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Fabrication of Biodegradable Poly(*d,l*-lactide-*co*-glycolide) Nanoparticles Containing Tamoxifen Citrate

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ABSTRACT

Biodegradable polymers have been extensively investigated as nano-carrier delivery systems in anti-cancer therapy. The anti-cancer drugs generally suffer from low aqueous solubility, short in vivo half-life and haphazard side effects. In this work, biodegradable poly(*d,l*-lactide-*co*-glycolide) nanoparticles (PLGA) containing tamoxifen citrate as a model anti-cancer drug were prepared using an o/w emulsification-solvent evaporation method. Dynamic light scattering (DLS), scanning electron microscopy (SEM) and analytical HPLC procedures were used to characterize the nanoparticles in terms of particle size, morphology and drug content. The characteristics of the nanoparticles including size, drug loading, and the efficiency of encapsulation were optimized by means of a full factorial experimental design over the influence of four different independent variables. Analyses of variance (ANOVA) were used to evaluate the optimized conditions for the preparation of nanoparticles. Based on the results, the most significant variables were homogenization speed and concentration of PLGA in organic phase with known total volume. Also, the interactions between the percentage of PVA and the amount of PLGA and organic phase volume were the most important cross-factor parameters. The optimum formulation condition with 192 nm mean size and 33 w/w% loading capacity was established by using 3 mg.mL^{-1} PLGA/dichloromethane in 7 w/v% PVA solution and 40% oil/water solvent ratio for emulsification at 24000 rpm homogenization rate. The results of this work facilitate the development of nano-carriers for tamoxifen delivery through optimization studies to control nanoparticles with specific properties and establish correlations between optimum production conditions and the required nano-carrier desired characteristics.

Key Words:

anti-cancer;
factorial design;
nanoparticle;
PLGA;
tamoxifen citrate.

INTRODUCTION

Advanced techniques in the field of drug delivery are emerging to increase the efficiency of pharmacotherapy by improving drug release administration performance [1]. Different classes of polymers have been introduced for preparation of polymeric delivery systems, which ideally should be biocompatible, biodegradable, processable and mechanically strong [2]. Among different classes of polymers, homopolymers of lactic acid (PLA) and copolymers of lactic and glycolic acids (PLGA) have been widely studied, due to their desirable properties and FDA approval [3-6]. Novel polymeric types of delivery systems can be highly beneficial for anti-cancer

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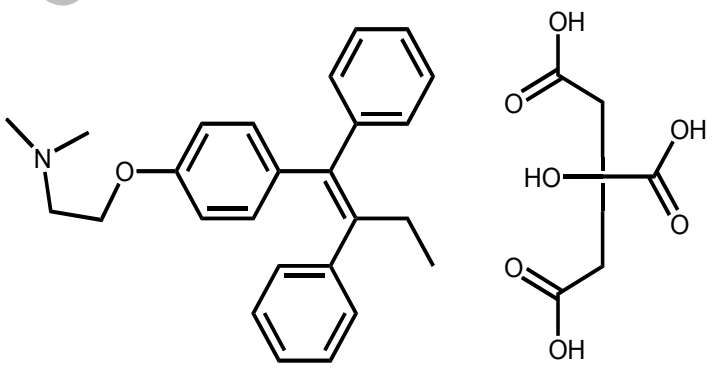
agents, since these drugs generally suffer from low solubility in biological systems, short in vivo half-life and disordered side effects. Cancer with six million annual deaths or 22.8% of the total mortalities is the leading cause of deaths worldwide. It is clear that the progress in cancer treatment has been slow and inefficient [7,8]. Breast cancer with 184450 new cases and 40930 deaths has been the most predominant cancer in the United States in 2008 [9]. Among novel drug delivery systems, polymeric nanoparticles which can be designed to control drug release in reaching target tissues seem to be promising in reducing dosage frequency and side effects in cancer therapy [10].

Polymeric nanoparticles are colloidal systems with diameters typically less than 1 μm in size and are formulated with a biodegradable polymer in which the therapeutic agent is entrapped in, adsorbed or chemically coupled onto the polymer matrix and released by different mechanisms [11-13]. Nanoparticles, can also improve the bioavailability of poorly absorbable drugs for oral delivery [14,15]. Moreover, they are able to permeate cells for cellular internalization and connective tissue permeation, and thus deliver the drug efficiently to the targeted tissues without clogging the capillaries [16,17]. The ability of polymeric nanoparticles to improve drug diffusion through biological barriers is a typical advantage for the delivery of anti-cancer agents. The enhanced endocytic activity and leaky vasculature in the tumor could result in accumulation of intravenously administered nanoparticles, protraction drug retention in tumor tissue, tumor growth reduction and prolongation of animal life [16,18]. Since it is difficult to assess the effect of the manufacturing parameters (variables) on the nanoparticle characteristics (responses), therefore establishing a statistical technique suitable for quantitative correlations between the variables and the responses seems to be necessary [19,20].

The objective of this work was to prepare and characterize tamoxifen loaded PLGA nanoparticles as a potential drug carrier for anti-cancer agents. Tamoxifen citrate (Scheme I) is used as a model anti-cancer drug with typical characteristics of this class of drugs, including low solubility in aqueous media, short in vivo half-life and haphazard side effects. It has been registered for breast cancer therapy for more than 25 years [21]. Interestingly, investigations about the tamoxifen loaded nanoparticles have been increased recently. For example, tamoxifen-loaded in poly(MePEG cyanoacrylate cohexadecyl cyanoacrylate) [22], poly(*D*-caprolactone) [23] and polyethylene glycol (PEG)-coated nanoparticles [24] with different therapeutic objectives have been reported. All these interests in particulate delivery of tamoxifen show potential advantages for using PLGA copolymers as a desirable matrix [3-6] to deliver this drug. Despite the previous report on the preparation of tamoxifen loaded PLGA microspheres [25] with totally different distribution profile in biological fluids, we have not



[(Z)-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-*N,N*-dimethylethanamine]

Scheme I. Chemical structure of tamoxifen citrate.

found similar study on tamoxifen loaded PLGA nanoparticles. The influence of different factors (single or combined) on the nanoparticles performance was studied by an experimental design technique.

EXPERIMENTAL

Materials

Tamoxifen citrate was obtained from Chemische Fabrik Berg Co., (Bitterfeld, Germany). PLGA copolymer (50/50; MW 40000-75000) was purchased from Boehringer Ingelheim Co. (Ridgefield, USA) and PVA copolymer (88% hydrolyzed, MW 22000) was purchased from Acros (New Jersey, USA). HPLC grade methanol was obtained from Caledon Co. (Georgetown, Canada), and water was purified by reverse osmosis (Milli-Q Millipore, France). Dichloromethane (DCM) was purchased from Qualigenes Co. (Bombay, India). Sodium 1-octane-sulphonate and glacial acetic acid were supplied by Merck (Darmstadt, Germany).

Preparation of Nanoparticles

Screening studies were carried out to set up the experimental conditions. Based on the amount of tamoxifen citrate solubility in dichloromethane (8.3 $mg.mL^{-1}$) a saturated organic phase was used in all parts of the work. In order to choose the best mixing time, some one-at-a-time experiments were performed from 1-30 min, while the other variables were fixed at their mid-levels. For each trial, the stability of the emulsion after 1 h and the particle size were studied. The results revealed that the most stable emulsion with the smallest particle size was prepared by 20 min homogenization. Based on our previous studies [32] and for determining the effects of two other important parameters, i.e., PVA concentration (2 and 3 w/v%) and homogenization speed (13500 and 24000 rpm) on preparation of drug loaded PLGA particles within the nanometer size range, these variables were taken at their two extremes of low and high levels.

For the rest of the study, the polymeric nano-particles were prepared by the following procedure: exact quantities of PLGA polymer and tamoxifen citrate (1 mg) were weighed and dissolved in dichloromethane. The organic phase was added into PVA aqueous solution and immediately homogenized using high speed homogenizer, (IKA, Ultra-Turax® T25 Basic, S25N-18G head, Germany) at room temperature for 20 min. The emulsion was stirred magnetically at room temperature overnight until the organic solvent was completely evaporated. Subsequently, nanoparticles were separated by centrifugation (Hettich, EBA-20, Germany). The separated nanoparticles were re-dispersed and centrifuged three times in distilled water to remove free drug and excess surfactants completely.

Experimental Design

Based on previous studies, the experiments were carried out by an o/w emulsification solvent evaporation method using a two-level full factorial design [20]. Design Expert 6.0.10 trial software was applied for designing the experiments. Three important responses including loading, efficiency and particle size were evaluated. Sixteen batches of nanoparticles were prepared according to the full factorial design process. Based on the results and the statistical models, the software offered optimum setting of formulation for the test conditions.

Particle size and Morphology

Nanoparticle mean diameter was measured by dynamic light scattering technique (DLS) using a Malvern NIBS nanosizer, (Worcestershire, UK). The analysis was performed at a temperature of 25°C using samples appropriately diluted with the ultra purified water in the range of 0.6 nm - 6 μm. The morphology of the nanoparticles was determined using scanning electron microscopy (SEM) (Phillips, the Netherlands). The samples were prepared on aluminium stabs and coated with gold prior to examination by SEM.

Drug Entrapment Studies

Whole nanoparticles prepared in each run were weighed and dissolved in 2 mL dichloromethane, which was then evaporated under a gentle stream of nitrogen gas and dissolved in 1 mL HPLC grade methanol for assay test. The concentration of tamoxifen citrate was analyzed by the reported USP

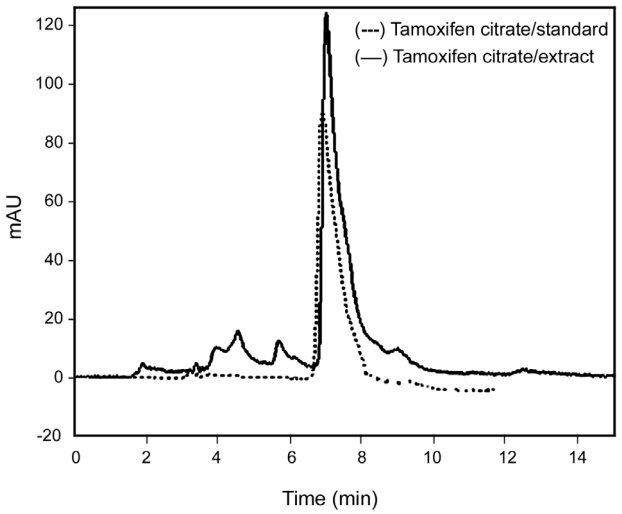


Figure 1. Tamoxifen citrate chromatogram.

HPLC method [26]. In brief; the HPLC system consisted of a model K-1001 solvent delivery equipped with a model PDA K-2700 ultraviolet detector at 254 nm (Knauer, Germany). The analytical column was (4.6×150 mm, 5 µm) CLC-phenyl L11, (Shimadzu, Japan). The mobile phase consisted of methanol solution containing 320 mL water, 2 mL glacial acetic acid, and 1.08 g sodium 1-octanesulphonate per liter. The retention time of tamoxifen was about 6.68 min (Figure 1). The amount of drug loading in nanoparticles and the nanoparticle recovery, also known as nanoparticle yield, was calculated using the following equations:

$$Loading(\%) = \frac{(weight\ of\ tamoxifen\ in\ sample)}{total\ weight\ of\ the\ sample} \times 100 \quad (1)$$

$$Efficiency(\%) = \frac{(loading \times total\ nano-particle\ weight(mg))}{added\ tamoxifen(mg)} \times 100 \quad (2)$$

RESULTS AND DISCUSSION

Following the intravenous administration, targeted drug delivery has to overcome many obstacles to reach the target tissue including the rapid opsonization and uptake of the injected carrier systems by the reticuloendothelial system (RES) in liver and spleen. In the case of nanoparticles (NPs), the intravenous distribution is significantly influenced by the particle's size and surface hydrophobicity, as particles higher than 300 nm are eliminated from blood. It is demonstrated that the higher drug bioavailability is obtained for nanoparticles in the size range 100-300 nm [27-29]. In the present work, the production techniques are introduced to control the size and drug loading of tamoxifen loaded PLGA nanoparticles. Also, to design a fabrication process to minimize drug losses during its processing, the efficiency of the production was evaluated using experimental design approaches.

PLGA nanoparticles were prepared by an o/w emulsification-solvent evaporation method, where the organic phase containing drug and polymer was added to an aqueous phase containing stabilizer. The resulting dispersion was immediately homogenized at high-speed and stirred over night at room temperature to remove the organic solvent [30]. This method may involve complex interfacial hydrodynamic phenomena. Among various factors, solute migration from the dispersed phase, concentration gradient near the interface and interfacial tension sensitivity towards solute concentration are the most important factors. The presence of surfactant may markedly complicate the situation since they act to suppress interfacial flow and the diffusion of dichloromethane to the aqueous phase. The main advantage of using surfactants in the process is the instantaneous and reproducible formation of nanometric, monodispersed nanospheres exhibiting a high drug loading capacity [31].

Four independent factors that affect the drug loading in o/w emulsion-solvent evaporation method, i.e., the surfactant concentration (A), the homogenization speed (B), the amount of polymer (C) and the volume of the organic phase (D) have been selected in this study. In order to adjust suitable levels of each factor, some primary studies were performed. For evaluation of the best levels, surface morphology and size of the resulted particles were considered. SEM studies on the resulting tamoxifen nanoparticles showed slick and spherical particles with apparent size of >1µm, as shown in Figure 2.

The results demonstrate that increasing PVA

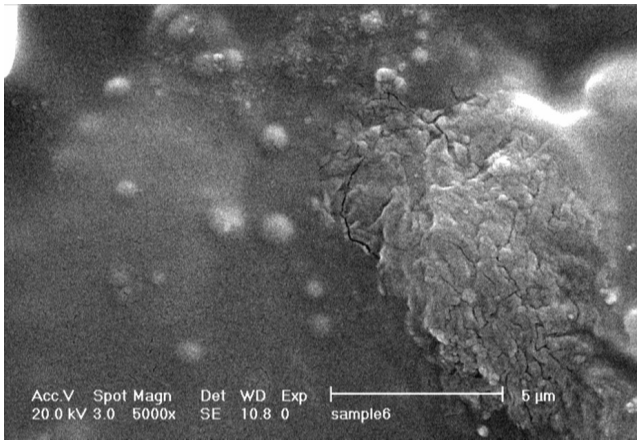


Figure 2. SEM Micrograph of tamoxifen loaded PLGA nanoparticles.

aqueous concentration and homogenization speed give rise to better particles, as can be seen in Figure 3, by comparing the size distribution curve for 2 w/v% PVA concentration and 13500 rpm homogenization speed and 3 w/v% PVA concentration and 24000 rpm homogenization speed in screening experiments which resulted in smaller particle size and narrower size distribution. To reduce further the particle size to less than 300 nm, PVA as a common surfactant in the preparation of PLGA nanoparticles was used with concentrations above 7.0 w/v% [32]. Also with a view of previous reports on PLGA nanoparticles formulation, the polymer concentration of 3 and

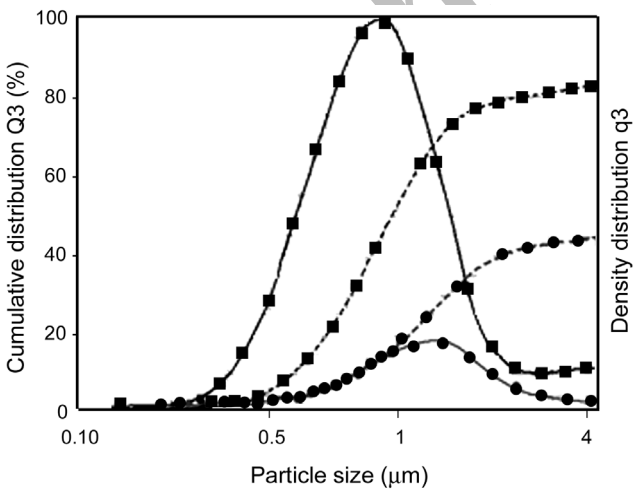


Figure 3. Particle size distributions of: PVA (2 w/v%) at homogenization speed of 13500 rpm (●) and PVA (3 w/v%) at homogenization speed of 24000 rpm (■).

Table 1. Selected variables and their levels.

Variables	Coded units	Levels	
		-1	+1
PVA (%)	A	7	10
Speed (rpm)	B	13500	24000
PLGA ($mg.mL^{-1}$)	C	3.00	5.00
Organic phase (mL)	D	20	40

5 $mg.mL^{-1}$ levels were selected. Finally, based on the instrumentation, the levels of homogenization process were selected to constant levels of 13500 and 24000 rpm, as given in Table 1.

The main objective in optimization studies is to determine the experimental conditions which yield the best responses. However, sometimes this is not so easy because experimental responses may be contradictory and require optimal compromises to be made to draw distinctions among them [33,34]. The selection of factors and levels in the design, which most affects drug loading, would be based on the results of some preliminary investigations. Based on the screening results, a technique of two-level factorial design offers the possibility of investigating four independent variables at two levels after only 16 experimental performances [20].

Since the experiments need at least two consecutive days to be run and in order to minimize the effect of uncontrolled within-a-day variables on the response, the experiments were performed based on the randomization law on 2 blocks. Corresponding design matrix and four responses are shown in Table 2. The influence of the above mentioned parameters on each calculated responses was evaluated by a full factorial experimental design. Using the above mentioned formulation technique in preparation of tamoxifen loaded PLGA nanoparticles a linear correlation between the individual response and the influential parameters can be established, as given in Table 3.

The results were calculated according to the ANOVA calculation (Table 4). The implication of the F value (the ratio of mean-squared error of each treatment to the one of residuals) depends on the degree of freedom of the model, as the effect of

Table 2. Full factorial design experiments and responses.

Batch No.	Ran.[1]	Block	Parameters				Responses		
			A	B	C	D	Loading (%)	Efficiency (g)	Particle size (nm)
1	15	2	-1	-1	-1	-1	18.12	0.07	202
2	6	1	1	-1	-1	-1	1.79	0.01	195
3	4	1	-1	1	-1	-1	33.81	0.08	132
4	14	2	1	1	-1	-1	17.44	0.07	117
5	8	1	-1	-1	1	-1	25.02	0.03	256
6	13	2	1	-1	1	-1	10.91	0.05	190
7	12	2	-1	1	1	-1	14.31	0.04	151
8	2	1	1	1	1	-1	29.45	0.03	139
9	7	1	-1	-1	-1	1	54.46	0.14	187
10	16	2	1	-1	-1	1	12.22	0.13	232
11	10	2	-1	1	-1	1	27.99	0.68	54
12	3	1	1	1	-1	1	33.56	0.08	156
13	11	2	-1	-1	1	1	14.62	0.13	188
14	1	1	1	-1	1	1	13.99	0.04	205
15	5	1	-1	1	1	1	13.48	0.05	143
16	9	2	1	1	1	1	19.89	0.09	120

(1) Randomization, A: surfactant (PVA) percentage; B: homogenization speed; C: PLGA concentration; D: organic phase volume.

Table 3. Final equation details in terms of influential factors.

Factors	Coefficient estimates					
	Loading		Efficiency		Particle size	
Intercept	+ -	7.03 0.90	+ -	0.97 3.28×10^{-3}	+ +	919.50 11.96
A	+	0.70	*NS*		-	285.66
B	-	0.64	*NS*		+	47.00
C	*NS*		+	5.04×10^{-3}	-	39.02
D	+	1.22	*NS*		*NS*	
AB	+	1.08	+	2.62×10^{-3}	-	90.55
AC	*NS*		*NS*		+	106.48
Equation power	0.65		0.01		1.33	

NS: non-significant model term. $[\text{Loading}]^{0.65} = -0.90\,A + 0.70\,B - 0.64\,C + 1.22\,AB + 1.08\,AC + 7.03$; $[\text{Particle size}]^{1.33} = 11.96\,A - 285.66\,B + 47.00\,C - 39.02\,D - 90.55\,AC + 106.48\,AD + 919.50$.

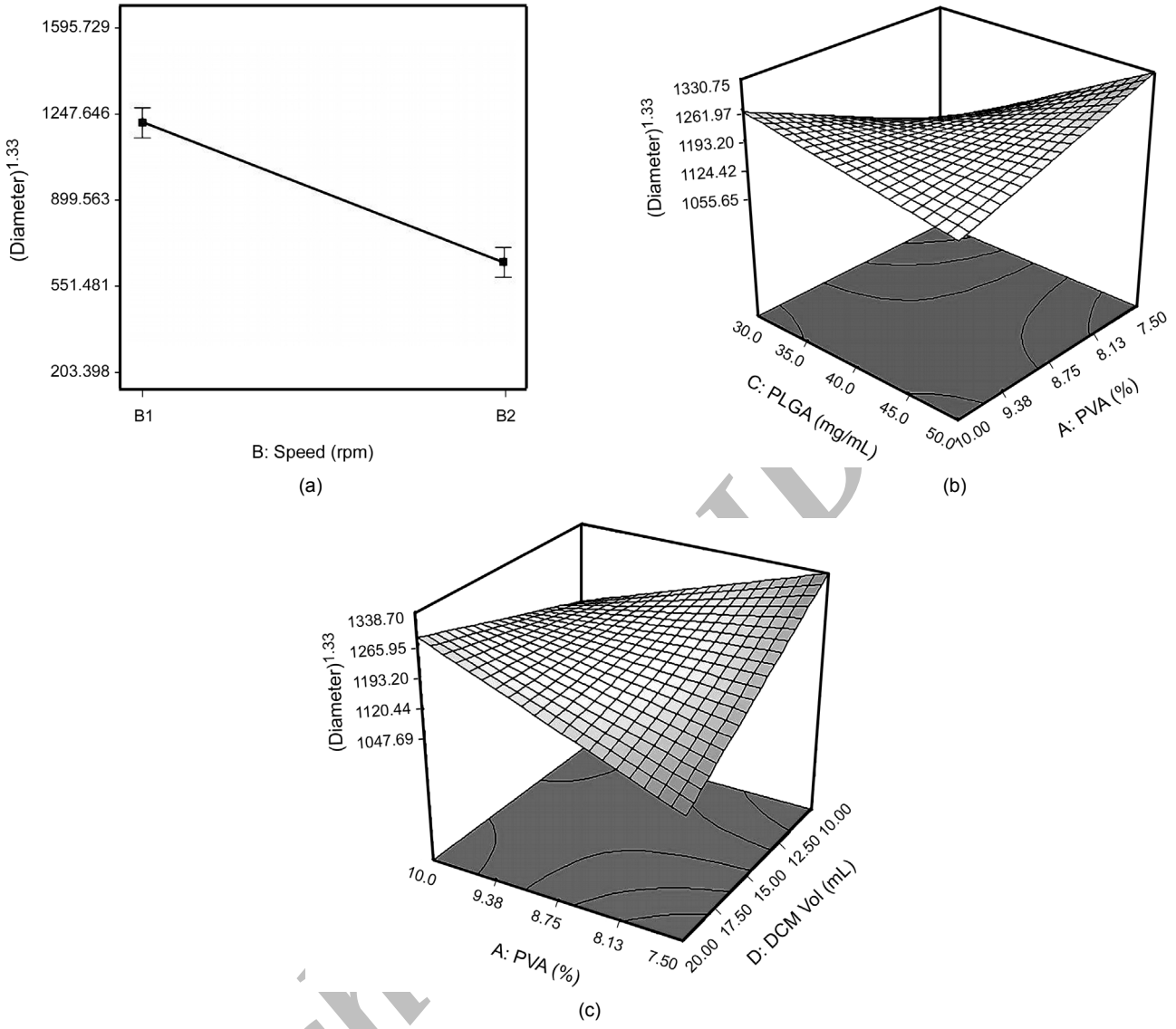


Figure 4. Response surfaces estimated from full factorial design: (a) homogenization speed, (b) amount of PLGA vs. PVA, and (c) PVA percentage vs. organic phase volume.

$p<0.0001$ in this column was statistically significant. The standard error of the estimate, the R-squared and the adjusted R-squared of each response are presented in Table 4. Based on the results, the most significant parameters were the homogenization speed and the concentration of PLGA in organic phase and its total volume. Also, the interactions between the percentage of PVA and the amount of PLGA and organic phase volume were the most important cross-factor parameters.

Figure 4 shows the response surface function developed by the model considering the effective parameters. It may be seen in Figure 4a that, increased mechanical stress which is followed by higher homogenization speed, (B2) makes smaller size particles. Also, increased PVA concentration with similar influence makes the particles smaller and the drug entrapment more efficient, (Figure 4b). Furthermore, the reduction of PLGA concentration in constant organic phase volume and higher PVA concentration, (Figure 4b) may give smaller particles.

In Figure 4c, with the reduction of the organic phase volume, it is noticed that the initial emulsion droplets contain higher concentration of PLGA and tamoxifen citrate, and consequently the mean particle size is increased while more efficient drug entrap-

Table 4. Analysis of variances for factorial design experiment.

Source	Loading			Efficiency			Particle size		
	SS [1]	Df [2]	Prob>F	SS	Df	Prob>F	SS	Df	Prob>F
Model	3.28	5	0.0328	6.89×10^{-4}	3	0.0039	1.68×10^{6}	6	0.0001
A	0.76	1	0.0574	1.72×10^{-4}	1	0.0157	2287.96	1	0.6687
B	*NS*			3.46 ×10-4	1	0.0970	1.31×10^{6}	1	< 0.0001
C	0.29	1	0.2107	*NS*			35350.88	1	0.1190
D	0.45	1	0.1291	*NS*			24358.46	1	0.1854
AC	0.92	1	0.0403	1.10×10^{-4}	1	0.0406	1.31×10^{5}	1	0.0099
AD	*NS*			*NS*			1.81×10^{5}	1	0.0042
Residual	33.79	9		3.99×10^{-4}	11		92807.52	6	
Cor total	113.90	15		1.36×10^{-3}	15		1.84×10^{6}	15	
Sig. model term	AC			D			B, AC, AD		
Std.Dev.	0.40			6.027×10^{-3}			107.71		
R-Squared	0.6937			0.6329			0.9477		
Adj R-Squared	0.5235			0.5328			0.9084		

(1) Sum of square, (2) degree of freedom. NS: non-significant model term.

ment is obtained.

Based on these results, the software has offered an optimum setting of conditions, which could lead to maximum loading with desired particle size. The optimum formulation was achieved by 7 w/v% of the emulsifier, 3.00 $mg.mL^{-1}$ of the polymer, 40 mL of DCM and 24000 rpm homogenization speed. The resulting tamoxifen nanoparticles show the best response in size with average of 192 nm, the best encapsulation efficiency of 82% and the optimum loading of about 33%. Our results, using optimization studies to control the particle size and drug loading, facilitate the development of nano-carriers for tamoxifen delivery.

CONCLUSION

In the present study, an efficient drug delivery system of PLGA nanoparticles containing tamoxifen citrate as a potent anti-cancer drug was prepared and characterized. For simultaneous analysis of different factors influencing the properties of the nanoparticles and to find optimum formulations, an experimental design method was employed to assess the system. The homogenization speed, organic phase volume and the interactions between the percentage of PVA and the amount of PLGA and organic phase volume statistically showed significant influence on the drug formulation. The optimum characteristics of mean particle size below 200 nm and over 80% of tamoxifen encapsulation efficiency could be achieved using experimental design technique.

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