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Formulation and Biocompatibility of Microemulsion-Based PMBN as an Efficient System for Paclitaxel Delivery

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Abstract

Introduction: Paclitaxel (PTX), an effective chemotherapeutic drug, is widely used to treat several types of cancers. However, its use is limited due to poor water solubility resulted in poor bioavailability. One of the main ways to increase the efficacy and endurance of medications depends on the carrier that used for it. In the current study, the microemulsions (MEs) were investigated based on Poly [2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-nitrophenyl-oxycrabonyl poly ethylene glycol-methacrylate (ME-ONP)] (PMBN) ability as a carrier for PTX to increase the half-life and its bioavailability. Also, the physicochemical properties of the ME system were evaluated.

Materials and Methods: PMBN, tween 80, triacetin, and glycerol were used as the drug carrier, surfactant, oil phase, and co-surfactant, respectively. The hemolytic activity was characterized using the RBC hemolysis assay to evaluate the blood compatibility of the MEs. In addition, the in vitro cytotoxic effect of PMBN-PTX-ME and PTX on MCF-7, 4T1, and HFF-2 cell lines was performed. PI and Annexin-V dyes were used for cell apoptosis.

Results: The conductivity of ME evaluated and the results showed 432 to 589 µS/cm. *In vitro,* the drug release study revealed that PTX has controlled release at different pH levels. Refractive Index (RI) and % transmittance of the MEs remained ranging from 1.43 to 1.53, and 89% to 98%, respectively. The MTT assay determined that PMBN-ME without PTX had no significant toxicity on HFF-2, MCF-7, and 4T-1 cell lines. Based on the apoptosis assay, treated cell lines with PMBN approximately remained alive.

Conclusions: The results revealed that ME-based on PMBN would be a promising drug delivery system for PTX drug delivery.

Keywords: Biocompatibility, PMBN polymer, Microemulsion, Paclitaxel

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Introduction

Paclitaxel (PTX) is an antineoplastic chemotherapeutic drug and is used for the treatment of many types of cancers including breast, ovarian, lung, bladder, prostate, melanoma, esophageal, AIDS-related Kaposi's sarcoma, as well as other types of solid tumor cancers. It can damage the microtubules structure induces apoptosis through a JNK-dependent pathway.[1] The major limitation of PTX administration in clinical applications is its poor water solubility (~0.4 µg/mL) and poor cellular permeability[2-4]; thus, for clinical administration, Cremophor1 EL (poly-oxyethylated castor oil) and ethanol (50:50 v/v) have been used in the commercial taxol formulation.[5] The use of heterogeneous non-ionic surfactant Cremophor EL causes serious side effects including anaphylactic hypersensitivity reactions, lipoprotein patterns, hyperlipidemia, reversal of P-glycoprotein (P-gp) activity, nephrotoxicity, neurotoxicity and cardiotoxicity.[6] In addition, Cremophor® EL can change the *in vivo* pharmacokinetic profile of PTX. It also can induce systemic and hematological toxicity through an oxidative stress-based mechanism, mainly inducing an oxidative damage.[7] PTX formulated in Cremophor EL has an unpredictable non-linear plasma pharmacokinetics. Another limitation of the clinical application of PTX is its substrate of P-gp, which can pump PTX out of cells and lead to drug resistance. Thus; some P-gp inhibitors like verapamil and PSC 833 were co-administered with PTX. Unfortunately, the results were disappointing to overcome drug resistance.[8]

To improve the therapeutic efficacy of PTX, it is necessary to develop a new drug delivery system to achieve the minimal side effects and improve its efficiency.[9,10] Today, an efficient drug delivery system based on nano-formulations of drugs can be used. These systems have several advantages such as enhancement of dissolution rate that can result in faster onset of action; elimination of toxicity; decrease in refractive

particle size, which enables to reduce in drug dosages and side effects as well as improving pharmacokinetic profile.[4,11-15] Many nano-formulation systems are used for anticancer drug delivery to selectively deliver various anticancer compounds to the tumor in a passive targeting manner.[8,16,17]

In recent years, biocompatible polymer-based drug delivery have been widely used for cancer treatment. Several studies introduce some new biopolymers such as 2-methacryloyloxyethyl phosphorylcholine (MPC) which contain the phosphorylcholine groups as bio-membranes which has excellent biocompatibility, lack of protein adsorption and platelet adhesion.[18] N-butyl methacrylate (n-BMA) units can behave as hydrophobic groups. Poly [2-methacryloyloxyethyl phosphoryl choline (MPC)-co-n-buthyl methacrylate (BMA)-co-p-nitrophenyl-oxycrabonyl poly ethylene glycol-methacrylate (ME-ONP)] called PMBN is a biocompatible terpolymer, which can be dissolved in water.[19] Due to incorporation of hydrophobic materials by n-BMA, it can be used as a carrier for PTX.[20] Using n-BMA polymer functionalized with ester groups as an excellent MEs system were designed and prepared for PTX (Figure 1).

MEs have been used in several pharmaceutical applications such as parenteral delivery,[21] oral drug delivery,[22] topical drug delivery,[23] ocular[24] and pulmonary delivery.[25] In the present study, MEs were formulated based PMBN as a PTX carrier, Tween 80 as a surfactant and glycerol as a co-surfactant. In order to investigate the physicochemical properties of MEs formulated, several parameters were evaluated including particle sizes, ζ-potential, atomic force microscopy (AFM), differential scanning calorimetry (DSC), PTX encapsulation efficiency, conductivity, refractive index (RI), the transmittance (%) and pH of the formulations. The *in vivo* cytotoxicity of microemulsions systems was performed using median lethal dose (LD50) investigations. Also, *in vitro* drug release, blood biocompatibility, as well as the cytotoxicity and apoptosis of the PMBN-ME and PMBN-PTX-ME were evaluated on MCF-7, 4T1 and HFF-2 cell lines by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay and flow cytometry analysis.[26]

Materials and Methods

Materials

The PMBN polymer was provided by Abnous Daroo Co. Iran. The PTX obtained by Sobhan Oncology Pharmaceutical Company (Iran). Triacetin, polyoxyethylene sorbitan mono-oleate (Tween 80), glycerol, dichloromethane (CH_2CL_2), Dimethyl sulfoxide (DMSO), PBS (phosphate buffer saline), sodium dodecyl sulfate (SDS) and MTT reagent were purchased from Sigma Aldrich Co., Germany. All cell lines used in this study were grown in DMEM and RPMI-1640 high glucose medium supplemented with 10% FBS (fetal bovine serum), which was provided by the Pasteur Institute of Iran.

Preparation of PMBN-ME Loaded With PTX

The biocompatible O/W ME system was prepared based on triacetin, Tween 80 and glycerol as oil phase, surfactant and co-surfactant respectively. The PMBN polymer was used as carriers of the hydrophobic drug PTX. One gram (8.33%). Tween 80 and 0.2 g glycerol (1.66%) were added in 7.76 g water (64.66%) and mixed together for 10 minutes as the aqueous phase. Then, to fabricate amid bond between PMBN and PTX, 0.01 g of PTX (0.08%) dissolved in 2 mL dichloromethane which was added to 0.03 g of PMBN polymer (0.25%) dissolved in 2.5 g (20.83%) double-distilled water as drop wise. The mixture of PTX and PMBN polymer was sonicated at 200 W under 40-45°C for 30 minutes (SJIA-950W; Ningbo Yin Zhou Sjia Lab Equipment Co Ltd, Ningbo, China) and then, was added to the aqueous phase (mixture of Tween 80, glycerol and water) under constant vigorous stirring until organic solvent (dichloromethane) was vapored. Finally, O/W ME was formed with titrated last solution with 0.5 g (4.16%) triacetin as dropwise and constant stirring under 40-45°C. The organic solvent was evaporated at room temperature for 45 min. Triacetin was served as oil phase to form the O/W ME system.

P*hysicochemical Characterizations*

FT-IR & UV-Vis Characterization of PMBN Loaded PTX

Fourier transform infrared (FT-IR) spectroscopy of PMBN, PTX and PMBN-PTX-ME were recorded by a spectrometer

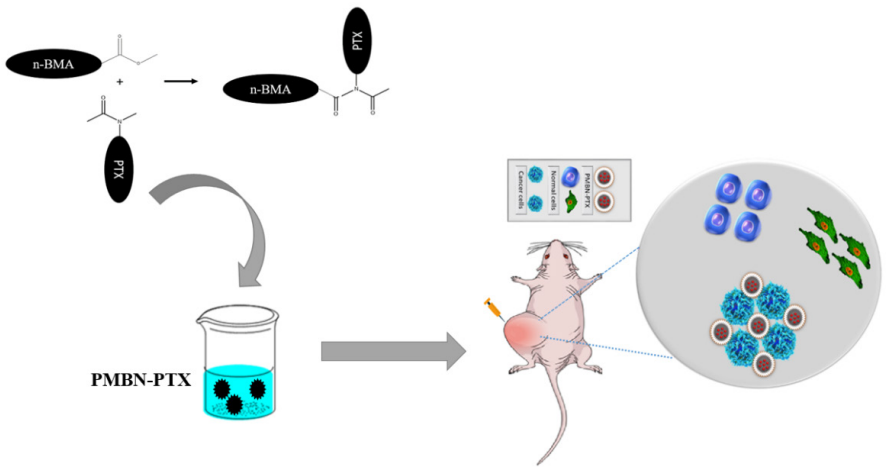

Figure 1. Schematic of PTX binding to PMBN Polymer and Illustration of PMBN-PTX in the Tumor Environment.

(Bruker IFS 66 v/s) at room temperature. Approximately 1.25 mg prepared sample was mixed by 250 mg potassium bromide (KBr) and passed in plate form. The spectra were scanned over the range of 400–4000 cm^{-1}. In addition, the absorption spectrum of the MEs system was examined by UV-Vis spectrophotometer to determine the components of PTX and PMBN in the MEs system formulation.

Particle Size and ζ-Potential Analysis of ME

The mean hydrodynamic size (Z-average) and size distribution (polydispersity index) of the O/W PMBN-ME in the presence and absence of PTX were assessed by Zetasizer nano-series instrument (Malvern Nano ZS®, Malvern Instruments Ltd, Worcestershire, UK) equipped with a solid-state He-Ne laser (λ=633) at 25°C with an angle of 90.0 degrees and the patented non-invasive backscatter technology. A 0.5 mL of ME were diluted with 1.5 mL ultra-pure water in a clean Malvern sample vial. The Z-average, PDI and **ζ**-potential of the ME were measured by Zetasizer Nano-Z (Malvern Instruments Ltd).

Morphology Analysis

The morphology MEs system was examined by AFM (JPK, Nano Wizard 2, Germany). The MEs were diluted by distilled water (1:5 ratio). The dilution ME was added to a mica substrate (1 cm^2) and allowed it to dry for 24 hours until fixing the sample. The AFM analysis was carried out with a Nanoscope IIIa Dimension 3000 atomic force microscope operated in tapping Also, the measurements were performed in the intermittent contact mode.

Differential Scanning Calorimetry

Thermal analysis was performed with a DSC (Mettler Toledo, model Star SW 9.30, Schwerzenbach, Switzerland). Approximately 10-15 mg of MEs samples were added into hermetic aluminum pans and quickly imprinted to prevent water evaporation from ME samples. Meantime, an empty hermetically sealed pan was used as a reference. The ME samples were exposed to a temperature ranging from 30-300°C (the flow rate of temperature at 15°C/min).

Conductivity Measurements

The conductivity meter Metrohm Model 712 was used for the measurement of MEs electric conductivity. This experiment was performed at room temperature (25±1°C). All measurements were repeated three times. In order to check the physical stability of the MEs system, the Z-average was investigated every 15 days for three months at room temperature by dynamic light scattering (DLS).

pH Determination of ME

The pH values for ME were assessed at 25°C by a Corning 220 pH meter (Cole-Palmer, Teddington, UK). This experiment was performed to investigate the influence of ME type and presence or absence of PTX on the pH as well as to compare the values with those of blood plasma.[27]

Refractive Index of ME

The RI of a ME system is a parameter to examine the speed of light transmission through the ME system. The RI for ME was assessed at 25°C using refractometer M46.17/63707 (Higler and Walts Ltd., England, UK).

Limpidity Test (Percent Transmittance)

The transparency of MEs system was determined by measuring the percentage of transmittance using spectrophotometer (Shimadzu, UV-160, Japan).[28] The percentage transmittance of the MEs system was measured at 633 nm with double distilled water taken as blank and three replicates were performed for each sample.[13]

The Paclitaxel Encapsulation Efficiency

The PTX encapsulation efficiency (EE %) of PMBN-PTX-ME was determined from the non-encapsulated free drug according to the described method by Bisht et al.[29] In brief, 50 µL of PMBN-PTX-ME was mixed with 450 µL deionized water and then the solution was diluted with deionized water 50 times. The solution was collected in a tube containing a dialysis membrane with the MW cut-off at 3000 Da, followed centrifuging at 12000 rpm (25°C) for 20 minutes. The free PTX which could pass through the membrane was collected, dried and dissolved in 1 mL dichloromethane and drug content was measured by spectrophotometer (Thermo Fisher Scientific, USA, Madison, model GENESYS™ 10S) at wavelengths of 230 nm. The PTX encapsulation efficiency (EE %) was calculated according to the following formulation:

$$EE\% = \frac{C_0 - C_1}{C_0} \times 100$$

C_0 is the total amount of PTX initially added and C_1 is the non-encapsulated amount of PTX in the ME sample.[30]

In Vitro Drug Release Studies

The *in vitro* PTX release from the PMBN-PTX ME and the free-PTX was carried out in 5% (w/v) tween 80 in sodium phosphate buffer solution media at two different pH release media. The pH 7.4 and pH 5.8 buffer solutions, simulated intestinal fluid and mimicked the cancer cell gap fluid, respectively. Initially, the dialysis tubing (Molecular weight cut-off >12000, HiMedia) was drenched in sterile water or phosphate buffer pH 7.4 overnight. The dialysis bag containing 480-µL suspension of PMBN-PTX ME was incubated in 20 mL PBS of different pH (pH 7.4 and pH 5.8) at 37°C under slight starring (100 rpm).

At predetermined time intervals, 0.5 mL of release solutions were withdrawn and replaced with the same volume of the fresh medium and then, analyzed the drug content at the wavelength of 230 nm according to the standard calibration curve.[31]

In Vitro Cytotoxicity Assay

Cell Culture

MCF-7, 4T1 and HFF-2 cell lines were provided by the Pasteur Institute of Iran. The MCF-7 and 4T1 were cultured in RPMI-1640 medium. HFF-2 was cultured in MDEM medium with 10% Penicillin-Streptomycin (10% Pen-Strep) and 10% heat-

inactivated FBS in an incubator at 37°C under an atmosphere of 5% CO_2 and 80% relative humidity.

Cytotoxicity and Cell Activity Studies

The HFF2, MCF-7, and 4T1 cells were cultured at the density of 6×10^3 cell/well in 96-well plates. After 48 hours (80% confluency), the culture medium was aspirated and replaced with 200 µL of media containing serial dilutions of treatment samples, including different PTX concentrations (0.625, 1.25, 2.5, 5, 10, 20, 40, 80 nM) and 200 µL culture medium as negative control were added into the wells. After incubation for 24 hours at 37°C, the drug-containing media was removed and replaced with 20 µL of the medium, including 5 mg/mL MTT solution, then treated cells were incubated at 37°C for 4 hours. The culture medium was removed and 150 µL of DMSO was added to each well to suspend the dark-blue formazan crystals while vigorously stirring the plates using an automated shaker. Cell viability was quantified by measuring the absorbance of the formazan at the wavelength of 570 nm with a microplate reader (Spectra Max 190).

The data were expressed as the percentages of viable cells compared to the survival of the control group (untreated cells as controls of 100% viability). The data were expressed as the mean ± standard deviation (SD) of five individual measurements.

Hemolysis Assay

Healthy human red blood cells (RBCs) were gifted from the blood bank of the Mousavi Hospital (Zanjan, Iran) which was re-suspended in isotonic PBS until diluted to 10% of their initial concentration.[32,33] Six different mass concentrations of PMBN-ME and PMBN-PTX ME (10, 40, 70, 100, 130 and 160 µg/mL) plus 1% SDS in positive controls and 500 µL PBS in negative controls were added into different micro tubes. Aliquots of 200 µL RBC were added to each tube. Then, 700 µL PBS was added in different tubes and incubated for 4 hours in a 37°C water bath. After incubation, the tubes were centrifuged for 10 minutes at 4000 g at room temperature. Finally, 200 µL of the supernatant was transferred to a 96-well plate. The absorbance was measured at 541 nm using a plate reader. The percentage of hemolysis was calculated as follows:

$$Hemolysis(\%) = \frac{\boldsymbol{A}_{sample} - \boldsymbol{A}_{negative}}{\boldsymbol{A}_{positive} - \boldsymbol{A}_{negative}} \times 100$$

In this equation, A_{sample} is the absorbance of the sample, $A_{positive}$ and $A_{negative}$ are the absorbance of the negative and the positive control samples.

Apoptosis Assay by Flow Cytometry

ApopNexin FITC assay detects the appearance of phosphatidylserine (PS) residues on the outer membrane of the cells which occurs as the early signs of apoptosis. Annexin-V which was frequently used in apoptosis investigation has a high affinity for PS and binds to cells with exposed PS. This means it can be used as a probe for inspected apoptosis. The signs of apoptosis were detected by an ApopNexin FITC apoptosis detection kit (Sigma Annexin-V, BioTek CO). The experiments were performed as described in the protocol of ApopNexin FITC kit. The PMBN-PTX-ME, PMBN, PTX, and the media as control were subjected into the cultured HFF2, MCF-7 and 4T1 cells and incubated for 24 h according to the ApopNexin FITC kit description.

LD50 Assay

The MEs were formulated using different chemicals (triacetin, Tween 80, PMBN and glycerol), which may have different toxic effects. The LD_{50}, as a toxin dose required to kill half of the individuals in a test population, is to evaluate the safety of the MEs system, an acute oral toxicity study was performed in 12 adult mice of an average weight of 30-35 g and were taken in an ideal laboratory condition as per OECD.[34] The LD_{50} assay was carried out at different concentration (ranging from 175 to 5000 mg/kg) of MEs system (PMBN-ME and PMBN-PTX ME), which were orally administered to each animal. All animals were weighed before administration, and one day and one week after the administration. Likewise, during the test, any physical activities and behavioral changes of animals were monitored. If all the animals survived after 24 hours, four other animals were treated at the highest does. If these animals survived too, the LD50 would be more than the dose limit and the test was stopped.

Statistical Analysis

Statistical analysis of the data was performed by the GraphPad Prism 7 software. The experiments were repeated at least three times, and the data were expressed as means ± SD. For normally distributed data, one-way analysis of variance (ANOVA) with repeated measures was used to compare within the same group and Tukey test for correction. Likewise, one-way ANOVA with Dunnett test was used to compare the mean between different groups. For the non-normally distributed data, the Kruskal-Wallis test and Fisher exact test were employed for correction and categorical variables, respectively. The P value < 0.05 and ns were considered as significant and not significant difference, respectively.

Results

Optimum formulation of O/W Microemulsion

The MEs system has an adequate ratio of organic phase (triacetin), aqueous phase (water, surfactant and co-surfactant) and PMBN polymer as a drug carrier. The results obtained by different fractions of ME composition have been summarized in Table 1. The F_4 and F_6 formulations were transparent in appearance. However, the F_4 formulation was selected for further studies, since this formulation has total amount of surfactant and co-surfactant lower than F_6 formulation. This is due to the toxicity of surfactant in high concentration. So, all of the samples were prepared according to the compositions ratio of the F_4 formulation.

Particle Size and ζ-Potential Analysis of Microemulsion

The Z-average and ζ-potential of drug-free ME (F_4) was analyzed by DLS (Table 2), which can be validated by the results of other studies that prepared similar ME formulations.[35] Figure 2a-b shows that the Z-average of the PMBN-PTX-ME and suggestion ME (F_4) were found to be around in 17.07 nm

Table 1. Composition and Evaluation of PMBN-ME Formulations

Formulations	PMBN (%W/W)	Tween 80(%W/W)	Glycerol (%W/W)	Triacetin (%W/W)	Water (%W/W)	State
F_1	0.24	6.40	2.16	4.00	87.20	Cloudy
F_2	0.24	6.40	1.28	3.98	88.10	Slightly Cloudy
F_3	0.24	8	2.40	6.36	83	Slightly Cloudy
F_4	0.24	8	1.60	4.00	86.16	Transparent
F_5	0.24	9.6	3.21	4.00	82.95	Slightly Cloudy
F_6	0.24	9.6	1.92	4.00	84.24	Transparent

Table 2. Evaluation of Particle Size, PDI and ζ-Potential PMBN-PTX-ME Compared to Free-PTX Formulations

Microemulsions Formulations	Particle Size (nm)	PDI	ζ-Potential (mV)
F_4	12.83	0.273	-6.77
PMBN-PTX-ME	17.99	0.359	-12.8

and 12.83 nm, respectively. The results were examined and no significant difference was observed in the Z-average particle size than suggestion ME after loading the drug. For all samples (suggestion ME and PMBN-PTX-ME), PDI values are in the range of 0.273 to 0.117, which indicated greater uniformity in particle size distribution, homogeneity and then their system stability.[36] In addition, ζ-potential of free-drug optimal MEs and PMBN-PTX-ME were determined and there was negative in the range of -6.77 mV to -12.8 mV (Figure 2c-d).

FT-IR & UV-Vis Characterization of PMBN – PTX-ME

The FTIR spectra of PTX, PMBN and PMBN-PTX-ME have been presented in Figure 3a. The FTIR spectroscopy was used to confirm that the PTX was successfully loaded on the PMBN. The FT-IR spectrum of pure PTX showed an absorption peak at 1500 to 1735 cm^{-1} which belonged to the aromatic C-H bonds. Likewise, absorption peaks at 3500 cm^{-1}, 2900 cm^{-1}, 1050 cm^{-1}, and 1630 cm^{-1} were attributed to the aromatic ring (C=C) stretching frequency, aromatic C-H bonds, and C=O amide stretching of PTX respectively. Further, the peak at 1451 cm^{-1} denotes the CH_2 scissoring mode of PTX.[23,24] In the FTIR spectrum of PMBN, the peaks were observed at 1735 cm^{-1} and 3400 cm^{-1} because of C=O amide and O-H groups stretching, respectively. Also, the aliphatic C-H stretch was observed at 2930 cm^{-1}. However, the peaks that appeared at 1086 cm^{-1} and 964 cm^{-1}, respectively, are referred to the $POCH_2$ and N^+ (CH_3).[37] In addition, for PMBN loaded with PTX, , the stretching of O-H groups in PTX spectrum observed at 1750 cm^{-1}. In the PMBN-PTX-ME case, it appeared at 1643 cm^{-1}. Such a shift might point out to the loading of the PTX in the PMBN (Figure 3a).

As all the characteristic absorption peak of PTX can be found in FT-IR spectra of the PMBN-PTX-ME, so, PMBN was successfully coated with PTX. The optical properties of PMBN-PTX ME were examined with UV-Vis absorption

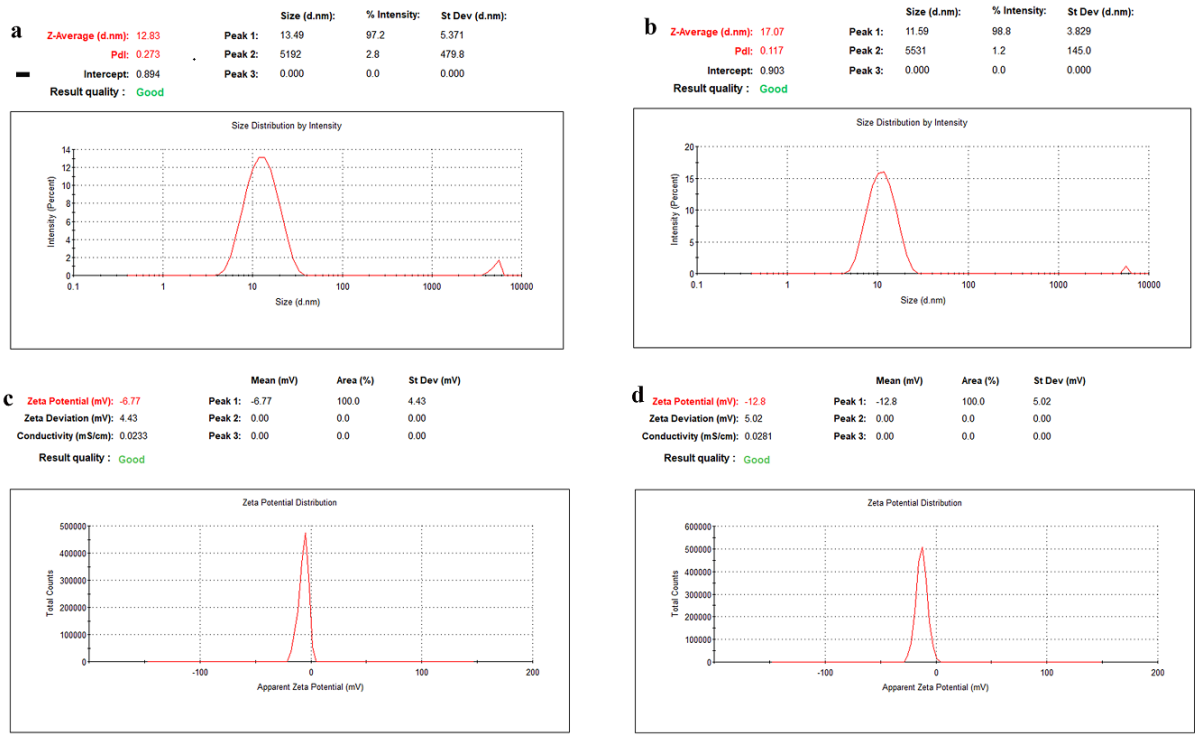

Figure 2. Particle Size Distribution for Free-Drug-ME (F_4) (a) and PMBN-PTX-ME (b), and Zeta Potential Curve Free-Drug-ME (F_4) (c) and PMBN-PTX-ME (d).

spectrophotometer (Figure 3b). The loading of PTX into the PMBN was examined using UV-Vis absorption. The UV-Vis spectrum peaks of the PMBN and PMBN-PTX ME were observed on 229.5 nm and 227.5 nm, respectively.

In the UV-Vis absorption spectrum of PTX, it can be seen that a characteristic absorption band centered at 230 nm, which has been assigned to the π-π^* transition. It can be concluded that the UV-Vis absorption spectrum of the PMBN-PTX ME confirms that the PTX was successfully loaded into PMBN.

Morphology Analysis

The morphology of ME and mechanical properties of the MEs system and assemblies determined by AFM was presented in Figure 4b. The ME particles had spherical shape with a smooth surface.[38] In detail, MEs particles exhibited a globular shape and homogeneous spherical morphology, which corroborated with the data DLS analysis (Table 2). Also, no roughness was observed on the particle surface.

Physicochemical Characterizations of Drug-Free and PMBN-PTX-ME

Encapsulation efficacy quantified according to the standard calibration curve in the concentration range of 2-20 µg/mL with the correlation coefficient of 0.9971. The limit of detection and limit of quantification were found to be 0.21 µg/mL and 0.65 µg/mL, respectively.

About 98.86% of the drug was encapsulated in the PMBN-

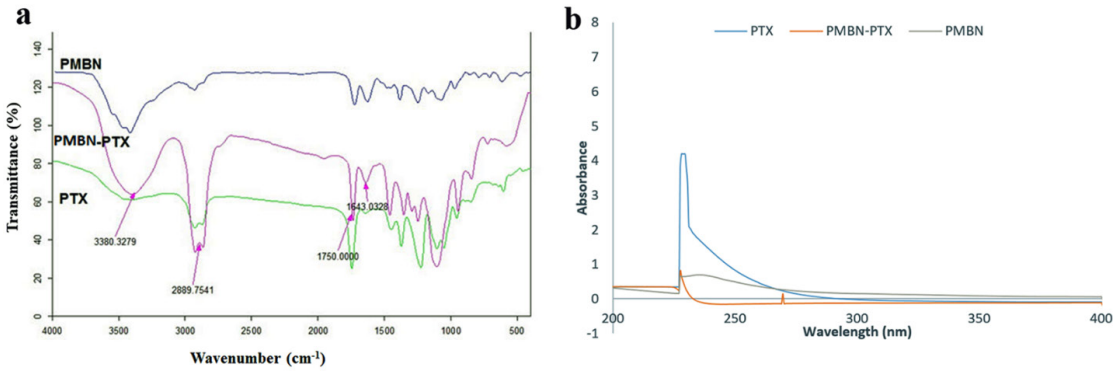

Figure 3. FT-IR Spectra of PMBN, PTX, and PMBN-PTX-ME (a) and UV-Vis Spectra of PTX, PMBN and PMBN-PTX ME (b).

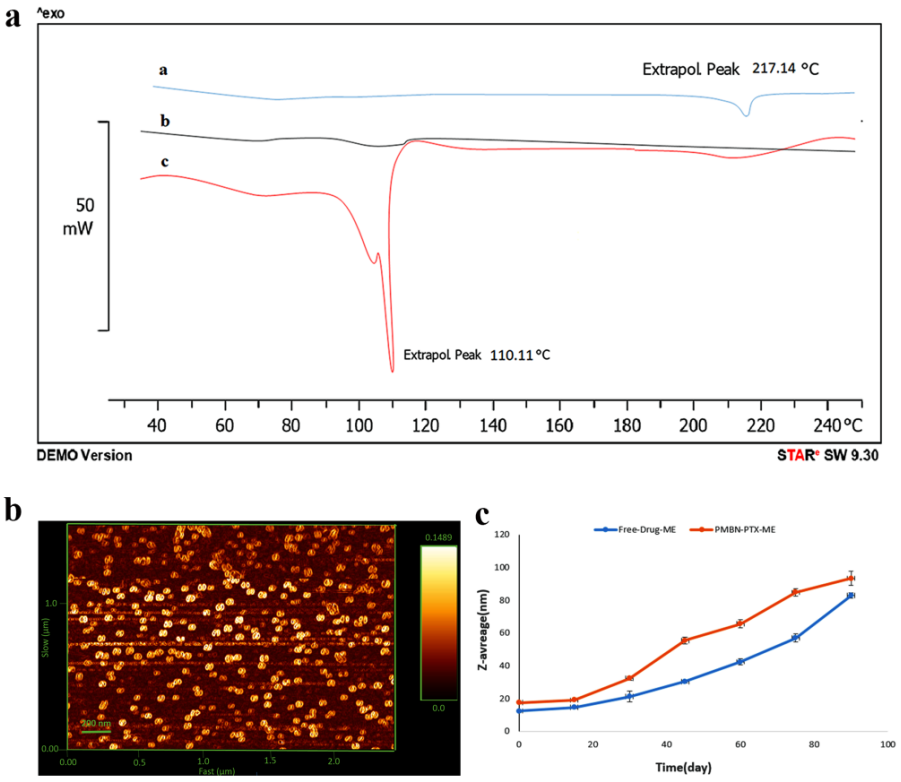

Figure 4. DSC Thermograms of PTX, PMBN-PTX-ME and PMBN (a), AFM Image of a PMBN-PTX-ME Prepared by Optimum Formulation (b) and Stability Curves of Free-Drug-ME and PMBN-PTX-ME (c).

PTX-ME which was determined by measuring the UV absorbance at 230 nm. The physicochemical characterizations of the MEs system were performed by conductivity, % transmittance, pH and RI (Table 3). The electrical conductivity coefficient of drug-free-ME (F_4) and PMBN-PTX-ME was revealed in 432 µS/cm and589 µS/cm, respectively. The high value of conductivity coefficient is characteristic of water in oil-in-water (w/o/w) type of ME.[39,40] The transmittance (%) of the drug-free ME (F_4) and PMBN-PTX ME was in the range of 89-98% with high percentage transmittance.[36] The results revealed that the ME systems were transparent and were considered as a single phase liquid.

The pH value of the PMBN-PTX ME was lower than PMBN-ME which has been attributed to the acidic chemical structure of PTX, which possesses hydroxyl groups in different positions. The RI of drug-free-ME (F_4) and PMBN-PTX-ME was around 1.43-1.53 which is close to the aqueous phase (1.333) and therefore is shown in the oil-in-water (O/W) type of microemulsions over 80% water as the continuous phase. Furthermore, the ME systems (drug-free and PMBN-PTX ME) stability were examined by evaluating their particle structure integrity as well as possibility of aggregation. Therefore, the Z-average of ME systems was monitored for three months as the main factor of the incubation time. As shown in Figure 4c, Z-average of free-drug suggestion MEs (PMBN-ME) and PMBN-PTX ME were not significantly increased throughout the measurement period. On the other hand, the differential scanning calorimetric (DSC) thermograms of the samples (PTX, PMBN, and PMBN-PTX ME) were performed to evaluate the thermal behavior of MEs system. In thermo-gram of PTX, an endothermic peak approximately at 217˚C shows the melting point of PTX. Also, the endothermic peak which appeared at 110˚C indicates the melting point of PMBN.

Figure 4-a shows no visible peak near the PTX and PMBN melting point for the PMBN-PTX ME thermo-gram, which confirms the encapsulation of PTX in ME. The data shows that the PTX and PMBN were amorphous in the ME systems.

In Vitro Drug Release

The dialysis bag method was employed to determine the release profile of the drug from the MEs system. This is the most versatile and popular method to study the drug release from nano carriers.[41] Figure 5 demonstrates the release profile of PTX through the PMBN-PTX-ME at two different pH buffer solutions (pH 7.4 and pH 5.8). In the first step, PTX was burst released from PMBN-PTX ME, which was mainly related to the release of PTX trapped into the aqueous phase.[42,43] In the second step, the PTX was released slower more than the first step and then continued up to 144 hours.

Table 3. Physicochemical Characterization of PMBN-PTX-ME and Optimum Formulation

Formulations	Conductivity (µS/cm)	pH	RI	%Transmittance
F_4	432	6.2	1.43	98
PMBN-PTX-ME	589	5.8	1.53	89

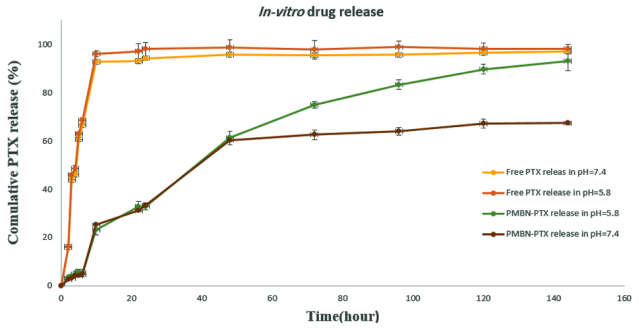

Figure 5. Accumulative Drug Release Profile of Free-PTX and PTX-PMBN-ME on Different pH Conditions.

This controlled release phenomenon is related to a slow rate of the formulation destruction. The cumulative amount of PTX which was released at pH 7.4 at 37˚C, was about 33.20% after 24 hours while 32.92% at pH 5.8 in 24 hours. Hence, after 24 hours there was no significant difference between the PTX release profiles at pH 7.4 and pH 5.8 buffer solutions. However, the percent of released PTX after 144 hours were 67.41% and 93.17% in the same media at pH (7.4) and pH (5.8), respectively. In addition, the free-PTX release was 97% and 98.3% in the same media at pH (7.4) and pH (5.8), respectively.

Cytotoxicity Analysis

For clinical and biomedical applications, cytotoxicity is one of the most important indicators that should be considered for the biocompatibility of any carrier. The cytotoxicity of blank MEs (PMBN-ME) was evaluated on HFF-2, MCF-7, and 4T-1 cell lines at different concentrations (80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 nM). They did not show any cytotoxic effect on the growth of cell lines. The MTT assays were performed at 24 hours to evaluate cell viability. All cells (HFF-2, MCF-7 and 4T-1 cells lines) were treated with different concentrations (80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 nM) of PTX and PMBN-PTX-ME after 24 hours. Untreated cells were used as a negative control.

In all cell lines, the inhibition rate of the PMBN-PTX-ME increased with an increase in the drug concentration from 0.625 nM to 80 nM (Figure 6a-c). Compared with untreated cells, cells exposed to PMBN-ME remained unchanged due to their outstanding biocompatibility of PMBN-ME. In comparison, when incubated with PMBN-PTX-ME, the amount of living cells obviously decreased, which is attributed to the cytotoxic effect of PTX released from the MEs system. Results revealed that cell toxicity is directly commensurate to PTX concentration in a 24 hours period time.

The PMBN-PTX-ME IC_{50} on MCF-7 and 4T1 were found 28 and 22 nM, respectively. Data were analyzed using ANOVA and $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P<0.001$ as considered significant, very significant, and highly significant respectively.

Hemolysis Assay

For clinical and biomedical applications, MEs systems should

move with the circulation into all the organs and tissues of the body. So, it is necessary to evaluate the biocompatibility of the ME systems.[44] The minimal hemolytic activity on human RBCs was indicated which PMBN-ME and PMBN-PTX ME are blood compatible. Blood compatibility was evaluated with *an in vitro* RBC induced hemolysis assay. The blood compatibility of PMBN-ME and PMBN-PTX ME were evaluated at a 10-130 µL concentration range. As shown in Figure 6d, increasing the mass concentration of PMBN and PMBN-PTX-ME up to 70 µg/mL was not significantly different in hemolysis percentage. The PMBN and PMBN PTX-ME in 160 µg/mL mass concentration have the hemolytic activity of 29.67 and 42.71% respectively. The results of the hemolysis assay revealed that the effect of PTX was not significant on the hemolytic activity of PMBN-PTX-ME.

Apoptosis Tests

To determine whether PTX-induce apoptosis, HFF2, MCF-7 and 4T1 cells were treated with PMBN-PTX-ME, PMBN, PTX, and the media as a control at 24 hours according to the manufacturer's protocol. Figure 7 presents a two-dimensional contour diagram of apoptosis (Annexin-V) versus necrosis (PI). As depicted in Figure 7, after 24 hours of incubation of all cells (HFF2, MCF-7 and 4T1 cells) with PMBN, the percent of live cells was significantly higher in comparison to the apoptotic or necrotic cells.

In contrast, the incubation of all cells with blank PTX was shown that the necrotic or apoptotic death in both MCF-7 and 4T-1 cells were significantly higher in comparison to the HFF-2 cells (as normal cells). On the other hand, the incubation of all cells with PMBN-PTX showed that the apoptotic death in MCF-7 cells was significantly higher in comparison with both HFF-2 (as normal cells) and 4T-1 cells. Although two different forms of cell death (apoptotic and necrotic) appeared in 4T1 following exposure to PMBN-PTX-ME, apoptosis was the major mechanism of cell death in MCF-7.

Median Lethal Dose (LD50)

The median lethal dose (LD 50) test was performed by an oral administration of MEs system on mice with the concentrations ranging from 17.5 to 5000 mg/kg. The maximum safe dose MEs system in mice was calculated in two groups. Control group, the saline has been used as an optional treatment; treatment groups, which were administrated with 17.5, 175, 1750, and 5000 mg/kg of MEs system. None of the mice died during a week, and after treatment with MEs system. It can be concluded that the MEs systems were non-toxic.

Discussion

Indeed, one of the scariest words is cancer. Cancer as a main human health threat is the second leading cause of death throughout the globe.[45] The treatment and diagnosis of cancer have been considered as a large portion of health spending, for example in the United States about $87.8 billion was spent in 2014 for cancer health care.[46] Although chemotherapy and surgery are the main procedures for treating cancer, several adverse effects, and low efficiency are common challenges that investigators encounter with.[47] Due to the problems mentioned, scientists have made great efforts to provide systems to mitigate these challenges.

One of the most well-known methods is the use of nano-formulation for drug delivery, which impressively increases the efficiency and has actually targeted the treatment of drugs.[48] Like other chemical drugs, PTX have several side effects

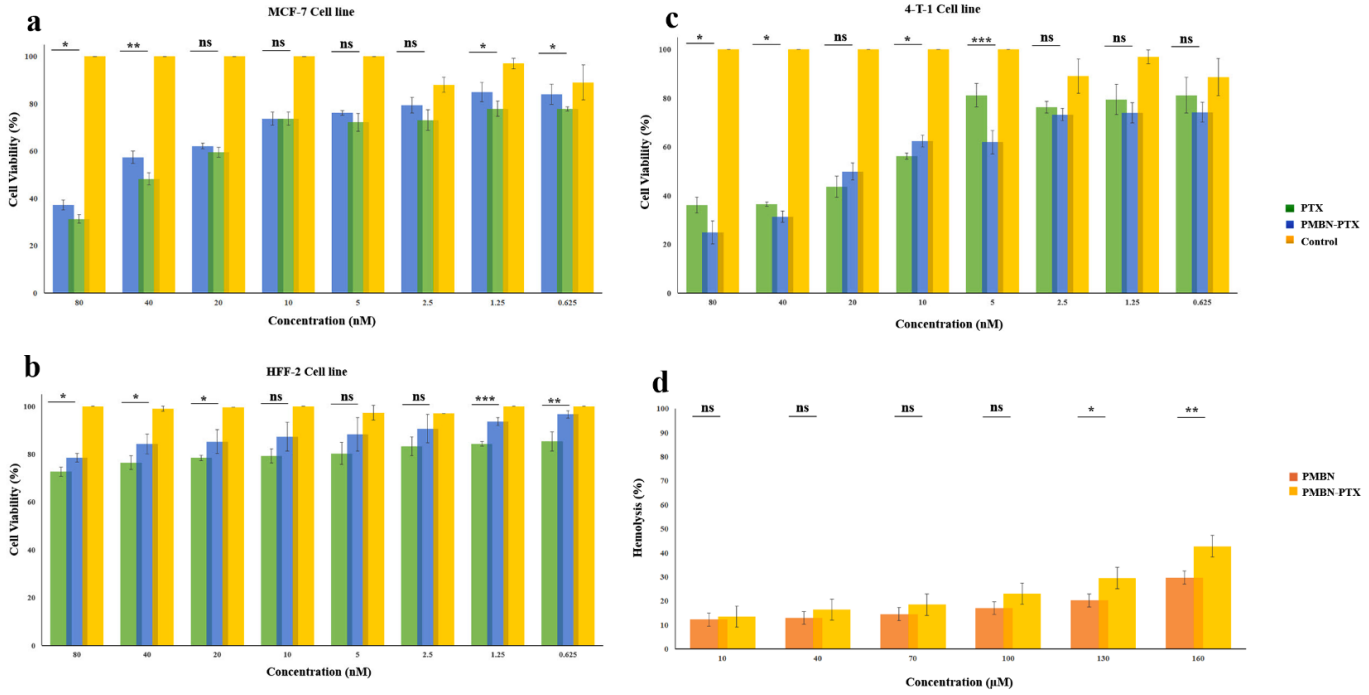

Figure 6. The Cell Viabilities of the PTX, PMBN-PTX-ME on MCF-7 Cells (a), HFF-2 cells (b), 4T-1(c) Cells and Percentage of Hemolysis Induced by PMBN and PMBN-PTX-ME for 4 h Under Different Mass Concentration and 37°C, Conditions (d).

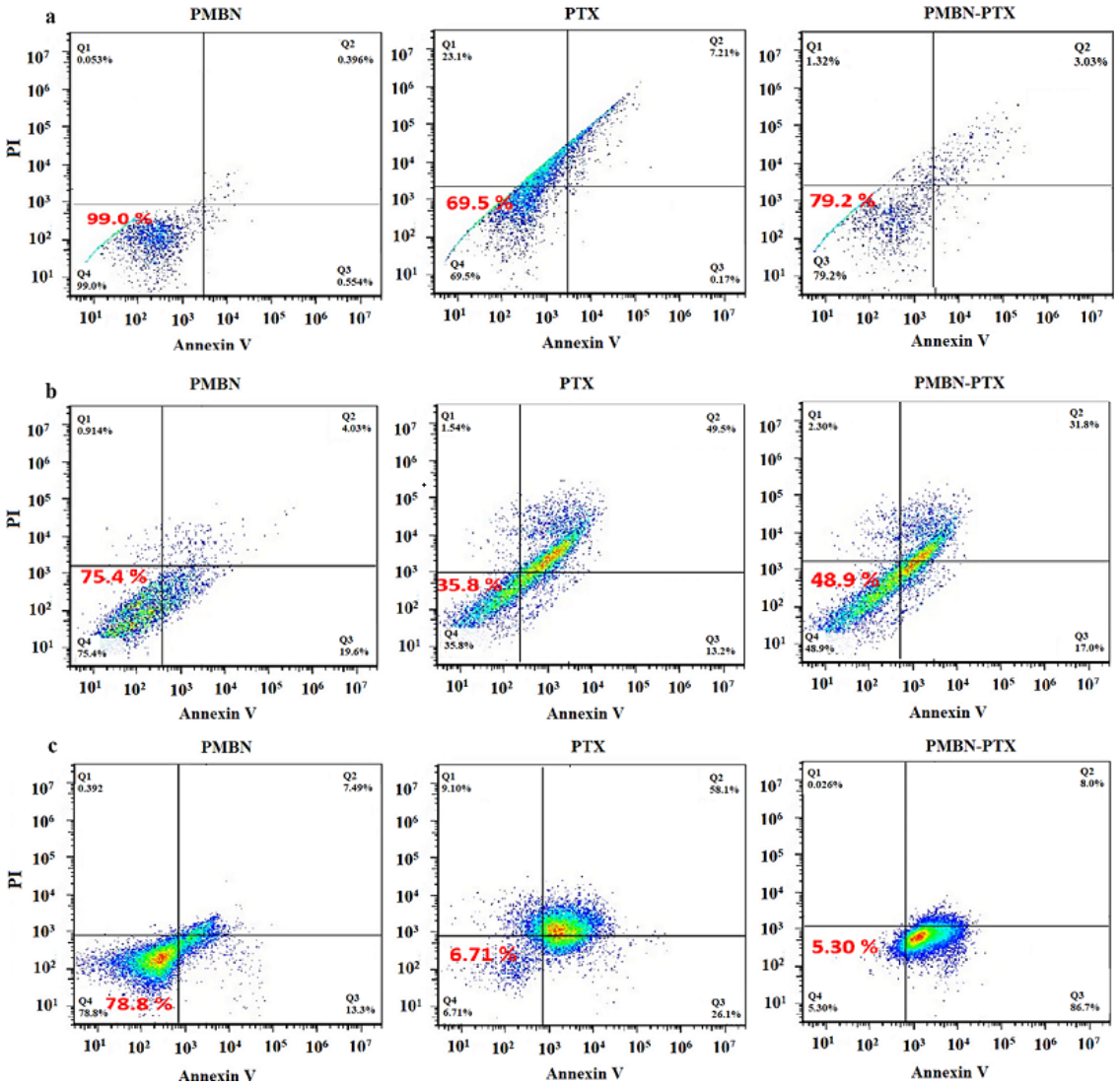

Figure 7. Live Cell, Apoptotic and Necrotic Populations (%) with PMBN, PTX and PMBN-PTX-ME on HFF-2 (a), 4T-1 (b) and MCF-7 (c) Cells Line Were Calculated From flow Cytometry Analysis.

such as nausea, vomiting, loss of appetite and neurotoxicity.[49] Some of the negative features, such as low solubility, reduce its effectiveness. To improve its performance, some modifications have been made and new versions of PTX such as Abraxane and Taxoprexin that fused PTX to albumin and fatty acid were introduced.[50,51] Other strategies for increasing the solubility and efficiency of PTX is nanoparticles. Paccal which is a Cremophor-free formulation and CTI 52010 are available systems based on nanoparticle drug delivery for PTX.[52]

Some studies confirmed PMBN as an appropriate carrier for PTX. For example, the EGF-PMBN-PTX system has a significant anti-tumor activity.[53] In addition, PMBN-IL2-PTX suppressed proliferation of cell lines with high-affinity IL-2 receptors.[54]

In this study, the MEs system based PMBN was successfully synthesized and characterized for PTX delivery. The biocompatible O/W ME system was prepared based on triacetin, Tween 80 (surfactant) and glycerol (co-surfactant) as oil phase. Tween 80 was selected as a surfactant due to its low toxicity, biocompatibility, and high HLB value, so, it is suitable for making O/W type of microemulsion.[36,55] In addition, glycerol was selected as co-surfactant because of its ability to create a large isotropic region.[36] Since ME surfactant molecules can intercalate with the co-surfactant molecules at the oil-water interface, so they can change the curvature of the droplet. Therefore, in ME composition, surfactant and co-surfactant type, as well as their concentrations are critical parameters. In a previous study, many important factors, such as the concentration of surfactant, co-surfactant, amount of triacetin, organic phase addition speed etc. were optimized to reach a perfect drug delivery system.[16] Now, with optimal conditions, the MEs system based on PMBN were studied as drug carrier.

In order to find the best formulation of O/W ME, different compositions of water, surfactant: co-surfactant were prepared (Table 1). The transparency appearance of microemulsions improved by increasing the Tween 80 ratio in each formulation.[56,57]

First, the average size, PDI, particle morphology, and

ζ-potential of MEs system was characterized. The results revealed that the MEs system has a homogenous size and small globular morphology with spherical ball shapes. In detail, MEs particles exhibited a globular shape and homogeneous spherical morphology, which corroborated by data DLS analysis (Table 2). The DLS results showed that the average size of MEs system was under 100 nm, which displays unique properties such as large specific surface areas, long circulation blood, and high efficiency of cellular uptake.

The surface charge can mainly affect the stability of the nanoparticles distribution. Surface charge is important in decisive whether the nanoparticles will cluster in blood flow or will stick to or interact with oppositely charged cell membrane. The plasma and blood cells constantly had a negative charge; NPs with slight negative surface charge can minimize nonspecific contact with these components through electrostatic interactions. The PDI value reflects the nanoparticle stability and uniformity of system; the high PDI values correspond to wide range of particle sizes, while the low PDI values imply samples consisting of homogeneous sized particles.[58] Also, **ζ** -potential is a scientific term for average electrostatic potential existing at the hydrodynamic plane of shear which is normally considered to be 0.2 nm from the surface. The ζ-potential also played a major role in cellular uptake, drug delivery targeting, skin drug delivery systems, tumor cells targeting, brain targeting, and multi drug resistance.[59]

The negative value of ζ-potential is related to hydroxyl groups of surfactants and the co-surfactant which contain oxygen atom with high electronegativity. On the other hand, it was found that the ζ-potential of PMBN-PTX ME had higher negative charge than that of the free-drug-MEs.[60]

The MEs system structure, PTX and PMBN in the MEs system and the chemical functional groups were confirmed by FT-IR. All chemical function groups in the composition of MEs were demonstrated in FT-IR spectrum. The physical interactions between the functional groups of PTX or PMBN with surfactant were observed by reducing or increasing the absorption peaks as well as shifts in some absorption peaks position of functional groups. In this study, The FT-IR results were in accordance to some previous studies.[31-63]

The physiochemical aspects of the MEs system including % transmission, RI, conductivity, pH and *in vitro* drug release were characterized. The RI and transmittance% factors of PMBN-ME was lower than that of PMBN-PTX ME. Due to the presence of PTX in the MEs system, the RI and transmittance% of the PMBN-ME was decreased. Thereby, the electrical conductivity was used to determine the type of microemulsions.[64] The conductivity is profoundly sensitive both to temperature and to composition. The composition of w/o/w ME systems have embraced, PMBN, tween 80, glycerol, and water. The w/o/w MEs systems have over 80% water as the continuous phase caused conductivity property. Meanwhile, the w/o (water-in-oil) systems having low conductivity. The pH value of PMBN-PTX ME was lower than PMBN-ME, which can be clouded because of the presence of acidic functional groups in the PTX chemical structure.[65] According to the release profile of PTX, the MEs system provided a sustained PTX release, which is pH sensitive. The polarity of the system was enhanced by reducing the pH of the buffer solution that led to improve the drug solubility. Meanwhile the tumor microenvironment and inside the endosome/lysosome are acidic (pH = 4.5–6.0) whereas the bloodstream is neutral, the favorable PTX release at acidic medium can decrease the side effect of nanoparticles to normal cells *in vivo*.[43]

According to the MTT, hemolysis and LD_{50} assay, the designed MEs system is biocompatible, safe and nontoxic. The MTT test proposed that these PMBN-ME are highly biocompatible and do not possess a toxic effect, and then, the PMBN-ME could be applied as a biocompatible carrier for biomedical applications.

Conclusions

In brief, we synthesized PMBN-ME as a stable, biocompatible, nontoxic, and promising nano-carrier for PTX delivery. To achieve the optimal microemulsion structure, the various amount of MEs compositions were examined (triacetin, surfactant, co-surfactant, and water). Finally, based on the MEs transparency appearance and then, particle size and ζ-potential, were selected of optimal formulations. It is believed that PMBN-PTX-ME is an efficient drug delivery for cancer treatment. Beyond *in vitro* evaluation, for more investigations, we need to deep investigate the potential of using PMBN-ME as a nano-carrier drug delivery *in vivo* evaluation.

Authors' Contributions

MG and SO performed the experiments; analyzed and interpreted the data. AS conceived and designed the experiments and performed the experiments. PMF contributed reagents, materials, analysis tools or data. HKM conceived and designed the experiments.

Conflict of Interest Disclosures

The authors declare no conflicts of interest.

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