chemistry proceedings

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Tocopherol-Doxorubicin Conjugate as a Lipid-Prodrug: Synthetic Methods, Self-Assembly, Breast Cancer Cell Inhibition, and Theoretical Analysis †

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† Presented at the 29th International Electronic Conference on Synthetic Organic Chemistry (ECSOC-29); Available online: https://sciforum.net/event/ecsoc-29.

Abstract

Developing natural lipid-based conjugates/prodrugs emerged as a promising topic in pharmaceutical chemistry and biomedicine. In this work, a natural antioxidant lipid, α-Tocopherol (vitamin E), was covalently connected with Doxorubicin (Dox) to synthesize a Toco-Dox conjugate through two approaches: Triphosgene activation (method A) and 4-nitrophenyl chloroformate (method B) activation. The latter method is non-volatile and generates safe-to-handle byproduct 4-nitrophenol, making it much less hazardous and more eco-friendly. Molecular structure of Toco-Dox was characterized by ^{1}H, ^{13}C NMR, FT-IR and MALDI-TOF-MS. Toco-Dox could self-assemble into nanoparticles in the DMSO/water mixture and Toco-Dox nanoparticles were further characterized by DLS. Moreover, the molecular properties of Toco-Dox were theoretically calculated or virtually analyzed (Dox as a control). In addition, unlike (free) Dox, Toco-Dox showed moderate MCF-7 breast cancer cell inhibition (cytotoxicity) and a cytoplasm localization behavior. This work provided an efficient approach to develop natural (fat-soluble) vitamin-based prodrug system for breast cancer chemotherapy.

Keywords: α-Tocopherol; Doxorubicin; Triphosgene; 4-nitrophenyl chloroformate; self-assembly; theoretical calculation; MCF-7; breast cancer

Academic Editor(s): Name

Published: date

Citation: Caires, L.; Maciel, D.; Castro, R.; Gonçalves, M.; Pereira, J.A.M.; Câmara, J.; Jaśkowska, J.; Rodrigues, J.; Tomás, H.; Sheng, R. Tocopherol-Doxorubicin Conjugate as a Lipid-Prodrug: Synthetic Methods, Self-Assembly, Breast Cancer Cell Inhibition, and Theoretical Analysis. *Chem. Proc.* **2025**, *volume number*, x. https://doi.org/10.3390/xxxxx

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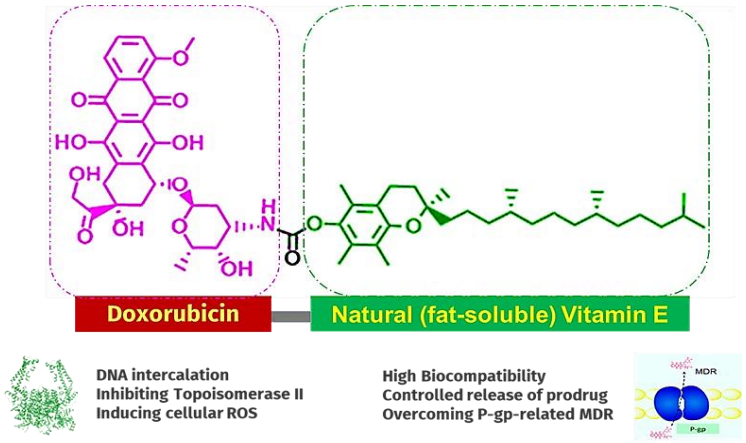
1. Introduction

Doxorubicin (Dox) is a topoisomerase II inhibitor and DNA intercalator that widely employed as a chemotherapeutic agent [1–5]. However, its clinical utility and application was largely limited by its severe toxicities, autophagy-induction, and multidrug resistance (MDR) effect [6]. To address these drawbacks, covalent chemical modifications have been employed to improve the bioavailability, therapeutic efficiency, and tumor selectivity of Dox [7,8]. Natural lipid-based chemical modification of Dox [9,10], especially at the primary amino group (–NH_2) on the daunosamine moiety, has been extensively

explored. The amide-containing lipid-Dox conjugates often exhibited improved physicochemical stability, enhanced intracellular uptake, controllable degradability and pharmacokinetics, and specific intracellular localization behaviors [11,12]. In previous works, our research team developed natural cholesterol (lipid)-Dox conjugate via degradable carbamate linkage and prepared its supramolecular (nano-) assemblies as anticancer nanoprodrugs, which showed controllable and quantitative drug loading capacity, high storage stability, tunable nanoparticle size and zeta potential, as well as enhanced breast cancer cell uptake and inhibition effects [13,14].

Among the natural lipid or lipid-like molecules [15,16], α-tocopherol, a fat-soluble vitamin, plays essential roles in many biochemical processes including membrane formation, signal transduction, hormone metabolism, and cellular communication. The physicochemical properties, biocompatibility, and biological activities of α-tocopherol make it a sustainable building block for prodrugs and nanotherapeutics [17].

In this work, we synthesized an α-tocopherol–based doxorubicin conjugate (Toco-Dox) by connecting Dox and α-tocopherol via a carbamate linkage (Scheme 1), in which DOX acts as a topoisomerase II inhibitor, DNA intercalator, and ROS inducer, meanwhile, α-tocopherol offers cell membrane-affinity, hydrophobicity, and the ability to overcome MDR. Accordingly, Toco-Dox was prepared via two strategies: triphosgene (method A) [13], and 4-nitrophenyl chloroformate (method B) activation [18] and was characterized by NMR and MALDI-TOF-MS. Toco-Dox could self-assemble in aqueous solution to form Toco-Dox nanoparticles [19], which were characterized by dynamic light scattering (DLS) analysis. Moreover, in silico theoretical analysis was conducted to predict the properties of Toco-Dox. Finally, the in vitro cytotoxicity of Toco-Dox against MCF-7 breast cancer cells and the intracellular localization was preliminarily investigated.



Scheme 1. Designation of the α-tocopherol–based doxorubicin conjugate (Toco-Dox).

2. Materials and Methods

2.1. Materials

All chemical reagents and solvents were directly utilized without further purification.

2.2. NMR and Mass Spectral Measurements

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on an Avance II+ 400 spectrometer (Bruker, Wissembourg, France) at 299 K (probe temperature). The chemical shifts (δ) are recorded in parts per million (ppm) and tetramethylsilane (TMS, δ = 0 ppm) as the internal chemical shift reference. The mass spectrum with electrospray ionization

technique (ESI-MS) was conducted on a Bruker Autoflex maX MALDI-TOF-Mass Spectrometer (Bremen, Germany, in positive mode).

2.3. Synthesis of Toco-Dox Conjugate

2.3.1. Method A (Triphosgene Activation)

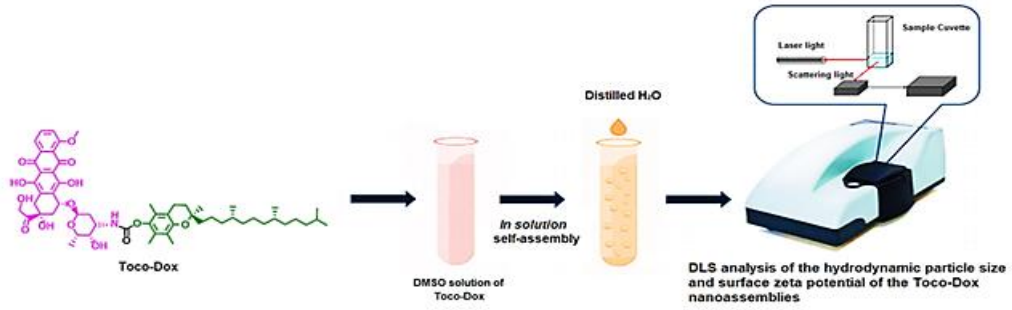
Tocopherol (1.0 equiv.) was dissolved in anhydrous dichloromethane under nitrogen atmosphere, followed by the addition of triphosgene (0.38 equiv.) with triethylamine (3.0 equiv.). The mixture was stirred at room temperature for 2 h. Doxorubicin hydrochloride (1.0 equiv.) with triethylamine (1.0 equiv.) was then added, and the reaction was stirred at room temperature for 12 h. After completion, the mixture was concentrated, and the crude product of Toco-Dox conjugate was purified by silica gel chromatography. (Note: highly toxic triphosgene or phosgene residuals should be carefully removed by treating excessive saturated $NaHCO_3$ solution before silica gel chromatography purification).

2.3.2. Method B (4-Nitrophenyl Chloroformate Activation)

Tocopherol (1.0 equiv.) was reacted with 4-nitrophenyl chloroformate (1.3 equiv.) and triethylamine (3.0 equiv.) and purified by silica gel column chromatography to generate the intermediate Tocopheryl-4-nitrophenolate. After that, doxorubicin hydrochloride (1.0 equiv., with 2 equiv. triethylamine) and Tocopheryl-4-nitrophenolate (1.3 equiv.) was added into DMSO/THF/MeOH solution, and the reaction was stirred for 12–16 h at 40–50 °C, and the crude product of Toco-Dox conjugate was concentrated and purified by silica gel chromatography.

2.4. In-Solution Self-Assembly of Toco-Dox and Dynamic Light Scattering (DLS) Analysis

Toco-Dox was self-assembled in a DMSO/distilled water (DMSO:H_2O = 1:9, v:v) mixture to form nanoparticles (final concentration 10 μg/mL). Hydrodynamic diameters of the resulting aggregates were measured using a Malvern Zetasizer Nano ZS (model ZEN3600, Worcestershire, UK) equipped with a 633 nm He–Ne laser. Measurements were performed in triplicate (n = 3) for each sample at 25 °C. (Scheme 2) Surface zeta (ζ) Potential of Toco-Dox nanoparticles were measured using disposable folded capillary cells (DTS1060C) and cuvettes (ZEN0040), on a Malvern Zetasizer Nano ZS at 25 °C.



Scheme 2. In-solution self-assembly to prepare Toco-Dox nanoparticles and DLS analysis.

2.5. In Vitro Cytotoxicity of Toco–DOX Conjugates in MCF-7 Cells

2.5.1. Cell Culture

MCF-7 breast cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and with 1% antibiotic–antimycotic (penicillin, streptomycin). Additionally, MCF-7 cells need to be complemented with 1% (*v*/*v*) 100× NEAA (Non-Essential-Amino Acids, Gibco) 1 mM sodium pyruvate (Sigma-Aldrich), and 3.3 μg/mL human insulin (Biomedicals) Cells were maintained at 37 °C in a humidified 5% CO_2

incubator (Nuaire) and sub-cultured every 3–5 days. For cytotoxicity assays, cells were seeded into 96-well plates at 5000 cells per well.

2.5.2. Resazurin Cytotoxicity Assay of Toco–Dox

Test solutions of the Toco–Dox and Dox (0–50 μg/mL, 2% DMSO in RPMI) were prepared and added to wells in quadruplicate (n = 4). MCF-7 cells were incubated for 48 h at 37 °C. After incubation, RPMI 1640 medium was replaced with 200 μL of 10% resazurin solution (in RPMI) per well. Plates were incubated for 3 h at 37 °C, and fluorescence was measured ($\lambda_{ex}/\lambda_{em}$ = 530 nm/590 nm) using a microplate reader. Cell viability was expressed as relative fluorescence units (RFU) compared to untreated controls.

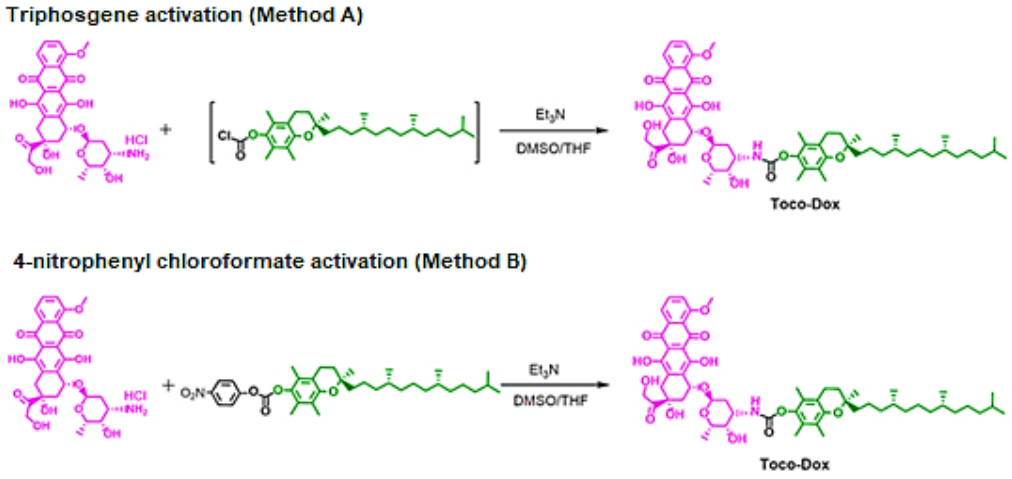
2.6. Confocal Fluorescence Imaging for Studying the Intracellular Localization of Toco-Dox

Following the Toco-Dox condition of resazurin assay, MCF-7 cells in 96-well plates were washed with PBS and fixed with 100 μL/well of 3.7% (*v*/*v*) formaldehyde for 20 min. After fixation, the wells were washed with PBS and stained with 100 μL of the respective fluorescent dyes (DAPI: cell nuclei staining. Alexa Fluor 488: cytoskeleton staining) for 20 min each. Intracellular localization of Toco-Dox was visualized using a Leica Mica Microhub confocal fluorescence microscope.

3. Results and Discussion

3.1. Synthesis of Toco-Dox via Triphosgene (Method A) and 4-Nitrophenyl Chloroformate (Method B) Activation

Herein, the Toco-Dox conjugate was synthesized by two different strategies: (traditional) triphosgene (Method A) and 4-nitrophenyl chloroformate (Method B) activation, the reactions were shown in Scheme 3.



Scheme 3. Synthesis of Toco-Dox using (traditional) triphosgene activation (Method A) and 4-nitrophenyl chloroformate activation (Method B).

Method A used 0.38 equiv. of triphosgene relative to α-tocopherol and the activation proceeded rapidly at room temperature. However, the in-situ generation of phosgene ($COCl_2$) may cause severe safety issues and handling risks. Notably, the triphosgene activation reaction also requires strictly anhydrous conditions and extensive post-reaction purification, which largely reduced the 'green chemical efficiency'. In contrast, method B used 1.2 equiv. of 4-nitrophenyl chloroformate and proceeded under mild heating (40–50 °C) with 61–70% conversion after 12–16 h. Despite the higher stoichiometric excess, method B is relatively safer and more eco-friendly. The hydrolysis of tocopheryl-4-

chloroformate intermediate produces only CO_2 gas and less-toxic 4-nitrophenol, which could be easily precipitated and handled upon addition of metal (e.g., Al^{3+}, Cu^{2+}) salts, thus minimized the production of hazardous byproducts and improved the sustainability. The detailed comparison of Method A and Method B was shown in Table 1.

Table 1. Comparison of Toco-Dox synthesis via triphosgene activation (Method A) and 4-nitrophenyl chloroformate activation (Method B).

Features	Method A: Triphosgene Activation	Method B: 4-Nitrophenyl Chloroformate Activation
Reagent ratio	0.38 equiv. to tocopherol	1.2 equiv. to tocopherol
Reaction rate	Fast	Moderate
Reaction temperature	Room temperature	Mild heating (40–50 °C)
Solvent conditions	Organic solvents (strictly anhydrous conditions)	Organic solvents (normal or ambient conditions)
Reaction Toxicity	In situ generates highly toxic phosgene gas	Produces less-toxic 4-nitrophenol and CO_2 gas by hydrolysis
Product handling or Purification	Requires careful handling to remove triphogene or phosgene	Safer to handle or purification
Product purity	High (>95% by UHPLC)	High (>95% by UHPLC)
Eco-friendliness	Low	Medium-high

The synthesized Toco-Dox was obtained as a dark red powder in the solid state (Figure 1). Toco-Dox is easy to be dissolved in $CHCl_3$ but almost insoluble in aqueous solution (PBS, 0.1 M, pH 7.4), indicating strong hydrophobicity of Toco-Dox conjugate. This observation was confirmed by thin-layer chromatography (TLC) analysis [20]. Toco-Dox exhibits a much higher R_f value (0.88) compared to free DOX (R_f = 0.05) under the same eluents ($CHCl_3$:CH_3OH = 10:1, *v*/*v*). The results implied that the tocopherol conjugation of Dox might facilitate the hydrophobic-induced assembly of Toco-Dox into nanoparticles and further enhance their cell membrane fusion, permeation, and cellular uptake ability.

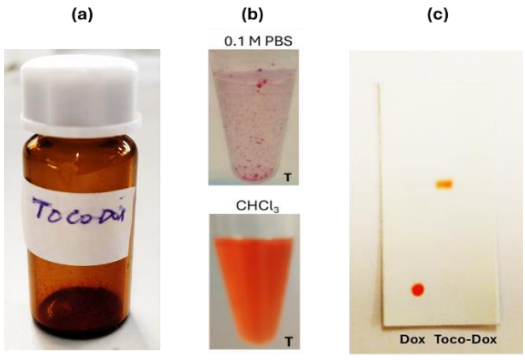
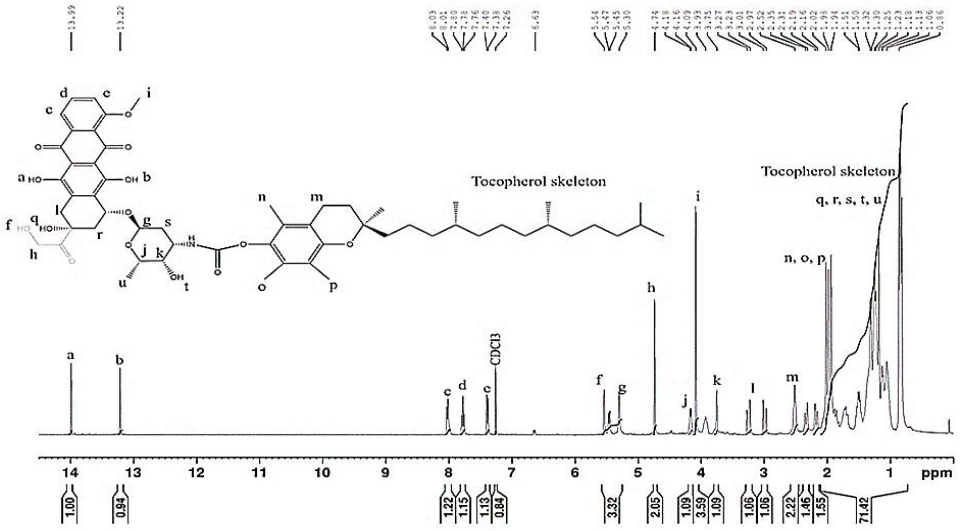


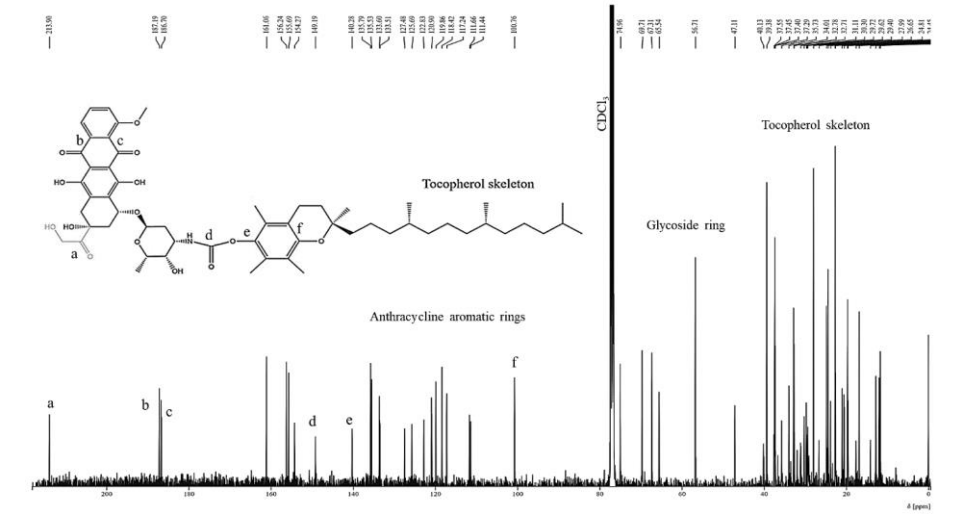
Figure 1. The synthesized Toco-Dox (**a**) in a solid state (dark red powder), (**b**) 0.2 mg Toco-Dox in 0.2 mL PBS (0.1 M, **top**) and $CHCl_3$ solution (**bottom**), and a molecular polarity comparison (**c**) of the synthesized Toco-DOX (right spot, R_f = 0.88) and free Dox (left spot, R_f = 0.05) on a silica gel 60 F254 aluminum TLC plate (eluent: $CHCl_3$:CH_3OH = 10:1, v:v).

3.2. NMR and MS Characterization of Toco-Dox

Molecular structure of the synthesized Toco-Dox was characterized by ^1H NMR, ^{13}C NMR, FTIR (data not shown) and MALDI-TOF-MS. As shown in Figure 2. The NMR and MS spectra confirmed the successful synthesis of the Toco-Dox conjugate with desired molecular structure.



(**a**)



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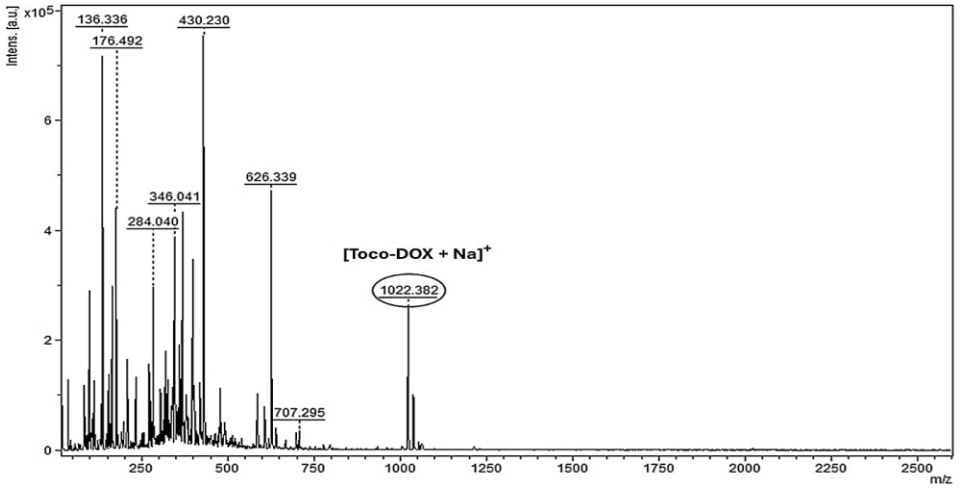


(**c**)

Figure 2. ^{1}H NMR (**a**), ^{13}C NMR (**b**) MALDI-TOF-MS (**c**) spectra of the synthesized Toco-Dox.

3.3. Theoretical Analysis and Calculation of the Synthesized Toco-Dox Conjugate

To better understand the molecular properties of Toco-Dox, theoretical analysis was conducted using SWISSADME and hyperchem 8.0 software. As shown in Table 2,

significant differences between the calculated properties of Dox and Toco-Dox could be observed. Conjugation with tocopherol largely increased the molecular weight from 543.52 g/mol to 1000.22 g/mol, thus remarkably enlarged the van der Waals surface area (492.72 → 916.66 Å^2), while the total polar surface area (206.07 → 227.61 Å^2) slightly increased. Toco-Dox showed higher molar refractivity (274.97) and lower molecular dipole moment (5.96 Debye), indicating its larger structure and higher hydrophobicity. Accordingly, the hydrophobicity/lipophilicity values (Log Po/w) drastically increased from 1.27 (Dox) to 12.62 (Toco-Dox), accompanied with a significant decrease of water solubility (Log S: −3.91 → −12.79), which might be benefit for cell membrane fusion and penetration.

Table 2. Theoretical analysis of the molecular properties of Dox and Toco-Dox.

Properties	Dox	Toco-Dox
Chemical formula	$C_{27}H_{29}NO_{11}$	$C_{57}H_{77}NO_{14}$
Molecular weight (g/mol)	543.52	1000.22
Total polar surface area (TPSA, Å^2)	206.07	227.61
Van der Waals Surface Area (Å^2)	492.72	916.66
Molar Refractivity	132.66	274.97
Molecular dipole moment (Debye)	11.05	5.96
Log Po/w (XLOGP3)	1.27	12.62
Water Solubility Log S(ESOL)	−3.91	−12.79

3.4. Self-Assembly of Toco-Dox in Aqueous Solutions and Characterization

Toco-Dox was self-assembled in both distilled water and RPMI culture medium, which could provide insight into the differences between ideal solution and in vitro biological conditions for nanoparticle formation. Toco-Dox nanoparticles formed in distilled water showed smaller hydrodynamic diameter of 117.5 ± 0.8 nm with a low PDI of 0.124 (Table 3). In contrast, when Toco-Dox assembled in RPMI cell culture medium, the particle size drastically increased to 2063.7 ± 290.0 nm with a high PDI of 0.642. It might be attributed to the ionic interactions of phenyl (-OH) groups in Toco-Dox with the electrolytes (e.g., Ca^{2+}, HPO_4^{2-}) in the RPMI medium, which reduced the Toco-Dox nanoparticles' stability and resulted in particle aggregation. These results suggest that the colloidal stability of Toco-Dox strongly depends on the ionic environment, which might significantly influence their related cellular uptake, trafficking, and intracellular distribution.

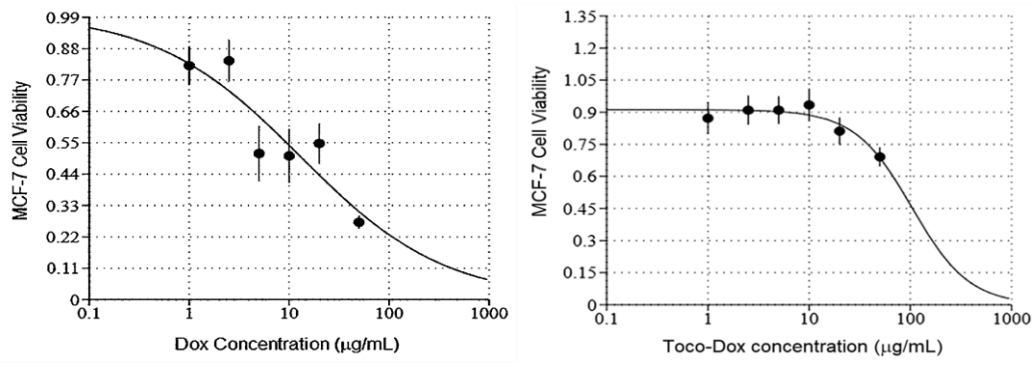
Table 3. Hydrodynamic particle size and polydispersity (PDI) of the self-assembled Toco-Dox nnanoparticles (final concentration 10 μg/mL) in different mediums.

Sample	Medium	Hydrodynamic Diameter (nm)	Polydispersity Index (PDI)
Toco-Dox	DMSO:H_2O = 1:9 (v:v)	117.5 ± 0.8	0.124
Toco-Dox	RPMI culture medium	2063.7 ± 290.0	0.642

3.5. In Vitro Cytotoxicity of Toco-Dox and Intracellular Localization

In vitro cytotoxicity of Dox and Toco-Dox was measured by resazurin assay (Figure 3), compared to free Dox, Toco-Dox exhibited less cytotoxicity/inhibition to MCF-7 cells within the range of 1–50 μg/mL. Interestingly, a hormetic (low concentration exposure induced stimulatory) effect on MCF-7 cell growth was observed at 1.0–10.0 μg/mL. The cytotoxicity/inhibition enhanced at 20–50 μg/mL (~84–68% viability), suggesting that Toco-Dox possesses degradability and enables slow release of Dox. UHPLC assay (data not shown here) suggests that the degradation of Toco-Dox prodrug is likely mediated by

enzymatic (cellular hydrolase-catalyzed) cleavage of the carbamate or amide linkages between the lipid chain and Dox, rather than simple acidic (H^+-based) hydrolysis.



(**a**) (**b**)

Figure 3. In vitro cytotoxicity of Dox (**a**) and Toco-Dox (**b**) at 0–50 μg/mL in MCF-7 cells (48 h) measured by resazurin assay. (Solid lines: the fitted trendlines of cytotoxicity).

The intracellular localization of Toco-Dox was preliminarily investigated (Figure 4), the Toco–Dox mainly localized in the cytoplasm of MCF-7 cells, while free Dox tend to localize inside cell nuclei [21,22]. The intracellular uptake trend of Toco-Dox and Dox correlates well with their observed cytotoxicity. After cellular uptake, Toco-Dox may undergo lysosomal or cytoplasmic enzyme-mediated cleavage, intracellularly releasing free Dox and subsequently inhibiting MCF-7 cell proliferation. Notably, unlike Dox, the cytotoxicity, cellular uptake, and intracellular localization of Toco–Dox suggested that the attached Tocopherol lipid chains play important roles in cellular behaviors.

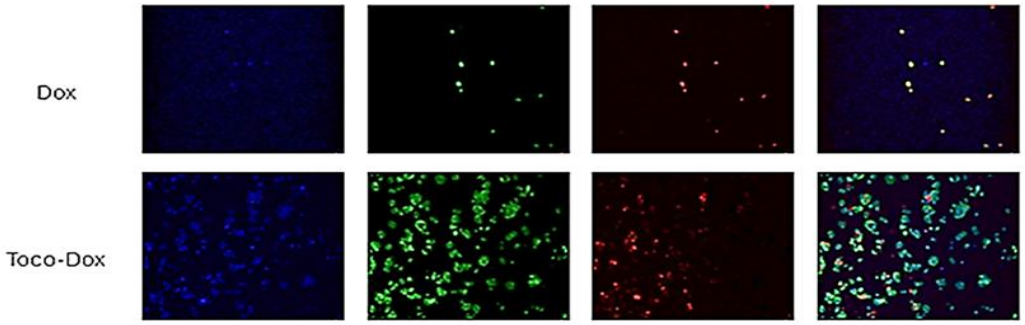


Figure 4. Intracellular fluorescent imaging of Dox and Toco-Dox in MCF-7 breast cancer cells (48 h incubation), free Dox was employed as a control. Blue fluorescence: DAPI-stained cell nuclei. Green fluorescence: Alexa Fluor 488-stained cytoskeleton, Red fluorescence: lipid-Dox and free Dox.

4. Conclusions

In summary, we synthesized a Toco-Dox conjugate through two approaches: Triphosgene activation (method A) and 4-nitrophenyl chloroformate activation (method B). Method B generates safe-to-handle byproduct 4-nitrophenol, making it less hazardous and more eco-friendly. Molecular structure of the Toco-Dox was characterized by ^{1}H, ^{13}C NMR, FT-IR and MALDI-TOF-MS. Toco-Dox could self-assemble into nanoparticles in the DMSO/water mixture. The molecular properties of Toco-Dox (Dox as a control) were in silico theoretically calculated and virtually analyzed. Moreover, cellular results showed that Toco-Dox have moderate (~84–68% viability at 20–50 μg/mL) MCF-7 inhibition ability and cytoplasm localization. This work provided an efficient approach to develop natural (fat-soluble) vitamin-based prodrug system for breast cancer chemotherapy.

Author Contributions: Conceptualization, R.S.; methodology, R.S.; software, J.J. and R.S.; validation, D.M., R.C. and M.G.; formal analysis, J.J.; investigation, L.C.; D.M.; R.C.; J.R.; J.J.; and R.S. resources, J.A.M.P.; J.C.; J.R.; H.T. and R.S. data curation, L.C.; D.M.; R.C.; J.R. and R.S. writing—original draft, L.C. and R.S. writing—review and editing, all the authors; visualization, L.C.; R.C. and R.S. supervision, D.M.; R.C. and R.S. project administration, H.T. and R.S.; funding acquisition, J.R. and R.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by FCT-Fundação para a Ciência e a Tecnologia (UIDP/00674/2020 and UIDB/00674/2020, and FCT individual employment grant 2021. 00453.CEECIND).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Acknowledgments: We thank FCT-Fundação para a Ciência e a Tecnologia (UIDP/00674/2020 and UIDB/00674/2020, FCT individual employment grant 2021.00453.CEECIND) and ARDITI-Agência Regional para o Desenvolvimento da Investigação Tecnologia e Inovação for the sponsorship.

Conflicts of Interest: The authors declare no conflicts of interest.

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