The Formation of Self-Assembled Nanoparticles Loaded with Doxorubicin and \( \text{d}-\text{Limonene} \) for Cancer Therapy

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**ABSTRACT:** Self-assembled nanoparticles present unique properties that have potential applications in the development of a successful drug delivery system. Doxorubicin (DOX) is an important anti-neoplastic anthracycline chemotherapeutic drug widely described. However, it suffers from serious dose-dependent cardiotoxicity. \( \text{d}-\text{Limonene} \) is a major constituent of numerous citrus oils that is considered a specific monoterpene against free radicals producing antioxidant activity. Herein, we aimed to design three types of self-assembled nanodelivery systems (nanoemulsion, niosomes, and polylactide nanoparticles) for loading both DOX and \( \text{d}-\text{limonene} \) to enhance the solubilization of \( \text{d}-\text{limonene} \) and provide antioxidant activity with excellent anticancer activity. As confirmed by dynamic light scattering and transmission electron microscopy, the nanoparticles were prepared successfully with diameter sizes of 52, 180, and 257 nm for the DOX-loaded nanoemulsion, niosomes, and polylactide nanoparticles, respectively. The zeta potential values were above \(-30\) mV in all cases, which confirms the formation of stable nanoparticles. The loading efficiency of DOX was the highest in the case of the DOX-loaded nanoemulsion (75.8%), followed by niosomes (62.8%), and the least was in the case of polylactide nanoparticles with a percentage of 50.2%. The in vitro release study of the DOX-loaded nanoparticles showed a sustained release profile of doxorubicin with the highest release in the case of DOX-loaded PDLLA nanoparticles. The kinetic release model for all developed nanoparticles was the Peppas–Sahlin model, demonstrating DOX release through Fickian diffusion phenomena. Moreover, all developed nanoparticles maintain the antioxidant activity of \( \text{d}-\text{limonene} \). The cytotoxicity study of the DOX-loaded nanoparticles showed concentration-dependent anticancer activity with excellent anticancer activity in the case of the DOX-loaded nanoemulsion and polylactide nanoparticles. These nanoparticles will be further studied in vivo to prove the cardioprotective effect of \( \text{d}-\text{limonene} \) in combination with DOX.

1. INTRODUCTION

Self-assembly is a process in which the building block molecules can organize themselves spontaneously via noncovalent interactions to obtain a macromolecular nanostructure with the minimum free energy of the system. This process is considered the most promising approach to preparing nanomaterials with a low cost and high yield. Depending on the molecular composition and the method of preparation, various nanostructures of various sizes could be generated. Self-assembled nanomaterials have been applied in a variety of scientific fields such as electronics, photonics, biology, and medicine. In medicine, self-assembled nanomaterials can be applied in tissue engineering, cancer immunotherapy, biosensing, antimicrobial therapy, anti-inflammatory drugs, the development of vaccines, and novel drug delivery systems. Many kinds of research have been developed to improve the properties of existing drugs by enhancing the water solubility of poorly soluble therapeutics, decreasing drug toxicity, improving drug pharmacokinetic profiles, and also developing a sustained release system of drugs. One of the most studied anticancer drugs is doxorubicin (DOX), which inhibits cancer growth by interrupting the topoisomerase II enzyme that is responsible for DNA replication and transcription. However, the clinical use of this potent anticancer drug is hampered due to its serious side effect, which is cumulative cardiotoxicity. The exact molecular mechanism regarding its cardiotoxicity is not well understood. However, various processes have been supposed to include the production of reactive oxygen species (ROS), dysfunction in the mitochondria, dysregulation of intracellular calcium, and p53-mediated cardiomyocyte death. Various studies showed that ROS production is...
considered the major cause of cardiotoxicity.\textsuperscript{22} Therefore, providing antioxidants with DOX therapy could reduce the cardiotoxicity side effect.\textsuperscript{23,24} Zhang et al. have incorporated resveratrol as an antioxidant with doxorubicin in solid lipid nanoparticles to reduce the DOX-induced cardiotoxicity. They observed that the heart rate, ejection fractions, and fractional shortening of Res-SLN-treated mice were higher than those of mice with cardiac toxicity induced by a single high-dose intraperitoneal injection of DOX.\textsuperscript{25} Another study was conducted by Shi et al., where they developed a submicron emulsion of liquiritigenin (Lq-SE) to reduce DOX-induced cardiotoxicity as Lq has the capability of being a strong radical scavenger and anti-inflammatory drug. They found that the Lq-SE reduced the release of serum cardiac enzymes, decreased oxidative stress, and enhanced antioxidant enzyme activity.\textsuperscript{26} Wi et al. developed liposomes encapsulating linalool nano-emulsions as an antioxidant with DOX to enhance anticancer activity and reduce side effects. The results showed an increase in cell death and inhibited tumor growth in comparison to DOX-liposomes. Moreover, the developed liposomes did not induce biochemical toxicity in comparison to healthy control mice.\textsuperscript{27}

\textit{D}-limonene is a monocylic monoterpene that is found in citrus oil and used as a flavoring agent. Recent studies have shown that \textit{D}-limonene possesses anti-oxidant activity and protects lymphocytes from oxidative stress induced by hydrogen peroxide.\textsuperscript{26,27} Moreover, numerous reports have shown that \textit{D}-limonene has anticancer activity on various types of cancer as it can trigger apoptosis and regulate the cell cycle.\textsuperscript{28,29} In addition, a study conducted by Rehman et al. showed that \textit{D}-limonene can be used as a protective agent against the nephrotoxic effect of DOX.\textsuperscript{30} However, \textit{D}-limonene suffered from low water solubility due to its hydrophobic nature, which hampered its use and efficacy.\textsuperscript{31} Therefore, it is necessary to develop a suitable delivery system to improve the solubility, bioavailability, and encapsulation of \textit{D}-limonene and DOX. Herein, we aim to prepare three different self-assembled nanomaterials (nanoemulsion, niosomes, and polylactide (PDLLA) nanoparticles) and study their capability to encapsulate \textit{D}-limonene and DOX. In vitro release, antioxidant, and anticancer activities were also investigated. We chose these different nanostructures to cover the most commonly developed nanoparticles loaded with DOX, which are surfactant-based micelles, lipid-based (niosomes), and polymeric-based nanoparticles (poly-\textit{D},\textit{L}-lactide). Moreover, these three nanostructures have shown an excellent capability to encapsulate hydrophilic and hydrophobic drugs in their cavities.\textsuperscript{32} Micelles are self-assembled nanostructures of amphiphilic compounds that have attracted great attention in drug delivery due to their capability to load hydrophobic and hydrophilic molecules and provide long blood circulation.\textsuperscript{32} Varshosaz et al. produced pluronic micelles loaded with DOX that showed concise particle size, sustained release of DOX, and interestingly higher cytotoxicity on breast cancer cells in comparison to the free drug.\textsuperscript{33} Niosomes become very popular for clinical use as they are an alternative to liposomes. They have almost similar structural and physical properties to liposomes, but they are formed from non-ionic surfactants instead of phospholipids, which provides higher chemical stability.\textsuperscript{34} Di Francesco et al. have developed DOX-loaded niosomes based on Tween 20 and Span 20 to treat metastatic breast cancers.\textsuperscript{35} Polymeric nanoparticles are widely used to enhance the pharmacokinetic properties of hydrophobic and hydrophilic drugs, provide sustained release properties, greater stability, and better oral bioavailability.\textsuperscript{37} The development of nanoparticles is commonly based on biodegradable polymers.\textsuperscript{38} Poly-\textit{D},\textit{L}-lactide is one of the most commonly used polymers that can safely be incorporated into the development of nanoparticles with minimal side effects.\textsuperscript{39} Ayen and Kumar studied the anticancer activity and the pharmacokinetic profile in vivo of the DOX-loaded polyethylene glycol–polylactide (PEG–PLA) nanoparticles in comparison to the commercially available LipoDox.\textsuperscript{40} They found that DOX loaded in PEG–PLA nanoparticles showed a better pharmacokinetic profile (higher AUC, longer blood circulation half-life, and less clearance) than free doxorubicin and comparable anticancer activity and cytotoxicity to the LipoDox. To our knowledge, there are no previous reports that encapsulate \textit{D}-limonene with DOX in these nanoformulations. Moreover, the comparison between these formulas has not been previously reported.

2. MATERIALS AND METHODS

2.1. Reagents and Materials. Doxorubicin HCl, poly(D,L-lactide) ester terminated (PDLA) (M<sub>n</sub> 18,000–28,000, inherent viscosity: 0.25–0.35 dL/g (0.1% in CHCl<sub>3</sub> at 25 °C)), \textit{D}-limonene, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Germany. Span 80 was purchased from Alfa Aesar, USA. MTS reagent, Trypan blue solution, and trypsin–EDTA solution 1× were purchased from Promega, USA.

2.2. Instrumentations. A Jenway 7315 spectrophotometer (UK) was used to measure the ultraviolet–visible (UV–vis) spectra using 10 mm quartz cuvettes. A water bath sonicator from Elmasonic S70Hz, Elma (Germany) was utilized. Dynamic light scattering (DLS) was used for hydrodynamic size and polydispersity measurements obtained at an angle of scattering of 90 degrees and a temperature of 25 °C (NanoBrook Omni, Brookhaven Instruments, USA). Zeta potential was determined in a NanoBrook Omni (Brookhaven Instruments, USA). Transmission electron microscope (TEM) images were obtained using a 60 kV Morgagni 286 transmission microscope (FEI Company, Eindhoven, Netherlands) at the University of Jordan. Uranyl acetate (2%) was used as a negative stain in the case of niosomes.

2.3. Preparation of Self-Assembled Nanoparticles.

2.3.1. Preparation of the O/W Nanoemulsion. The O/W nanoemulsion (NE) consists of two phases, the organic phase that includes Span 80 and \textit{D}-limonene and the aqueous phase that includes Tween 80 and Milli-Q water. The organic phase mixture was sonicated for 5 min and then added dropwise under mild stirring to the aqueous phase. The resultant flocculated NE was stirred for approximately 15 min and after that sonicated for 10 min to obtain the nanoemulsion encapsulated with \textit{D}-limonene. NE-DOX was prepared in the same method and by adding 2 mg of DOX in the aqueous phase.

In this study, the hydrophilic–lipophilic balance (HLB) value of the mixed surfactant used was adjusted at 12. The
HLB value for a mixture of Span 80 and Tween 80 can be determined by the following equation:

\[ \text{HLB} = 4.3x + 15(1 - x) \]

Here, \( x \) represents the weight fraction of Span 80 in the mixture. An HLB value of 15 (Tween 80) indicates a hydrophilic surfactant, while an HLB value of 4.3 (Span 80) indicates a lipophilic surfactant. Different amounts of mixed surfactants (500, 1000, and 1500 mg) containing a fixed amount of d-limonene (200 mg) were studied to determine the most appropriate amounts with the smallest particle size and a good polydispersity index.

2.3.2. Preparation of the Niosomes. The technique used to prepare niosomes was the thin film hydration technique. Two hundred milligrams of d-limonene with a cholesterol mixture with Tween 80 (100:100 mM ratio) were weighed adequately and then dissolved in a 10 mL chloroform mixture. Subsequently, the solvent was evaporated by a rotary evaporator until dried, obtaining a thin film of the lipid with a white appearance, followed by the addition of 10 mL of Milli-Q water to hydrate the film. The flask was heated to 55 °C for 60 min with stirring at 2 rpm to obtain a white suspension. After that, the obtained suspension was sonicated for 20 min to get fine vesicles and then kept at 4 °C for additional analysis. Niosomes-DOX was prepared in the same way and by adding 2 mg of DOX into the niosome mixture.

2.3.3. Preparation of the PDLLA Nanoparticles. A nanoprecipitation method previously developed in our group was used for this procedure. The organic phase, including 50 mg of PDLLA polymer, 5 mg of POE, and 200 mg of d-limonene, was dissolved in 5 mL of acetone. The aqueous phase was prepared by dissolving 3 g of 1% PVA in 10 mL of Milli-Q water. After that, the organic layer was added dropwise into the aqueous phase under mild stirring. The formed suspension was evaporated to get rid of the acetone solvent. Then, filtration of the nanoparticles was done by utilizing a 0.45 μm pore size membrane syringe filter to make a uniform particle size. The formed nanoparticles were stored at 4 °C. PDLLA NPs-DOX were prepared in the same method and by adding 2 mg of DOX to the aqueous phase.

2.4. DOX and d-limonene Loading Efficiency. Once DOX is loaded into the self-assembled nanoparticles, each type of nanoparticle was centrifuged at 5000 rpm for 10 min to remove the excess unloaded DOX amount. Then, the loaded amount of DOX was determined by UV−vis spectroscopy based on a developed calibration curve of DOX at \( \lambda_{\text{max}} = 485 \) nm.

\[
\text{loading efficiency of DOX (\%)} = \left( \frac{\text{amount of the loaded DOX}}{\text{total amount of DOX applied}} \right) \times 100\%
\]

The loaded amount of d-limonene in the developed nanoparticles was also determined by UV−vis spectroscopy using the constructed calibration curve of d-limonene at \( \lambda_{\text{max}} = 220 \) nm.

\[
\text{loading efficiency of d-limonene (\%)} = \left( \frac{\text{amount of the loaded d-limonene}}{\text{total amount of d-limonene applied}} \right) \times 100\%
\]

2.5. In Vitro DOX Release. An in vitro DOX release study was performed using the dialysis membrane method. The nanoparticles were introduced into a dialysis bag containing release media (phosphate buffer, pH = 7.4) that was subsequently sealed and placed in a large vessel containing release media (phosphate buffer, pH = 7.4) agitated with a magnetic stirrer for a defined time. At the predetermined time intervals, an aliquot from the solution outside the dialysis bags was collected to determine the released amount of DOX using the developed calibrated curve on UV−vis. In vitro dialysis was performed for 30 h.

2.6. DOX Release Kinetics. Several kinetic models were investigated to analyze the data of DOX release from the three developed self-assembled nanoparticles that were computed using DDsolver. The investigated models were the Korsmeyer−Peppas (\( m/m_{\infty} = k \times t^{n} \)), Peppas−Sahlin (\( m/m_{\infty} = k \times t^{n} + k_{c} \times t_{c}^{n} \)), and Higuchi models (\( m/m_{\infty} = k^{1/2} \)). The linear regression \( R^{2} \) and Akaike information criterion (AIC) were measured.

2.7. DPPH Radical Scavenging Assay. DPPH is a stable nitrogen-centered free radical that is conventionally used to determine the free radical scavenging activities of antioxidants present in a synthetic compound or plant extract. The DPPH scavenging assay was evaluated using the procedure of Blois with a slight modification by Gulcin. The samples dissolved in methanol were added to 1.0 mL of methanolic DPPH. The control samples and test samples were added to various test tubes. The mixtures were stirred vigorously at 25 °C and left to stand for 30 min in the dark. The absorbance of the obtained solutions was determined at 517 nm in a UV−vis spectroscopy against a blank sample. The radical scavenging activities were measured as a reduction in the absorbance of DPPH. When the absorbance values of the reaction mixture are low, it shows a greater free radical scavenging assay. The DPPH radical scavenging assay (%) was measured by utilizing the equation below:

\[
\text{DPPH radical scavenging assay (\%)} = \left( \frac{A_{0} - A_{t}}{A_{0}} \right) \times 100\%
\]

Here, \( A_{0} \) indicates the integral intensity of the DPPH signal of the control sample and \( A_{t} \) indicates the integral of the test sample.

2.8. Anticancer Activity. Cell Culture. The anticancer activity of the various nanoparticles was evaluated on liver cancer cells (HepG2). The cells were cultured in T-175 cell culture flasks added with a cell culture growth medium (CGM) consisting of Roswell Park Memorial Institute (RPMI) basal medium supported with FBS (10%), l-glutamine (1%), and penicillin/streptomycin (1%). The cells were stored in a standard cell culture incubator under special circumstances; 99% humidity, 5% CO\(_{2}\), and 37 °C.

During the subculturing stage, the medium was suctioned and then washed with an excess of Ca\(^{2+}\)-free phosphate-buffered saline. Afterward, the cells were incubated with 0.025% trypsin for 5 min inside the cell culture incubator until sufficient cells were separated from the flask. Subsequently, trypsin was inactivated by CGM, the cell suspension was assembled, and the viable cell count was measured using Trypan blue stain before adjusting the cell concentration to 50,000 cells/mL. Last, the cells were cultivated in a 96-well plate at 5000 cells/well. The cells were left to attach and accommodate the whole night before making any tests.
2.8.2. Cytotoxicity Test. A concentration-dependent cytotoxicity experiment was performed for the test substances for equivalent concentrations of DOX (1, 10, 30, 60, and 90 mcg/µL) under 7.4 pH conditions, where 100 µL of the test medium was used per well. After overnight incubation with the test conditions, 20 µL of MTS reagent was added to the whole wells and then incubated for 1 h in a cell culture incubator. Finally, the absorbance was determined at 490 nm by a plate reader.

3. RESULTS AND DISCUSSION

Doxorubicin is a potent anticancer drug that shows inhibitory activity on various pathways of cancer cell growth. However, this potent activity is accompanied by serious side effects. One of the important side effects is cumulative cytotoxicity caused by the formation of reactive intermediates and free radicals. D-Limonene is well known to have antioxidant activity and the ability to reduce the formation of harmful free radicals. Moreover, we took advantage of the enhanced permeability and retention phenomena (EPR) in the cancer tissues to develop various self-assembled nanoparticles that can passively target cancer cells loaded with DOX and D-limonene. In this project, we developed three types of self-assembled nanoparticles (nanoemulsion, niosomes, and polylactide nanoparticles) to study the capability of loading DOX and D-limonene in their cavities, the release profile of DOX, and its antioxidant and anticancer activity.

The preparation of the O/W nanoemulsion was based on two well-known surfactants, which are Tween 80 and Span 80. The reason behind choosing these surfactants is their high biocompatibility and low cytotoxicity. Span 80 is considered a lipophilic emulsifying agent, whereas Tween 80 is a hydrophilic one, and it is more soluble in water than in oil. To obtain a stable nanoemulsion, it needs to have a balance between the hydrophilic and lipophilic properties. Therefore, various mixtures of Span 80 and Tween 80 were used to have the appropriate HLB value. Low-HLB surfactants form a water-in-oil emulsion while high-HLB surfactants form an oil-in-water emulsion. Various studies have shown that surfactants with an HLB value of greater than 10 generally stabilize oil-in-water nanoemulsions. Herein, the HLB was adjusted to 12 according to the previous optimized study. D-Limonene was used as an antioxidant as explained previously to be included in the nanoemulsion formula, and we used the ultrasonic emulsification technique to produce a low-size and homogeneous nanoemulsion. Therefore, various O/W nanoemulsions were prepared with different amounts of mixed surfactant (500, 1000, and 1500 mg) containing a fixed amount of À-limonene (200 mg), and then the formed nanoparticles were analyzed by dynamic light scattering (DLS) to obtain the mean particle size with the polydispersity index (PDI). Table 1 demonstrates the size of the formed nanoemulsion concerning the various amounts of the mixed surfactants.

It was observed that the smallest particle size was obtained when 500 mg of the mixed surfactant was used with a hydrodynamic size of 34 nm. Therefore, this nanoemulsion was used to prepare the DOX-loaded nanoemulsion by adding 2 mg of DOX to the aqueous phase, obtaining a nanoemulsion with a particle size of 52 nm; this increase in size is probably due to the encapsulation of DOX in the core of the formed micelles, as has been observed previously in the literature. The formed nanoemulsion showed good polydispersity with a PDI of less than 0.3. Moreover, they were characterized by zeta potential analysis, obtaining −40 and −33.8 mV for the blank nanoemulsion and DOX-loaded nanoemulsion, respectively, which indicate a high surface charge repulsion that increases the nanoemulsion stability. To confirm the morphology of the formed nanoemulsion, a TEM analysis was performed. As we can observe in the TEM image of the DOX-loaded nanoemulsion in Figure 1, spherical nanoparticles were obtained with a mean size of 48 nm, which coincides with the DLS results.

The second developed self-assembled nanoparticles to encapsulate À-limonene and DOX are niosomes. Niosomes have a vesicular structure similar to liposomes but without the utilization of phospholipids. They are less expensive and more stable nanostructures compared to liposomes. They are formed from non-ionic surfactant vesicles (in our case, Tween 80) with the incorporation of cholesterol. Herein, we developed DOX-loaded niosomes containing À-limonene using the film-hydration method. The formed niosomes were characterized by DLS, zeta potential, and TEM. The DLS results showed the formation of blank niosomes 165 nm in size and 180 nm DOX-loaded niosomes. Both niosomes showed good polydispersity with a PDI of less than 0.3. The bigger size of niosomes in comparison to the nanoemulsion was expected as they are considered multi-bilayer nanoparticles. Moreover, the zeta potential analysis showed high negative values, with −32 and −37.29 mV for blank niosomes and DOX-loaded niosomes, respectively, indicating the high stability of the nanoformulations. Finally, the TEM image of the DOX-loaded niosomes (Figure 2) confirms the formation of perfect spherical vesicles with a mean size of 170 nm, which is a little less than the DLS results due to the shrinkage of the niosomes by the vacuum environment of the TEM conditions.

The third self-assembled nanoparticles developed for loading DOX and À-limonene is the poly-(DL)-lactide (PDLLA) nanoparticles. Poly-lactide is a synthetic polymer that is biocompatible and has obtained FDA approval for its application in drug delivery. We have previously developed a preparation method for PDLLA nanoparticles with a concise size in our research group. Herein, the PDLLA polymer was loaded with DOX and À-limonene, forming the nanoparticles using the nanoprecipitation technique. These nanoparticles were also characterized by DLS, zeta potential, and TEM. In this case, the blank PDLLA nanoparticles have 250 nm in size with a PDI value of 0.28 and zeta potential of −23.86 mV, which indicates good dispersity and stability. Upon incubation with DOX, the size increased slightly to 257 nm and reached PDI of 0.153, which indicates an improvement in polydispersity. Moreover, an improvement in the zeta potential of −35 mV has been observed. Moreover, the TEM image of DOX-loaded PDLLA nanoparticles (Figure 3) confirms the formation of the spherical nanoparticles with an average size of 240 nm.
The three developed DOX-loaded self-assembled nanoparticles showed good polydispersity with a PDI of less than 0.3 with the lowest PDI in the case of the niosomes. Moreover, the zeta potential analysis indicates that all self-assembled nanoparticles have high stability with a zeta potential above −30 mV, which will prevent the aggregation of the nanoparticles due to the repulsion of the surface charge. The nanoemulsion formula showed the smallest size of nanoparticles with a size of 52 nm. Table 2 summarizes the hydrodynamic size, PDI, and zeta potential results of all the developed nanoparticles. We have successfully prepared three different nanoformulations commonly used in the drug delivery and pharmaceutical industries. To our knowledge, there are no previous reports that studied the differences in drug loading capacity, the release profile of the encapsulated drug, and biological activity between these nanoformulations. Moreover, this comparison can provide sufficient information to identify the most successful formula to encapsulate doxorubicin and D-limonene. Therefore, we moved to analyze the effect of the size and composition of the nanoparticles on loading capacity, in vitro drug release, antioxidant capability, and anticancer activity.

The loaded amount of DOX and D-limonene in the developed nanoparticles was determined using spectrophotometry. A calibration curve of DOX was constructed at $\lambda_{\text{max}} = 485$ nm, another calibration curve of D-limonene was built at $\lambda_{\text{max}} = 220$ nm, and then the loading efficiency (%) was determined. In the case of NE-DOX, the loading efficiencies of...
DOX and d-limonene were 75.8 and 88.5%, respectively, followed by niosome-DOX, which was 62.8% for DOX and 86% for d-limonene, and lastly, in the case of PDLLA-DOX, the DOX and d-limonene loading efficiencies were 50.2 and 82%, respectively. The drug loading efficiency in the nanoparticles depends on various parameters including drug solubility in the nanoparticles, molecular weight, chemical interactions between the drug and the component of the nanoparticles, and the size of the nanoparticles.

In this project, the nanoemulsion demonstrated the highest loading efficiency. This could be attributed to the large surface area of the nanoemulsion, good chemical interaction, and solubility of DOX in the core of the nanoemulsion as both have proper hydrophilic properties.

For effective anticancer activity, DOX should be released from the core of the nanoparticles. For that reason, we have studied the release profiles of DOX from different developed nanoparticles at pH 7.4. In Figure 4, we can observe that almost the initial release of DOX from the three types was the same with some delay in the DOX-loaded nanoemulsion. The highest release was in the case of the DOX-loaded PDLLA nanoparticles with a 98% release of DOX, followed by the nanoemulsion with an 88% release and then the DOX-loaded niosomes with a 66% maximum release of DOX after 24 h. It can be observed that the sustained release behavior of DOX was released after 10 h. The differences in the in vitro release profile are due to the chemical interaction between DOX and the core of the different developed nanoparticles.

The possible release mechanisms of DOX from the developed nanoparticles could be attributed to the desorption of the drug from the surface of the nanoparticles, diffusion of the drug through the pores of the nanoparticle matrix, and erosion of the nanoparticle matrix. Release kinetic evaluation is important to understand the release behavior of the drug. The in vitro release profiles of DOX were assessed utilizing various kinetic models to find the best sustained-release model. The selection of the model was based on the calculated linear regression ($R^2$) and the Akaike information criterion (AIC). The most appropriate model should demonstrate an $R^2$ close to 1 and the lowest value of AIC. In our study, the most fitted kinetic release model for all developed nanoparticles was Peppas–Sahlin$^{47}$ with an obtained $R^2$ of above 0.99 and the lowest AIC. The Peppas–Sahlin model explains that the drug release mechanism happens through Fickian diffusion ($k_1$) and

| nanoparticles               | nanoemulsion (NE) | niosomes | PDLLA nanoparticles |
|-----------------------------|-------------------|----------|---------------------|
|                             | NE-Blank          | NE-DOX   | Niosome-Blank       | Niosome-DOX     | PDLLA-Blank | PDLLA-DOX |
| particle size (nm)          | 34                | 52       | 151.5               | 197             | 250         | 257       |
| PDI                         | 0.294             | 0.265    | 0.197               | 0.103           | 0.28        | 0.153     |
| zeta potential (mV)         | −40               | −33.82   | −32                 | −37.29          | −23.86      | −35       |

Figure 3. TEM image of the DOX-loaded PDLLA nanoparticles.

Figure 4. In vitro release profiles of DOX from the DOX-loaded niosome (black line), DOX-loaded nanoemulsion (red line), and DOX-loaded PDLLA NPs (blue line).
the relaxation of the polymer chain \( (k_r) \). When the \( k_r \) value is higher than \( k_p \), as in our case, the Fickian diffusion is the predominant factor in the drug release. Therefore, all developed DOX-loaded nanoparticles provide a sustained release behavior of DOX by the Fickian diffusion mechanism.

After the successful formation and loading of DOX and the sustained-release effect of DOX from the developed nanoparticles, their antioxidant activity was evaluated using the DPPH assay. The DPPH test is one of the most popular and frequently used methods for assessing the antioxidant activity of pure compounds. The method is simple, relatively inexpensive, quick, and efficient.\(^{27}\) DPPH is a stable free radical with a deep purple color and strong absorption of over 517 nm. The antioxidant reacts with the stable DPPH by donating an electron or a hydrogen atom and converting it to the reduced form of DPPH with the color changed to pale yellow. In this study, a DPPH activity assay was applied to determine the antioxidant capabilities of \( \text{d}-\)limonene and NPs loaded with DOX. \( \text{d}-\)Limonene is a well-known antioxidant that can reduce free radical formation and scavenge free radicals. Therefore, \( \text{d}-\)limonene is responsible for the free radical scavenging activity of the NPs loaded with DOX, which was spectrophotometrically evaluated. As shown in Table 3, it was noticed that these NPs retained the antioxidant activity of \( \text{d}-\)limonene, while DOX was loaded inside the NPs. Thus, these NPs, with their antioxidant activity, are highly expected to reduce the cardiotoxic effect of DOX caused by its generated free radicals.

The anticancer activity of the various DOX-loaded nanoparticles was investigated on liver cancer cells (HepG2) and liver normal cells (LX2). The cells were incubated with the loaded nanoparticles for 24 h; the viability test was evaluated using an MTS assay. Figure 5 shows the viability percentage of HepG2 and LX2 cells after incubation with DOX and the DOX-loaded nanoparticles at different concentrations. As can be noticed in Figure 5A, all tested compounds showed concentration-dependent anticancer activity. Moreover, all the DOX-loaded nanoparticles showed good anticancer activity with the highest activity in the case of the DOX-loaded PDLLA nanoparticles at all used concentrations. The results agreed with the release study as the highest release was in the case of DOX-loaded PDLLA nanoparticles. In addition, the DOX-loaded nanoemulsion showed excellent anticancer activity related to the DOX-loaded PDLLA nanoparticles. The effect of the developed nanoparticles on the LX2 liver normal cells demonstrated much less toxicity in comparison to doxorubicin as shown in Figure 5B. This could be attributed to DOX showing toxicity on normal cells due to the formation of free radicals. However, in this project, the encapsulation with \( \text{d}-\)limonene produces antioxidant activity that reduces the production of free radicals and decreases cytotoxicity as shown in the viability of the LX2 cells.\(^{30}\) Moreover, the passive targeting of the nanoparticles on tumor cells could be more investigated in vivo to take the advantage of the enhanced permeability and retention (EPR) phenomena that are found in cancer tissues.\(^{55}\) Therefore, we can conclude that both nanocarriers are good candidates for the effective delivery of DOX with \( \text{d}-\)limonene on liver cancer cells. These nanoparticles will be further studied for in vivo studies to prove the cardioprotective effect of \( \text{d}-\)limonene in combination with DOX.

### Table 3. Percentage of DPPH Scavenger Activity of \( \text{d}-\)Limonene and DOX-Loaded Nanoparticles

| tested compounds | \( \text{d}-\)limonene | NE-DOX | Niosome-DOX | PDLLA-DOX |
|------------------|------------------------|-------|-------------|------------|
| DPPH scavenging activity (%) | 63% | 56.8% | 60.48% | 59.57% |

A combinational system of \( \text{d}-\)limonene and the anticancer agent doxorubicin was successfully developed by encapsulating them into three types of self-assembled nanoparticles (nanoemulsion, niosomes, and PDLLA nanoparticles). The developed nanoparticles have been successfully characterized by several physicochemical techniques. The results revealed that DOX-loaded nanoemulsions and DOX-loaded PDLLA nanoparticles displayed effective anti-cancer activity and have low toxicity on LX2 normal cells. Moreover, the antioxidant activity of both these nanocarriers could help in reducing the cardiotoxicity of DOX. Therefore, these two nanoparticles could be promising candidates for enhanced anticancer therapy and can be further studied in vivo to prove the cardioprotective effect of \( \text{d}-\)limonene in combination with doxorubicin.

**Figure 5.** (A) Percent viability of HepG2 cancer cells. (B) Percent viability of LX2 normal cells treated with various concentrations of DOX and DOX-loaded nanoparticles in comparison to control.
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REFERENCES

(1) Yadav, S.; Sharma, A. K.; Kumar, P. Nanoscale Self-Assembly for Therapeutic Delivery. Frontiers in Bioengineering and Biotechnology 2020, 8, 127.
(2) Grzelczak, M.; Vermant, J.; Forst, E. M.; Liz-Marzán, L. M. Directed Self-Assembly of Nanoparticles. ACS Nano 2010, 4, 3591–3605.
(3) Varma, L. T.; Singh, N.; Gorain, B.; Choudhury, H.; Tambuwala, M. M.; Kesharwani, P.; Shukla, R. Recent Advances in Self-Assembled Nanoparticles for Drug Delivery. Curr. Drug Delivery 2020, 17, 279–291.
(4) Ghosh, S. K.; Böker, A. Self-Assembly of Nanoparticles in 2D and 3D: Recent Advances and Future Trends. Macromol. Chem. Phys. 2019, 220, 1900196.
(5) Yadav, A.; Gerislioglu, B.; Ahmadivand, A.; Kaushik, A.; Cheng, G. J.; Ouyang, Z.; Wang, Q.; Yadav, V. S.; Mishra, Y. K.; Wu, Y.; Liu, Y.; Ramakrishna, S. Controlled self-assembly of plasmon-based photonic nanocrystals for high performance photonic technologies. Nano Today 2021, 37, No. 101072.
(6) Lombardo, D.; Calandra, P.; Pasqua, L.; Magazù, S. Self-Assembly of Organic Nanomaterials and Biomaterials: The Bottom-Up Approach for Functional Nanostructures Formation and Advanced Applications. Materials 2020, 13, 1048.
(7) Amadi, E. V.; Venkataraman, A.; Papadopoulos, C. Nanoscale self-assembly: concepts, applications and challenges. Nanotechnology 2022, 33, 132001.
(8) Gholami, A.; Hashemi, S. A.; Yousefi, K.; Mousavi, S. M.; Chiang, W.-H.; Ramakrishna, S.; Marzadoodest, S.; Alizadeh, A.; Omidiﬁr, N.; Bebbudi, G.; Babapoor, A.; Li, X. 3D Nanostructures for Tissue Engineering, Cancer Therapy, and Gene Delivery. J. Nanomater. 2020, 2020, 1–24.
(9) Kittana, N.; Assali, M.; Zimmermann, W.-H.; Liaw, N.; Santos, G. L.; Rehman, A.; Lutz, S. Modulating the Biomechanical Properties of Engineered Connective Tissues by Chitosan-Coated Multilayer Carbon Nanotubes. Int. J. Nanomed. 2021, 16, 989–1000.
(10) Vincent, M. P.; Navidzadeh, J. O.; Bobbala, S.; Scott, E. A. Leveraging self-assembled nanobiomaterials for improved cancer immunotherapy. Cancer Cell 2022, 40, 255–276.
(11) Huang, S.; Song, Y.; He, Z.; Zhang, J.-R.; Zhu, J.-J. Self-assembled nanomaterials for biosensing and therapeutics: recent advances and challenges. Analyst 2021, 146, 2807–2817.
(12) Assali, M.; Almasri, M.; Kittana, N.; Alsouqi, D. Covalent Functionalization of Graphene Sheets with Different Moieties and Their Effects on Biological Activities. ACS Biomater. Sci. Eng. 2020, 6, 112–121.
(13) Carmona-Ribeiro, A. Self-Assembled Antimicrobial Nanomaterials. Int. J. Environ. Res. Public Health 2018, 15, 1408.
(14) Assali, M.; Shawahna, R.; Alhawareen, R.; Najareh, H.; Rabaya, O.; Faroun, M.; Ziyoud, A.; Hilal, H. Self-assembly of diclofenac prodrug into nanomicelles for enhancing the anti-inﬂammatory activity. RSC Adv. 2021, 11, 22433–22438.
(15) Assali, M.; Zohud, N. Design of multicomponent indothemacin-paracetamol and famotidine loaded nanoparticles for sustained and effective anti-inflammation therapy. Drug Dev. Res. 2021, 82, 448–457.
(16) Zaid, A. N.; Hassan, M.; Jaradat, N.; Assali, M.; Al-Abbassi, R.; Akkilany, A.; Abulateefeh, S. R. Formulation and characterization of combretastatin A4 loaded PLGA nanoparticles. Mater. Res. Express 2019, 6, 1250d7.
(17) Assali, M.; Kittana, N.; Qasem, S. A.; Adas, R.; Saleh, D.; Arar, A.; Zohud, O. Combretastatin A4-camptothecin micelles as combination therapy for effective anticancer activity. RSC Adv. 2019, 9, 1055–1061.
(18) Taymaz-Nikerel, H.; Karabekmez, M. E.; Eraslan, S.; Kirdar, B. Doxorubicin induces an extensive transcriptional and metabolic rewiring in yeast cells. Sci. Rep. 2018, 8, 13672.
(19) Renu, K.; Aberish, V. G.; PB, T. P.; Aranachalam, S. Molecular mechanism of doxorubicin-induced cardiomyopathy – An update. Eur. J. Pharmacol. 2018, 818, 241–253.
(20) Zhang, S.; Liu, X.; Bawa-Khalfe, T.; Liu, L.-S.; Lyu, Y. L.; Liu, L. F.; Yeh, E. T. H. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. Nat. Med. 2012, 18, 1639–1642.
(21) Gorini, S.; De Angelis, A.; Berrino, L.; Malara, N.; Rosano, G.; Ferraro, E. Chemotherapeutic Drugs and Mitochondrial Dysfunction: Focus on Doxorubicin, Trastuzumab, and Sunitinib. Oxid. Med. Cell. Longevity 2018, 2018, 1–15.
(22) Vincent, D. T.; Ibrahim, Y. F.; Espey, M. G.; Suzuki, Y. J. The role of antioxidants in the era of cardio-oncology. Cancer Chemother. Pharmacol. 2013, 72, 1157–1168.
(23) Zhang, L.; Zhu, K.; Zeng, H.; Zhang, J.; Pu, Y.; Wang, Z.; Zhang, T.; Wang, B. Resveratrol and Doxorubicin Induced-Cardiotoxicity Interactions: Evidence from In Vitro and In Vivo Studies. Oxid. Med. Cell. Longevity 2018, 2018, 1–24.
(24) Shi, C.; Wu, H.; Xu, K.; Cai, T.; Qin, K.; Wu, L.; Cai, B. Liqueiritigenin-Loaded Submicron Emulsion Protects Against Doxorubicin-Induced Cardiotoxicity via Antioxidant, Anti-Inflammatory, and Anti-Apoptotic Activity. Int. J. Nanomed. 2020, 15, 1101–1115.
(25) Wu, T. I.; Won, J. E.; Lee, C. M.; Lee, J.-W.; Kang, T. H.; Shin, B. C.; Han, H. D.; Park, Y.-M. Efficacy of Combination Therapy with Linalool and Doxorubicin Encapsulated by Liposomes as a Two-in-One Hybrid Carrier System for Epithelial Ovarian Carcinoma. Int. J. Nanomed. 2020, 15, 8427–8436.
(26) Himed, L.; Mermiz, S.; Monteagudo-Olivan, R.; Barkat, M.; Coronas, J. Antioxidant activity of the essential oil of citrus limon before and after its encapsulation in amorphous SiO2. Sci. Afr. 2019, 6, No. e00181.
(27) Roberto, D.; Micucci, P.; Sebastiani, T.; Graciela, F.; Anesini, C. Antioxidant Activity of Limonene on Normal Murine Lymphocytes: Relation to H2O2Modulation and Cell Proliferation. Basic Clin. Pharmacol. Toxicol. 2009, 38.
(28) Cirmi, S.; Mauger, A.; Ferlazzo, N.; Ganganzi, S.; Calapai, G.; Schumacher, U.; Navarra, M. Anticancer Potential of Citrus Juices and Their Extracts: A Systematic Review of Both Preclinical and Clinical Studies. Front. Pharmacol. 2017, 8, 420.
(30) Rehman, M. U.; Tahir, M.; Khan, A. Q.; Khan, R.; Oday, O. H.; Lateef, A.; Hassan, S. K.; Rashid, S.; Ali, N.; Zeeshan, M.; Sultana, S. d-limonene suppresses doxorubicin-induced oxidative stress and inflammation via repression of COX-2, iNOS, and NF-kB in kidneys of Wistar rats. Exp. Biol. Med. 2014, 239, 465–476.

(31) Karman, A. P.; Ebeler, S. E.; Nittin, N.; Dungan, S. R. Partitioning, solubility and solubilization of limonene into water or short-chain phosphatidylcholine solutions. J. Am. Oil Chem. Soc. 2021, 98, 979–992.

(32) Estanqueiro, M.; Amaral, M. H.; Conceição, J.; Sousa Lobo, J. M. Nanotechnological carriers for cancer chemotheraphy: The state of the art. Colloids Surf., B 2015, 126, 631–648.

(33) Varshosaz, J.; Hassanzadeh, F.; Sadeghi-aliabadi, H.; Larian, Z.; Rostami, M. Synthesis of Pluronic® F127-poly (methyl vinyl ether-alt-maleic acid) copolymer and production of its micelles for doxorubicin delivery in breast cancer. Chem. Eng. J. 2014, 240, 133–146.

(34) Kumar, G. P.; Rajeshwarrao, P. Nonionic surfactant vesicular systems for effective drug delivery—an overview. Acta Pharmaceutica Sinica B 2011, 1, 208–219.

(35) Uchegbu, I. F.; Florence, A. T. Non-ionic surfactant vesicles (niosomes): Physical and pharmaceutical chemistry. Adv. Colloid Interface Sci. 1995, 58, 1–55.

(36) Di Franceso, M.; Celia, C.; Cristiano, M. C.; d’Avanzo, N.; Ruozzi, B.; Miricciou, C.; Cosco, D.; Di Marzio, L.; Fresta, M. Doxorubicin Hydrochloride-Loaded Nonionic Surfactant Vesicles to Treat Metastatic and Non-Metastatic Breast Cancer. ACS Omega 2021, 6, 2973–2989.

(37) Babadi, D.; Dadashzadeh, F.; Osouli, M.; Abbasian, Z.; Daryabari, M. S.; Sadrai, S.; Haeri, A. Biopharmaceutical and pharmacokinetic aspects of nanocarrier-mediated oral delivery of poorly soluble drugs. Journal of Drug Delivery Science and Technology 2021, 62, No. 102324.

(38) Kumari, A.; Yadav, S. K.; Yadav, S. C. Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Surf., B 2010, 75, 1–18.

(39) Tong, R.; Gabrielson, N. P.; Fan, T. M.; Cheng, J. Polymeric nanomedicines based on poly(lactide) and poly(lactide-co-glycolide). Current Opinion in Solid State and Materials Science 2012, 16, 323–332.

(40) Ayen, W. Y.; Kumar, N. In Vivo Evaluation of Doxorubicin-Loaded (PEG3)-PLA Nanopolymerosomes (PolyDoxSome) Using DMBA-Induced Mammary Carcinoma Rat Model and Comparison with Marketed LipoDox™. Pharm. Res. 2012, 29, 2522–2533.

(41) Li, P.-H.; Chiang, B.-H. Process optimization and stability of d-limonene-in-water nanoemulsions prepared by ultrasonic emulsification using response surface methodology. Ultrason. Sonochem. 2012, 19, 192–197.

(42) Abdelbary, G.; El-Gendy, N. Niosome-encapsulated gentamicin for ophthalmic controlled delivery. AAPS PharmSciTech 2008, 9, 740–747.

(43) Assali, M.; Zaid, A. N.; Bani-Odeh, M.; Faroun, M.; Muzaffar, R.; Sawalha, H. Preparation and characterization of carvedilol-loaded poly(D,L)-lactide nanoparticles/microparticles as a sustained-release system. Int. J. Polyam. Mater. Polyam. Biomater. 2017, 66, 717–725.

(44) Shen, J.; Burgess, D. J. In vitro dissolution testing strategies for nanoparticle drug delivery systems: recent developments and challenges. Drug Delivery Transl. Res. 2013, 3, 409–415.

(45) Zhang, Y.; Huo, M.; Zhou, J.; Zou, A.; Li, W.; Yao, C.; Xie, S. DDSolver: An Add-In Program for Modeling and Comparison of Drug Dissolution Profiles. AAPS J. 2010, 12, 263–271.

(46) Rigter, P. L.; Peppas, N. A. A simple equation for description of solute release I. Fickian and non-fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs. J. Controlled Release 1987, 5, 23–36.

(47) Peppas, N. A.; Sahin, J. J. A simple equation for the description of solute release. III. Coupling of diffusion and relaxation. Int. J. Pharm. 1989, 57, 169–172.