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Doxorubicin-Loaded $(PEG)_3$-PLA Nanopolymersomes: Effect of Solvents and Process Parameters on Formulation Development and *In Vitro* Study

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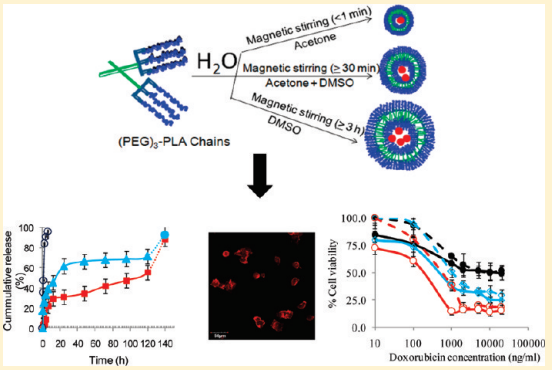
ABSTRACT: This study is focused on the preparation of doxorubicin-loaded nanopolymersomes (PolyDoxSome) and assessment of the effects of various solvents and process variables on the size and drug loading during preparation of formulation. PolyDoxSome was prepared by nanoprecipitation method using amphiphilic $(PEG)_3$-PLA copolymer, and the formation of polymersomes was assessed by dynamic light scattering and optical and transmission electron microscopy and evaluated for *in vitro* release profile and *in vitro* cytotoxicity. A systematic investigation indicated that solvent composition, order of addition, aqueous phase, copolymer concentration, and external energy input have significant influence on size and dispersity of PolyDoxSome. Under optimized conditions, PolyDoxSome had a size range of 130−180 nm with PDI < 0.2, a zeta potential ∼−8 mV, and a drug loading at ∼11% w/w with an encapsulation efficiency at ∼53% w/w. *In vitro* release profile of PolyDoxSome at 37 °C demonstrated that doxorubicin release was pH dependent and gave higher release at pH 5.5 in comparison to the release at pH 7.4 (similarity factor, f_2 < 50). PolyDoxSome exhibited enhanced cellular uptake of doxorubicin compared to free doxorubicin solution in MCF-7 cell line and showed a better cytotoxicity of doxorubicin at equivalent dose in nanopolymersomes. In conclusion, size and dispersity were strongly influenced by duration of magnetic stirring and overall composition of organic/aqueous media; however, size and dispersity were retained against different degrees of dilution. PolyDoxSome was able to control the release of doxorubicin in pH dependent manner and effectively deliver the drug in active form to MCF-7 breast cancer cells.



KEYWORDS: $(PEG)_3$-PLA, amphiphilic copolymer, nanopolymersomes, self-assembly, doxorubicin

1. INTRODUCTION

Polymersomes have emerged[1,2] as colloidal drug carriers in the past decade and have received growing attention. They are formed by the self-assembly of amphiphilic block copolymers containing two or more chemically distinct monomer sequences joined by a covalent bond that prevents blocks from macrophase separation upon dissolution. As demonstrated in previous studies, they have superior mechanical stability due to a thicker bilayer membrane wall[1,3,4] and thus provide better stability over liposomal carriers. They also have the possibility to tune physicochemical and biological properties by simply varying the type of copolymer blocks, their chain-length and the geometry of polymeric chains.[5,6] The aqueous core of polymersomes is separated from the outside medium by a hydrophobic membrane which makes them a vesicle-like structure with aqueous inner core surrounded by hydrophobic periphery and thus makes them suitable as drug carriers for hydrophobic, hydrophilic and amphoteric drug molecules.[2,7,8] Because of this property, they have tremendous potential applications in medicine, pharmacy and biotechnology.[3,9−13] They are in the size range of tens of nanometers to several micrometers in diameter, and their size can be optimized for specific applications.[2,3,14]

It is mentioned that the nanosize of drug carriers can facilitate the extravasations at tumor sites while avoiding renal clearance and nonspecific reticuloendothelial (RES) uptake. It is shown that <200 nm vesicles are favored for possible enhancement of extravasations into tumor tissues due to the EPR effect. This size range is sufficiently large (>60 nm) to prevents glomerular filtration and small (<200 nm) to delay/avoid RES uptake due to high radius of curvature that prevents efficient binding of opsonins.[15−18] Surface modification by PEG reduces the opsonization and minimizes the clearance by the RES, leading to longer blood circulation times and improved pharmacokinetic properties. At present, the most promising strategies in reducing RES uptake are to reduce the carrier size and to sterically stabilize the carrier with a layer of amphiphilic polymer chains such as PEG, which is a flexible, hydrophilic and weakly anionic or neutral macromolecule.[19−22] Over the past decade, a number of publications have been focused on morphology of these

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vesicles in solution and processing conditions which influence the encapsulation efficiencies of drug loaded polymersomes. Hence tailoring of vesicle size by experimental conditions and preparation methods is an important step in the development of nanoformulations.

For a drug formulation, size and drug loading are the critical parameters. Size is a key factor in the biodistribution of long-circulating carriers and in achieving therapeutic efficacy; whereas drug loading determines the efficacy of a formulation on therapeutic parameters. Encapsulation efficiency is directly linked to the commercial viability due to less wastage of the drug during processing steps of a particular formulation. These parameters, namely, size, drug loading and encapsulation efficiency, are discussed for many other particle based formulations.[23−29] The pioneering work on polymersomes was initiated by Discher's group. This group synthesized poly(ethyl ethylene)-*block*-poly(ethylene oxide) (PEE_{37}-*b*-PEO_{40}) and formed small vesicles (<200 nm) or larger vesicles (20−50 μm) and named polymersomes. They showed that prepared polymersomes were more stable than phospholipid bilayers and less permeable to water by several orders of magnitude with hydrophobic wall thickness of $\sim d$ = 8 nm compared to typical phospholipid bilayers (d = 3−4 nm).[1,30] Nevertheless, to the best of our knowledge, the effect of formulation and process variables on size and dispersity of polymersomes has not yet been studied in detail. In this study, we report the effect of solvents and processing conditions to control the size and dispersity of polymersomes and optimized conditions for preparing doxorubicin-loaded nanopolymersomes.

In the present work, amphiphilic copolymer $(PEG)_3$-PLA is synthesized using ester functionality and used for preparation of doxorubicin-loaded nanopolymersomes (PolyDoxSome). Earlier, these types of polymers were prepared using amide functionality and were explored for particulate drug delivery.[31] Recently, our group synthesized $(PEG)_3$-PLA with ester functionality and for the first time demonstrated its capability for vesicle formation[32] and its use for the preparation of amphotericin-B loaded polymersomes.[33] The self-assembly vesicle formation depends on the hydrophilic/hydrophobic balance of the polymer chain, and thus the effect of PEG chain length was very well studied for linear polymers by Discher and group;[1,30] however the effect of PEG chain length for branched polymers was studied by our group and discussed elsewhere.[34] In this report, the focus of the study is to identify important formulation and process variables during the preparation of PolyDoxSome, and to obtain an optimal condition for preparing these nanopolymersomes by the nanoprecipitation method. To investigate the influence of different solvents on characteristics of polymersomes, four water miscible organic solvents and their mixture are selected as organic phase based on their safety classification and ability to dissolve copolymer; and different aqueous phases are also taken in this study. Other experimental variables, namely, order of addition, dilution of preformed polymersomes, and copolymer concentration, are also studied to obtain the optimized size and drug loading of the proposed formulation. The optimized formulation was tested for drug release profiles, *in vitro* cytotoxicity and cell uptake studies using MCF-7 breast cancer cell lines.

2. EXPERIMENTAL SECTION

2.1. Materials. Doxorubicin hydrochloride was a generous gift from Dabur Pharmaceuticals, India, and used as received. Tris base/HCl, dimethyl sulfoxide, acetone, tetrahydrofuran, dimethylformamide and ethyl acetate were purchased from Sisco Research Laboratory, Mumbai, India. Citric acid (anhydrous) and toluene (anhydrous) were obtained from Central Drug House, New Delhi, India; mPEG1100 (anhydrous) was obtained from Fluka Chemica, USA, and dried by dissolution in anhydrous toluene through azeotropic distillation. Sodium hydride (sodium hydride suspension in paraffin oil as 60% for synthesis) was procured from Loba chemie, India; DL-lactide (anhydrous) was purchased from Purac Biochem, Gorinchem, The Netherlands. Stannous octanoate was obtained from Sigma-Aldrich, USA. Solvents for HPLC were purchased from J.T.Baker, USA, and used as received. Elga water is ultrapure water and the in house supply purified by Elga Stat, U.K. (resistance of 18 MΩ·cm at 25 °C). All other chemicals used in this study were analytical grade and used as received.

Table 1. Characteristics of the Copolymer Used for the Study

	hydrophilic wt fraction		mol wt[a] (kDa)			
copolymer	feed	practical[b]	M_n[b]	M_n[c]	M_w[c]	PDI[d]
$(PEG)_3$-PLA	1:5.5 (15%)	1:3 (26%)	12.05	10.00	17.50	1.75

[a] M_n value calculated from GPC was lower than that from ^{1}H NMR spectra. [b] Determined from integration of signals due to mPEG and PLA blocks on ^{1}H NMR spectra [c] Obtained by GPC with respect to polystyrene standards [d] PDI = Ratio of weight to number average molecular weight.

2.2. Synthesis of Copolymer. Synthesis of amphiphilic copolymer was carried out in two steps as described in our previous publication.[32] In this work, targeting molecular weight and hydrophilic weight fraction were different from copolymers synthesized in previous publication and thus polymer was synthesized for specific application. Briefly, citric acid and PEG were activated by reacting with thionyl chloride and NaH, respectively. Then, three PEG1100 chains were attached to three carboxylic acid groups of a citric acid molecule using esterification method to get $(PEG)_3$-PLA. Thus the obtained product $(PEG)_3$-citrate was purified and further used to undergo a reaction with DL-lactide using stannous octanoate as a catalyst to get a copolymer of $(PEG)_3$-PLA. Polymerization of DL-lactide to PLA chain was carried out on secondary hydroxyl group of citric acid using ring-opening polymerization. This polymerization was continued to get a targeted hydrophobic weight fraction of 85% in the prepared copolymer. Synthesized copolymer was characterized, and details are given in Table 1.

2.3. General Procedure for the Preparation of Polymersomes. There are many methods for the preparation of polymeric vesicles reported in the literature, such as film or bulk rehydration,[14,35] electroformation,[1] direct dissolution,[36] solvent injection,[33,37] emulsification[38,39] or precipitation[36,40] from solvent systems. In this work a nanoprecipitation method was used for the preparation of both blank or doxorubicin-loaded polymersomes as it has been found to be a valid and efficient alternative method to prepare nanopolymersomes.[24,25,36,40−42] In this method, amphiphilic block copolymer was dissolved in an appropriate water miscible organic solvent (10−20 mg/mL) and polymeric solution was injected to aqueous phase (or aqueous phase was injected to polymeric solution) to get a polymeric dispersion. This injection process was carried out under magnetic stirring (over a short period of time), and the polymeric dispersion was allowed to equilibrate for some time or until

the turbidity of the dispersion was stabilized (<20 min). The organic solvent or free drug of the polymeric dispersion was removed through dialysis (4−5 h) against used aqueous phase using a membrane (MWCO: 10,000 Da). This dialysis process was done under intermittent magnetic stirring to avoid clogging of the membrane. After dialysis, a stabilized polymeric dispersion (polymersomes in aqueous phase) was obtained, which was analyzed by dynamic light scattering, optical and transmission electron microscopy, and HPLC for size, dispersity, vesicular formation, and drug loading and encapsulation efficiency, respectively as described in sections 2.4 and 2.5.

2.4. Characterization of Polymersomes. Polymersomes were prepared in two size range of 2−10 μm and 50−250 nm. Though bigger size of polymersomes was not used for the proposed drug formulation, this could gave us an idea of the morphology of self-assembled structure and also for the drug location in polymeric vesicles and subsequent optimization of formulation conditions for nanopolymersomes. Hence, bigger polymersomes were prepared to visualize and confirm the vesicular morphology/drug localization using optical (TC 5500, Meji Techno, Japan) and confocal laser scanning microscopic (CLSM, Olympus Fluoview Fv 1000, Japan) techniques. For CLSM characterization, after self-assembly of vesicles, free doxorubicin was removed from external solution by centrifugation and subsequent washing. The pellet obtained was redispersed in 2 mL of Elga water, and about 10 μL of redispersed suspension was withdrawn for microscopic characterization. A light microscope with a magnification of 40× was used for blank polymersomes, whereas fluorescence images for doxorubicin loaded polymersomes were obtained by CLSM using a krypton−argon laser line (488 nm) for excitation of doxorubicin and a long pass filter (590 nm) for detection of emitted light.

Nanopolymersomes were assessed for their morphology using transmission electron microscopy (TEM) (Hitachi H-7500, Tokyo, Japan) operating at an accelerating voltage of 120 keV. For TEM characterization, a suspension of nanopolymersomes with a concentration of 1 mg/mL was applied dropwise onto a 400-mesh copper grid coated with carbon and negatively stained with 1% w/v phosphotungstic acid solution (PTA) (adjusted to pH 7.4 with NaOH).

Size, dispersity and zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS instrument (Malvern, Worchestershire, U.K.; Laser 4 mW He−Ne laser, operating at a wavelength of 633 nm) with 173° backscattering. The hydrodynamic diameter and distribution were determined using Cumulant and CONTIN analysis method. The electrophoretic mobility of the nanopolymersomes was measured by laser Doppler velocimetry (LDV) and a capillary zeta potential cell with gold electrodes. The electrophoretic mobility (μ) was converted to the zeta potential (ζ) using the Smoluchowski approximation. All measurements were carried out in triplicate and performed at 25 °C.

2.5. Determination of Doxorubicin Loading Capacity and Encapsulation Efficiency. Doxorubicin loading was carried out at 4:15 feed weight ratio (drug:copolymer, 21.0% w/w) using a nanoprecipitation method by codissolving the drug and copolymer in a mixture of DMSO and acetone in 1:4 (v/v) ratio and injecting this drug−copolymer solution to Tris buffer (10 mM, pH 7.4) or ethyl acetate saturated Elga water (pH 6.8). Unencapsulated free doxorubicin and organic solvents were removed by dialysis, and then the drug loaded polymersomes were dissolved in the mixture of DMSO and methanol in 1:4 (v/v) ratio followed by filtration (0.2 μm). The filtrate was used for analysis of doxorubicin in polymersomes. Analysis was done by reversed-phase (RP) HPLC using Shimadzu HPLC system with LC software coupled to RF-10AXL fluorescence detector (λex470, λem593). The separation was achieved on the C18 Inertsil ODS-3 V, 4.6 × 250 mm, 5 μm analytical column (GL Sciences Inc., Japan) maintained at 30 °C. Doxorubicin was isolated in isocratic at flow rate of 1.0 mL/min using a mobile phase consisting of acetonitrile and 50 mM acetate buffer; 35:65 (v/v) at pH 4.5. Loaded content was determined using the calibration curve established from standard solutions of doxorubicin in DMSO/methanol (1:4 v/v) mixture which was prepared by physical mixing of copolymer and doxorubicin in the same ratio to that of the formulation. Each experiment was carried out in triplicate, and mean values ± SD deviations were calculated using the following formulas:

$$\text{drug loading capacity (DL\%)} = \left(\frac{\text{weight of drug in polymersomes}}{\text{weight of polymersomes}}\right) \times 100 \quad (1)$$

$$\text{encapsulation efficiency (EE\%)} = \left(\frac{\text{weight of drug in polymersomes}}{\text{weight of feed drug}}\right) \times 100 \quad (2)$$

2.6. *In Vitro* Release Study. The *in vitro* release profile of doxorubicin from doxorubicin-loaded nanopolymersomes (PolyDoxSome) was determined at pH 7.4 (10 mM Tris buffer) or pH 5.5 (50 mM sodium acetate buffer) by a dialysis membrane method. For this study, a known quantity of PolyDoxSome (corresponding to 100 μg/mL of doxorubicin, 0.5 mL) dispersion and free doxorubicin solution of the same concentration and volume were prepared and transferred into a dialysis membrane (MWCO: 10,000 Da, supplied by Spectrum Laboratories, USA). The dialysis membrane bags were suspended to the appropriate buffers (20 mL) and maintained in a reciprocal shaker water bath at 37 ± 1 °C and 100 strokes per minute. In order to acquire and maintain sink conditions, a drug release study was performed at low drug loading content and with 0.5 mL of PolyDoxSome dispersion or doxorubicin solution (control) dialysis against 20 mL of release medium. At predetermined time intervals, aliquots of 1 mL of sample were withdrawn and replenished with the same volume of the fresh release medium. The amount of doxorubicin released to release medium was determined by HPLC as mentioned previously in section 2.5, and percent cumulative release was calculated using the following formula:

$$\text{percent cumulative release } (Q\%) = \left(\frac{C_n V + V_i \sum_{i=0}^{n-1} C_i}{\text{weight of polymersomes} \times \text{DL\%}}\right) \times 100 \quad (3)$$

where C_n is the sample concentration at T_n, V the total volume of release medium, V_i the sampling volume at T_i, C_i the sample concentration at T_i (both V_0 and C_0 are equal to zero), and DL % the percentage of drug loading.

2.7. Cell Culture, Internalization and Cytotoxicity. Cell culture experiments were performed on a breast cancer cell line (MCF-7) and were maintained in a humidified incubator (Shell Lab, Water jacketed CO_2 incubator) at 37 °C and 5% CO_2. MCF-7 cells cultured up to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic were used for the drug uptake and cytotoxicity studies.

For uptake and internalization study MCF-7 cells were seeded in 96 well plates at a density of 10^4 cells per well and incubated for 24 h before initiation of the uptake study. After that medium present in the wells was replaced with fresh medium containing 1, 5, and 10 μM free doxorubicin and PolyDoxSome (containing equivalent amount of doxorubicin) and incubated for various time points (0.5, 2, 4, 6, 8, 12, and 24 h). Then, medium was removed from the wells, cell monolayer was rinsed with PBS, trypsin-EDTA was added, and the samples were incubated for 10 min at 37 °C to allow cell detachment. Then, 10% Triton-X 100 in PBS was used to lyse cells. The amount of internalized doxorubicin from cell lysate was then measured by the HPLC method. In a separate experiment, for confocal microscopic study, cells were seeded at a density of 16×10^4 cells per petri dish (35 mm) containing 12 mm × 12 mm coverslips (SecureSlip Glass Coverslips, GRACE BIO-LABORATORIES and illustrated instructions for using SecureSlip was followed) for 24 h. Then, the samples were incubated with medium containing 10 μM free doxorubicin and PolyDoxSome (containing equivalent amount of doxorubicin) for 2, 6, and 24 h at 37 °C in 5% CO_2/95% air. Moreover, control samples (untreated MCF-7 cells) at the wavelength of doxorubicin have been captured if they show any basal cell fluorescence that can interfere and affect the test. After incubation, cells were washed and fixed by 4% formaldehyde for 15 min and stored at 4 °C until analysis. Coverslips with cells attached were transferred to slides and observed under confocal laser scanning microscope (CLSM) for uptake assessment with imaging software (Fluoview FV500). The images were acquired at 40× magnification using inverted stage microscope.

The cytotoxicity of blank nanopolymersomes, PolyDoxSome and free doxorubicin against MCF-7 cell lines was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cytotoxicity assay was performed in two ways: (1) immediate effect which refers to cytotoxic effect that was measured immediately after termination of treatment, and (2) delayed effect that refers to the drug effect that was measured after an additional growth period after removal of the drug from the medium, where drug containing medium was replaced with fresh medium at the end of treatment (6, 24, and 48 h) and the culture and cells were further incubated with drug-free growth medium until 72 h, irrespective of treatment durations. Briefly, human MCF-7 breast cancer cells were seeded in 96 well plates with density of 10^4 cells per well in culture medium. After 24 h incubation allowing cell recovery and attachment, medium was removed and the cells were further incubated with medium containing blank nanopolymersomes (copolymer concentration 5 mg/mL), free doxorubicin and PolyDoxSome with different concentrations of doxorubicin (0.01, 0.1, 1, 2, 5, 10, and 20 μM) for 6, 24, 48, and 72 h. Cell growth was monitored in a control group without addition of the blank polymersomes and without drug treatment. After predetermined time periods of 6, 24, 48, and 72 h of incubation at 37 °C, medium was piped out and 50 μL of MTT solution (500 μg/mL in media) was added to the wells and incubated at 37 °C. After 4 h of incubation the precipitates of formazan crystal were formed and solubilized in dimethyl sulfoxide (DMSO, 100 μL). The absorbance intensity was measured by microplate reader (PowerWave XS2, Bio Tek Instruments, Gen 5 software, USA) at λ_{max} 570 nm (background reading at λ_{max} 630 nm). Untreated cells were taken as control with 100% viability, and cytotoxicity was expressed as % reduction in cell viability, which was calculated from the ratio between the number of cells treated with doxorubicin or blank nanopolymersomes and that of nontreated cells (control):

$$\text{cell viability } (\%) = \left(\frac{\text{abs of samples}}{\text{abs of control}}\right) \times 100 \qquad (4)$$

where abs of sample is the absorbance intensity of the cells incubated with drug or blank nanopolymersomes and abs of control is the absorbance intensity of cells incubated with culture medium only (positive control).

The IC_{50}, the drug concentration at which inhibition of 50% cell growth was observed in comparison with that of control sample, was calculated by the curve fitting of the cell viability data. The cytotoxicity of doxorubicin solution and PolyDoxSome was analyzed and compared.

2.8. Statistical Analysis. All statistical significances of difference between data sets were determined using one-way ANOVA test at 95% confidence level (p-values <0.05 considered statistically significant). Data were expressed as mean ± SD.

3. RESULTS

3.1. Preparation of Polymersomes and Verification of Vesicular Structures. Polymersomes were prepared in two size ranges of 2−10 μm and 50−250 nm under different conditions of preparation and characterized for morphology and size using optical, CLSM, TEM and DLS techniques. The microscopic images of larger polymersomes elucidated well-defined vesicular structures and clearly indicated the ability of polymersomes to encapsulate doxorubicin inside aqueous lumen core as displayed in Figure 1A−C. Image in Figure 1A-ii displays a clear vesicular nature with the dark ring (red arrow) due to PLA block that forms a hydrophobic polymeric wall, the light interior aqueous lumen (white arrow) and white halo/aura (black arrow) due to surface PEG confirming the vesicular morphology. CLSM images and Z-sectioning of doxorubicin-loaded polymersomes (Figure 1B and Figure 1C) show the spherical and vesicular nature of the polymersomes. Z-Sectioning is used to display the change in intensity with varying height by optical slicing into the core from the outer shell corona of the vesicle indicating that doxorubicin is inside the aqueous core of the vesicle. Similarly, nanopolymersomes were prepared and evaluated for membrane thickness, morphology and type of self-assembly with TEM using contrast agent PTA negative staining (Figure 1D). PTA sodium salt, a staining agent for esters, was used to make PLA appear as the dark halos and PEG as the bright phase in TEM images. TEM evaluation confirmed the vesicular nature of nanopolymersomes with well-defined characteristics of vesicles showing dark halos and relatively bright interior aqueous region. The doxorubicin-loaded and blank nanopolymersomes were evaluated and compared for their morphology where doxorubicin-loaded nanopolymersomes showed a dense core due to the contribution of doxorubicin into a vesicular reservoir. From TEM evaluation the hydrophobic core of the membrane provides a contrast with a

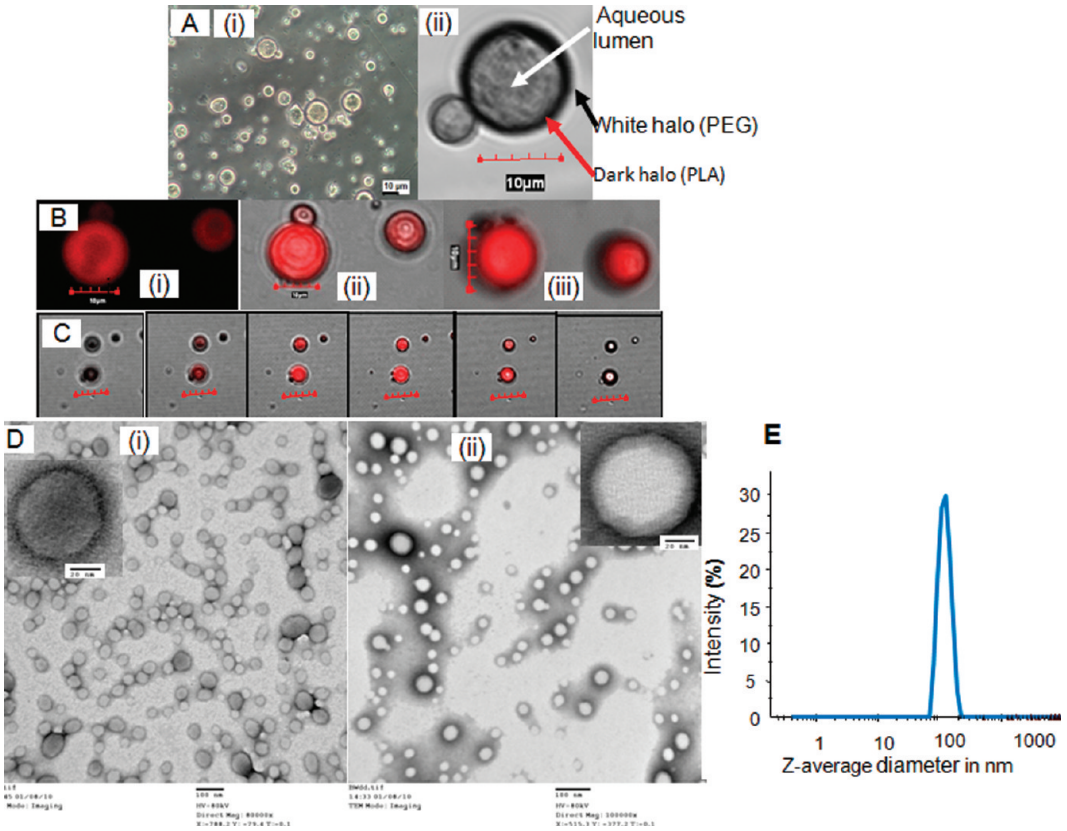

Figure 1. Microscopic and size analysis of polymersomes: (A) optical and DIC images; (B) CLSM images of doxorubicin loaded polymersomes; (C) *Z*-scanning of CLSM images corresponding to panel B; (D) TEM images of nanopolymersomes; (E) size analysis by DLS method. Panels A−C display microscopic images of blank or doxorubicin-loaded polymersomes and showed vesicular structure (the scale bar is 10 μm). (A) (i) and (ii) Optical microscopic and DIC images, respectively, of blank polymersomes prepared by injecting a solution of $(PEG)_3$-PLA in THF (15 mg/mL, 0.4 mL) to 5 mL of ethyl acetate saturated (8% v/v) phosphate buffer (0.1 M, pH 7.4) under stirring (1000 rpm). (B) CLSM images of polymersomes prepared by injecting a solution of $(PEG)_3$-PLA and doxorubicin in THF (0.4 mL) to 5 mL of ethyl acetate saturated phosphate buffer. (i) 2D images, (ii) overlapped images of CLSM and DIC, (iii) 3D images. (C) *Z*-Scanning CLSM images of doxorubicin-loaded polymersomes. (D) PTA stained TEM images of (i) doxorubicin-loaded; (ii) blank nanopolymersomes (scale bar: 100 and 20 nm for insets). (E) Size distribution by intensity for doxorubicin-loaded nanopolymersomes prepared by codissolving $(PEG)_3$-PLA copolymer and doxorubicin hydrochloride in DMSO/acetone (1:4) and rapidly injecting to Tris buffer (10 mM, pH 7.4) and measured with DLS (*Z*-average diameter, 129.1 nm; PDI, 0.13).

mean thickness of 9 ± 1.5 nm. Moreover, TEM images were circular/vesicular in nature and other morphologies like cylindrical vesicles or spherical micelles were not observed confirming that polymersomes are exclusive morphologies under given preparation method and conditions. The average diameter of nanopolymersomes observed with TEM was ∼40% smaller as compared to the measurements made by DLS (Figure 1E). This reduction in size could be explained by the fact that TEM operates at high temperature and leads to dryness of the sample, and subsequently it led to the shrinkage in size. Apart from this, the corona of the vesicular structure/polymersomes which is made by PEG chain cannot be visualized in TEM (probably due to the absence of dark staining of PEG chains) and thus it leads to a result of lesser size than the actual size.[43,44]

3.2. Influences of Solvents and Process Parameters on Size and Dispersity. As it is described in the above section, amphiphilic $(PEG)_3$-PLA copolymer was self-assembled into a vesicular morphology and the purpose of this study was to obtain the smallest size with a good size distribution. For this purpose, a number of formulation and process parameters that can be principal determinants to control the size and dispersity of polymersomes were studied with desired size range of 100 to 200 nm and low dispersity (PDI < 0.2). These variables include the organic solvents, aqueous phase, order of addition, dilution of preformed vesicles, copolymer concentration and energy input (speed of injection and magnetic stirring time).

3.2.1. Organic Solvents. To explore the influence of various solvents on size and dispersity of polymersomes, an amphiphilic copolymer solution was prepared in acetone, DMF, DMSO, THF, mixture of DMSO with THF or acetone at different mixing ratios (Table 2). The results presented in Figure 2 demonstrated that the average size of polymeric vesicles changed significantly with organic solvents used. The size obtained by DMSO was not significantly different from the size obtained using a mixture of DMSO and THF (1:1) (p-value >0.05) but significantly higher than the size obtained from a mixture of DMSO and acetone (1:1 or 1:4 ratios) (p-value <0.01). The sizes of polymersomes obtained from the solution of $(PEG)_3$-PLA in acetone or DMF were smaller than that of THF (p-value <0.05) or DMSO (p-value <0.001) with mean diameter less than 100 nm.

3.2.2. Order of Addition. Upon rapid injection of the organic solution to aqueous or opposite, well-defined nanopolymersomes were formed in a nanosize range (Figure 3). When polymersomes were prepared by injecting aqueous phase to

Table 2. Properties of Solvents Used in This Study[a]

solvents used	class	miscibility	PI	DC	RI	bp	den.	visc
acetone	3	100	5.1	21.0	1.36	56.0	0.78	0.32
DMF	2	100	6.4	38.0	1.43	153.0	0.94	0.92
DMSO	3	100	7.2	47.0	1.48	189.0	1.09	2.00
THF	3	100	4.0	7.5	1.40	66.0	0.88	0.55
ethyl acetate	3	8.7	4.4	6.0	1.37	77.0	0.89	0.45
DMSO + THF (1:1)	3	100						
DMSO + acetone (1:1)	3	100						
DMSO + acetone (1:4)	3	100						
water			9.0	80.0	1.33	100.0	1.0	

[a] PI, polarity index; DC dielectric constant; bp, boiling point (°C); RI, refractive index; den., density (g/mL); visc, viscosity (cP).

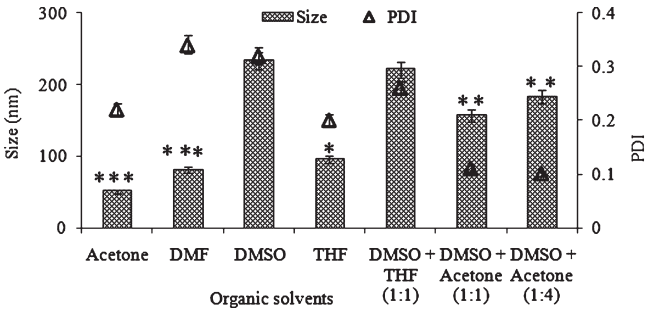

Figure 2. Effects of different organic solvents on size and PDI of polymersomes prepared by injecting $(PEG)_3$-PLA copolymer solution in different organic solvents (15 mg/mL, 0.4 mL) to 5 mL of Elga water/EA. For each column values presented are average values of three replicate experiments ($n = 3$) and error bars indicate standard deviations. Statistical differences between different solvents on size were expressed using one-way ANOVA−Tukey test. $^{*}p < 0.05$ vs acetone or DMF; $^{**}p < 0.01$ vs DMSO or DMSO/THF (1:1); $^{***}p < 0.001$ vs DMSO or DMSO/THF (1:1).

$(PEG)_3$-PLA solutions of acetone, DMF, THF or mixture of DMSO and acetone, relatively larger polymersomes with lower dispersity (more homogeneous) were obtained as compared to reverse order of injection (p-value <0.001). On the other hand, polymersomes prepared using DMSO and its mixture with THF resulted in smaller size (p-value <0.001) when aqueous phase was injected to $(PEG)_3$-PLA solution of organic phase. The average nanopolymersome size obtained in both orders of addition was in the range of 50−250 nm with narrow size distribution.

3.2.3. Dilution of Prepared Polymersomes. The effect of dilution on particle size has also been investigated in order to check the stability of nanopolymersomes under different degree of dilution using water as diluent. In this study it has been shown that once nanopolymersomes are formed by nanoprecipitation method, they are insensitive to dilution except minor increment, and no significant change is observed ($p > 0.05$) in all solvent systems, except DMSO (Table 3). Polymersomes prepared by injection of organic solution (DMSO) to aqueous phase significantly decreased in size upon 10 times dilution, while those prepared by reverse order of addition using DMSO significantly decreased their size upon all dilution factors. The nanopolymersomes prepared using different organic solvents other than DMSO were insensitive to different degrees of dilution at a maximum of 10 times dilution, regardless of order of injection.

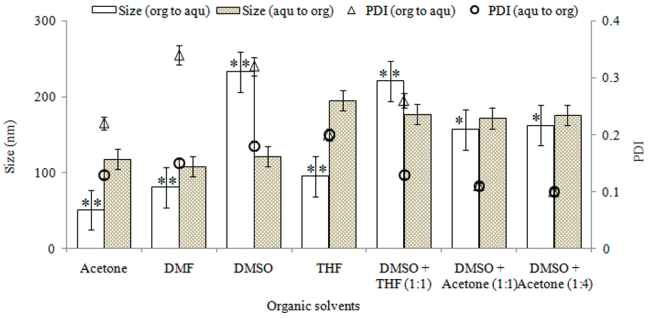

Figure 3. Effect of order of addition on size and PDI of polymersomes prepared by injecting $(PEG)_3$-PLA copolymer solution in different organic solvents (15 mg/mL, 0.4 mL) to 5 mL of Elga water/EA or reverse order of injection. For each column, values presented are average values of three replicate experiments ($n = 3$) and error bars indicate standard deviations. Statistical differences between orders of addition on size were expressed using one-way ANOVA−Tukey test). $^{*}p < 0.05$ and $^{**}p < 0.001$ vs aqueous to organic addition.

3.2.4. Aqueous Phases. The effect of aqueous phases has been investigated on size and dispersity of polymersomes prepared by injection of $(PEG)_3$-PLA solution of organic solvent to aqueous phase. The results are shown in Table 4. For this particular study, a mixture of DMSO and THF in a 1:1 ratio was used as organic solvent and injected to the aqueous phases. Moreover, aqueous phases saturated with ethyl acetate were explored, as ethyl acetate slows down the dissolution of water miscible organic solvents to facilitate self-assembly. Polymersomes prepared by injecting organic solution of $(PEG)_3$-PLA to Elga water resulted in smaller sizes (134.7 ± 4.2 nm) and higher dispersity (0.26) compared to polymersomes prepared by Elga water saturated with ethyl acetate (8% v/v) (177.1 ± 5.2 nm; 0.13). Apart from Elga water, different buffer systems such as Tris, phosphate, citrate and ammonium sulfate were also explored. Tris (10 mM, pH 7.4) buffer is found to give a desired size (171.6 ± 2.5) and PDI (0.05) as presented in Table 4. In the case of citric acid (300 mM, pH 4) and ammonium sulfate (250 mM, pH 7.0) buffers, nanopolymersomes were not formed and resulted in larger size and precipitation, respectively.

3.2.5. Copolymer Concentration. The effect of copolymer concentration on size and dispersity was studied at various concentrations of copolymer ranging from 10 to 30 mg/mL in organic solvent (i.e., 2−6 mg/mL in final dispersion of organic−aqueous phase). The results are shown in Figure 4. The size of polymersomes increased linearly from 140 to 250 nm upon increment of polymer concentration from 10 to 30 mg/mL keeping identical conditions. When the concentration of copolymer doubled from 15 to 30 mg/mL, the size of polymersomes increased by 140% (177 to 250 nm), whereas PDI increased from 0.15 to 0.33. At >30 mg/mL, the size and PDI increased substantially and size distribution was adversely affected giving more than one peak. It gave some precipitates which were observed at the bottom and remained undispersed following removal of organic solvent.

3.2.6. Speed of Injection and Magnetic Stirring. In order to investigate the influence of external energy on polymersomes' size and dispersity during formation of polymersomes, speed of injection and duration of magnetic stirring were varied. Regarding the speed of injection of organic solvent to aqueous phase or opposite, it was varied from dropwise addition to rapid injection (within few seconds). When it was added in a dropwise manner,

Table 3. Effect of Dilution by Water on Size and PDI of Polymersomes Prepared by (A) Injecting $(PEG)_3$-PLA Copolymer Solution in Different Organic Solvents (15 mg/mL, 0.4 mL) to 5 mL of Elga Water/EA and (B) Reverse Order of Injection[a]

	acetone		DMF		DMSO		THF		DMSO/ THF (1:1)		DMSO/ acetone (1:4)	
dilution factor	size (nm)	PDI	size	PDI	size	PDI	size	PDI	size	PDI	size	PDI
(A) Polymersomes Prepared by Injection of Organic Solution to Aqueous Phase												
no	50.6 ± 1.9	0.22 ± 0.01	80.5 ± 4.1	0.34 ± 0.02	232.7 ± 3.1	0.32 ± 0.02	95.2 ± 4.5	0.20 ± 0.02	220.6 ± 3.5	0.34 ± 0.02	133.3 ± 2.5	0.08 ± 0.01
2×	52.1 ± 2.6	0.23 ± 0.02	73.4 ± 2.9	0.36 ± 0.03	226.0 ± 3.5	0.31 ± 0.03	96.2 ± 2.7	0.30 ± 0.03	229.5 ± 2.5	0.32 ± 0.02	133.9 ± 2.9	0.11 ± 0.01
4×	51.0 ± 3.3	0.27 ± 0.01	104.3 ± 3.5	0.33 ± 0.02	232.9 ± 5.3	0.33 ± 0.01	97.3 ± 5.1	0.33 ± 0.02	255.0 ± 5.3	0.36 ± 0.03	138.5 ± 4.3	0.10 ± 0.01
10×	53.1 ± 2.9	0.26 ± 0.04	117.7 ± 4.5	0.34 ± 0.02	113.2 ± 2.9**	0.31 ± 0.02	100.8 ± 3.7	0.30 ± 0.01	263.0 ± 3.9	0.34 ± 0.03	164.0 ± 3.9	0.07 ± 0.01
(B) Polymersomes Prepared by Injection of Aqueous Phase to Organic Solution												
no	118.1 ± 2.9	0.13 ± 0.01	107.6 ± 4.1	0.15 ± 0.01	121.9 ± 4.1	0.18 ± 0.01	195.1 ± 5.3	0.20 ± 0.02	199.5 ± 4.3	0.17 ± 0.01	135.9 ± 4.5	0.10 ± 0.01
2×	115.6 ± 3.7	0.14 ± 0.01	95.1 ± 2.9	0.17 ± 0.02	106.8 ± 4.5**	0.18 ± 0.01	182.6 ± 3.7	0.18 ± 0.02	201.6 ± 4.7	0.18 ± 0.02	138.1 ± 3.7	0.10 ± 0.01
4×	116.4 ± 2.3	0.16 ± 0.01	96.6 ± 3.5	0.17 ± 0.01	101.1 ± 3.3**	0.19 ± 0.02	192.3 ± 6.1	0.20 ± 0.01	202.7 ± 5.1	0.21 ± 0.02	145.9 ± 6.3	0.01 ± 0.01
10×	116.5 ± 3.1	0.15 ± 0.01	88.2 ± 4.5	0.14 ± 0.01	97.7 ± 4.9**	0.15 ± 0.02	192.8 ± 4.5	0.19 ± 0.03	202.9 ± 5.5	0.19 ± 0.03	150.2 ± 3.9	0.10 ± 0.01

[a] Data presented are the average values of three replicate experiments ($n = 3$) followed by the standard deviation. Statistical differences between different degrees of dilution and no dilution (no) were expressed as *<0.05 and **<0.01 (one-way ANOVA−Tukey test). Polymersomes prepared by injection of organic solution (DMSO) to aqueous phase decreased in size (p-value <0.01) upon 10 times dilution, while those prepared by reverse order of addition using DMSO decreased their size upon all dilution factors (p-value <0.01).

Table 4. Effects of Different Aqueous Phases on Size and PDI of Polymersomes Prepared by Injecting $(PEG)_3$-PLA Copolymer Solution in DMSO/THF (15 mg/mL, 0.4 mL) to 5 mL of Aqueous Phases[a]

aqueous phase	size (nm) ± SD	PDI
Elga water	134.7 ± 4.2	0.26 ± 0.02
Elga water/ethyl acetate (8% v/v)	177.1 ± 5.2	0.13 ± 0.01
phosphate buffer (100 mM, pH 7.4)	302.6 ± 8.5	0.30 ± 0.02
phosphate buffer/ethyl acetate (8% v/v)	278.8 ± 6.3	0.30 ± 0.02
Tris buffer (10 mM, pH 7.4)	171.6 ± 2.5	0.05 ± 0.01
citric acid (300 mM, pH 4.0)	>2000.0	
ammonium sulfate (250 mM, pH 7.0)	precipitate	

[a] Data presented are the average values of three replicate experiments ($n = 3$) followed by the standard deviation.

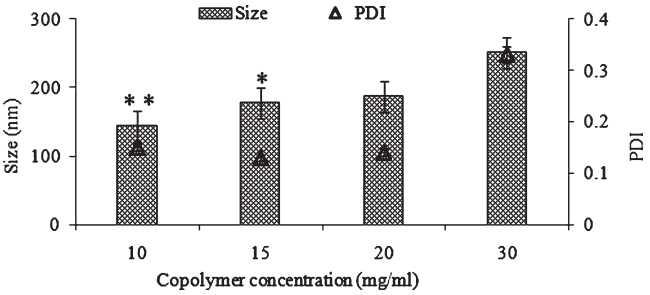

Figure 4. Influence of copolymer concentration on size and PDI of polymersomes prepared by injecting different concentrations of $(PEG)_3$-PLA copolymer solution in DMSO/THF (15 mg/mL, 0.4 mL) to 5 mL of Elga water/EA. For each column, values presented are average values of three replicate experiments ($n = 3$), and error bars indicate standard deviations. Statistical differences between copolymer concentrations on size were expressed using one-way ANOVA−Tukey test. *$p < 0.01$ and **$p < 0.001$ vs 30 mg/mL.

it led to larger polymersomes with high dispersity compared to a fast rate of addition. On the other hand, magnetic stirring is a common practice to be carried out to facilitate evaporation of solvent or during dialysis to prevent membrane clogging and maintain sink conditions. When stirring was allowed to proceed for 30 min or above, the size linearly increased to microsize range as the result is presented in Table 5. Observations of this study revealed that the same stirring speed with increasing stirring time gives larger polymersomes and after ∼3 h it resulted in a maximum size with the presence of precipitate. On the other hand when solution was not stirred at all, mixtures of nano- and micrometer size polymersomes with broad size range were obtained. Stirring at the time of solvent injection resulted in desired size range (100−200 nm) with homogeneous population characterized by narrow size distribution.

3.3. Drug Loading and Encapsulation Efficiency. Drug loading and encapsulation efficiency are important indices for drug delivery systems. This is especially true for expensive drugs, like doxorubicin. Drug loading content and encapsulation efficiency were determined using HPLC method. The results are shown in Table 6. Reasonable loading and encapsulation efficiency were obtained using Tris buffer (10 mM, pH 7.4) with average drug loading of 10.9 ± 0.49% (110 μg of drug/mg of nanopolymersomes) and encapsulation efficiency of 51.7 ± 2.3% as compared to Elga water saturated with ethyl acetate (pH 6.8). It is worthwhile to mention that the morphology and characteristic size of nanopolymersomes remain unchanged after doxorubicin is loaded as illustrated in TEM analysis (Figure 1).

3.4. *In vitro* Release Study. The *in vitro* release profile of doxorubicin from PolyDoxSome was determined at pH 7.4 (10 mM Tris buffer) or pH 5.5 (50 mM sodium acetate buffer) by a dialysis membrane method. Solution of free doxorubicin was also kept under similar conditions (to know if dialysis membrane hinders drug release) along with the release experiments and was treated as control in this study as shown in Figure 5. It is observed that free doxorubicin is released into bulk within 4 h, whereas only 15−25% of doxorubicin was released with the first 4 h. Doxorubicin-loaded nanopolymersomes showed a typical biphasic release profile in both release media. Release of the drug is relatively rapid in the first stage followed by a gradual decrease in release rate over a study period. Doxorubicin continued to be released from PolyDoxSome at a slower rate for over 5 days, after which the release rate was minimal. On comparison of doxorubicin

Table 5. Influence of Magnetic Stirring Time on Size and PDI of Polymersomes Prepared by Injecting $(PEG)_3$-PLA Copolymer Solution in DMSO/THF (15 mg/mL, 0.4 mL) to 5 mL of Elga Water/EA[a]

magnetic stirring time	size (nm) ± SD	PDI	*p*-values vs <1 min
<1 min (injection time)	177.1 ± 4.45	0.13 ± 0.01	
~30 min (self-assembly time)	565.3 ± 11.7	0.31 ± 0.02	0.001
≥3 h (solvent evaporation time)	>1000	1.00	0.0001

[a] Data presented are the average values of three replicate experiments ($n = 3$) followed by the standard deviation. Statistical analysis of magnetic stirring time on size was expressed using one-way ANOVA−Tukey test.

Table 6. Size, Loading and Encapsulation Efficiency of Doxorubicin in Nanopolymersomes Prepared Using Different Aqueous Phases[a]

	size (nm) ± SD		loading capacity (% w/w) ± SD		encapsulation efficiency (% w/w) ± SD	
theoretical loading	Tris buffer	water/EA	Tris buffer	water/EA	Tris buffer	water/EA
~21%	130 ± 4.45	301 ± 11.5	10.9 ± 0.49	5 ± 1.5	51.7 ± 2.3	25 ± 3.7

[a] Data presented are the average values of three replicate experiments ($n = 3$) followed by the standard deviation.

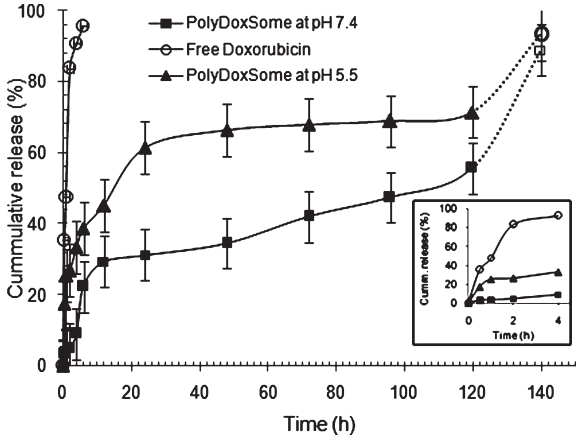

Figure 5. Cumulative *in vitro* release of free doxorubicin and PolyDoxSome ~130 nm at pH 7.4 (10 mM Tris buffer) or at pH 5.5 (50 mM sodium acetate buffer) at 37 °C using a membrane dialysis procedure over 5 days. All data points presented are average values of three replicate experiments ($n = 3$), and error bars indicate standard deviations. Release profile in acidic and basic media was compared by similarity factor ($f_2 < 50$). Dashed line indicates mass balance determined by hydrolyzing PolyDoxSome after 5 days. The same drug concentration was used (100 μg/mL, 500 μL) for free doxorubicin or PolyDoxSome (loading of ~11%). The inset indicates 4 h release profile of the experiment.

release at pH 5.5 and pH 7.4, the release of drug is faster at pH 5.5 (similarity factor, $f_2 < 50$).

3.5. *In Vitro* Cell Uptake and Cytotoxicity Study. In the present study internalization and cellular uptake studies were conducted at different doxorubicin concentrations and incubation times and were monitored by confocal microscopy and HPLC, respectively. Using the intrinsic fluorescence of doxorubicin, the internalization of PolyDoxSome in MCF-7 cell lines was visualized and compared to free doxorubicin by confocal laser scanning microscopy. Typical confocal microscopic pictures as shown in Figure 6 illustrate the distribution of internalized doxorubicin within cells with more intensity for PolyDoxSome, while MCF-7 cell lines, incubated with free doxorubicin, showed less intense fluorescence of internalized doxorubicin within cells. Moreover, control samples (untreated MCF-7 cells) at the wavelength of doxorubicin did not show any fluorescence as depicted in Figure 6 C,D. In agreement with confocal pictures, quantitative analysis of cellular uptake from lysed cells at different incubation times and concentrations of doxorubicin indicated that PolyDoxSome was internalized within cells to a greater extent relative to free doxorubicin (Figure 7). At constant doxorubicin concentrations (free doxorubicin or PolyDoxSome) cells were exposed with varying exposure times between 2 and 24 h. The results showed that the uptake was dependent on incubation time, which increased gradually with incubation time until saturation plateau phase is achieved at about 6−8 h (Figure 7 A). From the same study the uptake kinetics was shown by taking 6 h exposure that showed a linear correlation between intracellular and extracellular doxorubicin concentrations. The uptake of doxorubicin in either form was dependent on the concentration of doxorubicin and uptake increase with increase in the concentration, showing first order kinetics (Figure 7 B), while uptake efficiency decreased with an increase in concentration (Figure 7 C) in first order kinetics.

Cytotoxicity effect of free doxorubicin, PolyDoxSome and blank nanopolymersomes was tested. No cytotoxicity of the blank nanopolymersomes was observed at high concentration (100-fold of the concentration of blank polymersomes corresponding to that polymersomes containing 10 μM doxorubicin) as the cell viability did not decrease with reference to control over 72 h. Table 7 shows *in vitro* half-maximal inhibitory concentration (IC_{50}) values determined for free doxorubicin and PolyDoxSome on MCF-7 cell lines which were measured after 6, 24, 48, and 72 h of incubation times at different drug concentrations. The delayed effects were greater than the immediate effects with lower IC_{50} values for 6 and 24 h after treatment while there was no significant difference for 48 and 72 h incubation time. The cytotoxicity of PolyDoxSome was superior to free doxorubicin with low IC_{50} over a given incubation time in both immediate and delayed effects.

4. DISCUSSION

The present work is focused on examining several formulation and process variables that are suitable for optimizing the formulation with respect to the carrier size, dispersity and loading

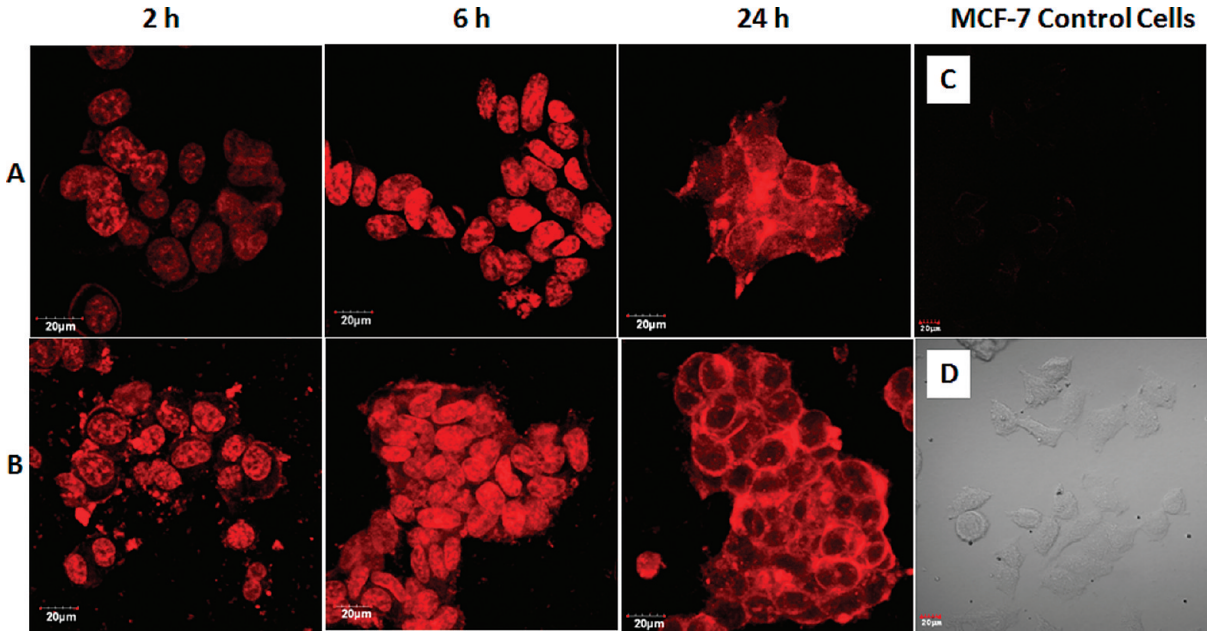

Figure 6. Confocal laser scanning microscopy (CLSM) images of MCF-7 cells: control or after drug exposure for 2, 6, and 24 h and untreated MCF-7 cells (from left to right): (A) free doxorubicin; (B) PolyDoxSome; (C) control cells under confocal image; (D) control cells under DIC image.

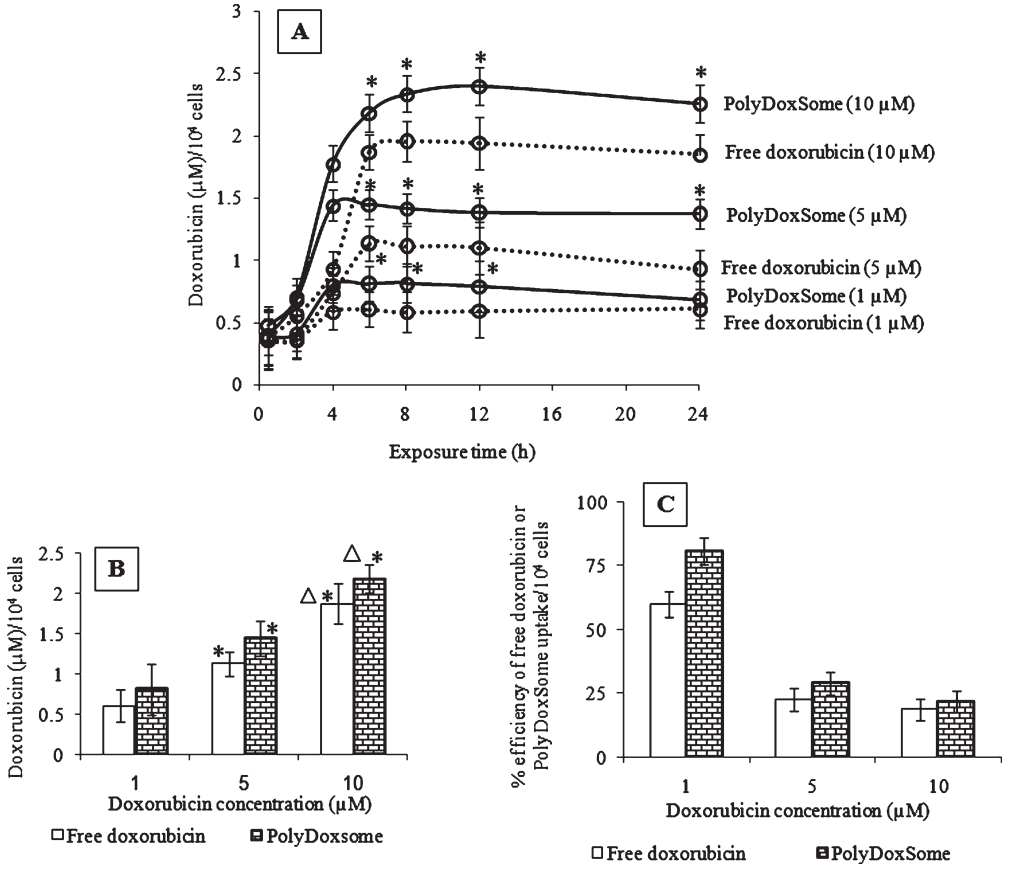

Figure 7. Intracellular uptake of doxorubicin by MCF-7 cells: (A) dependence of intracellular doxorubicin concentration on exposure time to constant doxorubicin concentration; (B) 6 h exposure time and determination of linear correlation between intracellular and extracellular doxorubicin concentration; (C) uptake efficiency. All data points or column values presented are average values of four replicate experiments ($n = 4$), and error bars indicate standard deviations. Statistical analysis was carried out using one-way ANOVA—Tukey test. *$p < 0.01$ vs 1 μM; $\Delta p < 0.05$ vs 5 μM.

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Table 7. *In Vitro* Half-Maximal Inhibitory Concentration (IC_{50}, μM) Values Determined for Free Doxorubicin and PolyDoxSome on MCF-7 Breast Cancer Cell Lines at Different Incubation Times and Drug Concentrations[a]

	immediate effect		delayed effect		*p*-values vs doxorubicin	
exposure time (h)	doxorubicin	PolyDoxSome	doxorubicin	PolyDoxSome	immediate	delayed
6	>20	>20	1.5	0.45		0.01
24	10	10	0.5	0.35		0.05
48	1	0.5	0.5	0.3	0.01	0.05
72	0.5	0.2	0.5	0.3	0.01	0.05

[a] IC_{50} values presented are the averages of four replicate experiments ($n = 4$). Statistical analysis on IC_{50} values was expressed using one-way ANOVA—Tukey test.

and encapsulation. The physicochemical characteristics of colloidal systems, namely, size, polydispersity and charge, are believed to influence the interaction with the cells and the biological milieu after *in vivo* administration. In this study various sizes of blank or doxorubicin-loaded polymersomes have been developed to characterize using DLS and different microscopic techniques. The self-assembly conditions of the copolymer depend on water—solvent, water—copolymer, and solvent—copolymer interactions which can modify the interdiffusion process between the two solvents. These interactions can be modulated by other, external processing conditions as described elsewhere. Hence independent study of each formulation and process variable is deemed necessary to meet a desired size and dispersity of polymersomes for targeted application. The method used in this work allowed the reproducible formation of homogeneous doxorubicin-loaded nanopolymersomes in the size range of 130—180 nm as shown by DLS technique and TEM observation. The average diameter of nanopolymersomes observed with TEM was ~40% smaller as compared to the measurements made by DLS; this could be due to the deflation while drying and processing conditions of TEM analysis.

It is observed that the size of nanopolymersomes is influenced by several formulative and process variables, with nature of solvent, order of addition, polymer concentration and external energy being its main determinants. The nature of solvent system can define the type of copolymer—solvent interactions and therefore can affect the size and morphology of self-assembled aggregates upon addition to aqueous phase by changing relative coil dimension of both membrane and corona chains. In general, the strength of polymer—solvent interaction is described by solubility and dielectric constant, and the polarity of the solvent influences the repulsion between the hydrophilic blocks.[45,46] The average sizes of nanopolymersomes obtained using DMSO and THF as organic solvents are relatively bigger compared to those obtained either using mixture of DMSO and acetone or acetone or DMF alone. This can be explained due to lower mixing rates of DMSO solution with aqueous phase attributed from higher viscosity of DMSO, whereas THF has lower miscibility with water compared to acetone or DMF attributed from its low dielectric constant and polarity index. It is likely that copolymer from highly polar and miscible organic solvent is rapidly exposed to a high water content upon solvent injection to aqueous phase that results in relatively faster precipitation and self-assembly to smaller polymersomes.

Apart from the influence of organic solvent, order of addition for the preparation of nanopolymersomes by nanoprecipitation has also been explored. Rapid injection of aqueous phase to organic phase gave polymersomes of bigger size compared to the reverse order of addition. A possible explanation for this can be due to lower mixing rates and low turbulence when a large amount of aqueous phase is added to a small volume of organic solution. Moreover, addition of water causes an increase in size in order to reduce the interfacial free energy between core and solvent in the corona (if the core is still mobile). Mobility of the core is possible in the presence of a significant proportion of organic solvent in solvent mixture since aqueous phase is injected to organic phase. Subsequently, as more aqueous phase is added, the solvent becomes increasingly poor for PLA block and the structure continues to increase in size, although addition of aqueous into organic phase of DMSO or mixture of DMSO and THF resulted in smaller size that can be explained due to faster rate of precipitation that resulted from dilution effect and decreased viscosity of DMSO and leading to kinetic locking of copolymer chains into formed self-assemblies. On the other hand, for other organic solvent systems, addition of organic phase to aqueous phase resulted in smaller sizes and relatively high dispersity polymersomes that can be explained due to rapid and turbulent conditions of frozen polymeric chains. Hence, addition of organic phase to aqueous phase was optimized for final formulation based on desired size and dispersity of formulation developed.

To investigate the effect of dilution of nanopolymersomes on size and polydispersity, polymersomes were diluted at various degrees of dilution using water. It is reported that, after vesicle formation, vesicles in mixed solvent systems increase in size due to the reduction of interfacial tension between hydrophobic vesicle wall (swelling with organic solvent) and external solvent system in the corona (if the core is still mobile).[47] In order to decrease the total interfacial free energy, fusion of smaller vesicles occurs with low growth rates and resulting in an increase in the vesicle size. Another interesting possibility is if solvent mixture is more polar because of large proportion of aqueous phase, as in the case of dilution, the core is immobile and hence vesicles are unable to reopen or fuse to larger ones and would be insensitive to dilution as is the case in this system. This might also be due to the shape of $(PEG)_3$-PLA where polymer has a larger hydrophobic chain with smaller PEG chains. This can facilitate the good locking of hydrophobic chains with small aqueous core made by small PEG chains, thus reopening of hydrophobic chain is difficult to fuse the vesicles with other vesicles to get the bigger size upon dilution.

In addition to organic solvents, the aqueous phase also has its effect on size and polydispersity of self-assembled structures. Hence, in the present work, different composition of aqueous phase was considered as the most critical step in the formation and size control of polymersomes as it is selective solvent for

hydrophilic PEG block (poor solvent for PLA block) and causes self-assembly (precipitation). The presence of ethyl acetate in Elga water slowed down the dissolution of water miscible organic solvents in water and hence resulted in a larger size and lower dispersity. Moreover, ethyl acetate is entrapped in the hydrophobic part of membrane during polymersome formation and softens the hydrophobic moiety, hence giving copolymer molecules adequate time and chain mobility to self-assemble to more homogeneous (less PDI), larger and stable polymersomes. On the other hand, the mean diameter of polymersomes obtained using buffer systems of citrate, phosphate and sulfate were larger compared to Tris buffer system and Elga water (with or without ethyl acetate saturation). This can be due to lower diffusion rates of organic solvents to buffers containing ions. If solvent mixture is more polar, as is the case of Elga water or Tris buffer, the hydrophobic core is immobile and particles are unable to reopen and fuse to larger ones. Hence it is observed that composition of aqueous phase should be more polar but nonionized to get smaller size and good dispersity of polymersomes.

To study the effect of copolymer concentration on size and polydispersity of polymersomes, copolymer with various concentrations was rapidly injected and characterized for size and polydispersity. There was a linear increment of size and polydispersity index as copolymer concentration increased. The effect of the polymer concentration on size and polydispersity appears to be due to higher resultant organic phase viscosity. High viscosity results in a more poorly dispersible copolymer solution to aqueous phase upon rapid injection that changes solvent diffusion kinetics and provides resistance to turbulence. Moreover, higher polymer concentration can lead to larger nanodroplet formation.

Similarly, the effect of external energy on size and polydispersity of polymersomes was investigated by varying the speed of injection and time of magnetic stirring. Injection speed has a significant effect on size and polydispersity of polymersomes as dropwise injection resulted in larger size of polymersomes that is believed to be caused by longer equilibration of the mixture that can allow vesicle fusion and growth. Additionally, dropwise addition of organic solvent into aqueous phase or opposite lacks turbulence of mixing for rapid induction of morphological rearrangement. On the other hand, magnetic stirring is a common practice to be carried out to facilitate evaporation of solvent or during dialysis to prevent membrane clogging and maintain sink conditions. In this study, when stirring time was allowed to proceed further for 30 min or above, the solution was strongly agitated and resulted in less reproducible larger polymersomes and copolymer precipitation as shown in Table 3. Extra stirring during polymersome formation can prevent the copolymer chains from adjusting fast enough to adopt a stable and nanosize vesicular form. On the other hand when solution was not stirred at all, mixtures of nano- and micrometer size polymersomes with broad size range were obtained. Hence, it is the finding of this study that vigorous stirring for a short period of time (only during injection) provides uniform nanosize range polymersomes. These results suggest that addition of external energy during formation of polymersomes (speed of injection or magnetic stirring) has a significant effect on size and dispersity of polymersomes, and results are in agreement with the other reports on different systems.[26,36,40]

Along with size optimization, the loading and encapsulation efficiency of the doxorubicin-loaded nanopolymersomes were studied under different conditions. Doxorubicin has pH dependent solubility and is completely ionized in acidic pH and thus is highly soluble. Proportion of nonionic state increased at pH 7.4 and is relatively less soluble. This solubility factor contributes the better encapsulation when Tris buffer is used at pH 7.4 because it reduces rapid diffusion of drug to outer aqueous phase. Moreover, the speed of self-assembly and nanoprecipitation that occurs in relatively high polar aqueous phase (Tris buffer) compared to Elga water saturated with ethyl acetate enables the drug to be rapidly entrapped, thus reducing its diffusion to the outer aqueous phase.

The *in vitro* release profile of doxorubicin from PolyDoxSome was studied under different conditions. Either the ability of doxorubicin to diffuse through the hydrophobic membrane of PolyDoxSome or degradation of the carrier membrane can affect the release profile of doxorubicin from PolyDoxSome. It is well reported and widely exploited that the hydrolysis of polyesters is accelerated by acidic pH which can change the hydrophobic—hydrophilic ratio in the polymer chain and thus may cause the degradation of polymersomes. In the same line of understanding, the release of doxorubicin from PolyDoxSome is relatively faster at acidic pH compared to neutral release medium, which is also in agreement with other studies.[27,28,34,41,48−52] This pH dependent release behavior is of particular interest in achieving the tumor targeted doxorubicin delivery with PolyDoxSome. Slower release rate of doxorubicin from PolyDoxSome at pH 7.4 demonstrated that most doxorubicin remains entrapped in PolyDoxSome for a considerable time period when injected PolyDoxSome stays in the plasma due to extended plasma circulation. This indicates that PolyDoxSome is able to deliver doxorubicin in a controlled manner over an extended period of time. From this finding it is anticipated that the faster release will occur once the PolyDoxSome reaches the tumor tissue (acidic microenvironment).

After formulation optimization, it is also important to know the *in vitro* efficacy of the formulation using related cell line, and thus the cytotoxic effect of PolyDoxSome is assessed using MCF-7 cell lines. The cytotoxic effects of doxorubicin would depend on the intracellular concentration and sustained retention of the drug by MCF-7 cell lines. In order to increase the cellular internalization of a drug, an appropriate concentration of the drug in the extracellular region should be maintained for a desired time period. The results of this study confirmed that free doxorubicin showed less cytotoxicity compared to PolyDoxSome at equivalent doxorubicin dose level. Blank nanopolymersomes did not show any toxicity, indicating that the superior cytotoxicity of PolyDoxSome is due to delivery mechanism. A steep increase in cytotoxicity in MCF-7 cell lines when PolyDoxSome is used indicates that PolyDoxSome is more potent than free drug. A possible explanation for the activity enhancement of PolyDoxSome can be attributed to different internalization mechanism. PolyDoxSome can be more readily internalized by different transport mechanism, such as endocytosis (energy dependent), while free doxorubicin was transported into cells only by a passive diffusion. Because of concentration dependent passive diffusion,[25,28,53] free doxorubicin was less effective at low concentration compared to PolyDoxSome which would be uptaken by endocytosis, an energy requiring process. Incubation time and concentration dependent cytotoxicity on MCF-7 cells was observed for both free doxorubicin and PolyDoxSome. Within 24 h of incubation, free doxorubicin did not show considerable inhibition effect on cell growth when doxorubicin concentration was maintained below 1 μM, whereas PolyDoxSome showed about 15% inhibition at concentration as low as

0.01 μM. In contrast, cell viability significantly decreased after incubation for 72 h against either free doxorubicin or PolyDoxSome in the concentration range of 1 to 20 μM. This indicates that over long incubation time there is sufficient doxorubicin internalization.

5. CONCLUSIONS

In this study, nanopolymersomes of various sizes could be obtained by nanoprecipitation method. To find out optimal conditions for nanoformulation development, a systematic investigation was carried out by changing different experimental parameters. It is worthwhile noting that the size and dispersity of nanopolymersomes were strongly influenced by the overall composition of organic and aqueous media. Moreover, order of addition of phases, copolymer concentration, and external energy input were found equally significantly to affect size and dispersity of polymersomes. However, they retained their size and dispersity against different degrees of dilution most probably due to immobility of the core upon addition of a large proportion of polar aqueous phase and hence vesicles are unable to reopen or fuse to larger ones. Under optimized conditions, PolyDoxSome in the size range of 130 to 180 nm with narrow size distribution (PDI < 0.2) were obtained where sterilization may conveniently be performed by membrane filtration using <200 nm sized filtration system. *In vitro* release profile that displayed slow doxorubicin release at physiological pH and fast release within more acidic release medium demonstrated the great potential of such carriers for anticancer chemotherapy. Thus, existing tumor pH variation in endolysosomal compartment after endocytosis and tumor tissue acidic microenvironment following extravasations would be an ideal trigger for the selective release of doxorubicin in tumor tissues or within tumor cells accomplishing tumor targeted delivery. *In vitro* cellular studies demonstrated higher cellular uptake of doxorubicin from PolyDoxSome than from doxorubicin in solution as observed through confocal microscopy and quantitatively determined from lysed cells and that the level of intracellular drug accumulation increased in a time and extracellular concentration dependent manner. Exposure of MCF-7 cells to PolyDoxSome resulted in higher cytotoxicity in comparison to the equivalent exposure concentrations of doxorubicin in solution, and cytotoxicity was dependent on concentration and exposure time. It was also observed that there was no further increase in polymersome/drug uptake by MCF-7 cells after 8 h of incubation time. All these results indicated a pronounced improvement in the polymersome uptake by MCF-7 cells and enhanced cytotoxic effect of doxorubicin at equivalent concentration when delivered in nanopolymersomes compared with the free drug.

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