European Journal of Pharmaceutical Sciences 138 (2019) 105037

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Development and optimization of a novel PLGA-Levan based drug delivery system for curcumin, using a quality-by-design approach

Fatemeh Bahadori[a], Zahra Eskandari[a,b,c,*], Nabiallah Ebrahimi[d], Muge Sennaroglu Bostan[e], Mehmet Sayip Eroğlu[e,f], Ebru Toksoy Oner[c,**]

[a] Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, BezmialemVakif University, Vatan Str. Adnan Menderes Blv. 34093, Fatih, Istanbul, Turkey
[b] Sabanci University, Orhanli-Tuzla, İstanbul 34956, Turkey
[c] IBSB-Industrial Biotechnology and Systems Biology Research Group, Department of Bioengineering, Marmara University, Goztepe Campus, 34722 Istanbul, Turkey
[d] Ibn Haldun University, Ulubatli Hasan Str. 34494 Basaksehir, Istanbul, Turkey
[e] Department of Chemical Engineering, Marmara University, Goztepe Campus, 34722 Istanbul, Turkey
[f] TUBITAK-UME, Chemistry Group Laboratories, Kocaeli, Turkey

ARTICLE INFO

Keywords:
Curcumin
Levan
PLGA
Quality by design
Nano drug delivery
Plackett–Burman
Box–Behnken

ABSTRACT

This study aimed to develop a PLGA, Levan-based drug delivery system (DDS) of Curcumin using a quality-by-design (QbD) approach to reveal how formulation parameters affect the critical quality attributes (CQAs) of this DDS and to present an optimal design. First, a risk assessment was conducted to determine the impact of various process parameters on the CQAs of the DDS (i.e., average particle size, ZP, encapsulation efficiency and polydispersity index). Plackett–Burman design revealed that potential risk factors were Levan molecular weight, PLGA amount and acetone amount. Then, the optimization of the DDS was achieved through a Box–Behnken Design. The optimum formulation was prepared using low molecular weight Levan (134 kDa), 51.51 mg PLGA and 10 ml acetone. The model was validated and the optimized formulation was further characterized using different physic-chemical methods. The study resulted in the most stable NP with a spherical and uniform shape and physical stability tests indicated its stability for at least 60 days at room temperature. In conclusion, this study was an effort for developing a DDS which solubilizes Curcumin in clinically applicable concentrations.

1. Introduction

Curcumin, a hydrophobic polyphenol derived from the turmeric spice, has been the focus of an extensive amount of research in recent years due to its vast array of pharmacological activities including cancer chemopreventive and chemotherapeutic activities. It has been shown to exert these anticancer activities by modulating a variety of molecular targets such as transcriptional factors, inflammatory cytokines, growth factors, receptors, kinases, other enzymes, and genes which regulate cell proliferation and apoptosis in a variety of different cancers including breast cancer (Gulcur et al., 2013). Curcumin has great therapeutic potential, but its low bioavailability (due to its poor water solubility, in vitro stability, and rapid in vivo metabolism) is a barrier to its clinical development (Anand et al., 2007). In addition to the chemotherapeutic effect of Curcumin, recent research has unveiled that Curcumin can also down-regulate multi drug resistance proteins and P-glycoprotein in cancer cells (Choi et al., 2008) and thus it has the potential to defend the multidrug resistance (MDR) development in cancer cells. Therefore, the development of a drug delivery system which solubilizes Curcumin in a stable and clinically applicable form and concentration and releases it at the target region is a great step toward the clinical application of this promising anticancer agent.

On the other hand, polymer-based nanoparticles (NP)s are frequently used to enhance drug bioavailability or to decrease side effects (Cooper and Harirforoosh, 2014). Poly lactide-*co*-glycolide (PLGA) is a polymer that is commonly used to prepare biocompatible NPs and has

Abbreviations: NP, nanoparticle; HL, *Halomonas* Levan; PLGA, poly lactide-*co*-glycolide; QbD, quality-by-design; DDS, drug delivery system; CQAs, critical quality attributes; DoE, statistical experimental designs; HMW, high molecular weight; MMW, Medium MW; LMW, Low MW; PDI, Poly Dispersity Index; DLS, dynamic light scattering; DSC, differential scanning calorimetry; PS, particle size

* Correspondence to: Z. Eskandari, Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, BezmialemVakif University, Vatan Str. Adnan Menderes Blv., 34093 Istanbul, Turkey.
** Corresponding author.
E-mail addresses: fbahadori@bezmialem.edu.tr (F. Bahadori), z.eskandari@sabanciuniv.edu (Z. Eskandari), msennaroglu@marmara.edu.tr (M.S. Bostan), mehmet.eroglu@marmara.edu.tr (M.S. Eroğlu), ebru.toksoy@marmara.edu.tr (E.T. Oner).

https://doi.org/10.1016/j.ejps.2019.105037
Received 30 April 2019; Received in revised form 30 July 2019; Accepted 2 August 2019
Available online 05 August 2019

been commercialized for a range of drug delivery systems (Muthu, 2009). PLGA is an FDA-approved biodegradable polymer and has been successfully applied in drug delivery systems. PLGA is known as the "gold standard" of biodegradable polymers applied in drug delivery systems (Hines and Kaplan, 2013).

Levans are fructan type polysaccharides produced from different bacterial cultures. These bacterial fructans are strongly biocompatible, adhesive and soluble in water and oil according to their chain structure (Oner et al., 2017). They are mainly consisting of linear β-(2–6) and partly banched β-(2–1) glycosidic linkages. (Öner et al., 2016). Among the fructanes, *Halomonas* Levan (HL) has favorable biological properties which is produced by halophilic *Halomonas smyrnensis* AAD6T cultures with linear ß-(2–6) glycosidic linkages (Kazak Sarilmiser and Toksoy Oner, 2014). Due to the favorable biological properties such as high cell adhesion and proliferation, non-cytotoxicity (Bondarenko et al., 2016; Calazans et al., 1997), antitumor activity and less immunological response, HL has been a particular interests for different studies of protein and active drug delivery, antioxidant and antitumor activity, tumor targeting and anticoagulant activity (Bostan et al., 2014; Erginer et al., 2016; Kazak Sarilmiser and Toksoy Oner, 2014; Osman et al., 2017; Sezer et al., 2011; Sezer et al., 2017).

Levan-based DDS has been demonstrated to form NPs through self-assembly (Tabernero et al., 2017). Levan has been the subject of many research studies. For instance, Kazak Sarilmiser and Toksoy Oner (2014) explored anticancer activity of oxidized Levan against various human cancer cell lines and reached the conclusion that increasing oxidation degree and dose enhances anticancer activity. Sezer et al. (2011) explored different biodegradable Levan-based NPs that had a range of charge, size, and release profiles.

Cancer cells are different from normal cells in many ways. One of them is the way cancer cells use more sugar-induced energy for their metabolism, proliferation and alteration of their shape to enhance extracellular adhesion capability. Usually, biopolymers are more preferred sources of energy for cancer cells in comparison to normal cells. Chitosan and hyaluronan have been popular cancer targeting biopolymers; however, the use of Levan for the same purposes is a recent event (Kazak Sarilmiser and Toksoy Oner, 2014; Osman et al., 2017; Sezer et al., 2011; Sezer et al., 2017). Levan, a sugar-based biopolymer, is a perfect candidate in DDSs because of the higher affinity of cancer cells toward sugar-based biopolymers.

Designing a drug delivery system of Curcumin using PLGA and Levan seems a promising and innovative approach that can lead to obtaining a more stable system and higher levels of bioavailability for Curcumin. Stability and level of aggregation of NPs are crucial parameters in their preparation since they highly influence their biotechnological applications. Due to the fact that factors such as size and chemical properties determine applicability of NPs, analyzing NP properties in different conditions is of importance. It has also been demonstrated that characterizing NPs in the related environment is an important step in toxicity evaluation (Fatisson et al., 2012). The research studies in this area show that stability of NPs in a given medium depends on an amalgamation of factors including surface properties and concentrations of NPs and media compositions and NP. As a result, in order to fully understand NPs interaction with other biological systems, characterizing and optimizing NPs in their reLevant environments is critical (Pavlin and Bregar, 2012).

Taking these into consideration, the main purpose of this research is to unravel the potentials of PLGA and Levan to be applied as carriers in a NP DDS containing Curcumin. We specifically aimed to develop a PLGA, Levan-based DDS of Curcumin using a quality-by-design (QbD) method to reveal how formulation parameters affects the critical quality attributes (CQAs) of this DDS and to present an optimal design. We aimed to develop and optimize a stable PLGA, Levan-based DDS of Curcumin with higher levels of in vitro stability and in vivo bioavailability.

Understanding the target product and the evaluation of the risks during the design and development of pharmaceutical forms are the main aims of QbD studies. This can be conducted by using different design of experiment tools (Rathore and Winkle, 2009). Traditional experimental methods are non-economical and sometimes non-feasible since it is needed to change a single factor and keep other involving factors fixed, which results in more experiments (Çelik et al., 2017). Moreover, factor interactions are not evaluated in traditional experimental methods. In contrast, statistical experimental designs (DoE) need fewer runs and present more reliable results. In addition, through data extrapolation and plotting of the findings, DoE can help in optimization of product and processes (Vanaja and Shobha Rani, 2007). Several experimental designs can be used if the aim is to decrease the number of runs and to acquire more useful data (Yerlikaya et al., 2013). When optimization of a process is the purpose, models like Box–Behnken Design are applied. We used this design in the present study.

2. Materials and methods

2.1. Materials

Curcumin (# SLBM5931V), PLGA (lactide:glycolide ratio of 50:50, acid and ester terminal groups, with molecular weights of 30,000–60,000 (High) (#P2191) and 24,000–38,000 (Low) (#739952)), acetone, and ethanol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tween 80 (T80) was purchased from Merck (#8.22187.0500), Germany. E471 (mono- and diglycerides) was purchased from Cagdas, Turkey (#201160613001). HL was produced by our group as will be explained in a separate section.

2.2. Hydrolysis of HL

HL was produced extracellularly from halophilic Halomonas sp. AAD6T strain using sucrose as nutrient recovered from the fermentation media of stationary phase cultures by ethanol precipitation and then purified through DEAE-Sepharose CL-6B Anion-Exchange Chromatography as described before (Kazak Sarilmiser and Toksoy Oner, 2014). HL is high molecular weight β (2-6)-linked linear fructan with a number average molecular weight of 4.200 kDa. Spectroscopic and chromatographic characterization data of HL are given in supplementary information file (S1–S4). Molecular weight of HL was one of the important parameters in this study. Thus, for the molecular weight optimization, HLs were prepared using the microwave-assisted acid hydrolysis method (Avsar et al., 2018; Erkorkmaz et al., 2018). Briefly, 5% (w/v) High Molecular Weight (HMW) Levan in acetic acid solution was subjected to microwave irradiation for 60 s to produce Medium MW (MMW) Levan and 120 s to produce Low MW (LMW) Levan at 60% power of the machine. Hydrolyzed samples were then precipitated with ethanol, dried in a vacuum oven and finally milled to prepare samples in powder form.

2.3. Characterization of Levan using MALLS-GPC, FT-IR and NMR

Multi-angle laser light scattering-gel permeation chromatography (MALSS-GPC) system was used to determine the molecular weight of Levan samples before and after hydrolysis. MALSS-GPC system consisted of PerkinElmer-series 200 GPC high pressure pump, injector, a Waters Ultrahydrogel Linear (0.78 × 30 cm, Waters) column, Wyatt Dawn Heleos light scattering (LS), and Wyatt Opti-Lab differential refractive index (RI) detectors at 654 nm. Analyses were performed at 25 °C and mobile phase was 0.1 M $NaNO_3$ in 2% acetic acid in water (v/v) with a flow rate of 1.0 ml/min. Sample concentrations were in the range of 0.5–2.0 mg/ml and all samples were filtered with a 0.2 μm filter prior to use. The specific refractive index (dn/dc) of low molecular weight HL was determined as 0.1370 ± 0.0028 ml/g in the solvent system. In RI measurements, all samples were injected to the instrument through 0.02 μm filter. MW of HMW Levan was 4.200 kD, MMW Levan

Table 1
The factors and their levels used in Plackett–Burman Design.

Factors	levels	
	-1	1
X_1: Curcumin (mg)	1	2
X_2: PLGA (mg)	50	80
X_3: Levan (mg)	50	80
X_4: PLGA molecular weight (kDa)	L[a]	H[a]
X_5: Levan molecular weight (kDa)	L[a]	H[a]
X_6: Surfactant type	T80	E471
X_7: Surfactant concentration (% v/v)	0.005	0.01
X_8: rpm	L[b]	H[b]
X_9: Dropping rate	Slow[c]	Fast[c]
X_{10}: Acetone amount (ml)	5	10

[a] L = Low and H = High (134 kDa and 4.200 kD as described in materials).
[b] L = 400 rpm and H = 580 rpm.
[c] Dropping rate of organic phase to aqueous phase. Slow: 15 drop/min. and Fast: 30 drop/min.

was 434 kDa and LMW Levan was 134 kDa (Supplementary Data S1).

FTIR is done according to explanations related to FTIR in characterization of NPs. NMR spectra was recorded on Varian Mercury-VX 400 MHz (400 MHz for ^{1}H and 100 MHz for ^{13}C). The FT-IR results show complete protection of functional groups of Levan after hydrolysis (Supplementary material S2). 2D ^{1}H NMR and 2D ^{1}H-^{13}C NMR (Supplementary material S3 and S4) of hydrolyzed Levan also proves that the hydrolysis process does not change the molecular structure of Levan. Correlations between neighboring protons (2D ^{1}H NMR) and protons and carbons (2D ^{1}H-^{13}C NMR) show protection of all OH groups and stability of β (2-6)-linked linear fructan units after hydrolysis.

2.4. Experimental design

2.4.1. Risk identification

Based on our experiences and prior literature, minimized average PS, Poly Dispersity Index (PDI) (Kumar et al., 2015; Shah et al., 2015), maximized ZP, and encapsulation efficiency (Patil et al., 2015) were selected as CQAs of the DDS since these factors are more probable to influence the therapeutic efficacy of the target DDS. Ten formulation and process variables (Table 1) which may affect the drug delivery system properties were specified and these variables were studied more.

2.4.2. Risk analysis: Plackett–Burman design

To screen the formulation parameters that affect the CQAs of the PLGA, Levan-based nano DDS of Curcumin, we used a Plackett–Burman statistical experimental design (Rahman et al., 2010). Ten factors were evaluated through twelve runs. The level of each selected parameter was selected according to some prior experiments and data (Almoustafa et al., 2017; Dhakar, 2012; Taghipour et al., 2014; Yushu and Venkatraman, 2006). These selected parameters as well as their levels are presented in Table 1.

Minitab 17 (Minitab Inc.; State College, PA, USA) was used for the generation and randomization of the design matrix and for subsequent statistical analyses (Table 2). The factors and their coded values are presented in Tables 1 and 2. The significance level and the factor coefficients were evaluated through ANOVA and multilinear regression analysis.

The experimental runs (i.e., formulations) were prepared in triplicate. As mentioned before, the dependent variables (CQAs) included average PS (Y_1), ZP (Y_2), encapsulation efficiency (Y_3) and PDI (Y_4).

2.4.3. The optimization process: Box–Behnken Design

Critical variables (i.e., Levan molecular weight, PLGA amount and acetone amount) were first identified through Plackett–Burman design. Then Box–Behnken Design with three factors and three levels was applied to optimize the formulation of the desired DDS (Ait-Amir et al., 2015; Yerlikaya et al., 2013). The medium levels of the selected factors were set as the midpoint of low and high levels that were directly adopted from the prior Plackett–Burman design (Table 3). Furthermore, the other seven factors were set at a fixed level in Box–Behnken Design since their influence on the response variables seemed statistically insignificant based on the results of Plackett–Burman design (Table 4).

Ten factors were evaluated through twelve runs. The level of each studied parameter was selected according to some prior experiments and data (Almoustafa et al., 2017; Dhakar, 2012; Taghipour et al., 2014). These selected parameters as well as their levels are presented in Table 1. Tween 80 which is an emulsifier and a surfactant. This is where E471 unlike tween 80, act only as surfactant and is not able to incorporate with forming droplets in water. Generally, 0.01 v/v % is considered as the safe limit for surfactants, half of this amount was checked to see if it is enough to achieve nanoparticles with the same quality. Rate of rotation of stirrer was set as low (400 rpm) and high (580 rpm) to check how mixing rate can affect the properties of nanoparticles. This is one of the properties that rarely is mentioned in literature. The high level is chosen according to the experiments in our lab so far and the 400 rpm is a mild rate of mixing in comparison with 580 rpm. Below these speeds acetone remains unmixed with aqueous media. Another factor that was investigated in this work was rate of dropping. 30 drop/min that is always used in our lab was considered as high and half of this amount was considered as low (15 drop/min) to

Table 2
Plackett–Burman Design experimental matrix (L = Low, H = High, S: Slow and F: Fast as described at Table 1).

Formulation Code	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}
PBD_1	1	50	50	L	L	T80	0.005	L	S	5
PBD_2	2	80	50	H	H	T80	0.010	L	S	5
PBD_3	2	80	80	L	H	E471	0.005	H	S	5
PBD_4	1	80	50	L	L	E471	0.010	H	S	10
PBD_5	2	50	80	H	L	E471	0.005	L	S	10
PBD_6	1	50	80	H	H	T80	0.010	H	S	10
PBD_7	1	50	50	H	H	E471	0.005	H	F	5
PBD_8	1	80	80	H	L	E471	0.010	L	F	5
PBD_9	1	80	80	L	H	T80	0.005	L	F	10
PBD_{10}	2	80	50	H	L	T80	0.005	H	F	10
PBD_{11}	2	50	80	L	L	T80	0.010	H	F	5
PBD_{12}	2	50	50	L	H	E471	0.010	L	F	10

Table 3
The factors and their levels used in Box–Behnken Design.

Factors	Levels		
X_1: Levan molecular weight (kDa)	Low[a]	Medium[a]	High[a]
X_2: PLGA amount (mg)	50	65	80
X_3: Acetone amount (ml)	5	7.5	10

[a] Low = 134 kDa, Medium = 434 kDa and High = 4.200 kD.

Table 4
The fixed levels of formulation and process parameters used in Box–Behnken Design.

Formulation and process parameter	Fixed level
Curcumin amount (mg)	1
Levan amount (mg)	80
PLGA molecular weight (kDa)	Low
Surfactant concentration (% v/v)	0.005
Surfactant type	T80
rpm[a]	High
Dropping rate[b]	Fast

[a] The magnetic stirrer speed = 580 rpm.
[b] Dropping rate of organic phase to aqueous phase = 30.

Table 5
Box–Behnken Design experimental matrix.

Formulation code	X_1[a]	X_2[a]	X_3[a]
BBD_1	Medium	65	7.5
BBD_2	Medium	50	5
BBD_3	Low	80	7.5
BBD_4	Medium	80	5
BBD_5	High	65	5
BBD_6	High	50	7.5
BBD_7	Low	65	5
BBD_8	Medium	65	7.5
BBD_9	Low	65	10
BBD_{10}	Medium	80	10
BBD_{11}	High	65	10
BBD_{12}	Low	50	7.5
BBD_{13}	High	80	7.5
BBD_{14}	Medium	65	7.5
BBD_{15}	Medium	50	10

[a] X_1: Levan molecular weight (High: 4.200 kD Medium: 434 kDa Low: 134 kDa); X_2: PLGA amount (mg); X_3: acetone amount (mg).

explore its effect on nanoparticles properties. Above 30 drop/min huge aggregates were recognized in our previous experiments and below 15 drop/min the period of experiment extended to long hours. Volume of acetone was doubled from 5 ml that is common to 10 ml to check any possible effect of increase in organic phase volume on nanoparticles' properties. Upper and lower limits of amount of Curcumin and PLGA were considered using optimized formulations in our laboratory and literature (Anand et al., 2010). Levan is introduced to this system for the first time in literature in this study and the examined amounts were determined by some pre-formulation studies (data are not shown). The effect of change in MW of carriers on properties of nanoparticle, commercial PLGA and homemade Levan with different MWs were determined using same pre-formulation studies.

Minitab 17 (Minitab Inc.; State College, PA, USA) was used to generate design matrix and to analyze the data (Table 5). The formulations were prepared in triplicate.

A polynomial model was established as follows after conducting a regression analysis for each of the responses:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where Y stands for the response, X_1–X_3 stand for the main effects of factors, X_1X_2, X_1X_3, X_2X_3 refer the interaction effects, ${X_1}^1$, ${X_2}^2$, ${X_3}^2$ stand for quadratic effects of factors, β_0 refers to the constant, and β_1–β_3 stand for the coefficients of the factors. To evaluate the significance of the factors on the responses, the *p* values of the regression coefficients (β_1–β_3) were identified. The significance of the model was also measured through ANOVA. Following the generation of the polynomial equations regarding the factors and responses, formulations were optimized in terms of average PS (Y_1), ZP (Y_2), encapsulation efficiency (Y_3) and PDI (Y_4) of the PLGA, Levan-based nano DDS of Curcumin using the developed mathematical model to specify the levels of Levan molecular weight (X_1), PLGA amount (X_2), and acetone amount (X_3). For this purpose, a design space was created using several contour plots and response surface graphs. To measure the correlation between the actual and predicted values of the responses, the optimized DDS formulation was prepared and evaluated. The optimum formulation was further characterized for its physical and chemical properties.

2.5. Preparation of the drug delivery system

The oil-in-water (o/w) emulsification-solvent evaporation method (with slight modification made on the previously reported process) was used for the preparation of NP formulations (Mathew et al., 2012; Mukerjee and Vishwanatha, 2009; Yallapu et al., 2010a). Formulations were prepared according to the Plackett–Burman Experimental Design Matrix (Table 2). In each case, the related amount of Curcumin (X_1) and PLGA (X_2) were separately dissolved in acetone (X_{10}) and then were mixed together. PLGAs with two different MW (X_4) were used. This mixture (organic phase) was dropped with two different speeds (X_9) into 20 ml aqueous phase which was stirring with different speeds (X_8) using magnetic stirrer (IKA Magnetic Stirrer, RCT basic). Aqueous media contained related amount of Levan (X_3) with two different MWs (X_5) in 19 ml of water and 1 ml of homogenized surfactant with different ratios (X_7). Two types of surfactants were used (X_6).

Evaporation of acetone was achieved through overnight stirring. For the solution that contained NPs to reach the volume of 20 ml, distilled water was added. The solution was then stored at 25 °C for more evaluations.

Dynamic Light Scattering (DLS) was used to check the size of particles and presence of aggregates (if there was any) immediately after experiments and after a few days. PDI of formulations obtained from DLS software were checked to assure monodispersity of formulations.

2.6. Determination of PS, Poly Dispersity Index (PDI) and ZP

DLS technique using Zetasizer (model ZEN 3600; Malvern Instrument, Inc., UK) at 25 °C was applied to determine PS and PDI of NPs. Default settings on the Zetasizer were used. In these settings, refractive index was 1.59, absorption was 0.010, water was the dispersant and measurement angle was 173. Measurements were performed five times, 3 min each and the data was analyzed in terms of number, intensity and volume distribution. Although, the “average size” presented by Zetasizer was used in QbD calculations (Eskandari et al., 2018). Laser Doppler micro-electrophoresis method using a folded capillary zeta cell (Malvern Instruments Ltd) was used to measure ZP.

2.7. Fourier-Transform Infrared Spectroscopy (FT-IR)

FT-IR was used to examine the molecular state of PLGA, Curcumin and Levan in the optimized formulation. Through 25 scans, spectra of PLGA, Curcumin, Levan, PLGA-Levan NPs, Curcumin loaded PLGA-Levan NPs were measured on an alpha platinum ATR (Bruker; USA). Experiments were performed from 400 to 4000 cm^{-1} at room temperature.

2.8. Differential scanning calorimetry (DSC)

Incorporation of components in the formulation was assessed using DSC. Ideally, the study should be done in aqueous system in micellar form. However, the ingredients of the system, including T80 and most importantly, water itself, cover a very high ratio of the mass of total system, leaving the amount of other components relatively low. So it was hard to observe significant and good peaks in aqueous system. To overcome this challenge, optimized formulation was prepared using the method mentioned above and lyophilized to eliminate water effect.

Measurements were conducted using Perkin Elmer Jade type differential scanning calorimeter under dynamic nitrogen (20 ml/min) at 5 °C/min heating rate.

2.9. Scanning electron microscopy

Morphology of synthesized PLGA-Levan-Curcumin NP analyzed by a scanning electron microscope, QuantomiX-102 capsule was used for imaging of particles. QX-102 capsules are suitable for imaging of particles in solutions like PLGA-Levan-Curcumin. Poly-L-Lysine was used as a positively charged polymer to coat the membrane according to the related protocol and then the optimized formulation was applied. Electron beam energy of 20 kV and magnification of x180K were used while taking SEM images.

2.10. Stability studies and calculation of encapsulation efficiency (EE %)

Stability of the optimized formulation and the same formulation without Levan was examined during storage for 60 days at 25 °C. PS and PDI, ZP were measured as explained above. Also a comparative release study was conducted to further analyze the differences between the obtained optimized and non-optimized formulations. For this end 1 ml of the optimized PLGA-Levan-Curcumin formulation and its equivalent formulation consisting of only PLGA-Curcumin were separately added to 99 ml of PBS buffer (pH = 7.4) and incubated at 37 °C. Curcumin released from the formulation was assumed to precipitate in the glass holder due to the low water solubility. Thus, the concentration of Curcumin at the upper part of the holder will decrease with time. Samples from the upper part of the release media were obtained for 5 days, diluted in ethanol to dis-assemble PLGA and Levan micelles and after a mild centrifugation, concentration of Curcumin was measured using fluorescence spectrophotometer (Ex. 425 nm, Em. 520 nm). One of the non-optimized formulations was chosen and the same experiments were conducted on it. The concentration of Curcumin was calculated using a pre-recorded concentration curve.

EE% was calculated based on the poor water solubility of Curcumin and was measured using above mentioned method.

To understand the effect of Levan in the enhancement of the solubility of Curcumin, similar to stability experiments, a formulation of the equivalents of all materials without Levan was prepared. The process of nano-formulation preparation was followed without using PLGA and Levan by dropping Curcumin dissolved in acetone on d.d. water. After formulation preparation and a short incubation in RT, samples from the upper parts of formulations and the water-Curcumin mixture were diluted with ethanol to dis-assemble the micelles or any aggregates and the concentration of the dissolved Curcumin was measured using fluorescence spectrophotometer.

3. Results

3.1. Experimental design

3.1.1. Risk identification: Plackett–Burman Design

Based on prior knowledge, minimized average PS, PDI, maximized ZP, and encapsulation efficiency were selected as CQAs of the PLGA, Levan-based DDS since these factors are likely to influence the therapeutic efficacy of the target DDS. Ten formulation and process variables (Table 1) which may affect the drug delivery system properties were specified and explored. The factors included Curcumin amount, PLGA amount, Levan amount, PLGA molecular weight, Levan molecular weight, surfactant type, surfactant concentration, rpm, injection rate, and acetone amount.

A Plackett–Burman experiment design with ten factors, two levels, and twelve runs was conducted to find the most significant factors. The examined formulations and the response values are presented in Table 6.

In the case of the average PS (Y_1), the three most significant factors were found to be Levan molecular weight (X_5), acetone amount (X_{10}), and PLGA amount (X_2), respectively (Table 7, Fig. 1). The R^2 value was 0.985, which indicated the fitness of the given model. However, in spite of the fact that the p value of the main effects acquired from ANOVA was 0.294 (hence not significant), yet these factors were explored in the next steps.

Table 6
The results of dependent variables obtained through Plackett–Burman Design.

Formulation code	Y_1: average particle size (nm)	Y_2: zeta potential (mV)	Y_3: encapsulation efficiency (%)	Y_4: PDI
PBD_1	140.60	23.00	85.97	0.19
PBD_2	202.70	13.80	54.01	0.17
PBD_3	205.30	33.20	91.97	0.23
PBD_4	152.30	27.20	94.65	0.27
PBD_5	132.00	15.40	67.40	0.24
PBD_6	156.70	8.21	92.32	0.19
PBD_7	180.80	23.90	89.39	0.31
PBD_8	144.40	18.20	96.94	0.15
PBD_9	170.80	30.80	74.46	0.15
PBD_{10}	121.40	8.43	57.81	0.16
PBD_{11}	132.90	20.00	72.96	0.24
PBD_{12}	165.10	20.50	81.93	0.29

As for ZP (Y_2), the three factors that were found to be the most significant ones included PLGA molecular weight (X_4), surfactant type (X_6), and surfactant concentration (X_7), respectively (Table 7) (Fig. 1). The R^2 value was 0.954, and implied that the model had a good fit. Nevertheless, in spite of the fact that the p value of the main effects reported through ANOVA was not significant (i.e., 0.494), yet the factors were examined in the next stages.

In case of the encapsulation efficiency (Y_3), Curcumin amount (X_1), Surfactant type (X_6), and PLGA molecular weight (X_4) were found the most significant parameters. The R^2 value was 0.895, which indicated model fit. However, in spite of the fact that the p value of the main effects acquired through ANOVA was 0.695 (hence not significant), yet these factors were explored in the next steps of the study.

For PDI (Y_4), the three factors that were shown to be significant were surfactant type (X_6), PLGA amount (X_2), and rpm (X_8), respectively (Table 7) (Fig. 1). The R^2 value was 0.931, which indicted the model was fit. However, in spite of the fact that the p value of the main effects acquired through ANOVA was not significant (i.e., 0.589), yet the factors were further examined in the next parts of the study through Box–Behnken Design.

3.1.2. Design model and data analysis: Box–Behnken Design

Box–Behnken design and responses form a total of 15 experiments are given in Table 8. The following quadratic, second-order equation was applied for the prediction of the maximum average size for the PLGA, Levan-based drug delivery system of Curcumin:

$$Y_1 = 207.6 - 3.2\,X_1 - 2.29\,X_2 - 5.15\,X_3 - 0.057\,X_1X_2 + 3.069 - 0.1020\,X_2X_3 + 7.31\,X_1^2 + 0.0296\,X_2^2 + 0.366\,X_3^2$$

Where, Y_1 is average size, and X_1, X_2 and X_3 stand for Levan molecular weight, PLGA amount and acetone amount, respectively. ANOVA was used to test the regression model, as reported for average size in Table 9. Based on the findings, a high F value (31.05) and a low p value (0.001) showed the statistical significance of the model equation. Statistical insignificance of lack-of-fit value of the model was observed (p = 0.842), indicating that the model fits. The coefficient R^2 value of 0.9824 verified the confidence level of the regression model, indicating that the model can explain 98.24% of variability in the response. The

Table 7
Statistical analysis of dependent variables of Plackett–Burman Design (X values described in Table 1).

Factors	Y_1: average particle Size (nm)		Y_2: zeta potential (mV)		Y_3: encapsulation efficiency (%)		Y_4: PDI	
	Coefficient	P value	Coefficient	P value	Coefficient	P value	Coefficient	P value
Constant	158.75	0.01	−20.22	0.05	79.98	0.04	0.22	0.04
X_1: Curcumin amount	1.15	0.78	1.66	0.49	−8.97	0.30	0.01	0.72
X_2: PLGA (mg)	7.4	0.26	−1.72	0.48	−1.68	0.77	−0.03	0.30
X_3: Levan (mg)	−1.73	0.68	−0.75	0.73	2.69	0.66	−0.01	0.49
X_4: PLGA molecular weight	−2.42	0.59	5.56	0.18	−3.67	0.57	−0.01	0.51
X_5: Levan molecular weight	21.48	0.09	−1.52	0.52	0.7	0.90	0.01	0.67
X_6: Surfactant type	4.57	0.39	−2.85	0.33	7.06	0.36	0.03	0.26
X_7: Surfactant concentration	0.27	0.95	2.24	0.40	2.15	0.72	0.00	0.91
X_8: rpm	−0.52	0.90	0.06	0.98	3.2	0.61	0.02	0.44
X_9: dropping rate	−6.18	0.30	−0.09	0.97	−1.07	0.85	0.00	0.94
X_{10}: Acetone (ml)	−9.03	0.22	1.8	0.47	−1.89	0.75	0.00	0.95

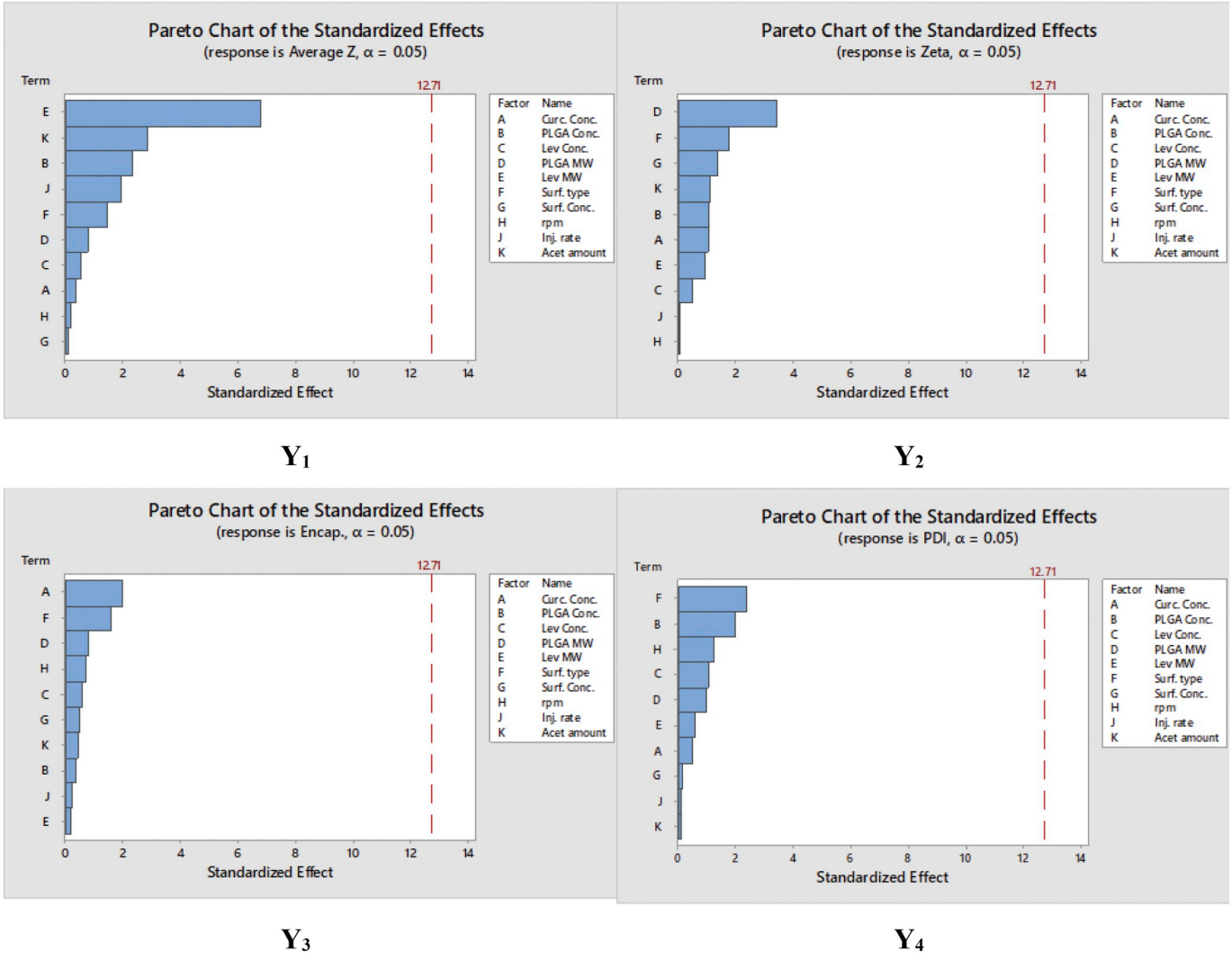


Fig. 1. The Pareto charts showing significant formulation and process variables on Y_1: average particle size, Y_2: zeta potential, Y_3: encapsulation efficiency and Y_4: PDI.

pred. R^2 value of 0.8897 and the adj. R^2 value of 0.9508 are in close harmony. This demonstrated an acceptable correlation between the observed values and the predicted ones.

The following equation was applied for the prediction of the maximum ZP for the desired drug delivery system:

$$Y_2 = 3.3 - 12.11\,X_1 + 0.227\,X_2 + 2.46\,X_3 + 0.1187\,X_1X_2 - 0.479\,X_2X_3 - 0.0280\,X_2X_3 - 5.450\,X_1^2 + 0.00110\,X_2^2 - 0.073\,X_3^2$$

Where, Y_2 is ZP, and X_1, X_2 and X_3 refer to Levan molecular weight, PLGA amount and acetone amount, respectively. The F value of the model (45.48) and low p value (0.000) implied that the model equation was significant, as shown in Table 10. The model's lack of fit ($p = 0.703$) was not significant. The coefficient R^2 value of 0.9879 means that the model could explain 98.79% of variability in ZP. The adj. $R^2 = 0.9662$ and the pred. $R^2 = 0.8989$ were close enough to each other.

Based on ANOVA results and the coefficient of determination, the model proved to be unsuitable to predict encapsulation efficiency. Although the p level was significant, the model's lack of fit was significant, implying that the model does not fit well. Therefore, encapsulation efficiency data were excluded in further assessment of the results and in optimization of the PLGA, Levan-based drug delivery system of Curcumin. Likewise, ANOVA results ($p > 0.05$) showed that the model was unsuitable in prediction of PDI. As a result, PDI data were also excluded in next evaluations. This approach is quite common in similar studies (Yerlikaya et al., 2013).

3.1.3. Optimization study: Box–Behnken Design

Results of Box–Behnken Design were used and Minitab 17 was used to specify the optimum conditions.

Expected limits were set as A- Maximized ZP and B-Minimized average size.

1- 3D surface plots for Y_1 (average size) as a function of

- Levan molecular weight and PLGA amount at fixed acetone amount,
- Levan molecular weight and acetone amount at fixed PLGA amount and
- PLGA amount and acetone amount at fixed Levan molecular weight

are displayed in Fig. 2, respectively.

2- 3D surface plots for Y_2 (ZP) as a function of

- Levan molecular weight and PLGA amount at fixed acetone amount,
- Levan molecular weight and acetone amount at fixed PLGA amount and
- PLGA amount and acetone amount at fixed Levan molecular weight

are shown in Fig. 2, respectively.

In the optimized formulation, the values of variables were found to be as X_1 = low, X_2 = 51.51 and X_3 = 10. The desirability value of this configuration was 0.96. Fig. 3 presents DLS results of particle size distribution of optimized formulation.

3.1.4. The accuracy of the optimized model

In order to examine the model accuracy for the optimum conditions, the optimized formulation was prepared in triplicate. Table 11 presents the predicted and experimental responses for the optimized formulation. Desirability of optimum formulation was 0.920. Desirability value between 0.8 and 1 is regarded as acceptable and excellent (Lazic, 2006).

Table 8
The results of dependent variables obtained through Box–Behnken Design.

Formulation code	Y_1: average particle size (nm)	Y_2: zeta potential (mV)	Y_3: encapsulation efficiency (%)	Y_4: PDI
BBD_1	115.2	24.97	39.20	0.12
BBD_2	123	21.10	48.40	0.10
BBD_3	127.1	26.43	35.11	0.08
BBD_4	154.6	27.43	40.01	0.11
BBD_5	152.3	11.20	82.94	0.18
BBD_6	134.2	6.32	85.03	0.25
BBD_7	133.5	26.03	43.61	0.13
BBD_8	122.2	23.20	43.17	0.11
BBD_9	83.21	26.10	48.04	0.12
BBD_{10}	118.9	23.10	33.88	0.92
BBD_{11}	132.7	6.48	100.00	0.21
BBD_{12}	102	24.60	45.28	0.16
BBD_{13}	155.9	15.27	96.97	0.18
BBD_{14}	110.1	21.90	40.31	0.11
BBD_{15}	102.6	20.97	100.00	0.11

Table 9
ANOVA results for response Y_1 (average size).

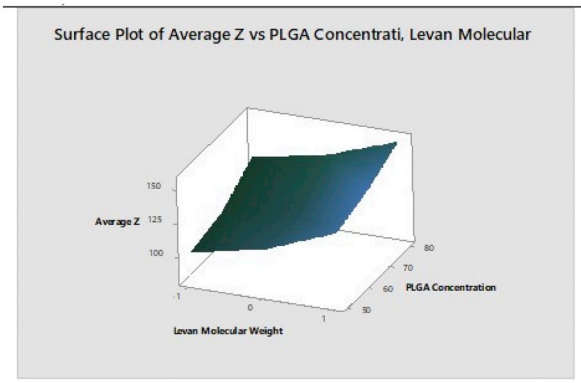
SOUECE	df	Adj SS	Adj MS	F-Value	p
Model	9	5833.00	648.11	31.05	0.001
X_1	1	2089.49	2089.49	100.1	< 0.001
X_2	1	1121.01	1121.01	53.7	0.001
X_3	1	1984.19	1984.19	95.05	0.000
X_1X_2	1	2.89	2.89	0.14	0.725
X_1X_3	1	235.47	235.47	11.28	0.020
X_2X_3	1	58.52	58.52	2.8	0.155
X_1^2	1	197.28	197.28	9.45	0.028
X_2^2	1	163.63	163.63	7.84	0.038
X_3^2	1	19.27	19.27	0.92	0.381
Error	5	104.37	20.87		
Lack of fit	3	30.56	10.19	0.28	0.842
Pure error	2	73.81	36.90		
Total	14	5937.37			

Note: $R^2 = 0.9824$, adj. $R^2 = 0.8897$ and pred. $R^2 = 0.9508$.
Abbreviations: adj., adjusted; ANOVA, analysis of variance; pred., predicted.

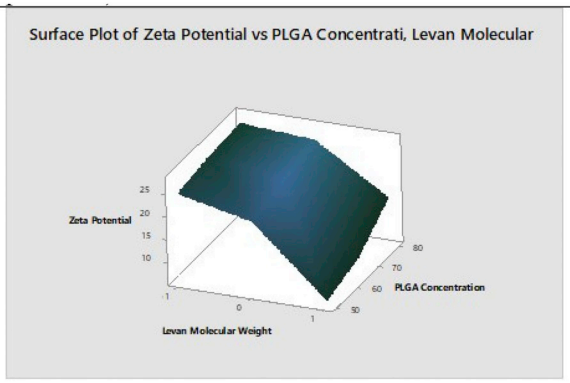
Table 10
ANOVA results for response Y_2 (zeta potential).

SOUECE	df	Adj SS	Adj MS	F-Value	p
Model	9	701.04	77.893	45.48	<0.001
X_1	1	510.242	510.242	297.9	<0.001
X_2	1	46.272	46.272	27.02	0.003
X_3	1	10.374	10.374	6.06	0.057
X_1X_2	1	12.674	12.674	7.4	0.042
X_1X_3	1	5.736	5.736	3.35	0.127
X_2X_3	1	4.41	4.41	2.57	0.169
X_1^2	1	109.654	109.654	64.02	0.000
X_2^2	1	0.227	0.227	0.13	0.731
X_3^2	1	0.763	0.763	0.45	0.534
Error	5	8.564	1.713		
Lack of fit	3	3.815	1.272	0.54	0.703
Pure error	2	4.749	2.375		
Total	14	709.604			

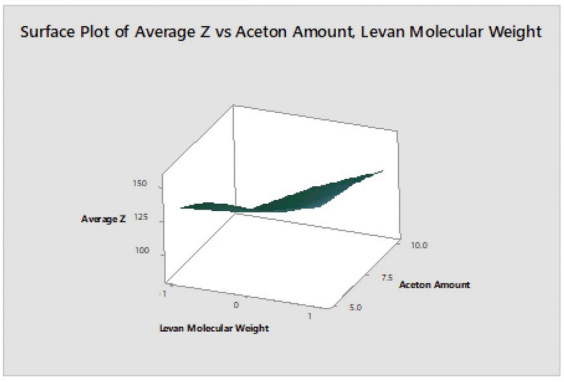
Note: $R^2 = 0.0.9879$, adj. $R^2 = 0.9662$ and pred. $R^2 = 0.8989$.
Abbreviations: adj., adjusted; ANOVA, analysis of variance; pred., predicted.



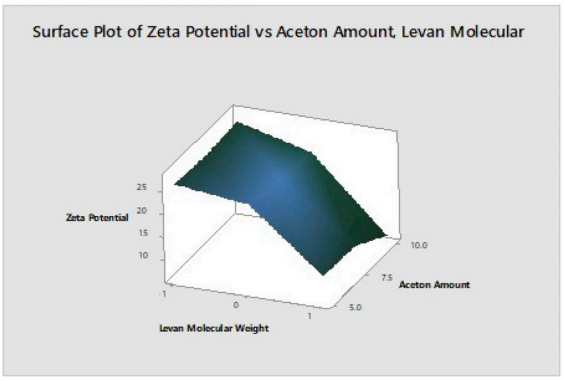
A: levan molecular weight and PLGA amount at fixed acetone amount



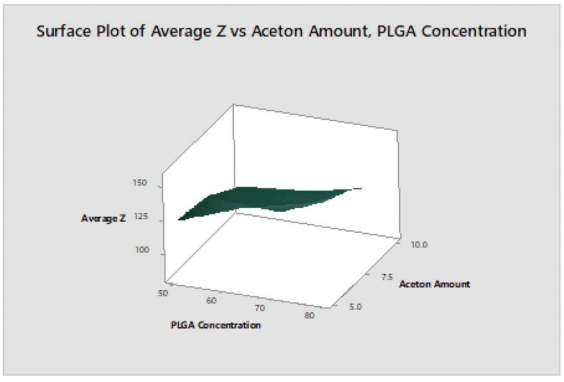
D: levan molecular weight and PLGA amount at fixed acetone amount



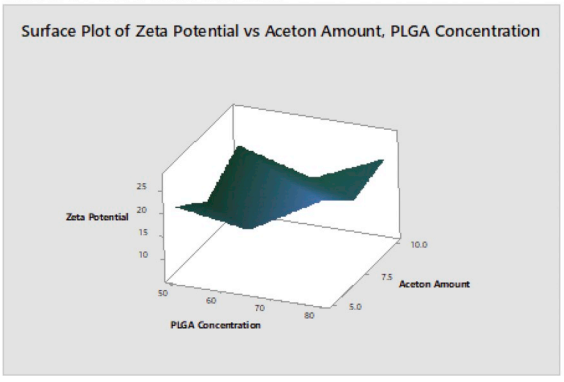
B: levan molecular weight and acetone amount at fixed PLGA amount



E: levan molecular weight and acetone amount at fixed PLGA amount and



C: PLGA amount and acetone amount at fixed levan molecular weight



F: PLGA amount and acetone amount at fixed levan molecular weight

Fig. 2. Response surface plots showing the effects of Levan molecular weight, PLGA amount and acetone amount on the average Z and Zeta potential of PLGA, Levan-based nano drug delivery system of Curcumin.

3.2. Fourier-Transform Infrared Spectroscopy (FT-IR)

Interactions between NPs' components were studied using IR. Curcumin revealed its signature peaks related to phenolic O—H stretching vibration at 3508 cm^{-1}, aromatic moiety C=C stretching ones at 1627 cm^{-1}, benzene ring stretching vibrations at 1597 cm^{-1}, C=O and C=C vibration peaks at 1504 cm^{-1}, olefinic C—H bending vibrations at 1426 cm^{-1}, aromatic C—O stretching related vibrations at 1271 cm^{-1} and C–O–C stretching vibration peaks at 1025 cm^{-1} (Yallapu et al., 2010b). The bands at 856, 885, 885 and 960 cm^{-1} were attributed to the bending vibrations of the C—H bond of RCH = CH2 alkene groups (Fig. 4-C) (Jyothi and Sravani, 2016).

FTIR spectra of PLGA (Fig. 4-A) shows stretching C=O at 1745 cm^{-1}. C—O of aliphatic polyesters are also shown in 1165 cm^{-1} and the 1083 cm^{-1} (Marques et al., 2013) symmetrical and asymmetrical peaks at 2980 and 2949 cm^{-1} are the ones that are the most significant and related to stretching of CH_2 and CH_3 groups. Bands of asymmetrical deformation of CH_3 are shown in 1375 cm^{-1} and CH_2 in 1450 cm^{-1} (Cibulková et al., 2006; Motta and Duek, 2006).

The broad absorption band of Levan at 3200 cm^{-1} is because of the OH stretching of fructofuranose rings as well as –CH_2-OH groups. The carbon–hydrogen (C − H) stretching vibration of fructose residues are responsible for the bands around 2850 cm^{-1}. Furthermore, “the sharp absorption bands that are observed at 950, 1000 and 1100 cm^{-1} are because of the C − O − C symmetric bending vibration of fructofuranose rings and glycosidic linkages” (Poli et al., 2009). A very weak peak at

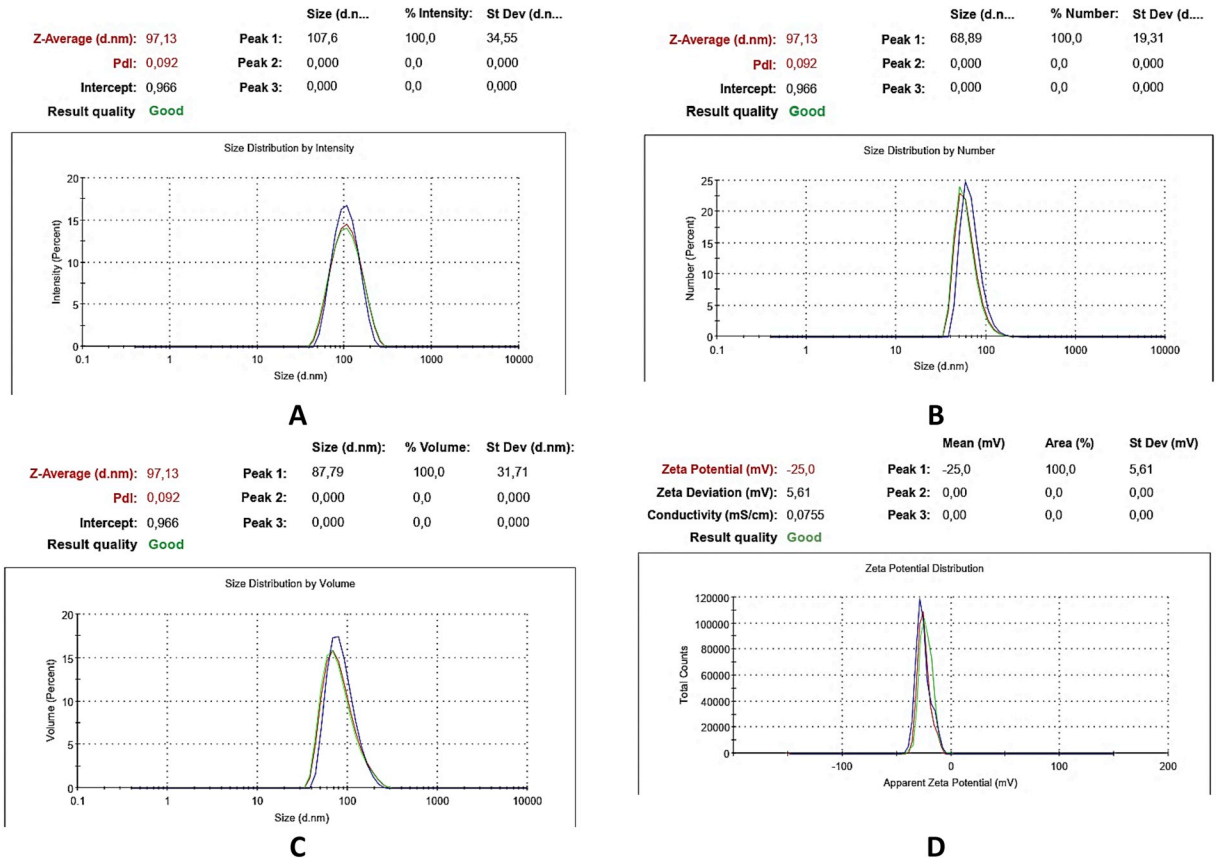


Fig. 3. DLS results of particle size distribution of optimized formulation A) By intensity (107 nm) B) By number (69 nm) C) By volume (88 nm) and D) Zeta potential (−25 mV)

Table 11
The observed and the predicted values of the optimum PLGA-Levan nano drug delivery system of curcumin based on desirability function.

Response	Observed	Predicted	Residual
Particle size (nm)	91.93	83.26	8.76
Zeta potential (mV)	25.7	26.08	0.38

1637 cm^{-1} is attributed to bending of C—O groups (Ibrahim et al., 2006). The increase in the intensity of the latter peak helped us in interpretation of PLGA-Levan FT-IR spectra (details in Discussion).

Successful encapsulation of Curcumin in PLG-Levan DDS could be deducted from absence of almost all peaks of Curcumin in the FT-IR spectra of PLGA-Levan-Curcumin including the absence of finger print area peaks, the lack of C═O, C═C and the lack of olefinic C—H (1504 cm^{-1} and 1426 $cm^{-1)}$ peaks. However, all peaks related to PLGA and Levan are presented in both PLG-Levan and PLGA-Levan-Curcumin formulations (details at Discussion), except one important change; in PLGA, the peak related to carbonyl group is present at 1749 but in PLGA-Levan the interaction between carbonyl of PLGA and hydroxyl groups of Levan limited the carbonyl group motions and shifted the ester-related peak to 1754 while decreased its intensity.

3.3. Differential scanning calorimetry (DSC)

DSC is known as a useful method for determination of melting temperature and melting enthalpy of polymers. The presence of the components and their physical state in the formulations were investigated using this technique. Fig. 5 shows the DSC curves of optimized formulation and its components, which was recorded after lyophilization. Curcumin is a small and crystalline molecule and has strong intermolecular interactions. In DSC curve of Curcumin (Fig. 5), an endothermic peak at 176.5 °C was due to the melting of Curcumin. The enthalpy value of this endotherm (ΔHm) was 82.1 j/g. Poly(lactide-*co*-glycolide) crystallinity and melting temperature mainly depend on the lactide/glycolide ratio and tacticity of the copolymer chain (Gilding and Reed, 1979). In DSC curve of PLGA, while a slight endotherm observed at 56.0 °C, which was attributed to the glass transition temperature (Tg), melting behavior was not seen in the DSC operational interval. On the other hand, in DSC curve of Levan (Fig. 5), as the first endothermic peak at around 100 °C was due to the removal of the free and bonded water, the second endothermic peak at 225 °C was because of the thermal decomposition. Levan has an amorphous structure and did not display a melting behavior. After emulsification of PLGA-Levan in the presence of Curcumin, and subsequent freeze drying, the melting endotherm of Curcumin disappeared. Additionally, the Tg of PLGA shifted to 39.0 °C from 56.0 °C in the formulation. These results could be taken as an evidence of the intermolecular interaction between Curcumin and PLGA-Levan which hindered the Curcumin crystallinity and resulted in the more homogeneous distribution of Curcumin in the formulation. This interaction was confirmed by IR results as well.

3.4. Scanning electron microscopy (SEM)

Because of scanning electron microscopy is operated under high vacuum, images of nano-sized micelles in an emulsion is almost impossible without changing their native state. However, the use of QuantomiX-102 imaging capsule containing imaging buffer provides observing images of the nano-sized micelles by SEM. Therefore, the PLGA-Levan based nano sized emulsion was fixed on poly(L-lysine) coated inner surface of QuantomiX-102 capsule through electrostatic interactions and the SEM images were taken with the help of imaging

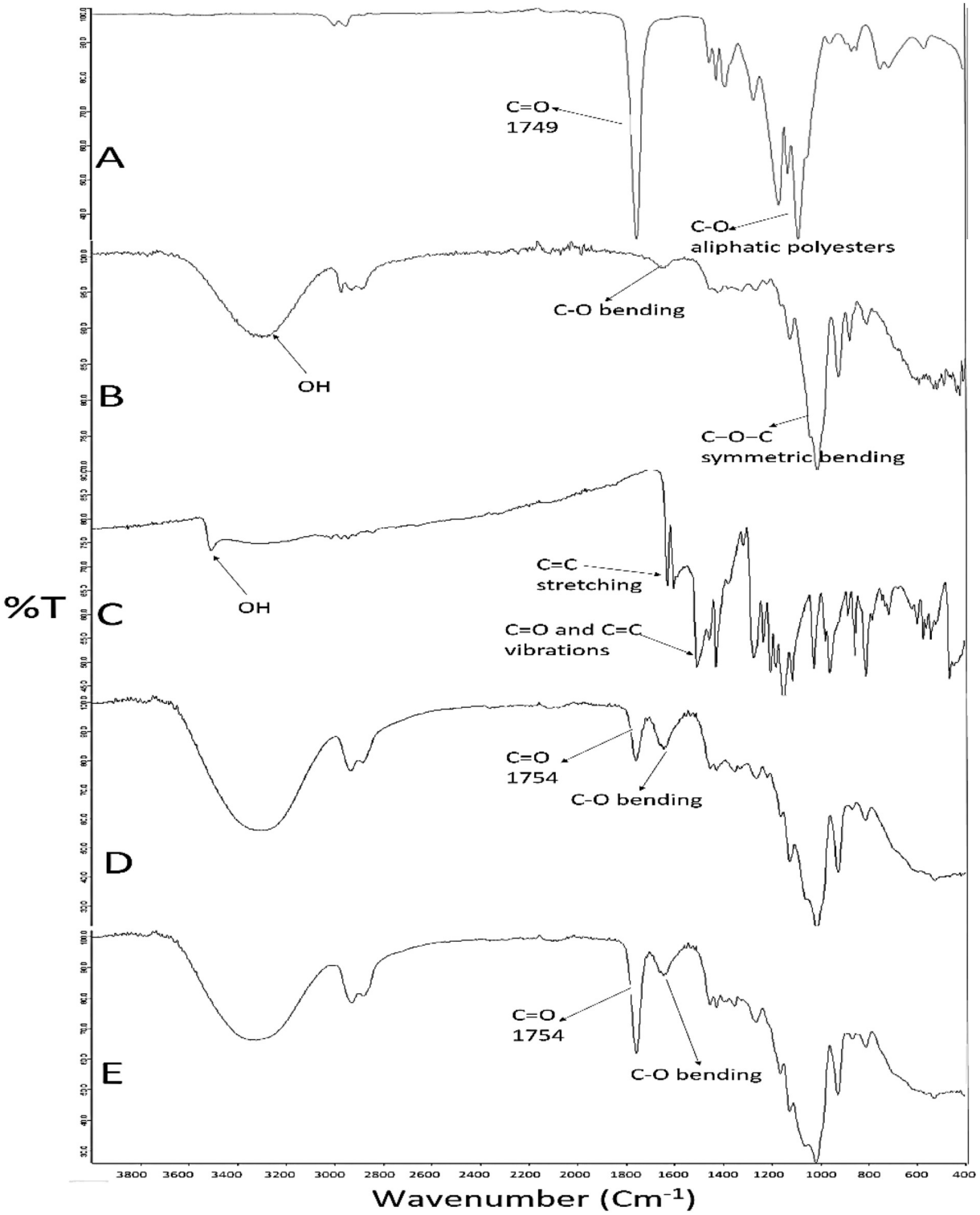


Fig. 4. FT-IR spectra of A) PLGA, B) Levan, C) Curcumin, D) PLGA-Levan and E) PLGA-Levan-Curcumin.

buffer at 10 kV acceleration voltage under high vacuum and low secondary electron mode at 18 K and 180 K magnifications. As shown in Fig. 6, all micelles had a spherical shape with varying PSs changing from 150 nm to less. However, the average PS was determined to be 92 nm using DLS. The PS of the micelles observed in SEM images are not consistent with the DLS measurements. This was due to the difficulty of taking images of the micelles in a wet state. In addition, while DLS gave us average PS values, the SEM images in Fig. 6 were only for large micelles in the emulsion.

3.5. Stability studies and calculation of encapsulation efficiency (EE %)

The optimized formulation was retained at 25 °C for 60 days and the changes in their PS, PDI and ZP were recorded. PS, PDI and ZP of particles after 60 days is given in Fig. 7. No considerable changes were observed in PS and ZP. PDI moderately increased over time, but the formulations were still completely monodispersed. Fig. 6 shows optimized formulation's results after 60 days. To investigate the effect of Levan on stability of optimized formulation a same formulation of it

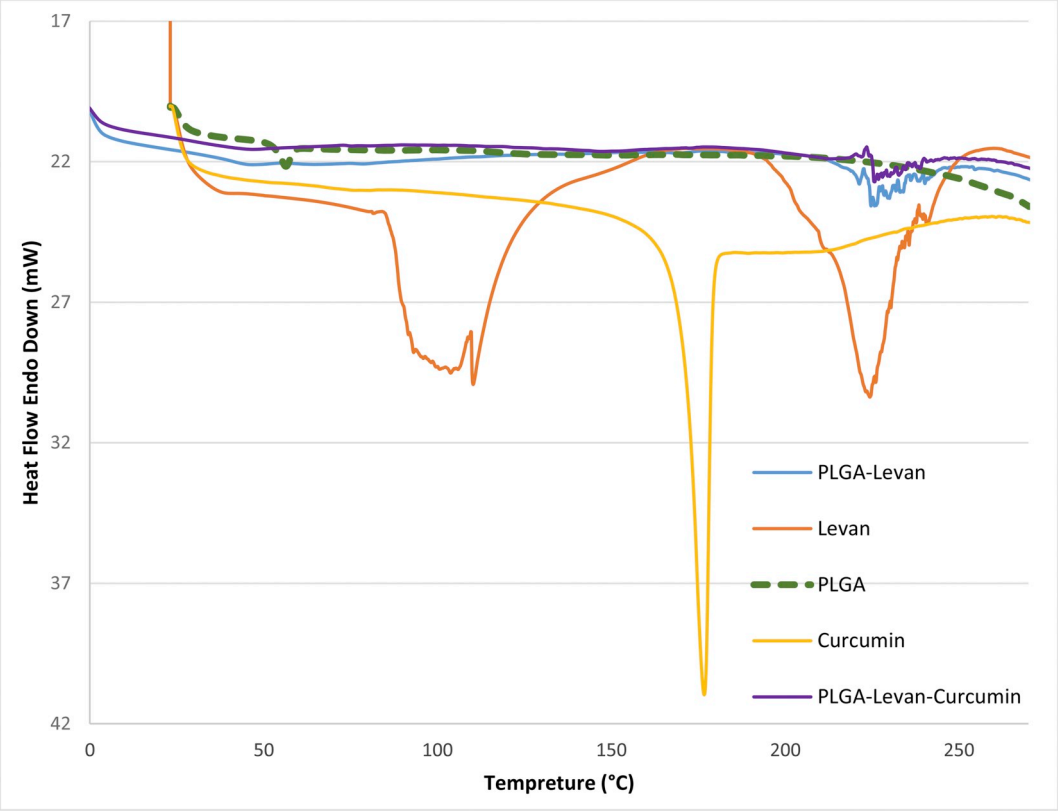


Fig. 5. DSC thermograms of Curcumin, PLGA, Levan, PLGA-Levan and PLGA-Levan-Curcumin nanoparticles after lyophilization

without Levan was prepared and its appearance and particle size and PDI was checked along with optimized formulation for 60 days (Table 12).

To compare the effect of Levan in the enhancement of the solubility of Curcumin with that of PLGA, formulations without using Levan and a mixture of Curcumin in water were prepared and their EE% were measured. The obtained results are presented in Supplementary data S7. As it could be seen PLGA-Levan and PLGA are almost equally incorporated in the enhancement of the solubility of Curcumin, this is where, the superior property of Levan is observed in particle size measurement studies, in which, 60 days of stability of PLGA-Levan-Curcumin nano formulation has been achieved without any deformation in the particle structure. Compared to the aqueous media which solubilizes around 10 ng of Curcumin, almost 2500 times solubility enhancement has been obtained for the Curcumin molecule.

The results of a comparative release study are available at Supplementary data (S8). During the period of 5 days, optimized PLGA-Levan-Curcumin formulation shows a constant release, while one of the non-optimized formulations chosen for this assay shows an increasing release behavior for Curcumin. These data are in good accordance with the results obtained from particle size measurements in which the stability of particles consisted of PLGA are decomposed in 15 days and PLGA-Levan particles are stable for 60 days.

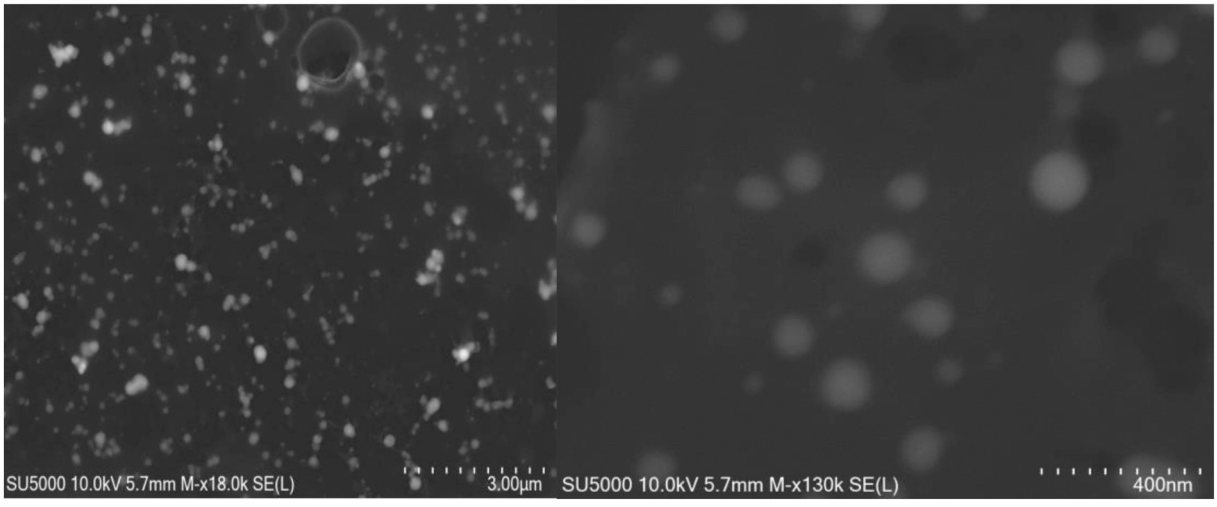


Fig. 6. SEM image of the optimized formulation

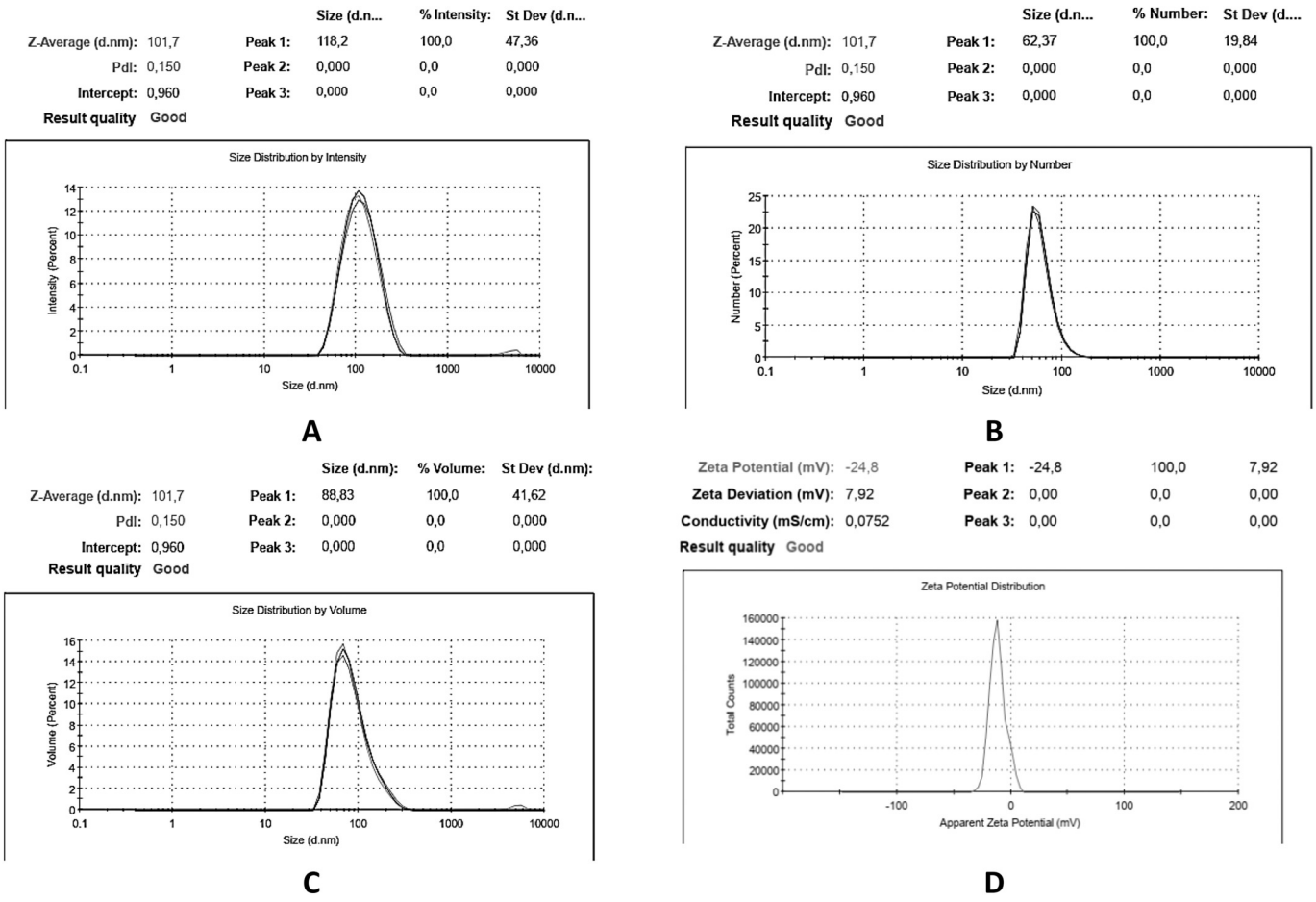


Fig. 7. DLS results of optimized formulation after 60 days A) By intensity (118 nm) B) By number (62 nm) C) By volume (89 nm) and D) Zeta potential (−24,8 mV).

Table 12
Stability of PLGA-Curcumin and PLGA-Levan-Curcumin based on size and dispersity (size are reported based on intensity% distribution and the graphics are available in Supplementary Information 5 and 6).

	1st day	7th day	15th day	30th day	60th day
PLGA-Curcumin	229	219	233	178	133
	–	–	–	653	232
	–	–	–	5049	5026
PLGA-Levan-Curcumin	97.13	97.79	102.3	103.1	101.7

4. Discussion

According to the results, surfactant type (X_6) was the variable which affected three responses at the same time; ZP (Y_2), encapsulation efficiency (Y_3) and PDI (Y_4). Tween 80 which is an emulsifier and a surfactant, stabilizes the emulsion droplets in the aqueous media after diffusion of organic solvent by reducing the surface tension between two phases (Fig. 8). This is where E471 as a surfactant, is only able to decrease the surface tension of aqueous media without interacting with the surface area of formed droplets. Previously Girotra et al. (2016) have come to the similar conclusion while Poloxamer 188 and Polyvinyl alcohol (PVA) were used in development of PLGA DDS. They have shown that Poloxamer 188 is incorporates with PLGA droplets in water resulting in obtaining smaller particles. Despite what Girotra et al. has reported, we did not find the amount of surfactant so effective in terms of PS, however, in accordance with their studies amount of PLGA was found to be a descriptive variable in PS determination using Box–Behnken Design.

In the equation Y_1, the negative sign of interaction X_1X_2 shows the decrease in PS with increase in levan MW and PLGA, while, the positive sign of interaction X_1X_3 points to the opposite effect of increasing Levan MW and acetone on PLGA particle size. We hypothesize that the size of organic solvent droplets in aqueous media is very definitive in the size of obtained PLGA particles (Girotra et al., 2016). Increasing the amount of polymer and polysaccharide in equal amounts of organic solvent, causes more interaction between these macro-molecules, resulting in formation of smaller particles, while increasing the amount of organic solvent, creates larger chambers for these macromolecules in aqueous media, which, consequently ends up with less interaction between them and obtaining larger particles.

To the best of our knowledge, this is the first report on the effect of different variables on the ZP of PLGA DDS. As it can be observed in the equation Y_2, ZP increases with PLGA and acetone amount while it decreases with Levan MW. As it has been shown in Fig. 8, Levan, being a hydrophilic macromolecule, incorporates with PLGA on the outer shell of the DDS (concluded by results obtained from physic-chemical characterization of the optimized formulation) shading the zeta-potential of particle raised from PLGA structure. It is well known that, cell membrane is negatively charged due to its phospholipid composition and hence cell internalization of DDS can be achieved using neutral or positively charged DDS (Bannunah et al., 2014). The incorporation of Levan, a non-toxic biocompatible and biodegradable macro-molecule with PLGA is a very important approach in this frame, which provides even more feasibility for PLGA DDS as an effective drug delivery system.

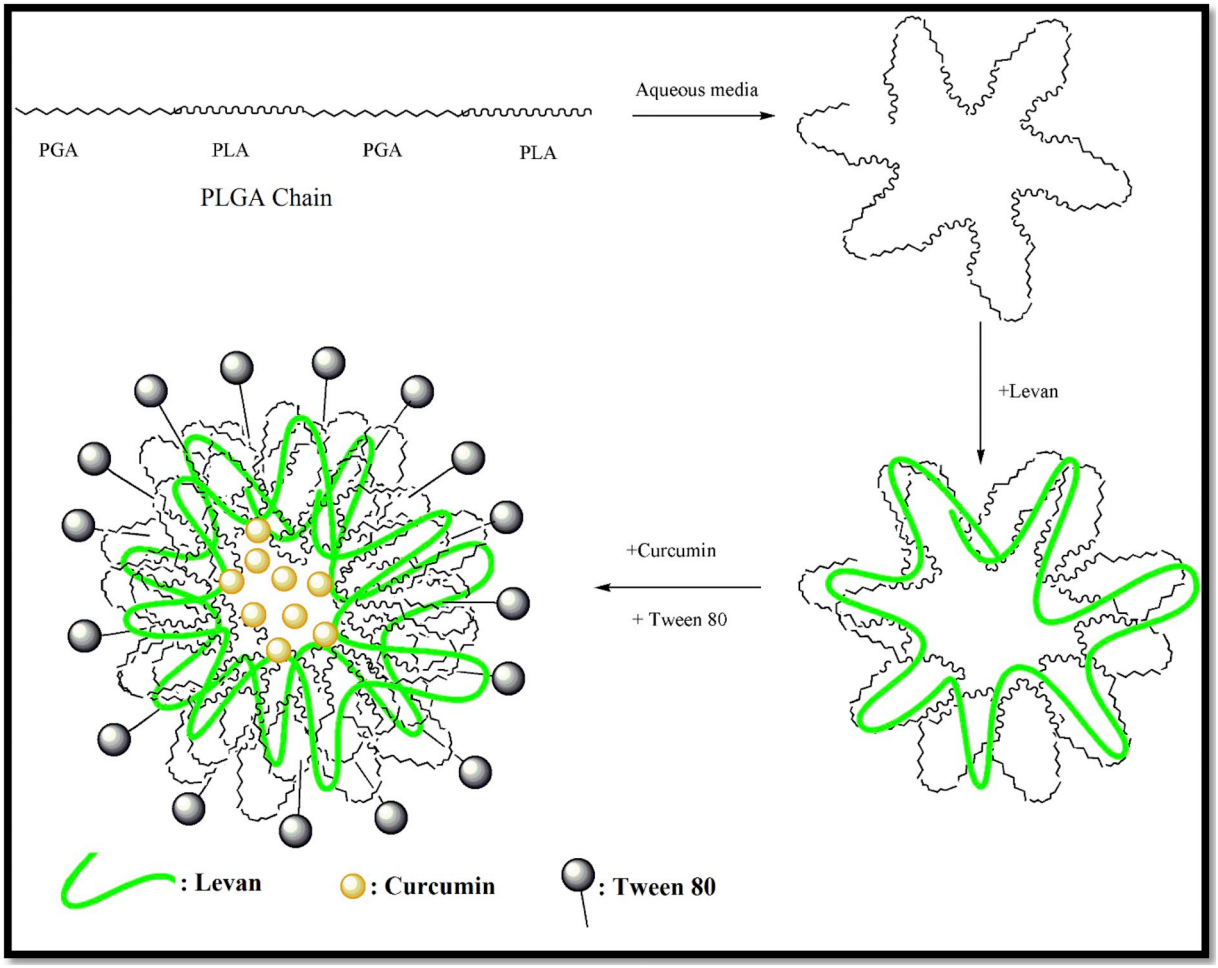


Fig. 8. Illustration of self-assembly process of PLGA in aqueous media, incorporation of Levan with PLGA on the shell and settlement of Curcumin in the hydrophobic core, while, surfactant (Tween 80) stabilizes each self-assembled DDS.

EE% probably is the factor which has been taken to the interest of nano drug delivery system developments the most. QbD investigations conducted in this study, revealed that this dependent variable is mostly affected by Curcumin amount, surfactant type and molecular weight of PLGA. Previously Dhakar (2012) have described the importance of first 10 min of emulsification in obtaining higher EE%. This period is defined as the solidification period of NP in aqueous media. The faster the solidification, the higher the EE%. It is obvious that higher molecular weight of PLGA accelerates particle solidification. Surfactant (type), as mentioned above, involves in stabilization of emulsified organic phase in the aqueous media and thus, facilitates diffusion of drug molecules in the hydrophobic environment of the drug delivery system, which consequently increases EE%. The higher amounts of Curcumin increases the hydrophobicity of the system and its diffusion rate in the PLGA-Levan DDS (Fig. 8), and, this explains its importance in EE%.

Finally, Poly-Dispersity Index (PDI) the most important variable in obtaining reproducible pharmacokinetic and pharmacodynamic outcomes from DDS is one other factor which rarely has been investigated in QbD studies of PLGA DDS. This dependent variable is affected by surfactant type (X_6), PLGA amount (X_2) and rpm (X_8). The influence of surfactant type and PLGA amount in the PDI value of Levan-PLGA DDS is raised from similar caused mentioned above and on Fig. 8. The speed of magnetic stirrer during particle preparation (rpm) gains importance in obtaining mono-dispersed particles (smaller PDI). This fact has previously been emphasized in different ways by several groups (Lindfors et al., 2007; Türeli et al., 2016). The particle formation starts in emulsified organic solvent by formation of a nuclei and continues by growing the particle around it. It has been reported that producing stable nanosuspensions requires fast nuclei formation rate but forming small PS needs slow growth rate. The limiting factor here is the mixing time. If mixing time that is faster than nuclei growth time can be achieved, small PSs that have narrow PS distributions can be produced (Türeli et al., 2016). This clearly indicates the importance of stirring speed of the system in gaining the smaller PDI.

Monitoring the PS distribution as a consequence of intensity and volume of scattered light using DLS for 60 days clearly shows the stability of obtained particles, while, their physicochemical characterization using FT-IR (Fig. 4) and DSC (Fig. 5) reveals the position of each component in DDS and SEM studies confirms the spherical shape of obtained particles. The stability of PLGA-levan Curcumin is also compared with that of OLGA-Curcumin (without incorporation with levan) and the results are shown in Table 12, along with Supplementary Information 5 and 6.

Mono-dispersed DDS monitored by DLS demonstrates complete incorporation of levan with PLGA. Very low values of PDI confirm this conclusion by pointing on presence of particles with narrow size dispersion.

The most significant change in the FT-IR spectrum of PLGA upon incorporation with levan (Fig. 4-A and D) is the reduction in the intensity of C═O stretching peak at 1745 cm^{-1}. Previously it has been

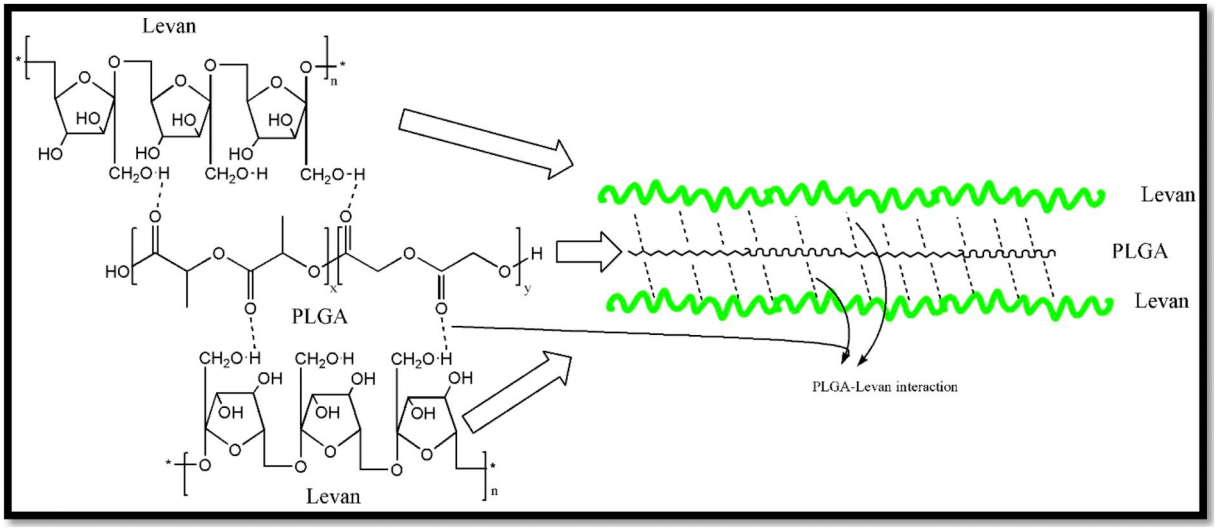


Fig. 9. Incorporation of functional groups of levan with those of PLGA resulted in coating PLGA chains by levan (concluded by interpretation of results obtained from DLS, FT-IR and DSC).

shown that preparation of Curcumin-PLGA nano-particles in presence of Sodium docecyl sulfate (SDS) causes similar effect on this peak (Pietra et al., 2017). The intensity of IR peaks is proportional to the changes in dipole moment that a bond undergoes during a vibration (Arrondo et al., 1993). This decrease in the band intensity clearly reveals the occupation of C═O group by the functional groups of another component in the system. Similarly, the increase in the intensity of C—O bending of levan at 1637 cm^{-1} indicates an increase in its dipole moment. We hypothesize that the interaction between the negatively charged oxygens of the C═O groups in PLGA with the positively charged hydrogens of O—H groups in levan, simultaneously causes a decrease in the dipole moment of C═O and increase in dipole moment of O—H groups. The increase in the intensity of OH vibration of levan at 3200 cm^{-1} in PLGA-levan FT-IR spectra compared to that of free Levan confirms this approach.

This allows us to illustrate PLGA-Levan incorporation as in Fig. 9. The very small increase in the intensity of C═O vibration of PLGA in PLGA-Levan-Curcumin FT-IR spectra (Fig. 4-E) could be attributed to occupation of some Levan O—H groups by the O—H groups of Curcumin, setting the carbonyl groups of PLGA free. This assumption is in well accordance with the results obtained from QbD studies and the monomodelar particles evidenced in DLS experiments.

The absence of all DSC thermograms related to PLGA, Levan and Curcumin in that of PLGA-Levan-Curcumin DDS, confirms that none of above mentioned components are in the crystalline form in the realized DDS, which is in well accordance with the results obtained from DLS and FT-IR.

5. Conclusion

Plackett–Burman and Box–Behnken statistical experimental designs were used to explore the influence of formulation variables on the properties of PLGA-Levan based DDS of Curcumin. FTIR and DSC confirmed the thorough incorporation of PLGA, Levan and Curcumin due to the involvement of all their functional groups. The stability studies revealed that the optimized PLGA-Levan based DDS of Curcumin was adequately stable. This is where PLGA-Curcumin were stable only for 15 days. The method reported in the study can be used to develop other DDSs and it can help to make the DDSs more bioavailable and to increase their circulation time in the body.

Acknowledgements

The authors have no competing interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2019.105037.

References

Ait-Amir, B., Pougnet, P., El Hami, A., 2015. Meta-Model Development. In: Embedded Mechatronic Systems 2. Elsevier, pp. 151–179.
Almoustafa, H.A., Alshawsh, M.A., Chik, Z., 2017. Technical aspects of preparing PEG-PLGA nanoparticles as carrier for chemotherapeutic agents by nanoprecipitation method. Int. J. Pharm. 533, 275–284.
Anand, P., Kunnumakkara, A.B., Newman, R.A., Aggarwal, B.B., 2007. Bioavailability of curcumin: problems and promises. Mol. Pharm. 4, 807.
Anand, P., Nair, H.B., Sung, B., Kunnumakkara, A.B., Yadav, V.R., Tekmal, R.R., Aggarwal, B.B., 2010. Design of Curcumin-loaded PLGA nanoparticles formulation with enhanced cellular uptake, and increased bioactivity in vitro and superior bioavailability in vivo. Biochem. Pharmacol. 79, 330–338.
Arrondo, J.L.R., Muga, A., Castresana, J., Goñi, F.M., 1993. Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy. Prog. Biophys. Mol. Biol. 59, 23–56.
Avsar, G., Agirbasli, D., Agirbasli, M.A., Gunduz, O., Oner, E.T., 2018. Levan based fibrous scaffolds electrospun via co-axial and single-needle techniques for tissue engineering applications. Carbohydr. Polym. 193, 316–325.
Bannunah, A.M., Vllasaliu, D., Lord, J., Stolnik, S., 2014. Mechanisms of nanoparticle internalization and transport across an intestinal epithelial cell model: effect of size and surface charge. Mol. Pharm. 11, 4363–4373.
Bondarenko, O.M., Ivask, A., Kahru, A., Vija, H., Titma, T., Visnapuu, M., Joost, U., Pudova, K., Adamberg, S., Visnapuu, T., 2016. Bacterial polysaccharide Levan as stabilizing, non-toxic and functional coating material for microelement-nanoparticles. Carbohydr. Polym. 136, 710–720.
Bostan, M.S., Mutlu, E.C., Kazak, H., Sinan Keskin, S., Oner, E.T., Eroglu, M.S., 2014. Comprehensive characterization of chitosan/PEO/Levan ternary blend films. Carbohydr. Polym. 102, 993–1000.
Calazans, G., Lopes, C., Lima, R., De Franc, F., 1997. Antitumour activities of levans produced by Zymomonas mobilis strains. Biotechnol. Lett. 19, 19–21.
Çelik, B., Sağıroğlu, A.A., Özdemir, S., 2017. Design, optimization and characterization of coenzyme Q10-and D-panthenyl triacetate-loaded liposomes. Int. J. Nanomedicine 12, 4869.
Choi, B.H., Kim, C.G., Lim, Y., Shin, S.Y., Lee, Y.H., 2008. Curcumin down-regulates the multidrug-resistance mdr1b gene by inhibiting the PI3K/Akt/NFκB pathway. Cancer Lett. 259, 111–118.
Cibulková, Z., Polovková, J., Lukeš, V., Klein, E., 2006. DSC and FTIR study of the gamma radiation effect on cis-1, 4-polyisoprene. J. Therm. Anal. Calorim. 84, 709–713.
Cooper, D.L., Harirforoosh, S., 2014. Effect of formulation variables on preparation of celecoxib loaded polylactide-co-glycolide nanoparticles. PLoS One 9, e113558.
Dhakar, R.C., 2012. From formulation variables to drug entrapment efficiency of microspheres: a technical review. Journal of Drug Delivery and Therapeutics 2.
Erginer, M., Akcay, A., Coskunkan, B., Morova, T., Rende, D., Bucak, S., Baysal, N., Ozisik,

R., Eroglu, M.S., Agirbasli, M., Toksoy Oner, E., 2016. Sulfated levan from Halomonas smyrnensis as a bioactive, heparin-mimetic glycan for cardiac tissue engineering applications. Carbohydr. Polym. 149, 289–296.
Erkorkmaz, B.A., Kırtel, O., Ateş Duru, Ö., Toksoy Öner, E.J.B., Engineering, B., 2018. Development of a cost-effective production process for Halomonas levan. Bioprocess Biosyst. Eng. 41, 1247–1259.
Eskandari, Z., Kazdal, F., Bahadori, F., Ebrahimi, N., 2018. Quality-by-design model in optimization of PEG-PLGA nano micelles for targeted cancer therapy. Journal of Drug Delivery Science and Technology 48, 393–402.
Fatisson, J., Quevedo, I.R., Wilkinson, K.J., Tufenkji, N., 2012. Physicochemical characterization of engineered nanoparticles under physiological conditions: effect of culture media components and particle surface coating. Colloids Surf. B: Biointerfaces 91, 198–204.
Gilding, D., Reed, A., 1979. Biodegradable polymers for use in surgery—polyglycolic/poly (actic acid) homo-and copolymers: 1. Polymer 20, 1459–1464.
Girotra, P., Singh, S.K., Kumar, G., 2016. Development of zolmitriptan loaded PLGA/poloxamer nanoparticles for migraine using quality by design approach. Int. J. Biol. Macromol. 85, 92–101.
Gulcur, E., Thaqi, M., Khaja, F., Kuzmis, A., Onyuksel, H., 2013. Curcumin in VIP-targeted sterically stabilized phospholipid nanomicelles: a novel therapeutic approach for breast cancer and breast cancer stem cells. Drug delivery and translational research 3.
Hines, D.J., Kaplan, D.L., 2013. Poly(lactic-co-glycolic) acid-controlled-release systems: experimental and modeling insights. Crit. Rev. Ther. Drug Carrier Syst. 30, 257–276.
Ibrahim, M., Alaam, M., El-Haes, H., Jalbout, A.F., Leon, A., 2006. Analysis of the structure and vibrational spectra of glucose and fructose. Ecletica Quim. 31, 15–21.
Jyothi, J., Sravani, R., 2016. Development of curcumin nanocrystals and evaluation of GI absorption efficiency in Comparison with curcumin and turmeric powder. World Journal of pharmacy and pharmaceutical sciences 5, 1990–2003.
Kazak Sarilmiser, H., Toksoy Oner, E., 2014. Investigation of anti-cancer activity of linear and aldehyde-activated levan from Halomonas smyrnensis AAD6T. Biochem. Eng. J. 92, 28–34.
Kumar, V.D., Verma, P.R.P., Singh, S.K., 2015. Development and evaluation of biodegradable polymeric nanoparticles for the effective delivery of quercetin using a quality by design approach. LWT-Food Science and Technology 61, 330–338.
Lazic, Z.R., 2006. Design of Experiments in Chemical Engineering: A Practical Guide. John Wiley & Sons.
Lindfors, L., Skantze, P., Skantze, U., Westergren, J., Olsson, U., 2007. Amorphous drug nanosuspensions. 3. Particle dissolution and crystal growth. Langmuir 23, 9866–9874.
Marques, D.R., Santos, L.A.d., Schopf, L.F., Fraga, J.C.S.d., 2013. Analysis of poly (lactic-co-glycolic acid)/poly (isoprene) polymeric blend for application as biomaterial. Polímeros 23, 579–584.
Mathew, A., Fukuda, T., Nagaoka, Y., Hasumura, T., Morimoto, H., Yoshida, Y., Maekawa, T., Venugopal, K., Kumar, D.S., 2012. Curcumin loaded-PLGA nanoparticles conjugated with Tet-1 peptide for potential use in Alzheimer's disease. PLoS One 7, e32616.
Motta, A.C., Duek, E.A., 2006. Síntese, caracterização e degradação "in vitro" do Poli (L-ácido láctico). Polímeros: Ciência e Tecnologia 16.
Mukerjee, A., Vishwanatha, J.K., 2009. Formulation, characterization and evaluation of curcumin-loaded PLGA nanospheres for cancer therapy. Anticancer Res. 29, 3867–3875.
Muthu, M., 2009. Nanoparticles based on PLGA and its co-polymer: an overview. Asian Journal of Pharmaceutics 3, 266.
Öner, E.T., Hernández, L., Combie, J., 2016. Review of levan polysaccharide: from a century of past experiences to future prospects. Biotechnol. Adv. 34, 827–844.
Oner, E.T., Hemberger, J., Demirci, T., Erginer, M., Yıldız, S.Y., 2017. Glycan-Based Nanocarriers in Drug Delivery, Drug Delivery Approaches and Nanosystems. 2. Apple Academic Press, pp. 191–228.
Osman, A., Oner, E.T., Eroglu, M.S., 2017. Novel levan and pNIPA temperature sensitive hydrogels for 5-ASA controlled release. Carbohydr. Polym. 165, 61–70.
Patil, H., Feng, X., Ye, X., Majumdar, S., Repka, M.A., 2015. Continuous production of fenofibrate solid lipid nanoparticles by hot-melt extrusion technology: a systematic study based on a quality by design approach. AAPS J. 17, 194–205.
Pavlin, M., Bregar, V.B., 2012. Stability of nanoparticle suspensions in different biologically relevant media. Digest Journal of Nanomaterials & Biostructures (DJNB) 7.
Pietra, R.C.C., Cruz, R.C., Melo, C.N., Rodrigues, L.B., Santos, P.C., Bretz, G.P.M., Soares, B.M., Sousa, G.R., Ferreira, M.V.L., Cisalpino, P.S., 2017. Evaluation of polymeric PLGA nanoparticles conjugated to curcumin for use in aPDT. Brazilian Journal of Pharmaceutical Sciences 53.
Poli, A., Kazak, H., Gürleyendağ, B., Tommonaro, G., Pieretti, G., Öner, E.T., Nicolaus, B., 2009. High level synthesis of levan by a novel Halomonas species growing on defined media. Carbohydr. Polym. 78, 651–657.
Rahman, Z., Zidan, A.S., Habib, M.J., Khan, M.A., 2010. Understanding the quality of protein loaded PLGA nanoparticles variability by Plackett–Burman design. Int. J. Pharm. 389, 186–194.
Rathore, A.S., Winkle, H., 2009. Quality by design for biopharmaceuticals. Nat. Biotechnol. 27, 26.
Sezer, A.D., Kazak, H., Öner, E.T., Akbuğa, J., 2011. Levan-based nanocarrier system for peptide and protein drug delivery: optimization and influence of experimental parameters on the nanoparticle characteristics. Carbohydr. Polym. 84, 358–363.
Sezer, A.D., Kazak Sarılmışer, H., Rayaman, E., Çevikbaş, A., Öner, E.T., Akbuğa, J., 2017. Development and characterization of vancomycin-loaded levan-based microparticular system for drug delivery. Pharm. Dev. Technol. 22, 627–634.
Shah, B., Khunt, D., Bhatt, H., Misra, M., Padh, H., 2015. Application of quality by design approach for intranasal delivery of rivastigmine loaded solid lipid nanoparticles: effect on formulation and characterization parameters. Eur. J. Pharm. Sci. 78, 54–66.
Tabernero, A., González-Garcinuño, Á., Sánchez-Álvarez, J.M., Galán, M.A., del Valle, E.M.M., 2017. Development of a nanoparticle system based on a fructose polymer: stability and drug release studies. Carbohydr. Polym. 160, 26–33.
Taghipour, B., Yakhchali, M., Haririan, I., Tamaddon, A., Samani, S.M., 2014. The effects of technical and compositional variables on the size and release profile of bovine serum albumin from PLGA based particulate systems. Research in pharmaceutical sciences 9, 407.
Türeli, N.G., Türeli, A.E., Schneider, M., 2016. Optimization of ciprofloxacin complex loaded PLGA nanoparticles for pulmonary treatment of cystic fibrosis infections: design of experiments approach. Int. J. Pharm. 515, 343–351.
Vanaja, K., Shobha Rani, R., 2007. Design of experiments: concept and applications of Plackett Burman design. Clin. Res. Regul. Aff. 24, 1–23.
Yallapu, M.M., Gupta, B.K., Jaggi, M., Chauhan, S.C., 2010a. Fabrication of curcumin encapsulated PLGA nanoparticles for improved therapeutic effects in metastatic cancer cells. J. Colloid Interface Sci. 351, 19–29.
Yallapu, M.M., Jaggi, M., Chauhan, S.C., 2010b. β-Cyclodextrin-curcumin self-assembly enhances curcumin delivery in prostate cancer cells. Colloids Surf. B: Biointerfaces 79, 113–125.
Yerlikaya, F., Ozgen, A., Vural, I., Guven, O., Karaagaoglu, E., Khan, M.A., Capan, Y., 2013. Development and evaluation of paclitaxel nanoparticles using a quality-by-design approach. J. Pharm. Sci. 102, 3748–3761.
Yushu, H., Venkatraman, S., 2006. The effect of process variables on the morphology and release characteristics of protein-loaded PLGA particles. J. Appl. Polym. Sci. 101, 3053–3061.