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Development, Optimization and *In Vitro* Evaluation of Etoposide loaded Lipid Polymer Hybrid Nanoparticles for Controlled Drug Delivery on Lung Cancer

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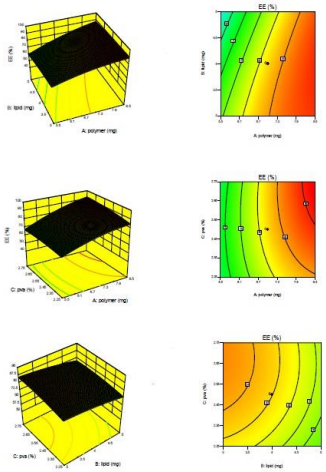
ABSTRACT

Lipid polymer hybrid nanoparticles [LPHNPs] are a system of nanoparticles which are a mixture of lipid monolayer shell and biodegradable polymer core developed for intelligent drug delivery of anti-cancer drugs. The present study aimed to develop and optimize LPHNPs for targeted delivery of *etoposide* anticancer drug through designing an experiment with Response Surface Methodology [RSM] and Central Composite Design [CCD] with quantitative control of three independent variables of lipid, polymer and polyvinyl alcohol [PVA] percentage which examined their effect on nanoparticle size and encapsulation efficiency [EE]. LPHNPs were prepared by one-step nanoprecipitation method. The results showed the optimizing of the tested values of polymer, lipid and %PVA. Lipid-polymer hybrid formulation was reported to be about 14% after 80 hr. The cytotoxicity effect of etoposide-containing LPHNPs for lung cancer cell lines A-549 and Calu6 showed higher antitumor activity compared with the free drug used. Further, the results showed that the high polymer concentration led to more space for drug enclosure and created a relatively compact matrix; therefore, the drug encapsulation efficiency increased as the amount of polymer increased. In LPHNPs, increasing the amount of polymer, lipid and increasing the percentage of PVA used in nanocarrier synthesis generally improved particle size and encapsulation efficiency. The results also showed that LPHNPs could be effective for the delivery of hydrophobic drugs such as etoposide.

GRAPHICAL ABSTRACT



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Introduction

Lung cancer is a disease characterized by the uncontrolled growth of cells in the lung tissue. If left untreated, cell growth can spread beyond the lungs by a process called metastasis to the nearby tissues or other parts of the body [1]. Lung cancers are classified according to their histological type. This classification is important for determining the type of disease management and predicting disease outcomes [2]. The vast majority of lung cancers are carcinomas, malignancies resulting from epithelial tissue. The most common symptoms are cough with bloody sputum, weight loss, and shortness of breath. The most common cause of lung cancer is prolonged exposure to tobacco smoke, which accounts for 90% of lung cancers [3]. Common treatments for this disease include surgery, chemotherapy, and radiation therapy. The limiting factor in cancer chemotherapy is currently the lack of selectivity of drugs against cancer cells [4]. High doses of these drugs are often required to achieve optimal levels of treatment in tumor tissues. Since most chemotherapy drugs used for cancer treatment have high molecular weight with a high volume of distribution, the application of high doses of chemotherapy agents causes healthy tissues in the body to be exposed to these drugs, leading to side effects. Poor specificity and resistance of tumors to the therapeutic agent is the main obstacle in the effective chemotherapy process [5].

Etoposide is an anti-cancer drug that prevents the spread of certain types of cancer by stopping cancer cells [6]. Etoposide is an enzyme topoisomerase II inhibitor that inhibits DNA production by acting on the pre-mitotic phase of cell division. Etoposide is applied in the treatment of lung and testicular carcinoma [7]. On the other hand, chemotherapy drugs such as etoposide are more effective in killing the rapidly dividing cells. Unfortunately, chemotherapy does not know the difference between cancer cells and normal cells. Normal cells will grow back and become healthy, but in the meantime, side effects occur [4]. In this regard, nanotechnology has been widely used to improve a variety of common treat;ments for cancer in recent years [8]. Due to the fact that the vessels around the tumor tissue are more permeable than the vessels of normal tissues and also need more oxygen and nutrients due to their higher growth rate, they have better drug absorption, which is called Enhanced Permeability and Retention [EPR] [4, 9]. Nanoparticles used for drug delivery include a variety of structures of different sizes, shapes, and materials, each has different drug loading capacity, release, cell targeting, and stability [10]. Nanoparticles can increase the intracellular concentration of chemotherapy drugs without causing cytotoxicity to other cells [11-14]. Drug systems in the delivery and release of drugs effective in the treatment of cancer diseases include liposomal nanoparticles, polymer micelles, dendrimers, carbon nanotubes, aptamers, quantum dots, and polymer composites [15].

A system of nanoparticles has been developed from a mixture of lipid monolayer shell and biodegradable polymer core developed for intelligent drug delivery of anti-cancer drugs [16]. Core-shell-type lipid-polymer hybrid nanoparticles [CSLPHNs], which are a combination of the mechanical advantages of degradable polymer nanoparticles and the biometric advantages of liposomes, have emerged as a strong and promising delivery medium [17]. In CSLPHNs, a degradable polymer core is surrounded by a shell composed of phospholipid layers. This hybrid architecture can provide advantages such as controllable particle size, surface performance, high drug loading, trapping multiple therapeutic drugs, regular drug release and good serum stability [18-20]. These nanoparticles are used for the loading of both hydrophobic and hydrophilic drugs [21]. New integrated lipid-polymer hybrid systems seek to reduce some limitations of liposomes and polymer nanoparticles. Briefly, the biometric properties of lipids and the architectural advantages of the polymer core represent a useful result as a new theory in drug delivery

systems. Polymer nanoparticles provide high drug loading, better biodegradability and stability characteristics, while lipid nanoparticles suggest the use of synthetic and natural lipids with better biocompatibility, long circulation and easy and convenient surface performance [22].

Hybrid nanoparticles are solid sub-micron particles composed of at least two components, lipids and polymers. Various bioactive molecules such as drugs, genes, proteins and intelligent ligands can be encapsulated, adsorbed or covalently bonded in hybrid systems. Common colloids of biodegradable polymers such as poly lactic co-glycolic acid [PLGA], poly caprolactone [PCL], dextran or albumin can be applied because they are biocompatible, biodegradable, non-toxic and have already been used in approved products [18, 19]. The most commonly used lipids are often zwitterionic, cationic, anionic and neutral phospholipids such as lecithin, DPPC, DPTAP, DOTAP and DOPT [23, 24].

The shell and core structure of the lipid-polymer hybrid system consists of three distinct elements, including the inner polymer core that contains the active therapeutic substance and affects the encapsulation and delivery of the drug; a layer of lipid as biocompatible shell surrounds the polymer core. In addition, it is a mold for surface modification and a barrier to prevent leakage of water-soluble drugs out of the core of a hybrid system [25, 26]; the outermost part of this system is the polymer polyethylene glycol coating that increases the shelf life of nanoparticles in the body and also acts as a protective layer of adsorbent detection proteins in the bloodstream [27]. The LLPHNPs preparation methods reported in most articles are solvent evaporation, one- and two-step emulsions and nanoprecipitation, followed by ultrasonic and self-assembly methods [28, 29]. In the present study, loading etoposide into LPHNPs was designed to develop and optimize this drug system through experimental design using a central composite design, which is one of the elements of the surface response methodology. In this study, poly lactic-co-glycolic acid [PLGA] was used for its biodegradability and high potential for loading hydrophobic drugs to encapsulate the drug [30, 31]. Phosphatidylcholine was used as a lipid to coat the polymer core, which helped the nanoparticles to diffuse better like a biological membrane [29].

Since little research has been done on etoposide and cell lines used in this study in the field of polymer lipid hybrid nanocarriers and its advantages over other common carriers, the results of this study can be very useful and effective for treatment of lung cancer. Therefore, the novelty of the present study is the use of a new carrier for etoposide to investigate better drug delivery for the treatment of lung cancer cell lines. These nanoparticles are evaluated in terms of particle size, size distribution, zeta potential, drug loading efficiency and encapsulation efficiency. Finally, the cytotoxicity of nanoparticles is evaluated by MTT method on two lung cancer cell lines A-549 and Calu6 and drug release kinetics.

Material and methods

Etoposide was purchased from KLAB, India. Cholesterol, phosphatidylcholine, MTT (0.5 mg/ml) and Polyvinyl Alcohol were purchased from Sigms, USA. PLGA with a molar ratio of 50:50 was purchased from Iran Polymer and Petrochemical Institute. The RPMI-1640 medium was purchased from Invitrogen, USA. Methanol and chloroform solvents were purchased from Merck, Germany. A-549 and Calu6 Cell lines were obtained from Pasteur Institute of Iran. All materials were analytical grade. Distilled water was used throughout the study.

Experiment design

CCD method is used to perform LPHNPs tests in Design Expert version 10. Hence the three factors of lipid content (A), polymer content (B) and polyvinyl alcohol (C) were studied as independent variables at three different levels (1, 0, -1), two axial points (+α, -α) and six replications at the central point were considered to estimate the experiment error and calculate

the repeatability. Physical and chemical properties such as particle size (Y_1) and drug loading efficiency (Y_2) were selected as dependent variables. The design parameters are shown in Table 1. The optimal goal is to optimize the independent variables (A, B, C), minimize the particle size (Y1) and increase the drug loading efficiency (Y_2) [32, 33].

Table 1: Levels of independent and dependent variables in experimental design by CCD method

Factor	Name	Units	Minimum	Maximum
X1	Polymer	mg/ml	4	10
X2	Lipid	mg/ml	2	6
X3	Pva	%	2	3
Response	Name	Units		
Y1	Size	nm		
Y2	EE	%		

Preparation of nanoparticles

The single-step nanoprecipitation method is used to prepare the LPHNPs formulation [26, 34 and 35], according to the reported values of the experimental design, which are 20 experiments. In this method, PLGA polymer and hydrophobic drug are dissolved in 1 ml of organic solvent such as chloroform or acetonitrile, which are suitable solvents for dissolving PLGA polymer, and an organic phase is formed. Then, the desired amounts of lipid is dissolved in 9 ml of aqueous phase to prepare the liposomal part of the formulation. The aqueous phase in this study included polyvinyl alcohol (PVA) surfactant. Once the aqueous phase is prepared, the resulting solution is placed on a magnetic stirrer heater at a temperature of 65 °C until the solution reaches a temperature of 65 °C while it is stirred for one hour to be dissolved completely. After the aqueous solution reaches the reaction temperature (65 °C), the organic solution containing the polymer and the drug is added to the aqueous phase drop by drop (under continuous stirring with a homogenizer at a speed of 10000 rpm). Then the resulting solution, which has a milky color, becomes vortex for 3 minutes. Finally, it is placed on a magnetic stirrer at room temperature for two hours to remove the existing organic solvent from the resulting solution. The resulting nanosuspension is then centrifuged using a 12 kDa Amicon filter and repeated three times to ensure that any organic solvent is removed from the suspension. The isolated particles have nanometer dimensions, which are suspended in deionized water to investigate their size and shape. The resulting solution is then placed in an ice bath and placed in a 60 Hz ultrasonic device for 4 minutes (five times, each time resting for 1 minute) to obtain more homogeneous nanoparticles. The same procedure is executed for the blank sample, which has no drug, except that the one removed from the organic phase.

LPHNPs specifications

Determination of nanoparticle size, particle size distribution index and zeta potential

Particle size, size distribution and zeta potential of particles are measured using a dynamic light scattering device (Zeta Sizer) (Nano ZS3600, Malvern Instrument Ltd, UK). The samples are placed in the analysis cell. The experiment is performed at ambient temperature with a detection angle of 90 °. Each experiment is repeated three times and the response of each experiment (nanoparticle size) is measured. The mean value of each of the measurement characteristics is calculated and their standard deviation is also calculated.

Nanoparticle morphology

Particle morphology is studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) Hitachi, Japan H9500 (JAPE) model. To prepare the sample for SEM,

after dilution using distilled water, a drop of the prepared LPHNPs is placed on a glass slide to dry and finally covered with a layer of gold. Also, the prepared sample is sampled with the device sampler, and injected into the device to check the morphology using TEM. Then, the morphology of the nanoparticles is evaluated by generating high-energy electron beams to pass them through the desired sample.

Determining the drug encapsulation efficiency in nanoparticles

Encapsulation Efficiency (EE) is the rate of weight of a drug encapsulated in a carrier system to the total added drug [36]. For this purpose, 1 ml of LPHNPs containing the drug and its blank formulation are centrifuged at 15000 rpm for 4 minutes at 4 °C. This is repeated three times, each time removing the clear supernatant from the bottom plate of the container and replacing it with deionized water. After centrifugation, the resulting clear supernatant containing the non-encapsulated drug in the nanocarrier is isolated and the optical density of this phase of the formulation is measured by spectrophotometer (UV-160IPC, Shimadzu, Japan) at 284 nm wavelength using a standard curve. The formula presented below has been used to determine % EE [37, 38]. To obtain the standard curve, different concentrations of Etoposide are prepared by serial dilution method and the light density is measured at 284 nm.

$$EE(\%) = \frac{(Actual\ amount\ of\ drug\ encapsulated\ in\ NPs)}{(Initial\ amount\ of\ drug\ used\ in\ NPs)} \times 100 \qquad (1)$$

Measurement of drug delivery rate (in vitro)

Dialysis bag technique is used to measure the delivery from LPHNPs containing Etoposide. Two ml of the Etoposide LPHNPs formulation, its control and the free drug are poured into three separate dialysis bags and the bags are immersed in a flask containing 25 ml of Phosphate-buffered saline (PBS, pH = 7.4). Each flask is then placed on a magnetic stirrer heater with a speed (100 rpm) for 80 h at 37 °C. At predetermined intervals, 2 ml of the phosphate buffer in the flask is removed and replaced with fresh phosphate buffer. Finally, the amount of Etoposide released in the phosphate buffer is measured by spectrophotometry at 284 nm wavelength and the Etoposide release rate is obtained using a standard curve. Drug release data are placed in different mathematical models and the drug delivery mechanism is determined using regression analysis.

In vitro cytotoxicity studies

Toxicity of LPHNPs containing Etoposide and standard drug is measured by MTT on A-549 and Calu6 cell lines. Cells were seeded in 96-well plates with a density of 1×10^4 cells in RPMI-1640 culture medium (containing 10% fetal bovine serum (FBS) and 1% penicillin / streptomycin antibiotic) with 5% CO_2 at 37 °C. They were incubated for 24 h at 37 °C and 5% CO_2. On removal of the supernatant, the cells were cultured with different concentrations of free drug and HLPNPs containing drug. Cytotoxicity was assessed after 24 and 48 of incubation. Absorption was measured at 570 nm by Elisa reader (BioTek Instrument, USA). The half maximal inhibitory concentration (Ic50) was determined by Equation (2) [12, 39]. In order to calculate the cell viability, Equation (3) was used as follows [40]:

$$Y = Ax + b;\ IC50 = (0.5-b)/a \qquad (2)$$

$$Cell\ Viability\ (\%) = \frac{\text{mean of absorbance value of the treatment group}}{\text{mean of absorbance value of the control group}} \times 100 \qquad (3)$$

Investigation of drug release kinetics

Different kinetic models including zero-order model (Equation 4), first-order model (Equation 5), Higuchi model (Equation 6) and Korsmeyer-Peppas model ((Equation 7) were used to model and determine the drug delivery mechanism from the optimal formulation of Etoposide-containing LPHNPs. These kinetic models were analyzed using Microsoft Office Excel (version 2010) to obtain the best model for in vitro release. In these equations, M_t / M_∞ represents the release fraction up to time t, K is the constant release rate and n is the delivery mode [41, 42].

$$M_t/M_\infty=K_{0t} \quad (4)$$

$$M_t/M_\infty=1-\exp(-K_{1t}) \quad (5)$$

$$M_t/M_\infty=K_H\sqrt{t} \quad (6)$$

$$M_t/M_\infty=K_{tn} \quad (7)$$

Statistical analysis

The results were expressed as significance ± SD (SD, n = 3). Statistical analysis was done using one-way analysis of variance (ANOVA), followed by Dunnett post hoc test using SPSS version 22.0. The significant statistical coefficient was determined at $p < .05$.

Result and Dissection

Analysis and optimization of Central Composite Design (CCD design)

Response Surface Methodology (RSM) is one of the statistical methods in process optimization that the target is affected by a number of variables. In RSM, the dependent variable (Y) and the independent variables are expressed by the following quadratic polynomial equation.

In Equation (8), the variable k represents the number of independent variables and Xi and Xj represent the independent variables. R_0 constant coefficient and R_i, R_{ii} and R_{ij} represent linear, square and interaction coefficients, respectively [43, 44].

$$Y = R_0 + \sum_{i=1}^{K} R_i X_i + \sum_{i=1}^{k} R_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{i \propto j}^{k} R_{ij} X_i X_j + \varepsilon \quad (8)$$

Statistical analysis based on RSM was used to predict the most appropriate model to describe the response surfaces (nanoparticle size and encapsulation efficiency). Each experiment was repeated 3 times and each time the response surfaces were determined, the results of which are shown in Table 2. The results of the experimental design showed that the designed system was affected in terms of lipid, polymer and polyvinyl alcohol, which led to high drug encapsulation efficiency and small nanoparticle size. Based on laboratory data, a quadratic polynomial model was proposed to relate independent factors and responses to predict optimal conditions. The quadratic equation for the particle size of LPHNPs (R_1) and the percentage of encapsulation efficiency (R_2) is as follows (Equations 9 and 10):

$$R1= -82.36184 + 8.84129\ A+ 115.83314\ B+ 195.60227\ C- 9.79167\ AB - 9.50000\ AC - 12.25000\ BC + 2.55152\ A^2 -0.63409\ B^2 - 19.54545\ C^2 \quad (9)$$

$$R2= -56.11667 + 5.31351A - 6.71269\ B+ 86.05227\ C+2.14167\ AB +4.90000\ AC - 1.05000\ BC - 1.35960\ A^2 – 1.19659\ B^2 - 21.74545\ C^2 \quad (10)$$

Table 2: Experimental runs, independent variables and measures responses of CCD experimental designs of the formulated HLPNPs

Run	A	B	C	% PS (nm)	% EE
1	0	0	0	75.5 ± 3.1	81.3 ± 1.9
2	1	1	1	61.2 ± 1.5	91.1 ± 1.2
3	0	0	0	76.9 ± 3.8	81.7 ± 2.4
4	0	0	0	77.1 ± 4.2	82.1 ± 1.9
5	2	0	0	65.1 ± 1.3	91.2 ± 2.5
6	0	0	0	76.2 ± 4.7	81.2 ± 1.8
7	-1	-1	-1	77.3 ± 4.3	71.1 ± 1.2
8	0	-2	0	62.6 ± 1.4	87.1 ± 1.1
9	-2	0	0	114.6 ± 5.4	48.7 ± 2.2
10	1	-1	1	62.1 ± 1.2	91.3 ± 1.1
11	1	1	-1	68.1 ± 1.8	83.3 ± 1.3
12	0	0	2	70.5 ± 3.2	79.4 ± 1.6
13	0	0	-2	75.2 ± 4.9	74.1 ± 2.3
14	0	2	0	85.5 ± 4.5	67.7 ± 2.8
15	1	-1	-1	69.1 ± 1.7	82.2 ± 2.6
16	-1	-1	1	78.3 ± 5.4	72.6 ± 2.5
17	-1	1	-1	107.6 ± 5.2	59.1 ± 1.8
18	0	0	0	72.5 ± 3.6	83.2 ± 1.9
19	-1	1	1	100.5 ± 3.7	59.8 ± 2.6
20	0	0	0	76.1 ± 5.5	83.1 ± 2.8

Data represent mean ± SD of 3 determination. Independent variable; A : Concentration of Lipid, B : Concentration of polymer, C : % PVA.
Dependent variable; %EE : entrapment efficiency, PS: particle size.

The coefficient and sign of each of the factors show the extent and manner of their impact. The positive sign of the factors indicates that as this value increases, the desired response also increases [42]. The results of analysis of the regression model variance for the responses are shown in Tables 3 and 4. Given the table of analysis of variance, the validity of the proposed model and the significance of the coefficients can be understood. The F value of the model in Tables, 137.21 for the R_1 and the value 122.33 for R_2 indicate the significance of the proposed model. Also, the value Prob>F is less than 0.0001, which shows the model terms are indispensable. In this case A, B, C, AB, AC, BC, A^2, B^2 and C^2 are indispensable. The P value of the model, which is equal to 0.0001, indicates that the model has an appropriate level of confidence. The P value for the Lack of Fit variables is 0.3983 and 0.0532 for the response surfaces R_1 and R_2, respectively, indicating that the model has good fit with the laboratory data. The quality of the fit is also determined by the R^2 term, where0.9920 and 0.9910 are calculated for R_1 and R_2, respectively. Adeq Precision also measures the signal-to-noise ratio. A ratio greater than 4 is accepted. In this study, for the response surfaces related to the size and percentage of encapsulation efficiency, it is equal to 42.775 and 40.749, respectively, which shows a suitable signal.

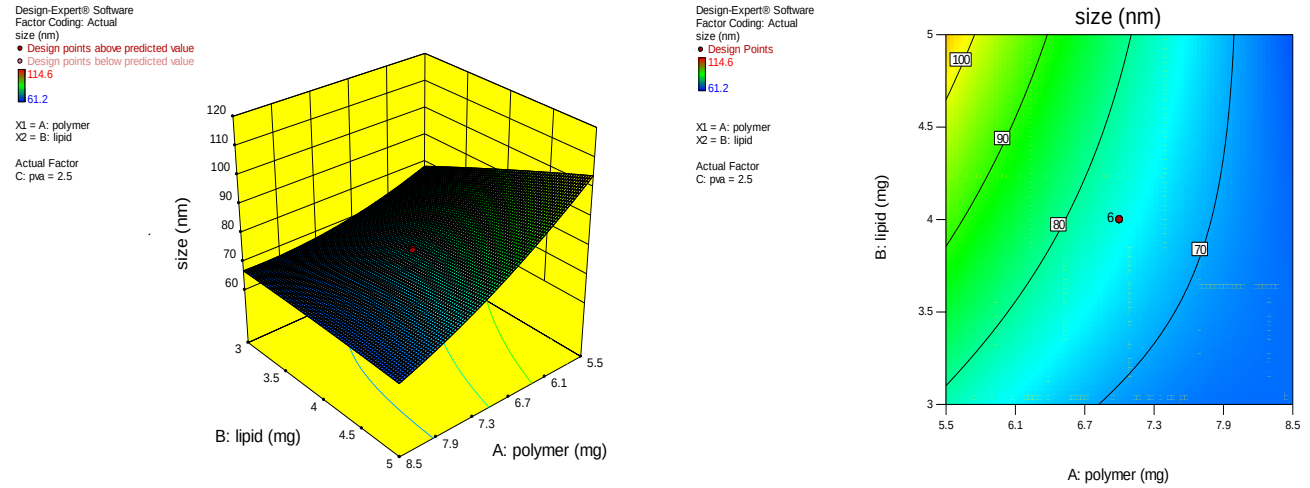
Table 3: Analysis of variance related to particle size response surface (R_1)

Source	Sum of Squares	df	Mean Squares	F Value	p-value Prob> F	
Model	3968.62	9	440.96	137.21	< 0.0001	Significant
C.V. %	1.50					
R-Squred	0.9920					
Adj.R-Squred	0.9847					
R-Squred	0.9920					
Adeq Precision	42.775					
Polymer(A)	2555.30	1	2555.30	795.13	< 0.0001	
Lipid(B)	580.81	1	580.81	180.73	< 0.0001	
PVA (C)	54.02	1	54.02	16.81	0.0021	
AB	369.92	1	369.92	115.11	< 0.0001	
AC	7.61	1	7.61	2.37	0.1550	
BC	8.00	1	8.00	2.49	0.1457	
A^2	312.01	1	312.01	97.01	< 0.0001	
B^2	4.59	1	4.59	1.43	0.2596	
C^2	13.30	1	13.30	4.14	0.0693	
Residual	32.14	10	3.21			
Lack of Fit	18.01	5	3.60	1.27	0.3983	not significant
Pure Error	14.13	5	2.83			
Cor Total	4000.75	19				

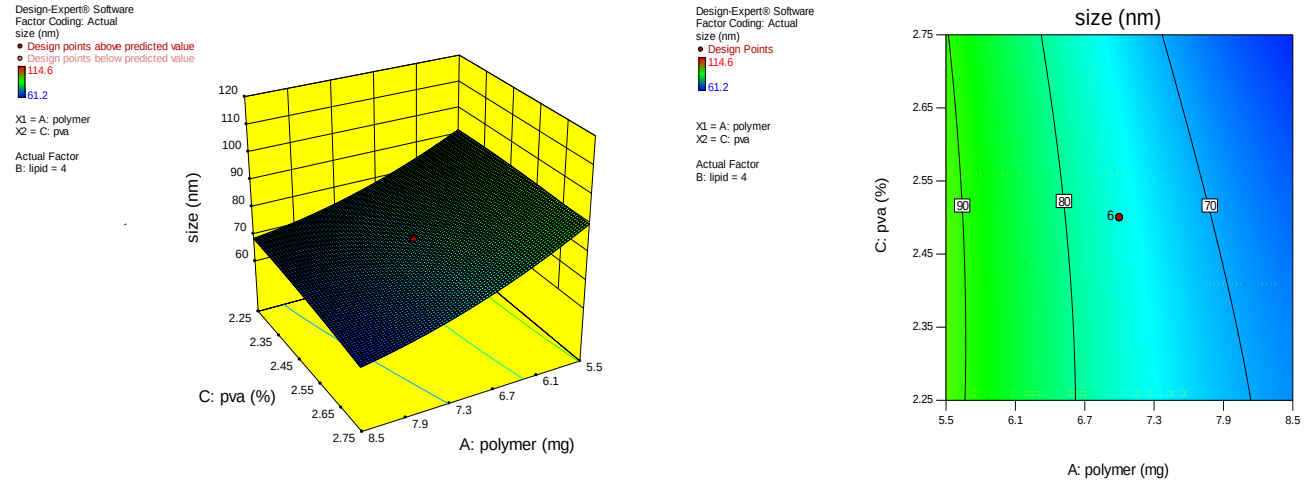
Table 4: Analysis of variance related to response surface of encapsulation efficiency percentage (R_2)

Source	Sum of Squares	df	Mean Squares		
Model	2476.65	9	275.18		
C.V. %	1.93				
R-Squred	0.9910				
Adj.R-Squred	0.9829				
Pred R-Squared	0.9352				
Adeq Precision	40.749				
Polymer(A)	1812.63	1	1812.63		
Lipid(B)	245.71	1	245.71		
PVA (C)	55.13	1	55.13		
AB	82.56	1	82.56		
AC	27.01	1	27.01		
BC	0.55	1	0.55		
A^2	235.29	1	235.29		
B^2	36.00	1	36.00		
C^2	46.44	1	46.44		
Residual	22.49	10	2.25		
Lack of Fit	18.67	5	3.73		
Pure Error	3.82	5	0.76		

The obtained quadratic equation shows that the optimal formulation can have a certain rate of lipids and polymers. Because according to the model, lipids, polymers and %PVA can have all three significant effects on increasing the encapsulation efficiency and reducing the particle size (Figure 1 and 2).



(a)



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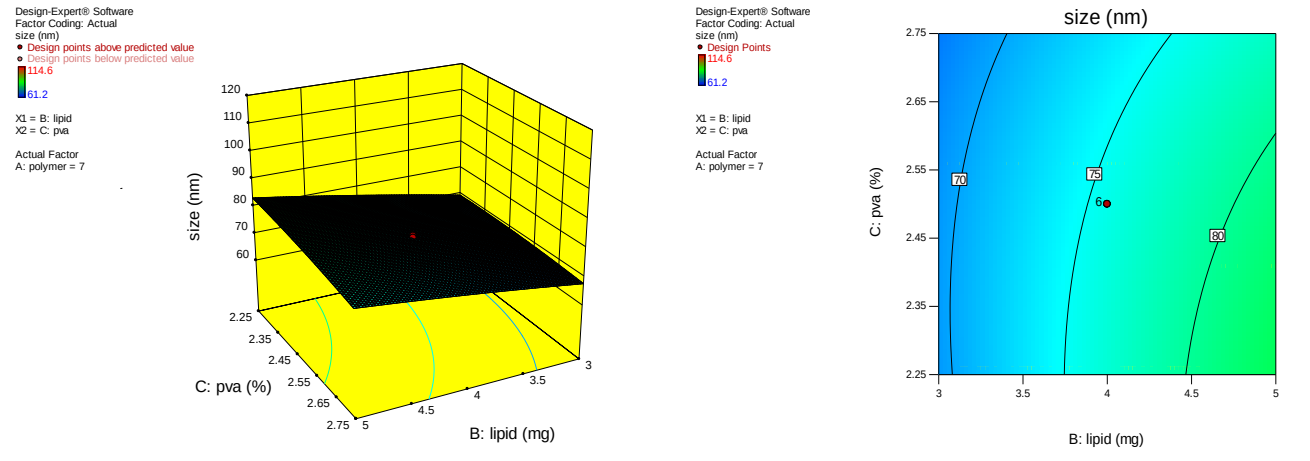
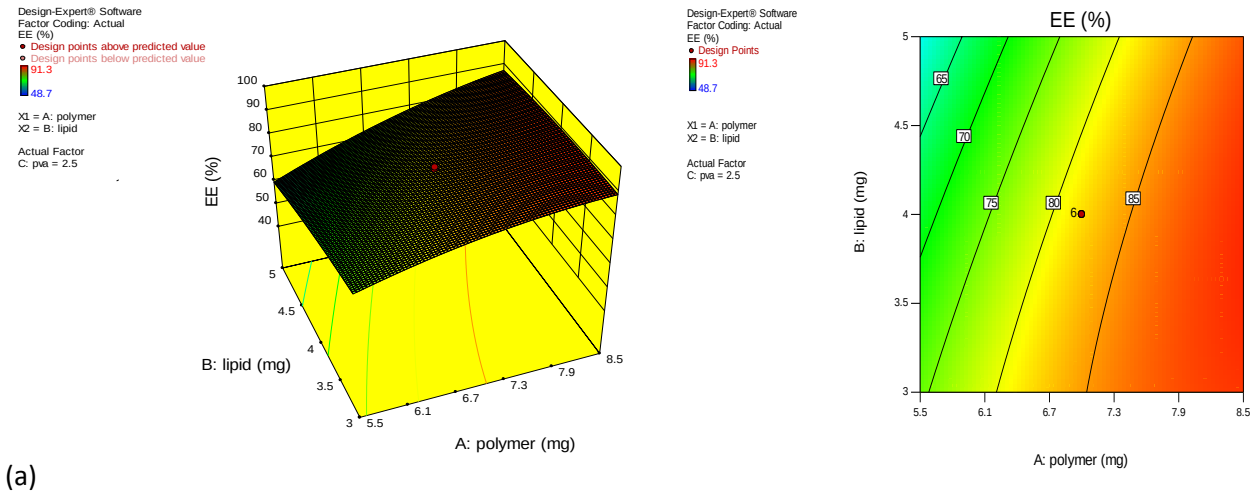
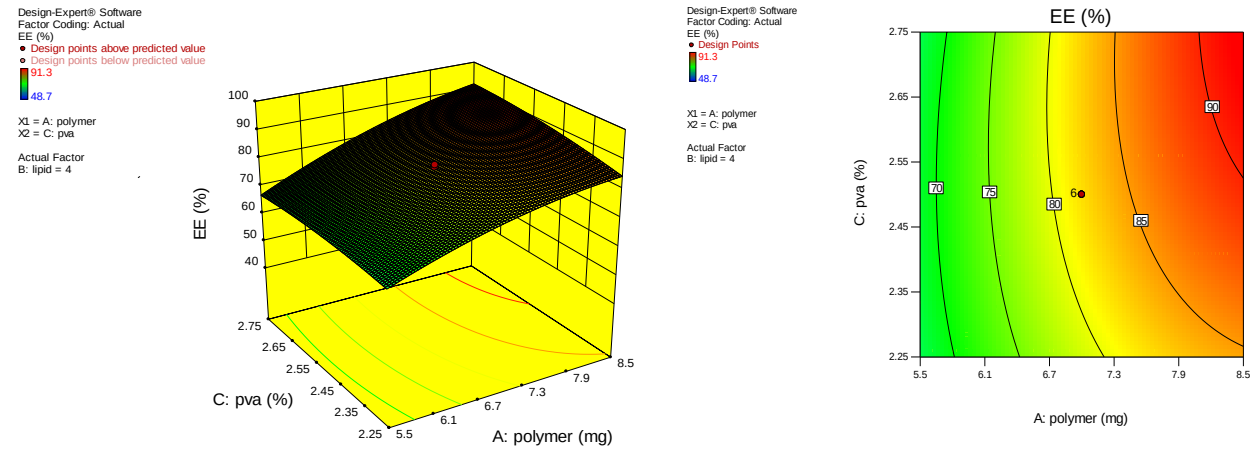


(c)

Figure 1: The way binary and ternary interactions affect the factors influencing particle size



(a)



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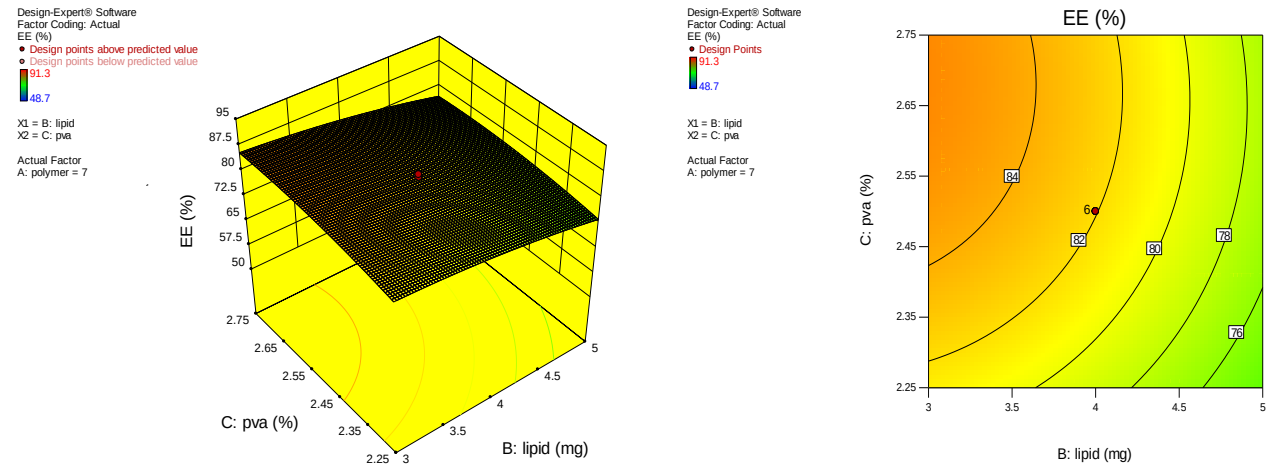


(c)

Figure 2: The way binary and ternary interactions affect the factors influencing encapsulation efficiency

Figure 1(a) shows the contour and 3D diagrams of the polymer and lipid content factors used in the fixed drug amount. As it is known, increasing polymer and decreasing lipid can reduce the

particle size and bring closer to the ideal point. Figure 1(b) shows the factors for the amount of polymer, the percentage of surfactant used in the amount of the fixed drug. Clearly, the increase of polymer as well as the increase of %PVA surfactant can reduce the particle size and get closer to the ideal point. Figure 1(c) shows a diagram of the interactions between the lipid and surfactant used in the fixed drug content. As it is known, smaller particle sizes can be achieved in average contents of lipid and increasing the percentage of polyvinyl alcohol used as a surfactant.

Figure 2(a) shows contour and 3D diagrams related to polymer, lipid content factors, Figure 2(b) is related to polymer content factors, surfactant percentage and Figure 2(c) shows the interaction of lipid consumption interactions and surfactant used in the fixed drug content. As it is known, increasing polymer and decreasing lipid can increase the percentage of drug loading efficiency and bring it closer to the ideal point, also increasing the rate of polymer. Also increasing the percentage of PVA surfactant can improve the percentage of drug loading efficiency and get it closer to the ideal point. Due to high concentration of polymer, it creates more space to encapsulate the drug and produces a relatively compact matrix, and with moderate amounts of lipids and increasing the percentage of polyvinyl alcohol used as a surfactant, the percentage of drug loading efficiency can be higher.

The optimal number of factors affecting the particle size, i.e. the ratio of the polymer, lipid and PVA surfactant contents used by the model, is estimated (Figure 3). Based on the optimal values predicted by the software and the actual values performed in the laboratory after three repetitions, the results are reported in Table 5.

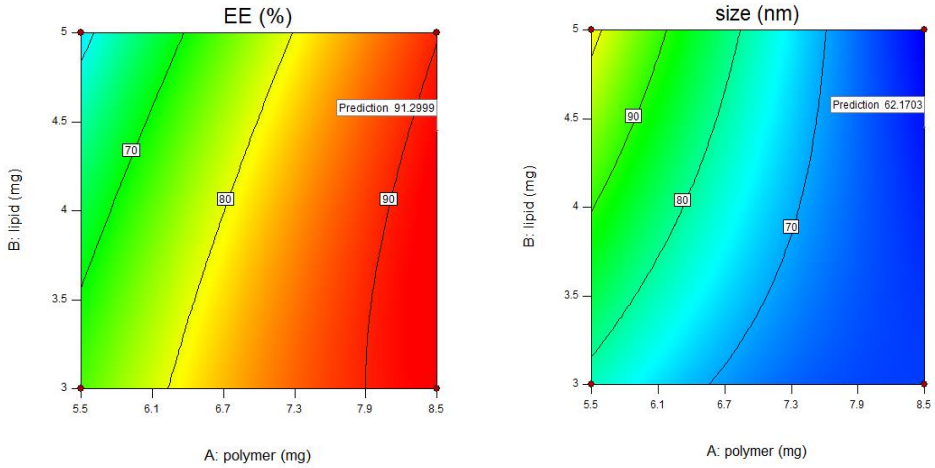


Figure 3: Values optimized by the proposed model

Table 5: Reported results of particle size and drug loading efficiency based on optimization value and actual values obtained from experiments

	Polymer (mg)	Lipid (mg)	%PVA	Particle Size(nm)	%EE
Predcited formulation	8.49	4.44	2.75	62.17	91.29
Actual Optimized formulation	8.5	4.5	2.75	61.51±2.1	90.51±3.2

Determination of nanoparticle specifications

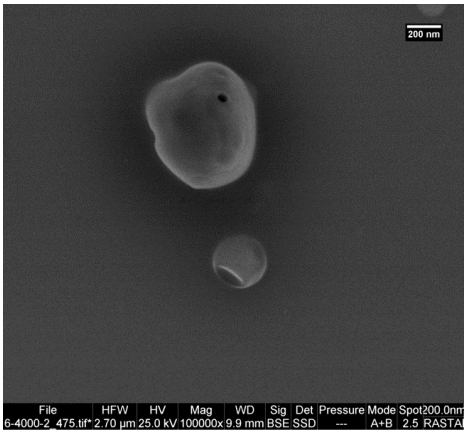
Particle size analysis and distribution (HLPNPs)

After examining the response surfaces by data analysis of variance, numerical optimization was performed by applying the desired constraints and specifications for the independent variables, followed by the desired response surfaces with the values predicted by the software. Then, the formulation of composite nanoparticles with the predicted values was prepared and the size of nanoparticles, particle distribution and zeta potential of LPHNPs related to the optimal point

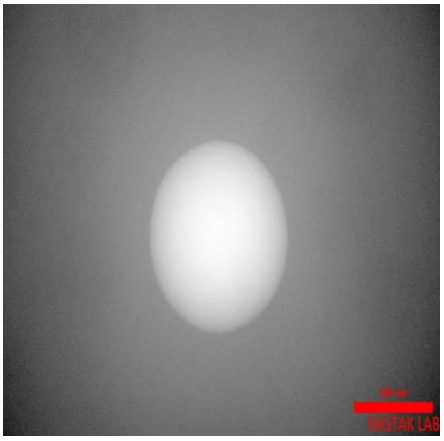
obtained from the model estimate were 61.51 ± 2.10 nm, 0.38 ± 0.14 and -29.6. 0.43 mv and blank values were 59.1 ± 1.8 nm, 0.28 ± 0.12 and -23.6 ± 0.28 mv, respectively.

Morphology of optimal formulation

The size of LPHNPs is an important factor for their diffusion into cells and tissues. Smaller nanoparticles are generally absorbed more slowly by the reticuloendothelial system (RES); therefore, they have longer circulation times, slower delivery time, and longer half-lives. The morphology of the nanoparticles by SEM and TEM is shown in Figure 4. The images showed that the nanoparticles had a smooth surface and a monodisperse and integrated pattern, and the vesicles were spherical.



(a)



(b)

Figure 4: Morphology of LPHNPs by (a): SEM image and (b): TEM image

Estimation of the efficiency of the encapsulated drug

EE is defined as the ratio of the drug in NPs to the total amount of drug added to the formulation. The encapsulation percentage was calculated based on the standard drug formulation curve. Based on Equation 1, the percentage of EE was equal to 90.51 ± 3.2.

In vitro study of delivered drug

Dialysis was performed to determine the delivery behavior of in vitro LPHNPs containing Etoposide and free Etoposide to investigate the effect of LPHNPs on drug delivery. Etoposide delivery from LPHNPs was measured in phosphate buffered saline (pH = 7.4) at various time intervals over 80 h (Figure 5). The results showed that 14% of the drug in the formulation of LPHNPs was delivered in phosphate buffer within 80 hr. The delivery of Etoposide was examined using spectrophotometry and the results showed that the delivery of Etoposide LPHNPs was slower than that of free Etoposide at the same time (80 hr).

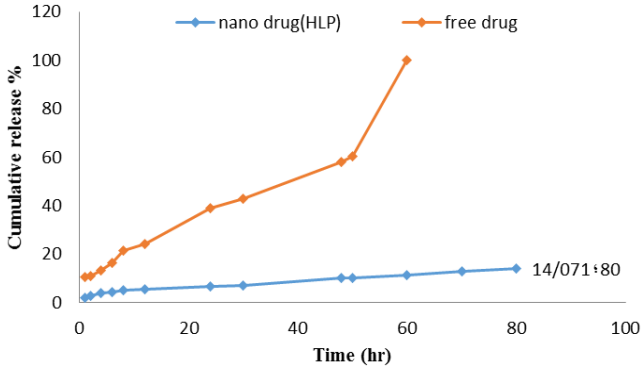


Figure 5: In vitro delivery profile of free Etoposide formulation and LPHNPs containing Etoposide in phosphate buffered saline (pH 7.4,) at 37 °C (n = 3) and specified time

In vitro toxicity and cell stability of Etoposide-containing hybrid nanoparticles

Different concentrations of drug-containing NP were tested by MTT, and all the experiments were repeated three times to increase the accuracy of the results. In vitro toxicity activity and capability of HLPNPs and free drug were evaluated using MTT test on two cell lines A-549 and Calu6. The values of Ic50 of Etoposide containing LPHNPs and free Etoposide for the cells are given in Figure 6. The test results in Figure 7 for each cell line show that the drug and the drug loaded in the hybrid carrier showed a dose-dependent toxicity factor in the cell lines. However, the blank nanoparticles have no effect on the cell and show similar results as untreated cells ($p>0.5$) because NPs were selected at a safe concentration with a non-toxic effect on the cells. The toxicity of LPHNPs can be attributed to the toxic effect of Etoposide. Toxicity increased with increasing concentration, indicating that drug concentration plays an important role in the in vitro toxicity of Etoposide. These results indicate that the toxicity of the drug loaded on nanoparticles is significantly more on cancer cells ($p<0.05$) than that of free drugs and compared with the hybrid carrier, better results were obtained from the nanocarrier effect. All the data are presented as significant ± SD (n = 3).

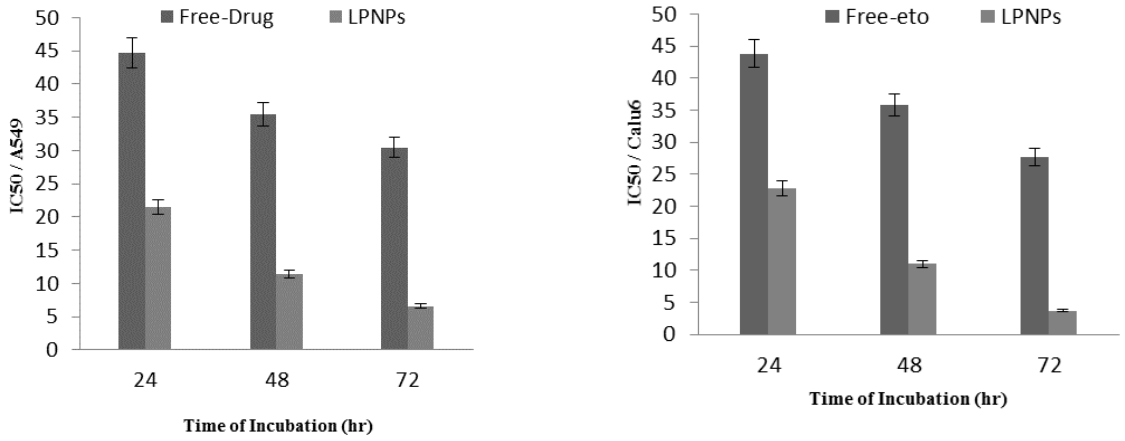


Figure 6: IC50 value in terms of (mg / ml) Etoposide- containing LPHNPs and free Etoposide in cell lines A-549 and Calu6 during two incubation times at 37 °C under 5% CO_2

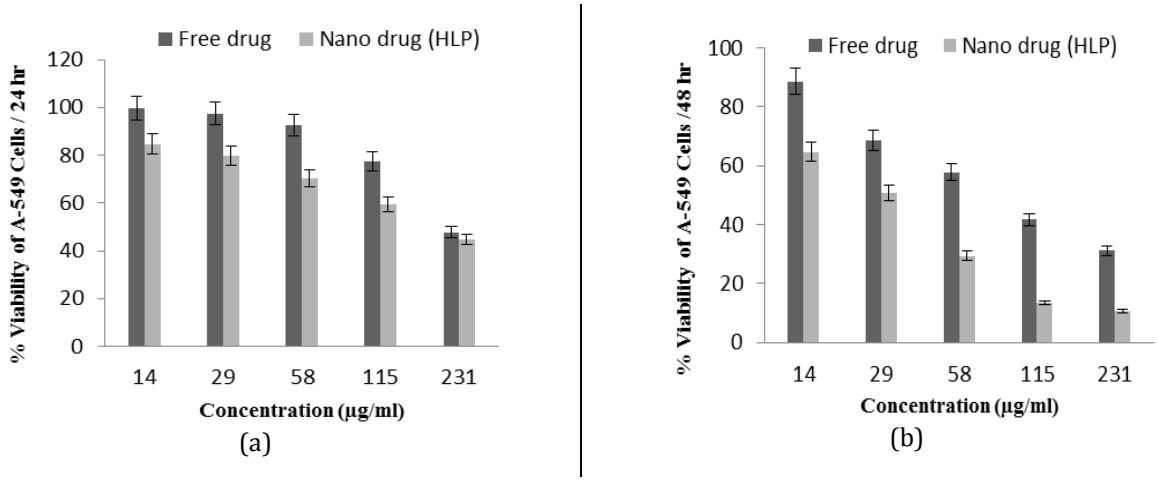


Figure 7: Cell Viability of lipid-polymer hybrid nanocarrier containing Etoposide and free drug in Calu6 and A-549 cell lines in two incubation times at 37 °C under 5% CO_2. a) After 24 hr incubation b) After 48 hr incubation.

Investigation of Drug release kinetics in hybrid nanoparticles

Different kinetic models (zero order, first order, Hiuchi and Korsmeyer-Peppas) were used to best describe the mechanism of drug delivery from nanoparticles. The values of correlation coefficient (R^2) for each of the kinetic models are given in Table 6 to select the most appropriate model to describe the drug delivery mechanism. The results show that the delivery of Etoposide from LPHNPs follows the Higuchi equation and shows a controlled intruded delivery. On the other hand, by matching the laboratory data in the Peppas equation, a constant n is obtained, which predicts the mechanism of drug delivery from the nanoparticles. (If $n < 0.5$, we will have a Fick diffusion and if $n \leq 10 \geq 0.5$, we will have non-Fick's diffusion) [45]. According to the value reported for the constant n, which is the slope of the Korsmeyer-Peppas diagram in Table 6, it can be seen that the mechanism of drug delivery from the nanoparticle system follows Fick's law [46].

Table 6: Calculated parameters to investigate delivery kinetics

Type of Model	slop	Intersept	R^2
Zero Order	0.1388	3.1124	0.95
First Order	0.0201	1.2544	0.83
Higuchi	1.4232	0.7148	0.99
Korsmeyer-peppas	0.4294	0.6905	0.99

Lung cancer is a disease characterized by uncontrolled cell growth in lung tissues [47]. If left untreated, cell growth can spread beyond the lungs in a process called metastasis into nearby tissues or other organs [48]. On the other hand, because lung cancer is latent in the first five years and the person realizes the progression of the disease and infection after a large part of the cells become involved in the cancer, it is vital to look for possible treatment strategies to increase performance of chemotherapy drugs, especially Etoposide, the anti-cancer drug, to treat damaged tissues.

In the present study, we managed to produce hybrid nanoparticles that have a core shell structure where lipid shell surrounds a polymer core. The single-step nanoprecipitation method is a fast and reproducible method for the preparation of LLPHNPs [49], which has been used in this study. In this method, in order to form the organic phase, the polymer and the drug are dissolved in an organic solvent such as chloroform. The organic phase is then added dropwise to the aqueous phase containing lipid and finally an emulsion is formed. The main basis of this method is the precipitation of the polymer from a lipophilic solution which is a combination of polar solvent and water. Specifically, the used PLGA polymer precipitates as a hydrophobic core to encapsulate low water-soluble drugs [50, 51].

Design Expert 10 was used to evaluate the factors affecting the encapsulation efficiency (%EE) and particle size in lipid polymer hybrid nanocarriers based on different amounts of PLGA polymer, lipid and PVA surfactant according to the CCD method. 20 experiments including 14 experiments designed by CCD method plus 6 central points were performed at this stage. Contour and 3D diagrams of the polymer and lipid content factors used in the amount of fixed drug are shown in Figures 1 and 2, as it is clear that the increase in polymer and the average amount of lipid can reduce the particle size and push it close to the ideal point [42]. On the other hand, increasing the polymer as well as increasing the percentage of PVA surfactant can reduce the particle size. This decrease in particle size can be explained by the fact that at high concentrations of surfactant, the molecules of this substance tend to mass and have a sufficient amount to cover the composite nanoparticles, so the surfactant activity increases and has a

significant effect on the size of nanoparticles [52]. Also, in relation to the drug encapsulation efficiency, increasing the polymer and moderate amounts of lipids can increase the efficiency percentage. Still, increasing the polymer, and increasing the percentage of PVA surfactant can improve the drug encapsulation efficiency and approach the ideal point. Due to the high concentration of polymer, it creates more space to encapsulate the drug and produces a relatively compact matrix [53]. This structure has particles in the range of 50 to 200 nm, and the smaller the particle size, the easier it is to diffuse into cancer tissues and cells. In this study, the particle distribution size, zeta potential of LPHNPs related to the optimal point obtained from the model design estimate were 61.51 ± 2.1 nm, 0.38 ± 0.14 and -29.6 ± 0.43 mv, respectively, and the blank values were 59.1 ± 1.8 nm, 0.28 ± 0.12 and -23.6 ± 0.28 mv, respectively. It can be concluded that smaller hybrid structures offer better particles. The negative zeta potential creates a large repulsive force among the nanoparticles, prevents the accumulation of nanoparticles and makes them stable [12, 54].

SEM and TEM results showed that the prepared nanoparticles had a smooth, uniform surface and a monodisperse integrated pattern and the vesicles were spherical, indicating slow drug delivery. However, our results were somewhat consistent with similar studies in this field [42]. In these studies, it was found that the structure of LPHNPs had a uniform spherical structure and have a suitable morphology [37, 55].

Drug delivery from nanoparticles is an influential factor in diagnosing the biological effects of the carriers used. The images related to the delivery rate of the free drug in comparison with the drug loaded in the LPHNPs are shown in Figure 7 for a period of 80 hr to evaluate the delivery. It was observed that in comparison with the delivery rate of the free drug, the drug delivery rate was much slower than HLPNPs. Therefore, the results showed that HLPNPs had the ability to encapsulate drugs [36]. The results of drug delivery in hybrid nanoparticles showed that within 80 hr, 14% of the drug encapsulated in the hybrid nanocarrier and 100% free drug were delivered in the same time period. The study of delivery experiments showed that the delivery process consisted of two different phases, a fast and a slow delivery. These results were also reported in other studies [17, 56]. In the early hours of the assessment, an explosive delivery of the drug was observed and then the delivery rate decreased over time.

Drug encapsulation efficiency in nanoparticles is important. According to the standard curve and spectrophotometric method for hybrid nanoparticles whose efficiency was done by design of experiments and during 20 different experiments, considering the optimized point, EE showed that it had an efficiency of 90.51 ± 3.2. The rate of drug encapsulation efficiency in this nano-carrier indicates its high encapsulation power, which are approved [57]. In this study, experiments were designed and performed to measure the cellular cytotoxicity of Etoposide in standard form and its preparation with lipid-polymer hybrid form of drug by MTT method. The results showed that the drug-free (blank) formulation had no toxic effect on the two cell lines A-549 and Calu6. The IC50 value of the LPHNPs containing drug is lower compared with the free drug, so the survival rate of cancer cells with the hybrid technique is further reduced and indicates the superiority of this nanocarrier over the free drug. Similar results have been reported by previous studies [58], which indicate the effect of prepared nanocarriers on the pathway of cancer cell death and the effect on cytotoxicity.

Drug release kinetics in carrier was found to follow the delivery kinetics in the form of Higuchi. On the other hand, according to Table 6, the Higuchi model had an acceptable value of R^2 compared with other models. The Higuchi model was chosen for these carriers. According to the results, Higuchi model was selected according to the high R^2 rate. In addition, considering that the slope of the Korsmeyer-Peppas diagram showed the same value of n and considering that its value was less than $n < 0.5$, so the delivery mechanism

in these nanocarriers was as Fick and we had a Fick's diffusion.

According to the results obtained in the present study and the study of in vitro drug delivery, the need for further studies of in vivo nanodrugs, which is dependent on the natural system of living organisms, is felt. Researchers are therefore advised to examine the formulation prepared using the present study in vivo and compare the results. In this way, the ability of nanodrugs can be better confirmed compared with the standard drug on lung cancer.

Conclusion

Etoposide was significantly and successfully loaded on LPHNPs using single-step nanoprecipitation technique. The nanoparticles prepared by this technique were evaluated in terms of size, size distribution, zeta potential, encapsulation efficiency and cytotoxicity. The results showed that the synthesized nanoparticles had a high encapsulation efficiency. Because the hydrophobic nature of Etoposide can help the drug to be highly encapsulated, this is strengthened by the presence of lipids in the nanoparticle matrix and reduces drug leakage from the nanoparticle core. Because the environment outside the polymer is liquid, and if the drug is hydrophilic, the probability of the drug leaking out of the core and reducing efficiency would increase. Therefore, the lipid-polymer hybrid structure is a more suitable structure for hydrophobic drugs such as Etoposide. In general, in lipid-polymer hybrid nanocarriers, increasing the amount of polymer, mean amounts of lipid content and increasing the %PVA used in the synthesis of nanocarriers improved particle size and encapsulation efficiency. These nanocarriers also improved the cytotoxic effects and increased the efficiency of nanoparticles on lung cancer cell lines. In fact, the results of this study showed that the hybrid formulation of lipid polymer from Etoposide had significant antitumor activity and could become a promising new formulation for the treatment of lung cancer in humans.

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Conflict of Interest

We have no conflicts of interest to disclose.

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