparatus was applied with a specific quartz cell

$$\text{Hemolysis (\%)} = \frac{\text{Absorbance (test)} - \text{Absorbance (}-\text{ve control)}}{\text{Absorbance (}+\text{ve control)} - \text{Absorbance (}-\text{ve control)}} \times 100 \quad (1)$$

consisting of 3 electrodes; (1) a working electrode: a glassy carbon electrode (GCE), (2) a reference electrode: a silver electrode and (3) an auxiliary electrode: a platinum wire, Fig. 1 [21].

Fresh blood (10ml) was collected from a healthy person and added to EDTA tubes. The voltammetric cell was filled with diluted blood (1:10 ratio of deionized water) and flooded the electrodes in diluted blood. These electrodes detected the electrochemical reactions by the cyclic voltammogram and viewed them on the connected personalized computer [22].

RESULTS AND DISCUSSION

This study aimed to concentrate on decreasing the hemotoxic effect of doxorubicin chemotherapy. An experimental effort has been taken to achieve this task; depending on the nanotechnology.

Characterization of Chitosan Nanoparticles (CsN)

The synthesized CsN was prepared according to the ionic gelation process. Fig. 2 shows the AFM Z-Axis image that revealed the (3D) surface of the prepared CsN which appeared regular in shape. The scanning was applied on an area (8.98 × 8.98μm) to measure the nano-size distribution of CsN and to reveal the surface arrangement.

According to the AFM results, the size and frequency of the prepared CsN was estimated as appeared in the histogram and Abbott curve, Fig. 3. Most of the prepared CsN was less than 57 nm in size with an average of approximately 33 nm. This average is smaller than that reported by previous studies [23, 24].

The morphology of the prepared CsN was examined by FE-SEM and TEM analysis. The CsN were showed nano-sized spherical uniform particles and regular in shape, as shown in Fig. 4. The CsN was utilized as a nano-carrier for doxorubicin due to several properties including; nontoxicity, biodegradability, biocompatibility, mucoadhesivity, and hemocompatibility [25]. Additionally, the solubility of CsN at neutral or alkaline pH values (such as for the blood) is negligible, which makes it a perfect choice for delivering DOXO without releasing it in the blood

Table 1. The preparation conditions of CsN, CsN-PEG and DOXO-CsN-PEG polymers.

Preparation parameters	Preparation Conditions		
	Preparation of CsN	Preparation of CsN-PEG	Preparation of DOXO-CsN-PEG
Concentration	1% Cs dissolved in 1% acetic acid solution	1% CsN dissolved in DW	1% CsN-PEG dissolved in DW
pH	5	-	6.5
1st stirring	1000 RPM for 48 h	250 RPM for 30 h	250 RPM for 30m
Temperature	Room Temperature	Room Temperature	Room Temperature
PEG	-	5% (350RPM, 30m)	-
DOXO	-	-	5μg/ml (250RPM for 60m)
TPP	0.4%	0.4%	0.4%
2nd stirring	350 RPM for 30m	250 RPM for 30m	1000 RPM for 48 h
1st Centrifugation	10,000RPM for 30m	10,000RPM for 30m	10,000RPM for 30m
Washing with DW	Once	Once	Twice
2nd Centrifugation	5,000RPM for 10m	5,000RPM for 10m	5,000RPM for 10m
Sediment Lyophilization	Lyophilized	Lyophilized	Lyophilized

[26].

Characterization of CsN coated with Polyethylene Glycol (CsN-PEG)

The PEG is a hydrophilic and nontoxic polymer. It was used to enhance the hydrophilicity of CsN and increase the stability of an encapsulated DOXO drug [27]. Furthermore, PEG protects CsN from phagocytosis by blocking the positive charges on their surface [28].The AFM result of the CsN-PEG polymers revealed a reduction in the size of nanoparticles to about 21nm Fig. 5. This is

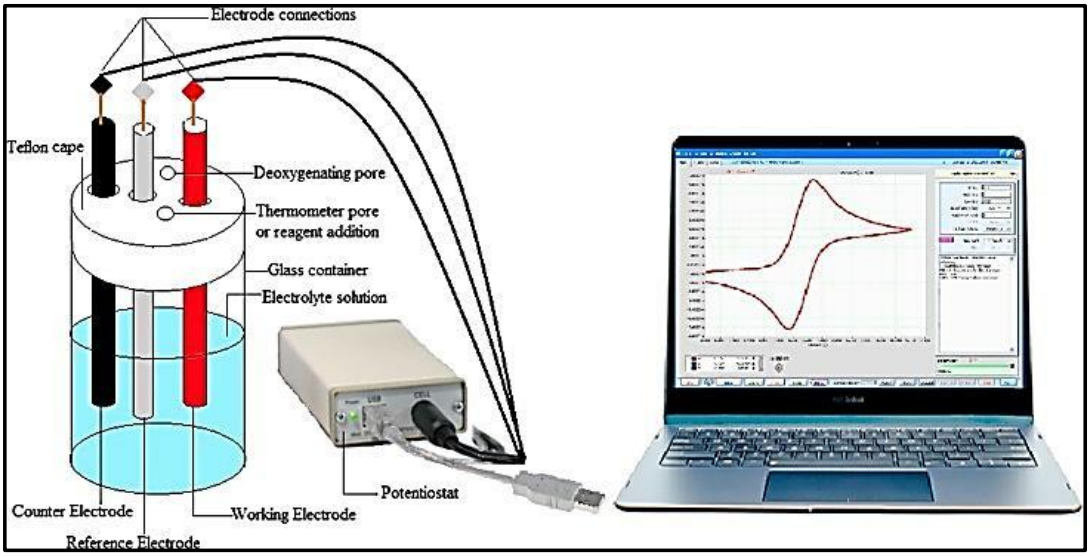

Fig. 1. Setup for the Cyclic Voltammetry [18].

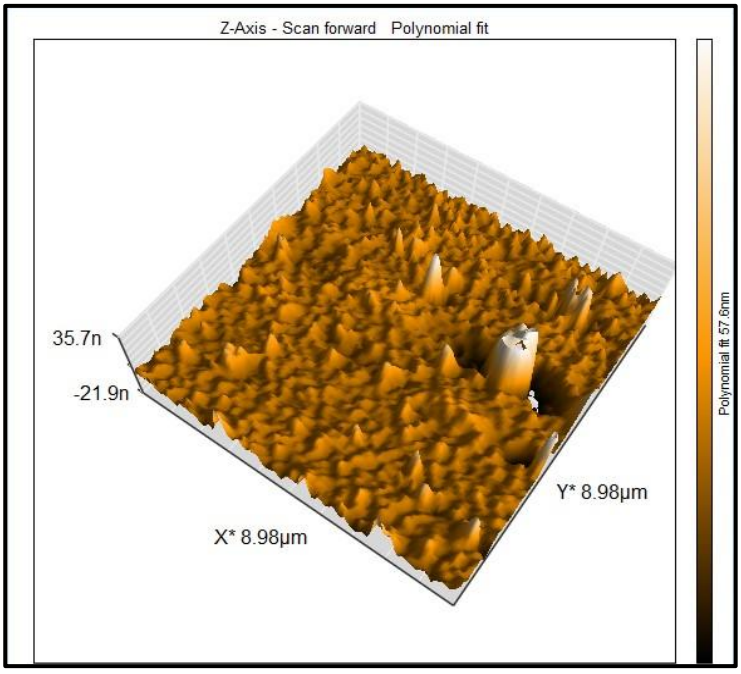

Fig. 2. AFM results (Z-Axis) of CsN.

considered an indicator of the successful cross-linking between chitosan and PEG due to the ability of PEG to form an interpenetrating network that applies pressure on the surfaces of CsN [29].

This cross-linking was confirmed by the FE-SEM and TEM results, Fig. 6. The FE-SEM of CsN-PEG polymers showed spherical uniform particles and regular in shape. In TEM analysis, the morphology of CsN-PEG appeared as spherical nanoparticles with light a brush-like shape around the nanoparticles which referred to crosslinking of CsN with PEG.

Characterization of Doxorubicin coated with CsN-PEG (DOXO-CsN-PEG)

DOXO is the most effective chemotherapy against several types of cancers, despite it has various side effects on the general human body due to the inability to distinguish between cancer cells and normal cells. These side effects include the blood profile such as myelosuppression, anemia, neutropenia, and thrombocytopenia [30]. The coating of DOXO with CsN-PEG can minimize the effects on normal cells and make it targeted to cancerous cells [31]. The encapsulation of DOXO

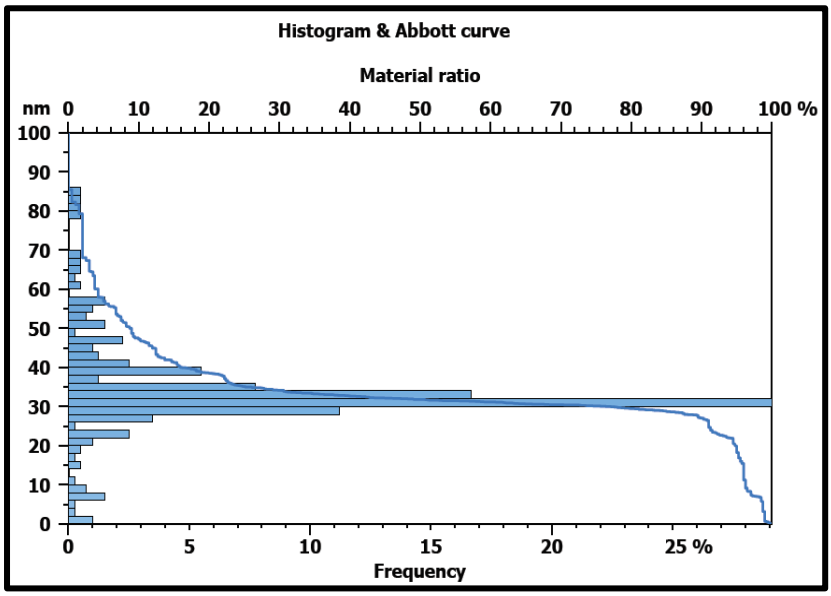

Fig. 3. Histogram and Abbot Curve of CsNs by AFM analysis.

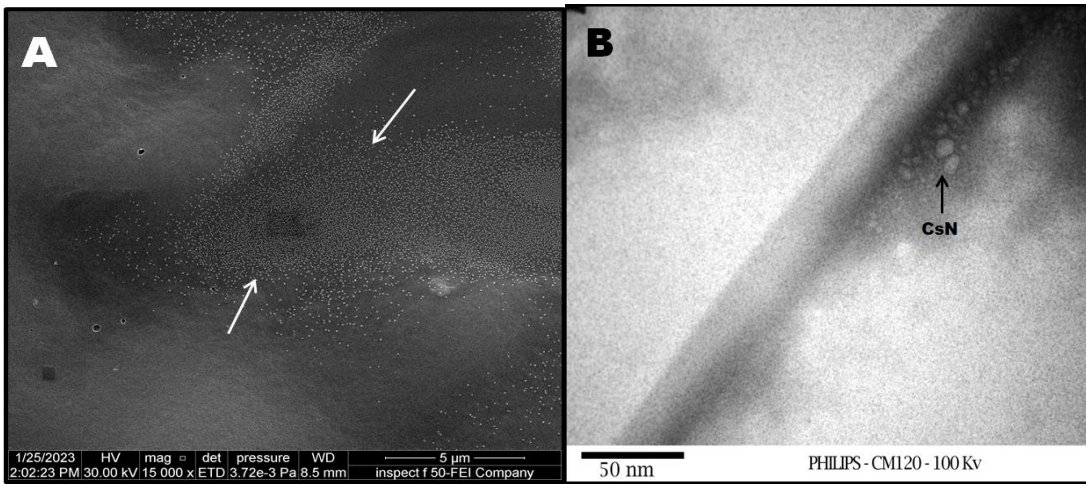

Fig. 4. (A) FE-SEM result, and (B) TEM result of CsN.

with CsN-PEG was done at pH 6.5. This supports the entrapment of DOXO core inside the CsN-PEG polymers through intracellular hydrogen interactions which are promoted by two factors; the deprotonating of chitosan at 6.6 and the hydrophilic nature of DOXO [32]. This encapsulating of DOXO with CsN-PEG was demonstrated with increasing the size of the prepared particle to about 103nm, Fig. 7.

Furthermore, the morphology of the DOXO-CsN-PEG polymers was examined by FE-SEM analysis that revealed large and irregular dark core shape of DOXO surrounded with an irregular light shadow due to coating with CsN-PEG polymers, as appeared in Fig. 8.

Cytotoxicity on Human Blood Cells

Since PEG is rapidly hydrolyzed at a pH ranging from 5 to 6 which is identical to pH in the tumor microenvironment, this facilitates the DOXO

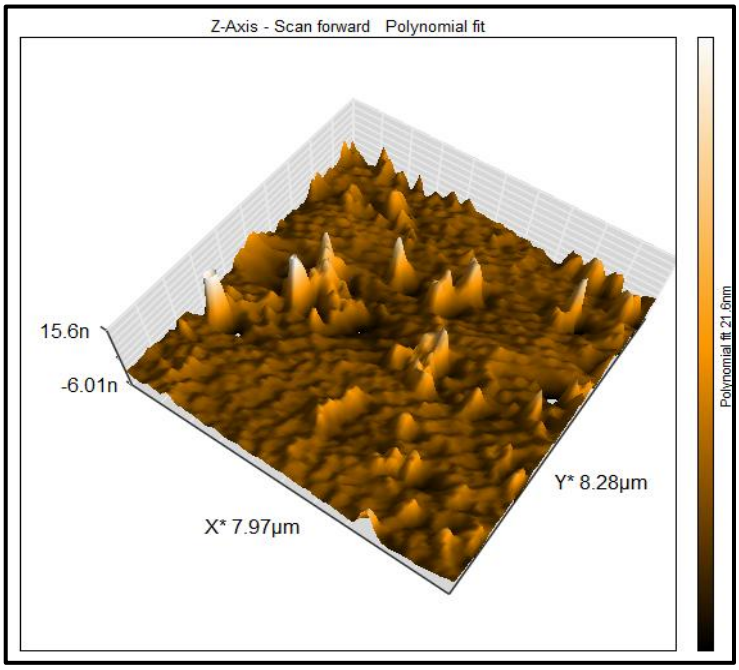

Fig. 5. AFM results (Z-Axis) of CsN-PEG.

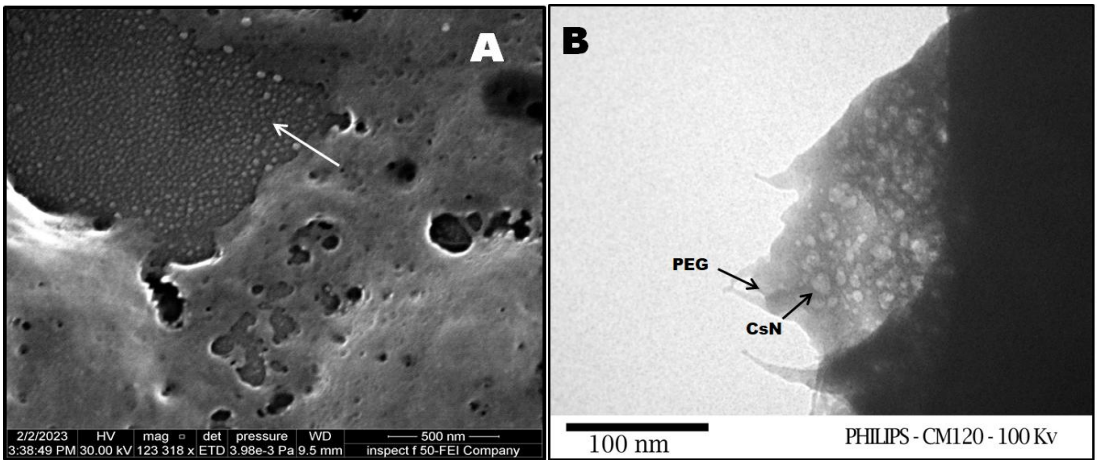

Fig. 6. (A) FE-SEM result, and (B) TEM result of CsN-PEG.

release in cancerous tissues. On the other hand, it prevents releasing the entrapped DOXO in blood and normal tissues (pH 7.4) [33,34]. To confirm this theory, the cytotoxicity on human blood cells was studied for DOXO before and after coating. This was done by determining the hemolysis activity and the redox reactions of the prepared polymers on the blood.

Hemolysis Test

The hemolysis test is usually utilized to investigate the nanoparticles' effects on RBC integrity and determine the optimal use for administration. This assessment was measured the concentration of released hemoglobin from the damaged RBCs. The oxygenated hemoglobin can be measured by a spectrophotometer [35].

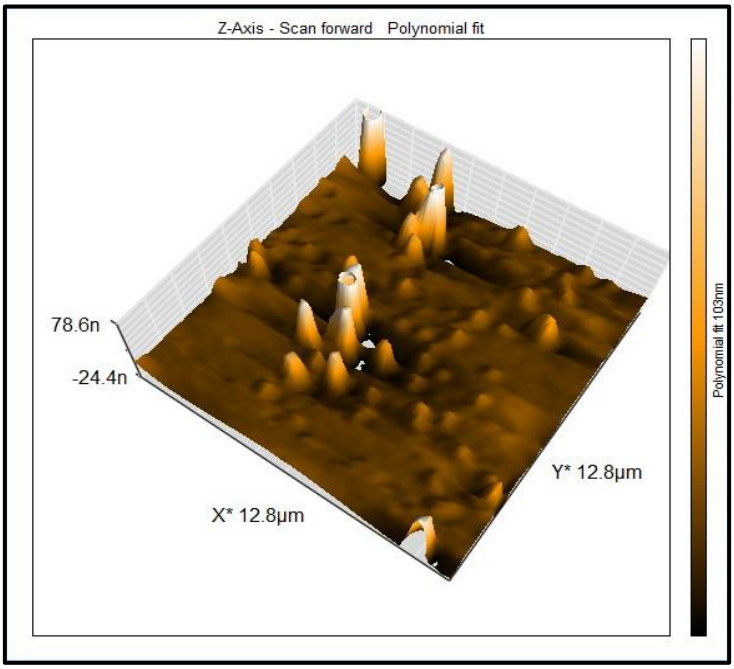

Fig. 7. AFM results (Z-Axis) of DOXO-CsNs-PEG polymers.

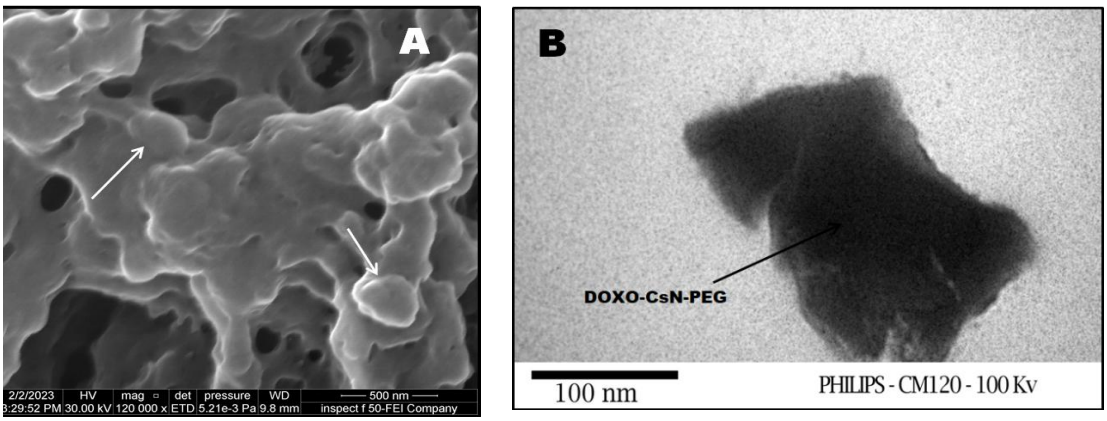

Fig. 8. (A) FE-SEM result, and (B) TEM result of DOXO-CsN-PEG polymer.

The formulated DOXO-CsN-PEG polymer showed negligible or no hemolysis percentage (less than 5%) at all studied concentrations, as shown in Fig. 9.This percentage is considered within the safety limit for intravenous administration of medications [36], therefore confirming the safety of the formulated polymer on blood. On the other side, the hemolysis percentage was highly elevated 13.2% at 31.25µg/ml to 75.5% at 1000µg/ml when treated with DOXO.

DOXO causes hemolysis of RBCs by its interaction with the lipid cell membrane and the cytoskeleton of RBCs [37]. It inhibits the actin polymerization, which decreases the rigidity of RBCs by reducing their mechanical strength. It also inhibits the Na-K-ATPase activity, which causes volume regulation disruption [37]. On the Contrary, the formulated DOXO-CsN-PEG polymer appeared less hemolysis

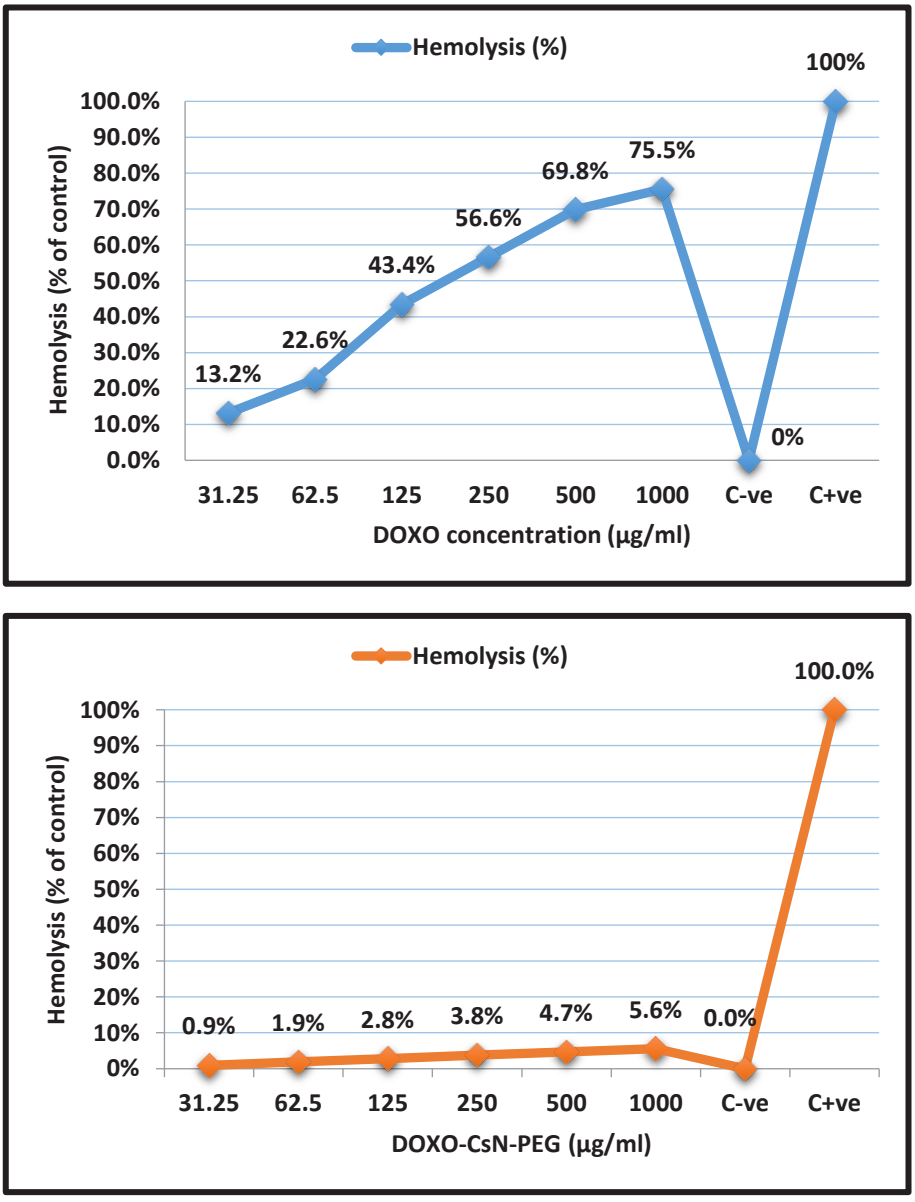

Fig. 9. The hemolysis percentage of blood after incubated with (Upper) Doxorubicin and (Lower) DOXO-CsN-PEG at (31.25, 62.5,125, 250, 500 and 1000 µg/ml).

than the non-coated DOXO. These results could be attributed to the fact that the large groups of CsN and PEG on the surface of the drugs limited the touching with the RBCs, thereby decreasing the interaction of DOXO with the RBCs cell [38]. Additionally, the FDA approval for utilizing

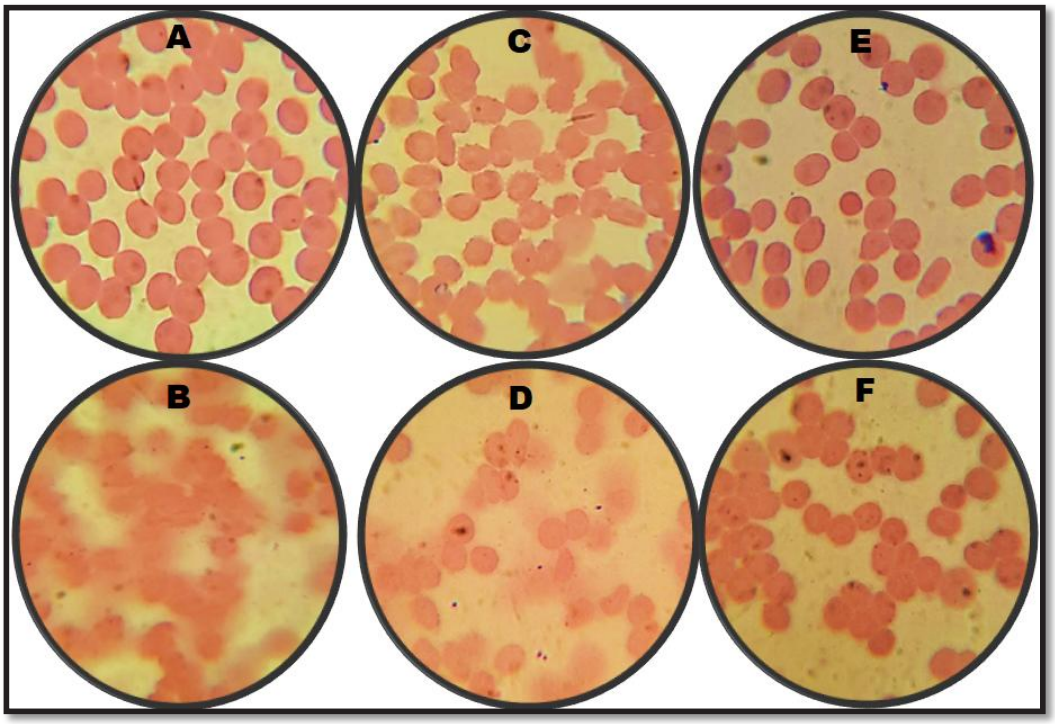

Fig. 10. Light Microscopic images for RBCs after treating (for 1hr at 37Cº) with; (A) Control negative (B) Control positive (C) Doxorubicin 31.25 µg/ml (D) Doxorubicin 1000 µg/ml (E) DOXO-CsN-PEG 31.25 µg/ml, and (F) DOXO-CsN-PEG 1000 µg/ml.

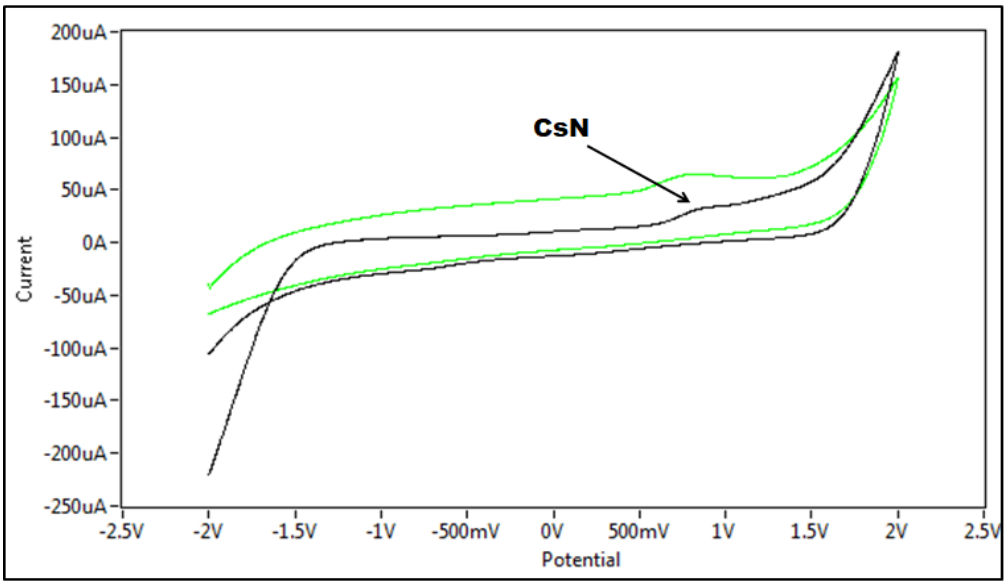

Fig. 11. Cyclic voltammogram for CsN at scan rate of 0.1 Vsec-1 in blood medium; (Black line) of CsN, and (Green line) of blood.

PEG2000-DSPE in the preparation of Doxil® is evidence of its blood compatibility and safety [39].

Fig. 10 shows microscopic images of RBCs after incubation with DOXO (at 31.25 and 1000 µg/ml) and DOXO-CsN-PEG (at 31.25 and 1000 µg/ml) in comparison with positive and negative controls. The DOXO-CsN-PEG images showed no changes in the RBC forms similar to negative control Figs. 10E and F). Conversely, the marked form alterations were shown for RBCs treated with DOXO at 31.25 µg/ml, such as lysis of the cells, shrinkage, and shape deformation, as shown in

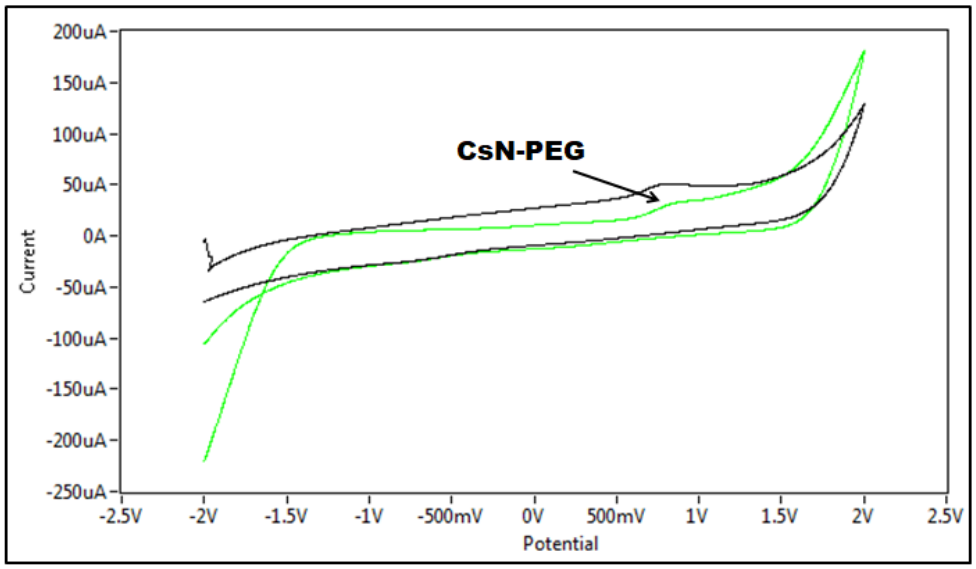

Fig. 12. Cyclic voltammogram for CsN-PEG at scan rate of 0.1 Vsec-1 in blood medium; (Green line) of CsN-PEG and (Black line) of blood.

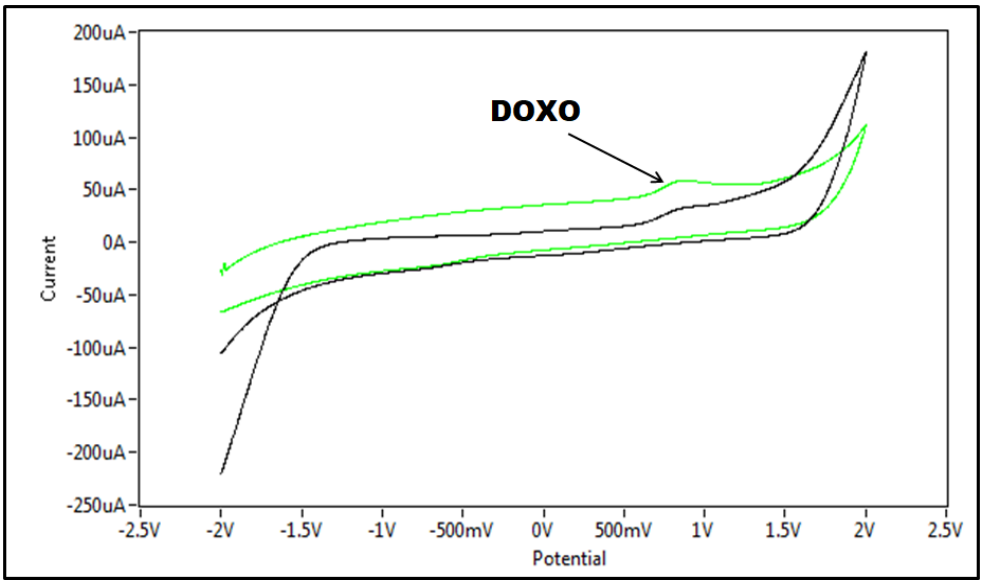

Fig. 13. Cyclic voltammogram for doxorubicin at scan rate of 0.1 Vsec-1 in blood medium; (Green line) of doxorubicin, and (Black line) of blood.

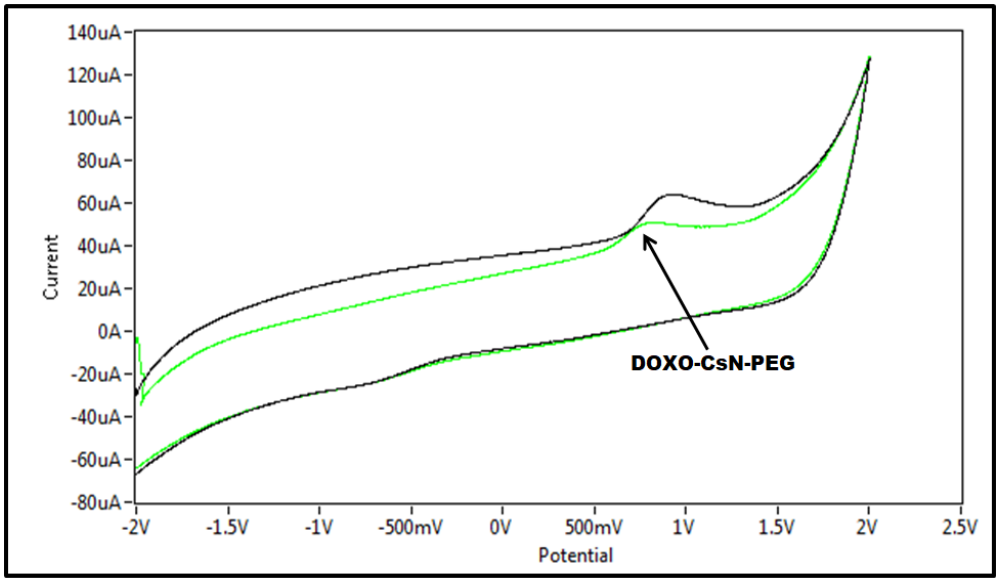

Fig. 14. Cyclic voltammogram for DOXO-CsN-PEG at scan rate of 0.1 Vsec-1 in blood medium. (Green line) of DOXO-CsN-PEG, and (black line) of blood.

(Fig. 10 C). Additionally, highly noticed hemolysis was recorded for RBCs treated with DOXO at 1000 µg/ml. All the hemolysis test results on RBCs suggested that the DOXO-CsN-PEG are highly compatible with RBCs and more safe than DOXO on the human blood.

The Redox Reactions by Cyclic Voltammetry

Using the CV technique, the electrochemical properties of the prepared CsN, CsN-PEG, and DOXO-CsN-PEG in the blood were studied. The oxidation peak of blood was reduced from 70 µA to 20 µA when treated with CsN, as shown in Fig. 11. This electrochemical behavior of the prepared CsN confirmed that it can be considered as a good antioxidant agent in blood [40,41].

Furthermore, the oxidation peak of blood was also decreased when treated with CsN-PEG (from 50 µA to 25 µA), as shown in Fig. 12. This referred to the antioxidant role of this polymer on blood.

On the other side, the electrochemical properties were also studied for DOXO before and after coating with CsN-PEG polymers. The results showed that the oxidative peak of blood was elevated from 30 µA to 60 µA when treated with DOXO before coating, Fig. 13. This result referred that DOXO had an oxidative effect on blood. In contrast, the oxidative peak of blood was decreased from 65 µA to 50 µA after treatment with DOXO-CsN-PEG polymers, (Fig. 14). These results confirm the gold standard role of CsN-PEG polymers in reducing the toxicity of DOXO on normal tissue such as blood [42].

CONCLUSION

This study concluded that CsN and CsN-PEG polymers can act as antioxidant compounds for the blood medium. Additionally, the DOXO chemotherapy was converted from an oxidative agent into an antioxidant of blood when coated with CsN-PEG polymers. Eventually, these results confirm the gold standard role of CsN-PEG polymers in decreasing the toxicity of DOXO on normal tissue such as blood.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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