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Formulation, Optimization And Characterization Of Folate-Conjugated Polymeric Nanoparticles Of Paclitaxel

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ABSTRACT

The rationale of present study is to formulate and characterize folate-conjugated poly(lactide-co-glycolide)-block-poly(ethyleneglycol)(PLGA-PEG-FOL) nanoparticles loaded with Paclitaxel . Folate conjugated nanoparticles can be used as vehicles to assist active targeting of various anti-cancer drugs to target cancer cells. PLGA–PEG–FOL was synthesized as follows: activation of PLGA, conjugation of activated PLGA with PEG-bis-amine, and conjugation of FA to PLGA–PEG.

Characterization of the synthesized conjugate was done by using FTIR and ^{1}HNMR. Drug loaded nanoparticles was prepared by the Double emulsion solvent evaporation(DESE). After the formulation of polymeric nanoparticles, various parameters like particle size, polydispersity index and zeta potential were measured. Morphology was observed using scanning electron microscopy. Flow Cytometry was used to evaluate the uptake of optimized Folate conjugated NPs by the breast cancer cells (MCF7). The results showed that FITC labelled PPF-NPs were internalized into the MCF7 cells effectively.

Keywords: Paclitaxel, PVA, Soluplus®, Anticancer, Nanoparticle, Folic acid

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INTRODUCTION

Recently Folate receptors targeted drug delivery system is emerging as an alternative choice for the treatment and imaging of various types of cancers. Due to its very minute size and capacity of high binding affinity for cell surface folate receptors (FR), folate conjugates can deliver a range of drug molecules to pathologic cells affecting normal tissues.

A variety of nanoparticulate systems are used to deliver Paclitaxel to solid tumors, including dendrimers, liposomes, polymeric nanoparticles, micelles, lipid emulsions, niosomes, and self-emulsifying drug delivery systems (SEDDS). In the field of targeted delivery, polymeric nanoparticles made of PLGA are highly preferred due to the fact that they are biodegradable, compatible and highly effective. In addition to being approved by the FDA, PLGA nanoparticles can also be targeted at tumors by absorbing them through Enhanced Permeation and Retention (EPR) effect in solid tumors, which in turn allows them to easily penetrate through the vascular system through dripping endothelial tissue found on the tumor's periphery [1, 2]

A variety of drug carriers such as nanoparticles [3), liposomes [4], lipid nanoparticles [5], polymeric micelles [6] and] have been successfully linked to Folic acid for targeted delivery of drugs to cancer cells.

MATERIALS AND METHODS

Paclitaxel was received as a gift from Fresenius Kabi Oncology Limited. Polymers PLGA 85:15 was purchased from Sigma Aldrich and Soluplus® was received as gift by BASF (USA). Poly vinyl alcohol (cold water soluble) was obtained from Himedia Laboratory Pvt. Ltd., Mumbai. All other chemicals used in the study were of the analytical reagent grade.

Drug Excipients Compatibility study:

Compatibility of Paclitaxel with other major excipients used in preparations of nanoparticles was done by using FTIR (Alpha, Bruker, Ettlingen, Germany and DSC (Perkin, DSC 4000). FTIR spectra and DSC thermograms obtained after study were analysed for any possible interaction.

Preparation of Paclitaxel loaded PLGA-PEG-FOL(PPF) nanoparticles:

A new way of preparing paclitaxel loaded PPF nanoparticles has been devised by using the DESE method (Double Emulsion Solvent Evaporation) as previously reported [7, 8] with some modifications. Different ratios of PVA (Polyvinyl Alcohol) and Soluplus® were used to prepare nanoparticles. For the primary emulsion, PVA (2.0%w/v) and Soluplus® (0.25%w/v) were used

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as stabilisers, and for the secondary emulsion, PVA (1.0 %w/v) and Soluplus® (0.03 %w/v) were used as stabilisers. In formulations F2 and F5 mixtures of PVA and Soluplus® were used as stabilisers. In the preparation of F2 and F5, 0.25 % w/v PVA was used as a primary emulsifier and 1.5 % w/v PVA was used as a secondary emulsifier.

By dissolving PPF and drug in 2ml dichloromethane, 1ml 2.5 %w/v PVA was added dropwise, followed by homogenization at 18,000 rpm to create a double emulsion to produce nanoparticles. In order to make the secondary emulsion, 75 ml of PVA at 1.5% (w/v) is added to the primary emulsion and homogenised at 18,000 rpm again. A double emulsion was then prepared by placing the emulsion in a sonicator for 45 minutes and stirring it gently overnight for the purpose of evaporating the organic solvent and solidifying the nanoparticles. Using centrifugation at 5,000 rpm for 5 minutes, we separated the larger nanoparticles from the supernatant, which was collected and centrifuged again at 15,000 rpm for 30 minutes to obtain nanoparticles of desired sizes. In order to remove free drug from the surface of the nanoparticles, the nanoparticles were re-suspended with distilled water and centrifuged to remove excess stabilisers (PVA and Soluplus®). The washing process was repeated twice. A deep freezer was then used for storage of the separated nanoparticles at -40°C and eventually lyophilization was carried out to preserve them. Detail composition of six formulations of PPF nanoparticles as in Table 1.

Table 1: Composition of Paclitaxel loaded PLGA-PEG-FOL(PPF) nanoparticles

Formulation code	Paclitaxel (mg)	PPF (mg)	Polyvinyl alcohol (%w/v)		Soluplus® (%)	
			Primary	Secondary	Primary	Secondary
F1	10	100	2.0	1.0	---	---
F2	10	100	---	1.0	0.25	---
F3	10	100	---	---	0.25	0.03
F4	10	50	2.0	1.0	---	---
F5	10	50	---	1.0	0.25	---
F6	10	50	---	---	0.25	0.03

CHARACTERIZATION OF PACLITAXEL LOADED PPF NANOPARTICLES

Drug Loading and Entrapment Efficiency

A centrifuge tube containing 2mL of acetonitrile and 2 mg of Paclitaxel loaded nanoparticles was used to measure drug loading and entrapment efficiency. Thereafter, it was continuously shaken in an incubator shaker at 37°C for 3–4 hours until it was cooled to room temperature. In order to separate the continuous phase from the dispersed phase, centrifugation was used. A spectrophotometric analysis at 219 nm was performed on the supernatant collected after the

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reaction was completed, and the released drug was quantified [4, 5]. According to the following equations, drug loading and entrapment efficiency percentages were calculated:

$$\text{Actual Drug loading}(\%) = \frac{\text{Amount of drug present in nanoparticles}}{\text{weighed of nanoparticles sample analysed}} \text{x100}$$

$$\text{Entrapment efficiency}(\%) = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \text{x100}$$

Particle Size Analysis and Zeta Potential Measurement (ZP):

The particle size, its distribution, and the zeta potential of Paclitaxel-loaded PPF nanoparticles were analysed using a Malvern NANO ZS90 instrument, which employs a solid-state laser using dynamic light scattering (DLS) to measure particle size, particle distribution, and zeta potential of Paclitaxel-loaded PPF nanoparticles. It has been found that when freeze dried nanoparticles are suspended together in double distilled water by sonication, the average size of the hydrodynamic particles, the size distribution, the polydispersity index, and the zeta potential of the particles can be determined [11, 12].

Scanning electron microscopy (SEM) and Transmission electron microscopy(TEM) for determining surface morphology:

In order to assess the surface morphology of the nanoparticles, the shape and morphology of the prepared nanoparticles were examined by scanning electron microscopy (Hitachi SEM (S-3600N)). The nanoparticle sample was mounted on metal stubs using double-sided adhesive carbon tape that was adhered to the metal and fractured with a razor blade to obtain the appropriate amount of nanoparticles. Using secondary electron emissive SEM under an argon atmosphere, gold was sputter-coated on the samples and morphology was observed under this condition. It was demonstrated that the detailed morphology of nanoparticles could be visualized and depicted using transmission electron microscopy (TEM) JEM CX 100 operating at 200kv with a point-to-point resolution that could visualize and depict the morphology of nanoparticles. Drying the samples on carbon coated grids is followed by staining them negatively with 2% aqueous uranyl acetate solution after they have been dried. PPF nanoparticles are shown to be formed and sized in a similar manner to other nanoparticles through combination of both bright field imaging and multi-mode imaging at varying magnifications [11, 13].

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In Vitro Drug Release Study

In phosphate buffer pH 7.4, drug release studies of the formulated PPF nanoparticles were conducted [14]. In Eppendorf tubes containing 5 mg of freezedried nanoparticles, 2ml of phosphate buffer was added and the tubes were kept at 37°C in an incubator. After shaking the samples at 120 rotations/minute for 0 hours, 1 hours, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, 36 hours and 48 hours, they were centrifuged, and 0.5 ml of supernatant was collected. To maintain the same conditions, 0.5 ml of the withdrawn samples were replaced with fresh phosphate buffer solution. A spectrophotometer at 219 nm was used to determine the release of the drug from the samples.

In Vitro Drug Release Kinetic Study:

In order to understand its pharmacokinetic models, it is necessary to evaluate the mechanism by which drug is released from nanoparticles as well as its corresponding kinetics. Data obtained from the in vitro drug release studies were analyzed using a range of kinetic equations such as zero order, first order, etc., and graphs were compiled based on these equations. In order to determine the value of r2 and k, a regression analysis was performed on the corresponding linear plots [14].

Cellular Uptake of nanoparticles

The cellular uptake of NPs was qualitatively detected by fluorescence microscopy and flow cytometry.

RESULTS

Drug excipient compatibility study

An FTIR analysis was done on paclitaxel, individual major excipients, physical mixtures of the major excipients and paclitaxel, as well as paclitaxel-loaded polymeric nanoparticles to explore the compatibility between the paclitaxel and the excipients. As shown in figure 1, FTIR spectra are presented. A comparison was made among the DSC thermograms obtained for pure drug, physical mixtures of drug polymers, and lyophilized polymeric nanoparticles. This can be seen in Figure 2

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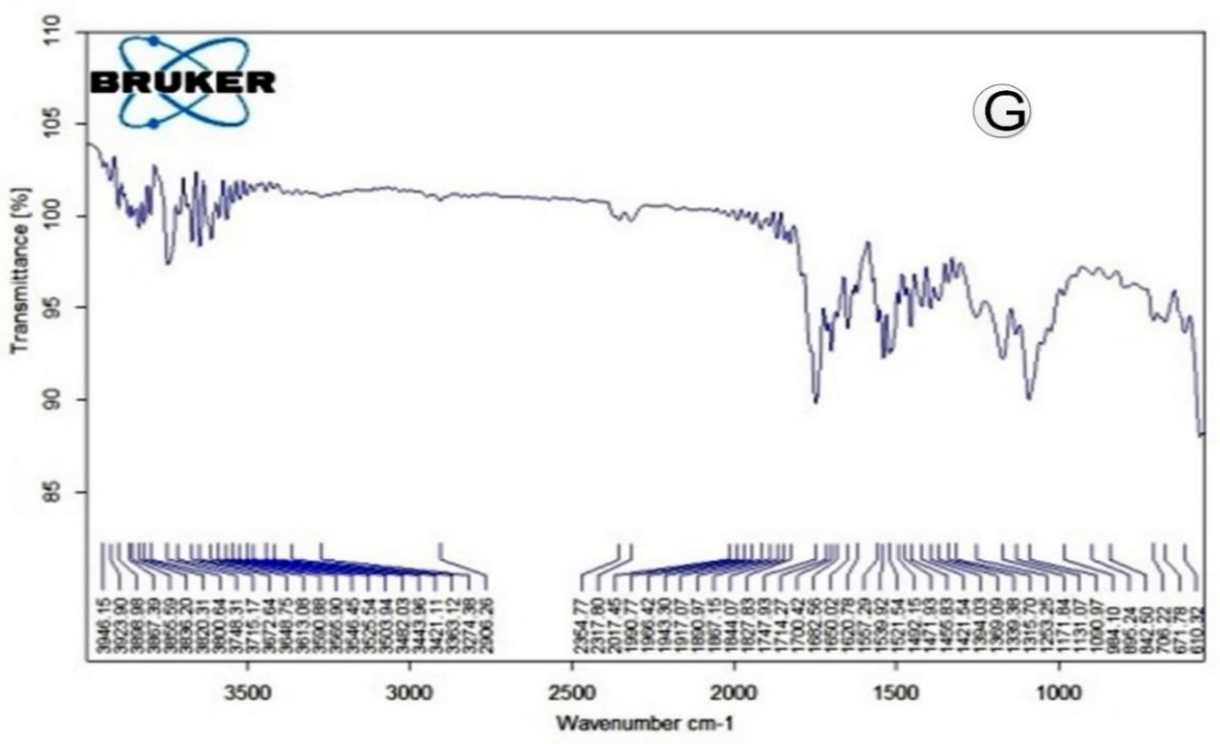

Figure 1. Fourier transform infrared (FTIR) spectra of drug with excipients (Paclitaxel, PLGA, PVA, Soluplus®, FOL)

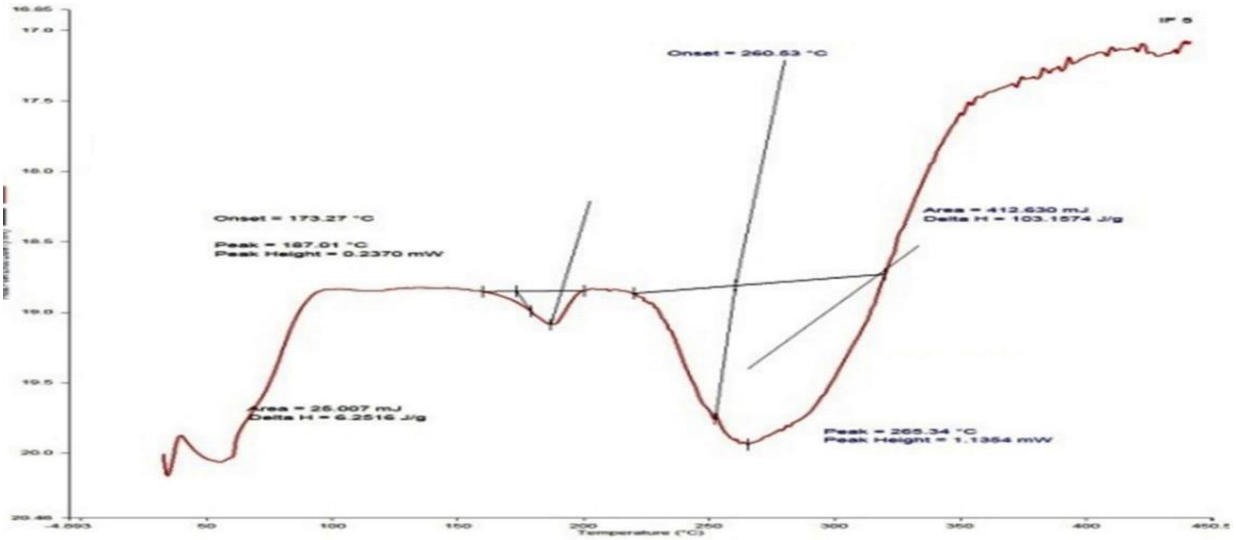

Figure 2. Differential scanning calorimetry thermograms of physical mixture of drug with excipients containing Paclitaxel, PLGA, PVA, FOL

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Preparation of Paclitaxel loaded PPF nanoparticles

Using a double-emulsion solvent evaporation method, polymeric nanoparticles loaded with Paclitaxel were prepared by using the PPF polymer and stabilizing them using different types and ratios of stabilizers, such as PVA, Soluplus®, and mixtures of both PVA and Soluplus®. There were various concentrations of drug polymer ratios that were used in the formulation process, as well as different concentrations of stabilizers that were used in the formulation process. Following the preparation of the formulations, the size, surface, and release characteristics of the formulations were evaluated.

Determination of entrapment efficiency and drug loading

The entrapment efficiency (%) and drug loading (%) of the nanoparticles F1-F6 ranges from 53.20±0.18 % to 66.41±0.14% and 4.13±0.18% to 10.26±0.20% respectively. Results for all the formulations are shown in Table 2.

Table 2: Characteristics of Polymeric nanoparticles

Formulation code	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Drug loading (%)	Entrapment efficiency (%)
				(Mean ± SD) *	
F1	495.8	0.615	-4.09	4.51±0.16	59.63±0.15
F2	401	0.460	-7.18	4.13±0.18	55.55±0.21
F3	395	0.444	-6.65	4.45±0.13	59.45±0.19
F4	328	0.558	-14.4	10.26±0.20	66.41±0.14
F5	430	0.358	-2.07	8.08±0.13	53.20±0.18
F6	313	0.726	-5.81	9.45±0.12	62.16±0.14

*n=3

Determination of nanoparticles size and its surface properties:

As can be seen in Table 2, the particle size distribution of the prepared PPF nanoparticles F1-F6 ranges from 313 nm to 495.8 nm.

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Evaluation of surface morphology of nanoparticles:

As shown in the SEM and TEM images, the nanoparticles were found to be spherical and had smooth surfaces as shown in the Figures 3 to 4 (Figure 3 to 4).

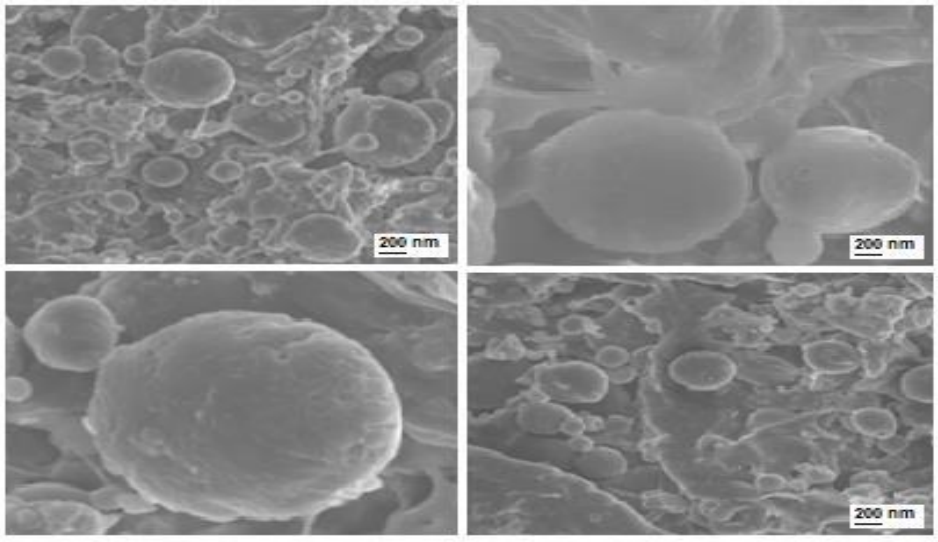

Figure 3. SEM images Paclitaxel loaded Polymeric Nanoparticles (F4)

In vitro drug release and pharmacokinetic modeling of Paclitaxel loaded PPF nanoparticles

This study was conducted in phosphate buffer (pH 7.4) to study the drug release from polymeric nanoparticles loaded with Paclitaxel. As shown in Table 3, the cumulative percentage of drugs released over a period of time has been calculated and presented. Cumulative percentage drugs released verses time profile graph is shown in figure 5.

Table 3. In vitro drug release data from PPF nanoparticles

Time (hours)	**Cumulative percentage drug release (Mean ± SD)***					
	F1`	**F2**	**F3**	**F4**	**F5**	**F6**
0	0	0	0	0	0	0
1	19.90±0.09	19.25±0.15	18.82±0.13	22.28±0.08	17.98±0.13	21.56±0.07
3	43.34±0.06	41.28±0.10	40.27±0.11	47.97±0.13	38.27±0.03	47.04±0.05

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6	45.08±0.10	43.61±0.11	43.91±0.15	53.32±0.08	39.56±0.12	50.85±0.09
9	49.73±0.09	47.02±0.08	48.33±0.13	57.61±0.10	42.46±0.14	53.76±0.06
12	53.39±0.16	50.16±0.07	50.96±0.07	59.76±0.11	45.53±0.16	56.32±0.11
24	58.56±0.11	53.20±0.09	55.41±0.17	64.75±0.06	48.74±0.18	60.45±0.09
36	64.66±0.08	57.35±0.13	60.44±0.10	70.50±0.15	52.44±0.17	65.87±0.05
48	68.94±0.10	61.05±0.10	64.10±0.12	75.10±0.11	55.55±0.18	72.88±0.08

*n=3

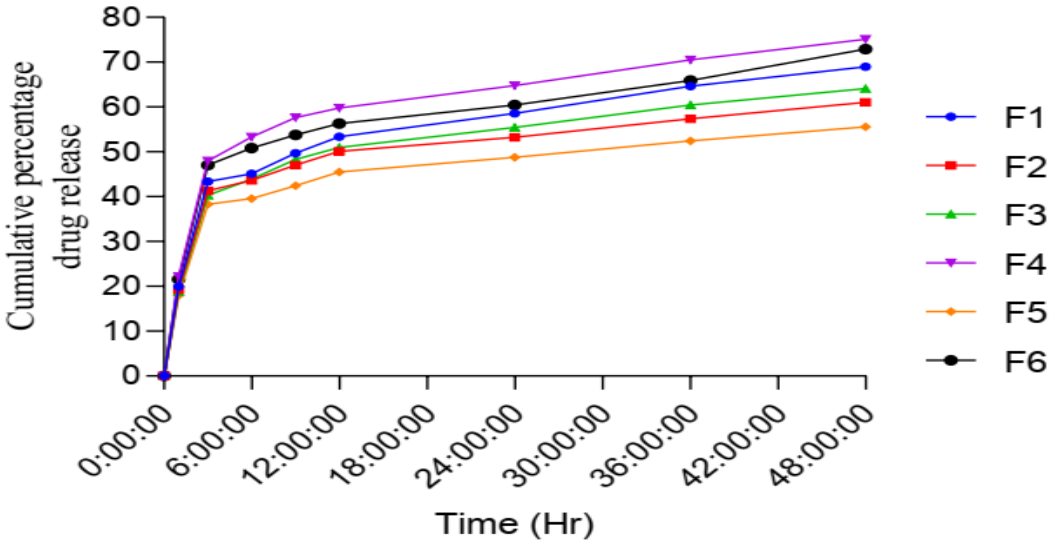

Figure 4. Graph depicting the cumulative percent drug released vs time

DISCUSSION

Drug excipient compatibility study

According to the interpretation of the FTIR spectra of individual drug spectra and excipients spectra as well as their physical mixture and final formulation, there were no chemical interactions between the drug and excipients, as all the important drug peaks were observed in the mixture of drug and excipients as well as polymeric nanoparticles containing Paclitaxel. The DSC thermograms for Paclitaxel and PPF show a broad endotherm at 220.6°C for Paclitaxel and 51.38°C for PPF. The presence of a drug peak at the same temperature in the DSC thermogram of both the physical mixture and the drug-loaded formulation indicates that the drug is compatible with the polymer. The present formulations did not result in any interactions that

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could have altered the individuality of the drug, and therefore, the current components are compatible with each other inthe current formulations as a finished product.

Preparation of Paclitaxel loaded PPF nanoparticles

In order to formulate paclitaxel loaded polymeric nanoparticles with optimum size, zeta potential, polydispersity index, entrapment efficiency, and in vitro drug release characteristics, six formulations of PPF nanoparticles with PVA and Soluplus® as stabilizers were formulated by double emulsion solvent evaporation in order to obtain the desired size, zeta potential, polydispersity index, and entrapment efficiency. Compared to other formulations, nanoparticles formulated using the PVA stabilizer (F4) showed a higher level of drug loading and entrapment efficiency when compared to other formulations.

Determination of entrapment efficiency and drug loading

The percentage entrapment efficiency of all the formulation was varied in the range from 53.20±0.18% to 66.41±0.14% and percentage drug loading was found in the range from 4.13±0.18% to 10.26±0.20%. A significant effect of drug polymer ratio and stabilizer concentration on entrapment efficiency and drug loading was observed from the values of entrapment efficiency and drug loading. It was found that the drug loading and entrapment efficiency was greater for 10:50 ratio (drug: PPF) than for 10:100 ratio, and nanoparticles prepared with PVA as stabilizer demonstrated greater drug entrapment efficiency than those prepared with Soluplus®. According to this finding, the amount of polymer incorporated in the formulation does not directly relate to the loading and entrapment ratio of the drug. There are a number of factors that affect this process, including the optimum ratio of drug and polymer to be used, along with the stabilizer and homogenization speed, among others.

Determination of nanoparticles size and its surface properties

The results of the particle size data revealed that the drug polymer ratio and the concentration of surfactant have a significant impact on the particle size of3561 nanoparticles. As a result, the average diameter of all the drug-loaded formulations (F1 to F6) varied from 323.5 nm to 497.3 nm on average. It is also suggested that PVA might be an effective stabilizer for the production of nanoparticles with the desired size, based on these results. An analysis of particle size also showed that as the polymer concentration increased, the particle size of the drug-polymer formulation increased, as in the case of formulation F1-F3(10:100) drug-polymer ratio in comparison to formulation F4-F6 (10:50) drug polymer ratio. In earlier studies, it has been hypothesized that an increase in polymer concentration in the organic phase could result in a decrease in shear stress during homogenization because the viscosity of the polymer increases

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with increasing polymer concentration. Therefore, as the viscosity of the organic phase decreases, the dispersion of the organic phase with the aqueous phase also decreases, leading to an increase in the particle size [13]. It was found that the polydispersity index of the formulation containing the drug varied between 0.358 and 0.728 on average. It was evident from the PDI value of the nanoparticles that the nanoparticles had a homogeneous distribution during the formation process. It has been determined that the surface charge of Paclitaxel loaded PPF nanoparticles can be determined by zeta potential (ZP) analysis. As can be seen from the zeta potentials of the nanoparticles F1 to F6, the zeta potentials ranged from -2.07 to -14.1. A zeta potential range of -30 mV to +30 mV indicates that those nanoparticles do not aggregate rapidly to form a cluster and retain their original size for a considerably longer period of time [9].

Evaluation of surface morphology of nanoparticles

Upon examination of the SEM images of the nanoparticles loaded with Paclitaxel, it becomes evident that the particles were of submicron size and that they were distributed homogeneously throughout the particles. TEM images indicate that the drug has been distributed in particulate form throughout the body of the nanoparticles, which is consistent with the results of the polydispersity index.

Flow Cytometry: Uptake of NPs by the breast cancer cells (MCF7)

To investigate whether Paclitaxel-encapsulated NPs were internalized into the breast cancer cell line MCF7, cellular uptake of FITC labeled PPF-NPs were analyzed by flowcytometry and fluorescent imaging microscopy. The uptake rate of FITC labelled PPF-NPs was significantly ($p < 0.05$) higher than untreated controls (Figure 5a). It is assumed that the adding up of folate agent on the surface of NPs would augment the uptake of NPs by FR positive cells. The Uptake of FITC labeled FITC labeled PPF-NPs were also evaluated by fluorescent imaging microscopy (Figure 5b). The results showed that FITC labelled PPF-NPs were internalized into the MCF7 cells effectively.

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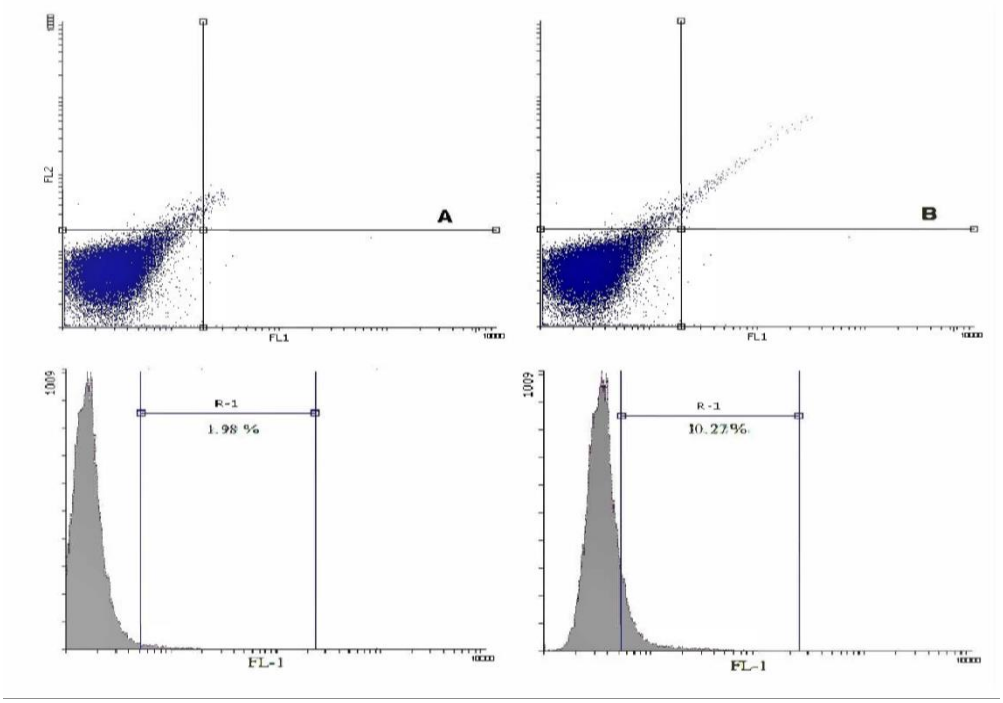

Figure 5a. Results from flow cytometry indicating the uptake of PPF-NPs by the breast cancer cells (MCF7). A = Control cells and B = PPF-NPs treated cells

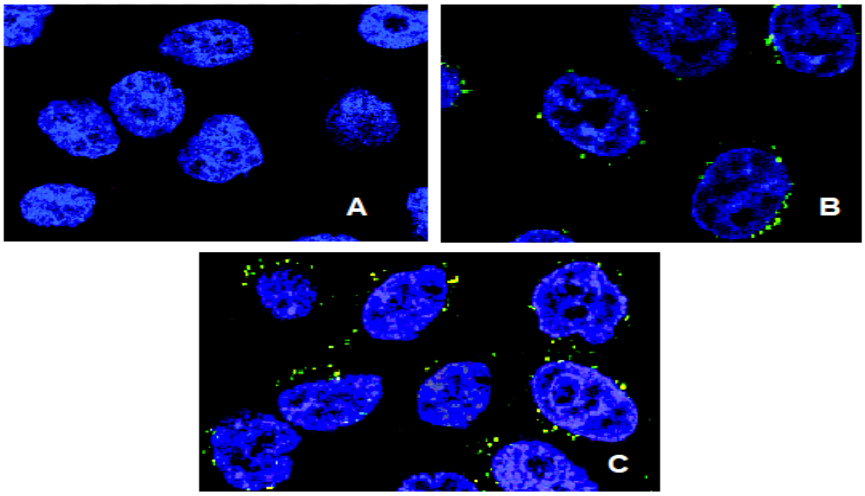

Figure 5b. Results of fluorescent imaging microscopy showing the internalization PPF-NPs into the MCF7 cells. A = Control cells and B & C= PPF-NPs treated cells

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CONCLUSION

In the present study we aimed to use folic acid as the targeting agent conjugated to Polymeric nanoparticle for targeting folate receptor binding sites on the surface of cancer cells. Paclitaxel loaded PPF nanoparticles were prepared by double emulsion-solvent evaporation method using PVA, Soluplus®, and mixtures of PVA and Soluplus® as stabilizers and characterized for their physiochemical properties. Following the characterization, formulation (F4) was selected as the best formulation on the basis of its characterization. Furthermore, the selected formulation was further characterized for their morphological properties using transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

PLGA–PEG–FOL (PPF) Conjugated Polymeric nanoparticles were observed to be spherical in TEM and SEM images. It was found that at the end of 48 hours, the cumulative amount of drug released from lyophilized paclitaxel loaded polymeric nanoparticles, in formulation F4, was 75.10±0.11 %, which was higher than that released from other formulations. On the basis of in-vitro drug release study the formulation F4 was selected as the best and optimum nanoformulation. Hence it can be concluded that Paclitaxel loaded PPF nanoparticles may act as an effective and promising anticancer drug delivery system. Flow Cytometry results showed that FITC labelled PPF-NPs were internalized into the MCF7 cells effectively.

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