Quality-By-Design Model in Optimization of PEG-PLGA Nano Micelles for Targeted Cancer Therapy

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Short Title:
QbD Model in Optimization of PEG-PLGA Nano Micelles

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ABSTRACT

Poly (D,L-Lactic-co-Glycolic acid) (PLGA) is a biodegradable and biocompatible polymer approved by FDA for clinical uses. Surface functionalization of self-assembly micelles made of PLGA with Poly-Ethylene Glycol (PEG) improves its stability and half-life in blood circulation via inhibiting adsorption of proteins on the surface and consequently decreasing opsonization rate. The purpose of present study was to optimize PEG amount absorbed on PLGA (PEGabsPLGA) micelles by application of quality by design approach. Based on risk assessment, effect of three variables including PLGA concentration, PEG concentration and molecular weight (MW) of PLGA were studied. Central composite design was implemented for design of experimentation with 26 runs. The PEGabsPLGA nano drug delivery system (NDDS), produced by o/w method, was optimized according to particle size, polydispersity index (PDI) and zeta potential values. Validation of the model was successfully performed with three representative formulations from the design space. As a result, 43.79 mg of PLGA with MW of 30,000-60,000 was incorporated with 12.61 mg of PEG to obtain a 69 nm NDDS (predicted 67.72 nm) with the PDI value equal to 0.124 (predicted 0.112). The results successfully led to the preparation of the most stable nanoparticles which were stable at room temperature for six months.

1. INTRODUCTION

Drug delivery systems (DDS) have been introduced to increase the effectiveness of a drug system through increasing the permeability, solubility and metabolic stability of a drug molecule [1]. DDS are the results of interdisciplinary research which combines different fields such as polymer science, pharmaceutics and molecular biology [2]. DDS are basically designed to transport pharmaceutical agents to the systemic circulation and to control the pharmacokinetics, pharmacodynamics, non-immunogenicity, non-specific toxicity and bio-recognition of target site [3]. DDS are more advantageous than traditional systems in their tendency to deliver the pharmaceutical agents more selectively to a desired site, elimination of over- or underdosing, increase in patient compliance, prevention of side effects and consistent absorption within the desired cell [4].

Ideal polymeric DDS should have specific properties such as adequate drug loading, compatibility with the drug, proper molecular weight and controlled release [5]. These properties are important in the prevention of rapid body clearance and the creation of affinity to cell surface and receptors to allow pinocytic uptake by the target cells. These properties are favorable for selective targeting, stability, non-immunogenicity, reproducible zero-order drug release, controlled biodegradability and non-toxicity [5]. At the moment, no such ideal DDS exist that reveal all these characteristics. Nevertheless, an acceptable product with approximate zero-order release and controlled degradation kinetics can be achieved through physicochemical modification of the polymer or the drug. To reach this purpose, an array of research is being conducted to develop biodegradable polymeric nanoparticles specifically designed for drug delivery [6]. Poly (lactic-co-glycolic acid) (PLGA) is one of the most effectively used biodegradable polymers in this process which is able to form nano sized micelles in aqueous media. PLGA has been approved by the US FDA and the European Medicines Agency (EMA) in varying DDS in humans [7-9]. But, the body treats hydrophobic particles as foreign and the reticulo-endothelial system (RES) removes these from the blood stream and takes them up in the liver or the spleen [10]. This is one of the most significant

biological obstacles to nanoparticle-based controlled drug delivery. To address these limitations, a number of methods of surface modifications have been developed to create nanoparticles that are not recognized by the RES. Nano-micelles can be coated with molecules that conceal the hydrophobicity by providing a hydrophilic layer at the surface [11]. The most general method for surface modification is use of the hydrophilic and non-ionic polymer polyethylene glycol (PEG) [12, 13]. The existence of PEG on the PLGA nanomicelles imparts extra functionality through the use of polymeric nanoparticles [14]. Some studies suggest synthesis of PLGA–PEG–PLGA nano-micells [15].

Considering these facts, the main objective of this study is to optimize a nano drug delivery system using PEG absorbed on PLGA (PEGabsPLGA) by application of quality by design (QbD) approach. We specifically aimed to develop PEGabsPLGA nanomicellar formulations using a QbD approach to understand the influences of formulation and process parameters on the critical quality attributes (CQAs) of these nanoparticles and to establish a design space. Since the process of absorption of PEG on micelles doesn't contain any complex chemical reactions, it may be used for a better adoption of commercial chemotherapeutic nano drug delivery systems.

In a QbD study, the main purpose is to develop a detailed process and product understanding, which can be reached through well-established design of experiment tools [16]. Conventional experimental methods have many disadvantages. Changing a single experimental factor at a time and keeping other factors constant leads to more experiments and makes the process non-feasible [17]. Furthermore, this eliminates the possibility of evaluating factor interactions. Statistical experimental designs (DoE) provide more accurate results with fewer runs compared with the conventional approaches. In addition, optimization of product and process is possible by DoE based on extrapolation of data and plotting of the results [18]. There are several experimental designs in the literature that can be applied to reduce the number of studies while obtaining more useful data [19]. If the goal is to mathematically estimate a response or to precisely optimize a process, models such as Central Composite Design (CCD) are used. This design has been used in the current study.

2. MATERIALS AND METHODS

2.1. Materials

Poly Ethylene Glycol-2000 methyl ether (# 202509), PLGA (Lactide:Glycolide ratio of 50:50), with molecular weights of 30,000-60,000 (High) (#P2191) and 24,000-38,000 (Low)(#739952) were purchased from Sigma-Aldrich USA. Acetone, ethanol and Tween 80 were purchased from Merck (#8.22187.0500), Darmstadt, Germany.

2.2. Experimental design

PEGabsPLGA drug delivery system was optimized by the response surface methodology. According to preliminary studies and literature review [20-22], PLGA amount, PEG amount and PLGA molecular weight were selected as three important factors. Three factors were studied at 5 different levels (-α, -1, 0, +1, +α) using a CCD. The α value of 1.414 was chosen to maintain rotatability and orthogonality of the design. Total 26 experiments were carried out with 10 center points, 8 axial points and 8 cube points (Table 1).

Table 1. Selected variables in central composite design

Factors	Levels				
X_1: PLGA amount (mg)	43.79	50	65	80	86.21
X_2: PEG amount (mg)	1.89	5	12.5	20	23.11
X_3: PLGA molecular weight	30,000-60,000 (High)		24,000-38,000 (Low)		

Minitab 17 (Minitab Inc.; State College, PA, USA) software was used for experiment design and statistical analysis. Response was predicted by two quadratic polynomial equations, each one representing one of the two levels (shown by i) of the categorical variable (i.e., PLGA molecular weight):

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$$

where Y_i is the response for each level of PLGA molecular weight (i.e., Low and High), X_1 and X_2 are the main effects of factors, X_1X_2 is the interaction effects of factors, X_1^1 and X_2^2 are quadratic effects of factors, β_0 is the constant, and β_1 and β_2 are the coefficients of the factors. Analysis of variance (ANOVA) was performed to evaluate the effect of independent variables on the responses, and P=0.05 was considered to be statistically significant. Predicted (pred.) and adjusted (adj.) correlation coefficient (R^2) was calculated to evaluate the fitness of model. After generating the polynomial equations regarding the factors and responses, formulation and process were optimized with respect to average particle size (Y_1), zeta potential (Y_2) and PDI (Y_3) of the PEGabsPLGA nano drug delivery system using the developed mathematical model to determine the levels of PLGA amount (X_1) and PEG amount (X_2) and PLGA molecular weight (X_3). For this, a design space was constructed using several contour plots and response surface graphs. The optimized drug delivery system formulation was prepared and tested to evaluate the correlation between the predicted and the actual values of the responses. The optimum formulation was further characterized for its physicochemical properties.

2.3. Preparation of the Drug Delivery System

The preparation of nanoparticles was based on simple oil-in-water (o/w) emulsification-solvent evaporation method [23]. Briefly, PEG and PLGA were dissolved in 5 ml of acetone based on Table 2. The obtained solvents were mixed and dropped into 20 ml stirring aqueous phase containing 1 ml of homogenized (0.05%) Tween 80 and 19 ml water.

Acetone was evaporated overnight. The resulting suspension containing PEGabsPLGA nanoparticles were reached to 20 ml with distilled water and were kept at 25°C for further tests.

2.4. Determination of Particle Size (PS), Polydispersity Index (PDI) and Zeta Potential (ZP)

PS and PDI of nanoparticles were determined by dynamic light scattering (DLS) technique using Zetasizer (model ZEN 3600; Malvern Instrument, Inc., London, UK) at 25°C. Default setting on the Zetasizer was used, i.e. refractive index of 1.59, absorption of 0.010, water as the dispersant and

measurement angle of 173. Measurements were repeated 5 times, 3 minutes each and data were analyzed by number, intensity and volume distribution, however, the "zeta average" reported by the operating system of the zetasizer was used in QDB calculations [24]. ZP was determined by laser Doppler micro-electrophoresis method by using a folded capillary zeta cell (Malvern Instruments Ltd).

2.5. Fourier Transform Infrared Spectroscopy

To evaluate molecular state of PEG and PLGA, Fourier transform infrared (FTIR) spectra of PEG, PLGA, PEGabsPLGA nanoparticles were recorded on an alpha platinum ATR (Bruker; USA) using 25 scans. Measurements were conducted at room temperature between 400 and 4000 cm^{-1} [10].

2.6. Atomic Force Microscopy

Atomic force microscopy (AFM) study was performed to evaluate the particle shape, size, and distribution of nanoparticles. For AFM imaging, a High performance AFM (NanoMagnetics, Ankara, Turkey) equipped with a PPP-NCLAuD cantilever in dynamic mode was used. In order to stabilize the particles on mica, an origami buffer was used. A droplet of origami buffer was bedded on the mica and after 30 minutes, a droplet of formulation was added and imaged after 30 minutes [25, 26].

2.7. Stability Studies

Stability of optimized formulation was evaluated during storage at 25°C for 60 days. PS and PDI, ZP were measured as described.

2.8. Determination the Amount of PEG Incorporated with PLGA

Self-assembly is defined as "processes that involve pre-existing components, are reversible, and can be controlled by the proper design of the components" [27]. Thus systems such as PEG-PLGA are equilibrated reversible systems. In such systems the hydrophobic and hydrophilic chains dissolve in aqueous media in an amount equal to their water solubility. Excess amount incorporates with other chains to make self-assembly systems. The chains in aqueous media continuously enter to the micellar system and leave it. Thus, continuously there are constant amount of amphiphilic materials

outside of the micelles in aqueous media. Previously it has been shown that the materials which are not incorporated in the structure of micelles could be separated from them by centrifugation in high speed and low temperature [28]. The optimized particle was prepared according to above mentioned method. After evaporation of acetone the volume of the system was recovered to 20ml using distilled water and centrifuged at 4°C, $38,203 \times$ g for 10 min. Supernatants were collected and lyophilized at -80°C and 0.03 mbar. Obtained fluffy material was weighted and accepted as the constant amount of polymeric material not incorporated with micelles. The experiment was repeated in triplicates and the FTIR spectra of dried supernatant was recorded to verify its chemical structure.

3. RESULTS

3.1. Experimental Design

3.1.1. Design Model and Data Analysis

CCD setup and obtained responses from a total of 26 experiments are presented in Table 2. The model fit was assessed and the results showed good model fit for size and PDI. However, for Zeta potential, lack of fit of the model (p=0.0302) was significant. In other words, the model was not suitable in prediction of Zeta potential and Zeta potential was not included in the optimization process.

The following quadratic, second-order equations were used to predict the minimum particle size for the PEGabsPLGA nano drug delivery system:

When PLGA molecular weight is 30,000-60,000 (High):

$$Y_{1,1} = -144.2 + 5.92 \ X_1 + 3.46 \ X_2 - 0.0324 \ X_1 X_2 - 0.0348 \ X_1^2 - 0.0413 X_2^2$$

When PLGA molecular weight is 24,000-38,000 (Low):

$$Y_{2,2} = -109.2 + + 5.54 \ X_1 + 2.82 \ X_2 - 0.0324 \ X_1 X_2 - 0.0348 \ X_1^2 - 0.0413 \ X_2^2$$

where $Y_{1,1}$ and $Y_{2,2}$ are particle size for different levels of PLGA molecular weight, while X_1 and X_2 are coded values for PLGA amount and PEG amount, respectively. Regression model was tested by ANOVA

as presented for average size in Table 3. According to the results, a low p value (0.005) indicated that the model equation was statistically significant. Lack-of-fit value of the model was statistically insignificant (p=2.85), which implies that the model fits well. Confidence level of the regression model was verified by a coefficient R^2 value of 0.6737 that indicated 67.37% of variability in the response can be explained by this model.

The following second equation was used to predict the minimize PDI for the PEGabsPLGA nano drug delivery system:

When PLGA molecular weight is 30,000-60,000 (High):

$$Y_{3,1} = 0.840 - 0.02068 \ X_1 - 0.02502 \ X_2 + 0.000211 \ X_1 X_2 + 0.000154 \ X_1^2 - 0.04 + 0.000517 \ X_2^2$$

When PLGA molecular weight is 24,000-38,000 (Low):

$$Y_{3,2} = 1.033 - 0.02335 \ X_1 - 0.02715 \ X_2 + 0.000211 \ X_1 X_2 + 0.000154 \ X_1^2 + 0.000517 \ X_2^2$$

where $Y_{3,1}$ and $Y_{3,2}$ are PDI for different levels of PLGA molecular weight, while X_1 and X_2 are coded values for PLGA amount and PEG amount, respectively. The low p value (0.022) implied the significance of the model equation as displayed in Table 4. Lack of fit of the model (p=0.286) was not significant. According to the coefficient R^2 value of 0.5975, 59.75% of variability in PDI could be explained by the model.

Table 2. Central Composite Design Experimental Matrix (X_1: PLGA amount (mg), X_2: PEG amount (mg) and X_3: PLGA molecular weight *)

Run	variable			Responses		
	X_1	X_2	X_3	Av. Z	Zeta	PDI
CCD$_1$	65.00	12.50	Low	102.50	20.90	0.10
CCD$_2$	65.00	12.50	Low	107.80	13.80	0.09
CCD$_3$	43.79	12.50	Low	86.73	30.67	0.10
CCD$_4$	65.00	12.50	Low	105.60	24.17	0.09
CCD$_5$	80.00	5.00	Low	119.60	22.87	0.12
CCD$_6$	65.00	12.50	High	109.30	5.81	0.11
CCD$_7$	50.00	20.00	High	74.75	8.90	0.14
CCD$_8$	65.00	12.50	Low	113.00	26.80	0.10
CCD$_9$	65.00	12.50	High	107.90	5.85	0.11

CCD$_{10}$	80.00	5.00	High	102.20	5.62	0.17
CCD$_{11}$	65.00	12.50	Low	90.48	23.13	0.10
CCD$_{12}$	65.00	12.50	High	111.70	6.10	0.01
CCD$_{13}$	65.00	12.50	High	115.90	6.67	0.01
CCD$_{14}$	50.00	20.00	Low	87.17	33.17	0.14
CCD$_{15}$	80.00	20.00	Low	98.40	29.43	0.15
CCD$_{16}$	50.00	5.00	Low	90.02	23.87	0.21
CCD$_{17}$	86.21	12.50	Low	110.30	36.80	0.09
CCD$_{18}$	50.00	5.00	High	60.10	9.39	0.12
CCD$_{19}$	65.00	23.11	High	111.90	7.63	0.12
CCD$_{20}$	65.00	23.11	Low	98.04	24.50	0.12
CCD$_{21}$	43.79	12.50	High	70.93	0.23	0.14
CCD$_{22}$	65.00	1.89	Low	94.60	0.47	0.11
CCD$_{23}$	80.00	20.00	High	106.00	6.85	0.26
CCD$_{24}$	86.21	12.50	High	104.00	5.71	0.19
CCD$_{25}$	65.00	1.89	High	111.40	6.64	0.13
CCD$_{26}$	65.00	12.50	High	91.08	3.18	0.10

*: Low: 24,000-38,000, High: 30,000-60,000

Table 3. ANOVA results for PDI

SOURCE	SUM OF SQUARES	df	MEAN SQUARE	F VALUE	P Value
Model	0.039	8	0.005	3.15	0.022
X$_1$	0.001	1	0.001	0.82	0.377
X$_2$	0.000	1	0.000	0.18	0.678
X$_3$	0.000	1	0.000	0.2	0.659
X_1^2	0.017	1	0.017	10.77	0.004
X_2^2	0.012	1	0.012	7.61	0.013
X$_1$X$_2$	0.005	1	0.005	2.92	0.106
X$_1$X$_3$	0.006	1	0.006	4.16	0.057
X$_2$X$_3$	0.001	1	0.001	0.67	0.426
Lack of fit	0.017	9	0.002	1.51	0.286
Pure error	0.010	8	0.001	3.15	0.022
Total	0.065	25			

Table 4. ANOVA results for size

SOURCE	SUM OF SQUARES	df	MEAN SQUARE	F VALUE	P Value
Model	3592.16	8	449.02	4.39	0.005
X$_1$	2358.58	1	2358.58	23.04	0
X$_2$	0	1	0	0	0.999
X$_3$	28.2	1	28.2	0.28	0.606
X_1^2	850.79	1	850.79	8.31	0.01
X_2^2	75.04	1	75.04	0.73	0.404
X$_1$X$_2$	106.58	1	106.58	1.04	0.322
X$_1$X$_3$	132.11	1	132.11	1.29	0.272
X$_2$X$_3$	91.88	1	91.88	0.9	0.357
Lack of fit	1096.29	9	121.81	1.51	0.285
Pure error	643.66	8	80.46	4.39	0.005

Total	5332.1	25			

3.1.2. Optimization Study

Optimum conditions were determined by Minitab 17 software based on the obtained results from CCD study. Desired limits were set as minimized size and PDI. Coded variables for optimized formulation were found as $X_1=43.79$, $X_2=12.60$ and X_3=High.

Three-dimensional surface and contour plots for PDI and size as a function of PEG amount and PLGA amount with PLGA molecular weight fixed as high are presented in Fig. 1.
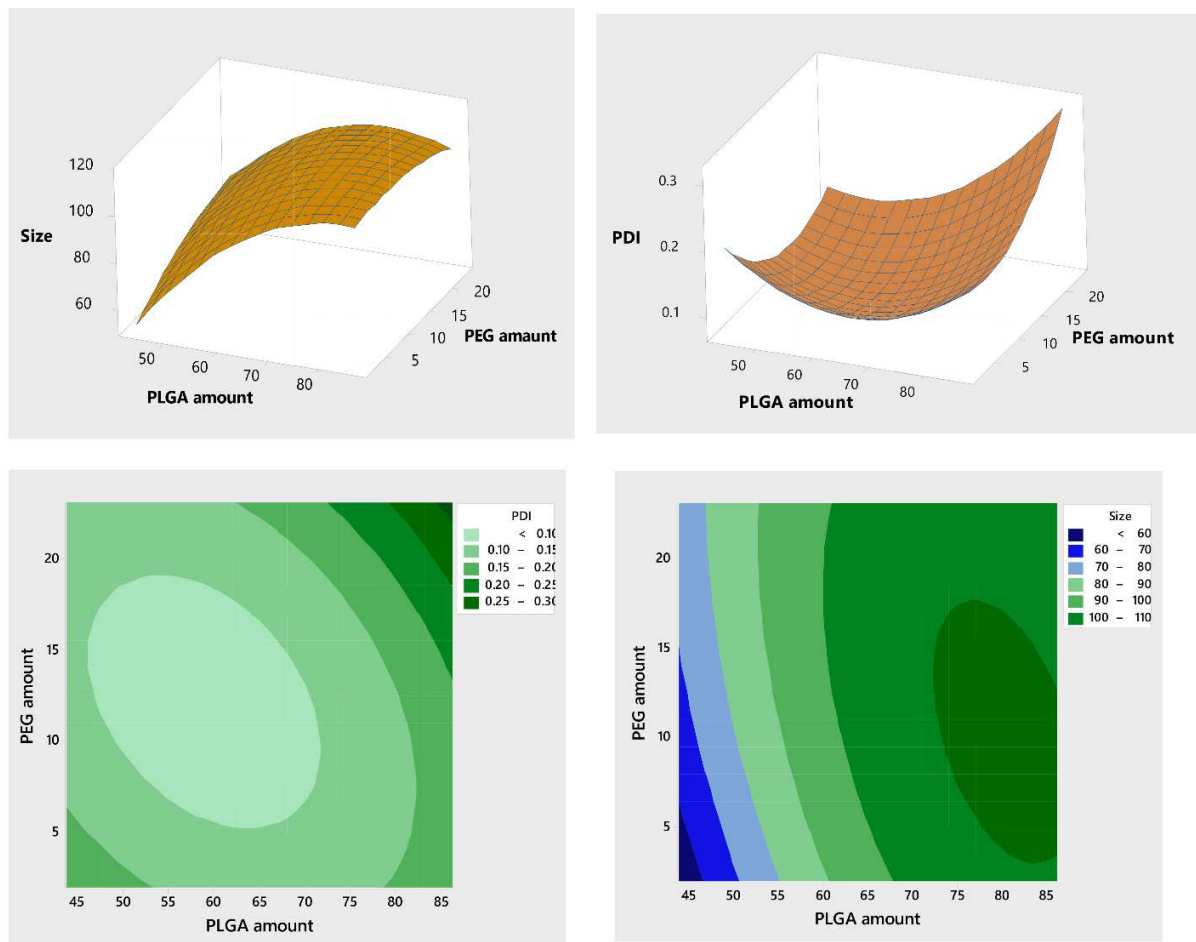
Fig. 1. Contour and response surface plots showing the effects of PLGA amount and PEG amount (while PLGA molecular weight is fixed as 30,000-60,000 (High)) on the average particle size and PDI of the PLGA-PEG drug delivery system.

The optimized formulation (i.e., PLGA amount =43.79, PEG amount =12.61 and PLGA molecular weight =High) was prepared in triplicate to evaluate the model accuracy for the optimum conditions. Predicted and experimental responses for optimized variables are presented in Table 5. Observed experimental values were in close agreement with the predicted values. Coordination between the results indicated the significance and validity of the model.

Table 5. The Observed and the Predicted Values of the Optimum PEGabsPLGA Nano Drug Delivery System Based on Desirability Function

Response	Observed	Predicted	Residual
Particle size (nm)	69.0	67.72	1.28
PDI	0.124	0.112	0.012

3.1.3. Determination of Particle Size (PS), Polydispersity Index (PDI) and Zeta Potential (ZP)

PS, PDI and ZP measurements were conducted for all nanoparticle formulations prepared for studying the experimental design. PLGA and PEG amount ranging from 43.79 to 86.21 and 1.88 to 23.11, respectively, led to obtaining nanoparticles with size distribution between 60.10 and 119.60 nm, while PDI of these nanoparticle was below 0.213, revealing narrow size distribution and uniform particle formation. ZP results varied between -0.23 and −36.80 mV, which shows direct dependence of this value on the PEG amount. The results of optimized formulation were verified three times at laboratory. Fig. 2 shows results of one of them.
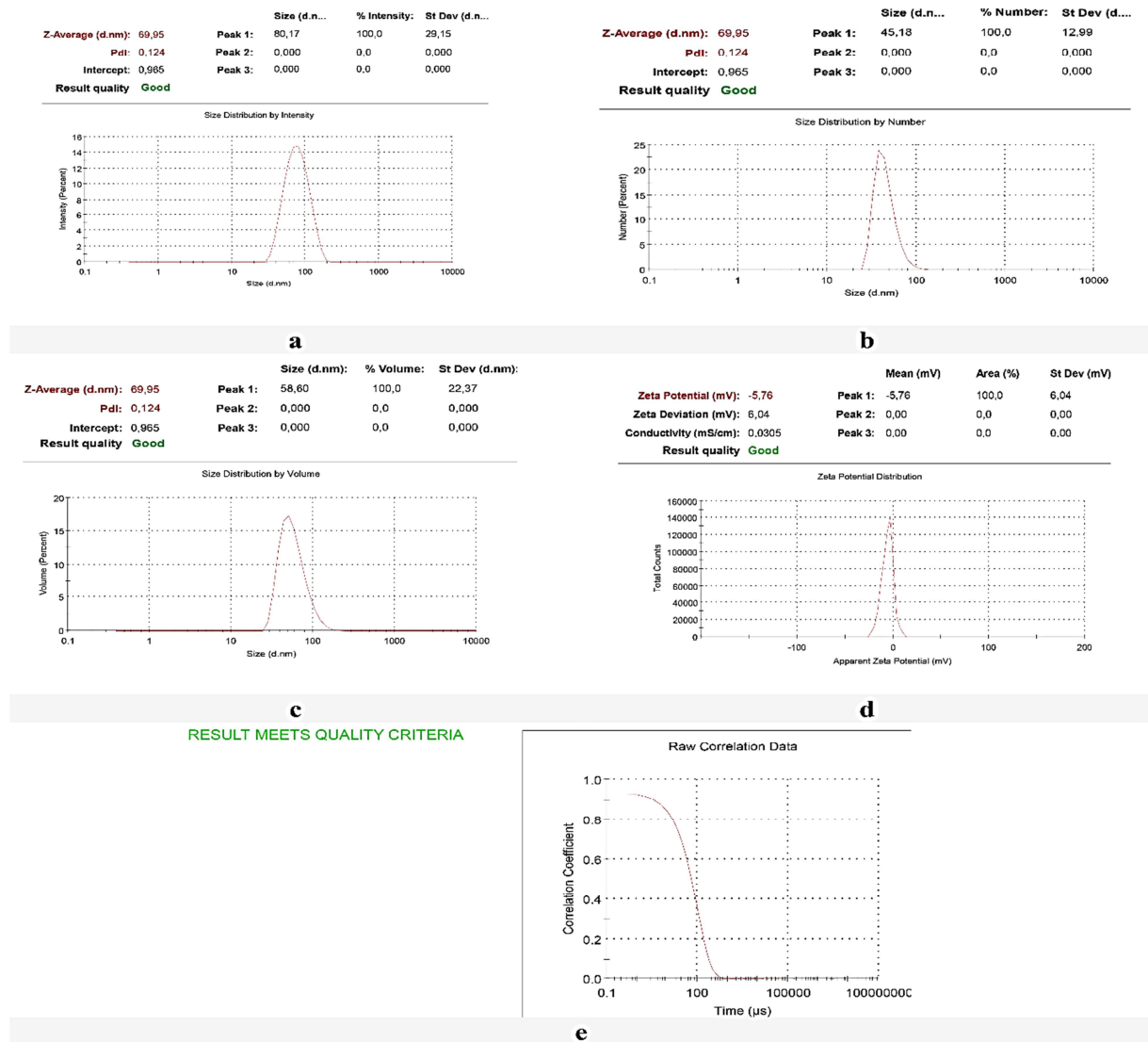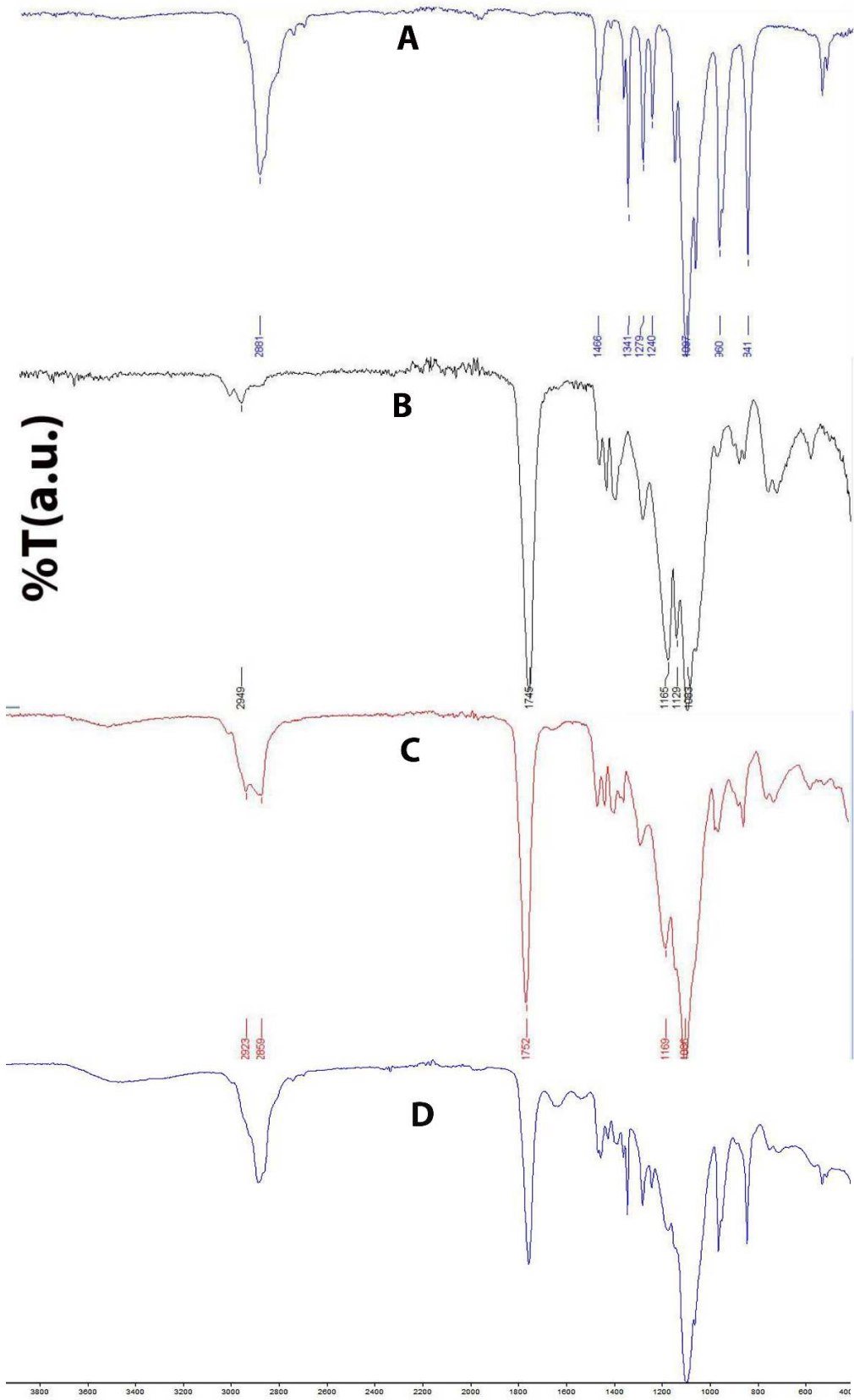
Fig.2: DLS results of particle size distribution of optimized formulation measured in terms of **a:** % intensity: 80 nm , **b:** %number: 45 nm, **c:** %volume: 58 nm and **d:** zeta potential: -5.76 mv and finally **e:** time history of particle sizing all of which, indicates obtaining mono-dispersed particles with no hidden aggregates

PS is an important parameter for membrane penetration in all systemic and local applications and it has been investigated by different research groups to determine its optimum range. Generally, it has been concluded that particles below 200 nm are sufficient for delivery to deeper layers [29-31].

3.2. Fourier Transform Infrared Spectroscopy

To investigate the interaction between PEG and PLGA, FTIR spectra of PEG, PLGA, PEGabsPLGA nanoparticles were acquired (Fig. 3). In the spectrum of PEG, the regular PEG absorption band around 3450 cm^{-1} showing the OH stretching band is absent due to the methoxide derivative which was used in formulation process. The aliphatic C-H stretching bands at 2881 cm^{-1}, C-H bending vibrations at 1466 and 1341 cm^{-1} and C-O-C stretching vibrations at 1279 and 1005 cm^{-1} were observed, which are consistent with the data in the previous reports [32]. At the FTIR spectra of PLGA, the peak generated by symmetrical and asymmetrical stretching of CH$_2$ and CH$_3$ groups between 2980 and 2949 cm^{-1} were the most significant ones, while bands of C=O stretching (1745 cm^{-1}) asymmetrical deformation of CH$_3$ were presented in 1375cm^{-1} and CH$_2$ in 1450 cm^{-1} were also presented. The regular C-O stretching of aliphatic polyesters are also presented in 1165 cm^{-1} and the 1083 cm^{-1} [33].

While all above mentioned peaks of PEG and PLGA are presented in spectra of PEGabsPLGA nano-particles as well, there are some minor changes and shifts by which it is possible to conclude the successful incorporation of these two polymer with each other and also the regions of which they are incorporated. The C-H and/or CH$_3$ stretching bands of PLGA and PEG have been shifted from 2881 and 2949 to 2923-2850 combining at a single region. The decrease at the intensity of C-H stretching peak of PEG and increase in that of PLGA reveals the incorporation of these two polymers in an inclusive manner. Not only the hydrophilic glycolic units of PLGA are interacted (represented by the CH$_2$ stretching peaks) but also the hydrophobic lactic members are incorporated with PEG. The very small shifting of the peaks at this region to lower wavelength shows the firm incorporation between PLGA and PEG with consequently causes need of higher energy for the C-H bonds to be stretched. This is where the C=O stretching peak of PLGA has been up-shifted to 1752 points at the ease of bending this bond by IR, which could be caused by occupying the aliphatic regions of PLGA by PEG which consequently causes the free stretching of this carbonyl group.

%T(a.u.)

A
2881
1466
1341
1279
1240
1097
960
841

B
2949
1745
1165
1129
1083

C
2923
2859
1752
1169
1085

D

Fig. 3: IR results of A) PEG B) PLGA, C) PEGabsPLGA and D) the chemical composition of supernatants for determination of amount of PEG and PLGA incorporated

3.3. Atomic Force Microscopy

AFM study was performed to evaluate the particle shape, size, and distribution of PEGabsPLGA nanoparticles (Fig. 4). The nanoparticles were found to be spherical, nonporous, smooth, and homogenously dispersed. The particle size distribution of nanoparticles showed correlation with the results obtained from DLS studies, i.e., ≈ 45nm.
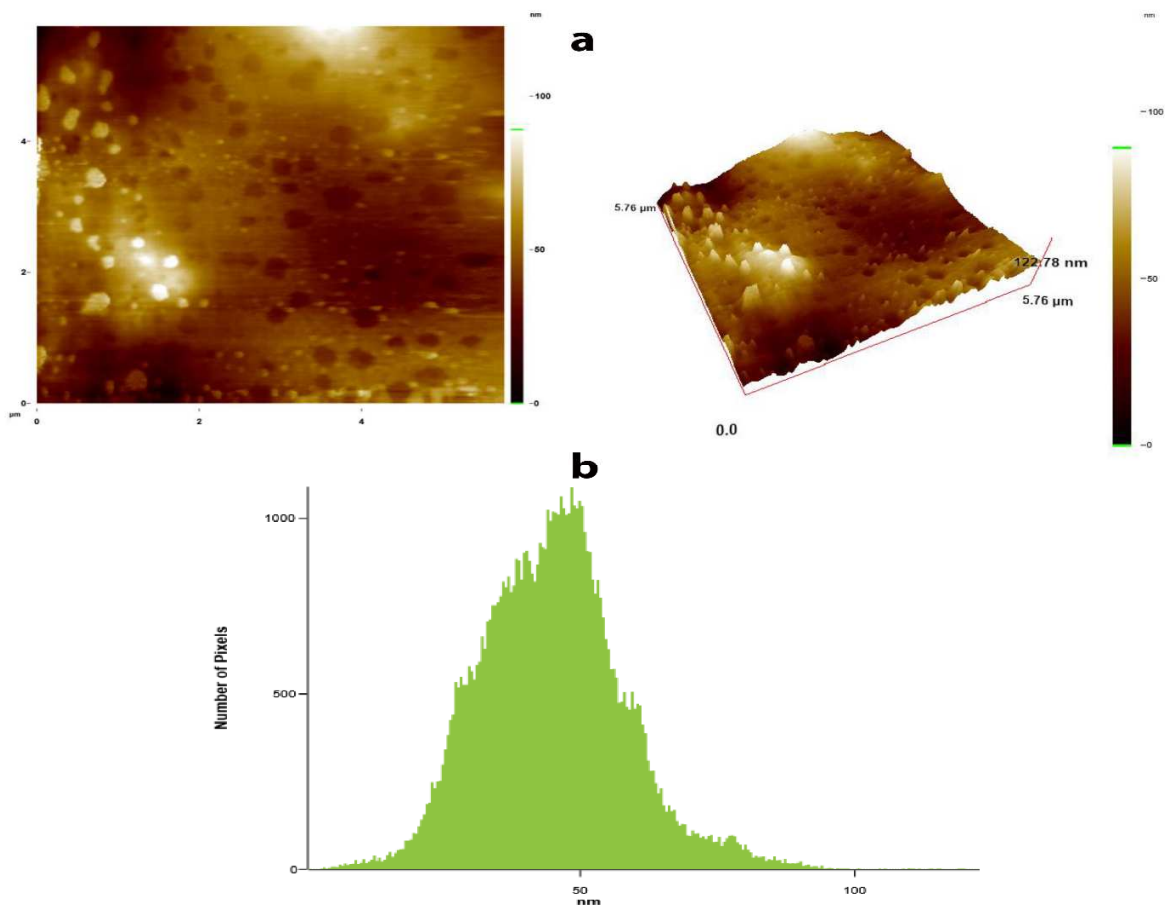
Fig. 4: AFM **a:** topography and **b:** histogram of optimized formulation of PEGabsPLGA nanoparticles indicating the particle size $\approx$ 45nm

3.4. Stability studies

PS, PDI and ZP changes of formulations stored at 25°C during 60 days were monitored. PS, PDI and ZP

of particles after 60 days are shown in Fig.5. There is not any considerable change in PS and ZP. PDI is slightly increased with time but it is still completely mono-dispersed. Fig. 6 shows the results of optimized formulation after 60 days.

3.5. Determination the Amount of PEG Incorporated with PLGA

Some studies use polymers to decrease the surface tension of water and ease the micelle formation. These polymers must be removed from the system to avoid toxicity. Polyvinyl alcohol is one of those polymers. It has been shown that centrifuge at high speed and low temperature is able to remove PVA from system without causing any disruption in micellar structures [28]. Thus we centrifuged the optimized formulation and separated the supernatants to understand the composition and amount of the PEG and/or PLGA which are not incorporated with micelles in a certain period of time. As we mentioned in Optimization Study results the optimized formulation is consisted of 43.79 mg of PLGA with MW of 30,000-60,000 and 12.61 mg PEG. Since each 20 ml of formulation contains almost 3 mg of Tween 80 the optimized formulation is totally weighted 59.4 mg. The amount of supernatant was around 22.2 mg which is almost 37.4% of whoe materials used.

FTIR studies showed that both PLGA and PEG are presented in the lyophilized supernatant (Figure 3, D). The higher intensity of peaks related to PEG in mixture shows that the amount of PEG is higher than PLGA in mixture. This shows that almost 37.4% of the polymeric material is continuously exchanged between micellar aggregates and aqueous media and not only hydrophilic PEG is attended in this exchange but also amphiphilic PLGA is circulated inside and out of micelles as well.
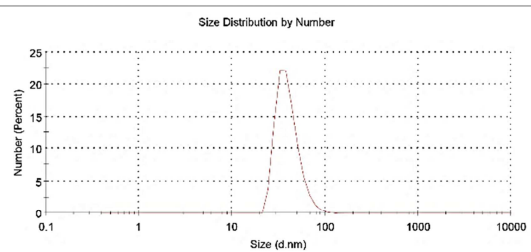
a

	Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 70,93	Peak 1: 83,82	100,0	34,94
Pdl: 0,145	Peak 2: 0,000	0,0	0,000
Intercept: 0,964	Peak 3: 0,000	0,0	0,000
Result quality Good			

Size Distribution by Intensity

b

	Size (d.n...	% Number:	St Dev (d....
Z-Average (d.nm): 70,93	Peak 1: 40,44	100,0	12,54
Pdl: 0,145	Peak 2: 0,000	0,0	0,000
Intercept: 0,964	Peak 3: 0,000	0,0	0,000
Result quality Good			

Size Distribution by Number

c
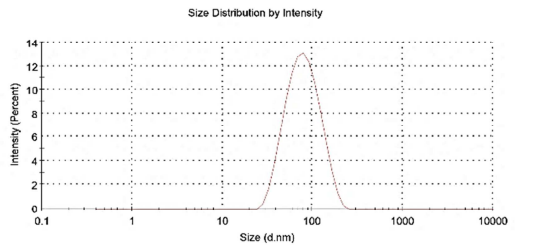
	Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm): 70,93	Peak 1: 55,79	100,0	24,76
Pdl: 0,145	Peak 2: 0,000	0,0	0,000
Intercept: 0,964	Peak 3: 0,000	0,0	0,000
Result quality Good			

Size Distribution by Volume

d
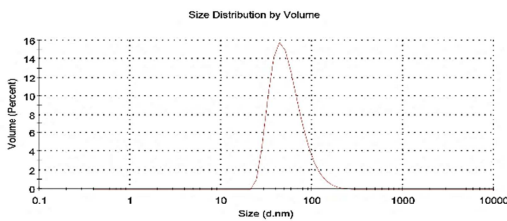
	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -5,06	Peak 1: -5,06	100,0	7,53
Zeta Deviation (mV): 7,53	Peak 2: 0,00	0,0	0,00
Conductivity (mS/cm): 0,0950	Peak 3: 0,00	0,0	0,00
Result quality Good			

Zeta Potential Distribution

e

Raw Correlation Data
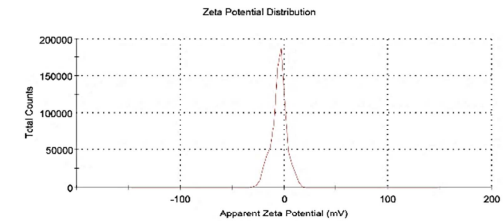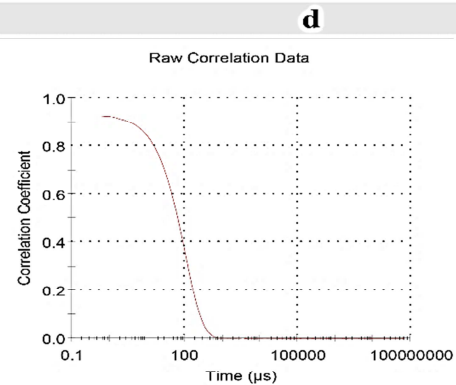
Fig. 5: DLS results of optimized formulation after 60 days of incubation at room temperature measured in terms of **a:** % intensity: 83 nm, **b:** %number: 40 nm, **c:** %volume: 56 nm and **d:** zeta potential: -5.06 mv and finally **e:** time history of particle sizing all of which, indicate stability of obtained optimized nano-micelle at the end of stability studies

4. DISCUSSION

While there are numerous studies made on PLGA-PEG NDDS, the optimized ratio of absorbed PEG to PLGA is not explored yet. Koopaei et. al [34] have chemically conjugated PLGA (50:50, MW 4800) with PEG 5000. They have optimized the process of nanoparticle preparation, by choosing the polymer concentration, drug concentration and ratio of the organic to aqueous solvent as the independent variables to obtain particles with size of 118nm. Probably using the PEG with MW much higher than the one in our study is what has caused obtaining particles with bigger size. However, the ratio of PEG to PLGA has been chosen as a constant (1:4, W/W) and it was not included in optimization process [34]. In another study, ocular administration of dexibuprofen using PEGylated PLGA Diblock copolymer nanospheres has been optimized according to pH, drug and surfactant concentrations as variables while the amount of polymer was kept constant (90 mg) [35]. This study has emphasized the importance of pH in production of mono-dispersed NDDS consisted of PLGA-PEG and the size of obtained particles is reported as "under 200 nm" which is much higher than currently reporting data. However, their results regarding reduction in the zeta potential of NDDS from -20mV [36] to -5 mV by PEGylation of PLGA micelles is in complete agreement with our current study.

In the current study, the validity of obtained results has been tested by chasing the stability of produced nano-micelles for six months. The size and mono disperse behaviour of nano-micelles was considered as the determinants of stability. The symmetrical shape of size diagrams obtained from DLS indicates the spherical shape of all produced nano-micelles [37, 38]. Neither their shape nor their size does not show any difference at the stability assay time period.

Regarding the dominance of the intensity and the volume of the light scattered by shapeless aggregates compared to that of spherical particles [38], the presence of aggregation in the aqueous system could be best detected by %volume and %intensity distribution measurements. The mono-disperse behaviour of obtained optimised PEGabsPLGA nano-micelle was evidenced using these parameters, which showed the same profile at the beginning and the end of stability assay.

As it is well known, the amphiphilic materials such as PLGA form self-assembly systems in aqueous media [39]. Particles made by these systems are consisted in the presence of water and disassociate upon drying. To the best of our knowledge, the AFM imaging of a self-assembly system is performed in this study for the first time by using origami buffer. The results of particle size obtained from AFM assay of the optimised formulation is in well coordination with DLS results as ≈ 45nm.

Results obtained from responses of variables X_1 (PLGA amount (mg)), X_2 (PEG amount (mg)) and X_3 (PLGA molecular weight) show that the size of nano-micelles increases in accordance with the increase in the amount of PLGA, not with its MW. In other words, 65 mg low MW PLGA with 12.5 mg PEG results in obtaining 102.5 nm particles while, the same amounts of PEG with low MW 86.21 mg PLGA results in obtaining 110.3 nm particles. However, same amounts of high MW PLGA and PEG result in obtaining 104.00 nm particles.

The results of FTIR indicate the complete incorporation of PLGA with PEG in all functional group regions. Thus, we report the complete incorporation of PLGA with PEG resulting in formation of mono dispersed stable particles, which provides a suitable base for uploading hydrophobic drug molecules.

5. CONCLUSION

In this study, the effects of formulation and process variables on the properties of PEG-PLGA drug delivery system were evaluated by the use of Central Composite Design. The physicochemical tests such as FTIR revealed the complete interaction of PLGA and PEG chains by involving all of their functional groups in this mutual effect. The stability studies showed that the optimized drug delivery system was sufficiently stable. The same method can be applied for the development of other drug delivery systems in order to increase bioavailability and circulation time in the body. Hundreds of tumour targeting and/or sustained release nano formulations have been developed based on PLGA nano micelles, which obviously would face problems such as opsonisation and fast clearance in body. Replacement of PLGA with PEG chemically conjugated PLGA would cause basic modifications in these formulations, thus, here in this study we presented the optimized amount of PEG which is needed to be absorbed on certain amount of

PLGA without any need for chemical reactions.

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