

Development of 5-FU Loaded Poly Lactic-Co-Glycolic Acid Nanoparticles for Treatment of Lung Cancer

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Abstract

Non-Small Cell Lung Cancer (NSCLC) accounts for about 84% of all lung cancer types diagnosed so far. Every year, regardless of gender, the NSCLC targets many communities worldwide. 5-Fluorouracil (5-FU) is a uracil-analog anticancer compound. This drug tends to annihilate multiple tumour cells. But 5-FU's most significant obstacle is that it gets very easily metabolized in the blood, which eventually leads to lower anticancer activity. Therefore, a perfect drug delivery system is needed to overcome all the associated challenges.

In this experiment, an attempt was made to prepare 5-FU loaded poly lactic-co-glycolic acid nanoparticles using solvent evaporation method and subsequently observed the effect of molecular weight of poly lactic-co-glycolic acid, loading of poly lactic-co-glycolic acid, sonication period on the cytotoxic effect of 10 % w/w 5-FU loaded PLGA nanoparticles against human A549 Isogenic cell line.

In this experiment, two points are more evident: first, poly lactic-co-glycolic acid has a major impact on 5-FU release due to higher degradation and rate of diffusion in nanoparticle solution; and second, nanoparticles with a larger surface area and smaller particle size have a lower half-maximal inhibitory concentration (IC₅₀) value. The IC₅₀ of all nanoparticles was significantly higher (p=0.0145) than that of the free 5-FU controlled group (8.34Nm). The cytotoxicity would be greater if the IC₅₀ value was lower. Nanoparticles with an 18-minute sonication time was found to be more cytotoxic than those with PLGA nanoparticles containing 12% polyvinyl alcohol.

In this experiment 10% w/w 5-FU loaded poly lactic-co-glycolic acid nanoparticles was prepared for laboratory research to translational research for the treatment of lung cancer.

Keywords: Non-small cell lung cancer (NSCLC), 5-FU, PLGA, Polymeric nanoparticles, A549 Isogenic cell line, IC₅₀ value

Introduction

Lung cancer has the highest mortality rate of all cancer types worldwide⁽¹⁻³⁾. Lung cancer is a term used to describe both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC)⁽⁴⁾. There are almost 87% of these two NSCLC events. It has a very poor prognosis, the greatest problem for NSCLC; only 15% of patients live up to 5 years with a greater chance of recurrence⁽⁵⁾. Chemotherapy and radiotherapy, which also have severe complications, i.e., poor selectivity, resistance, tissue toxicity, myelosuppression, etc., are currently available for any lung cancer treatment⁽⁶⁾. The normal iv administration of chemotherapeutic medicaments demands a high dose with the consequence of savior toxicity; the chemotherapeutic drugs eventually kills cancerous cells as well as adjacent healthy cells⁽⁷⁾. There are many benefits of encapsulating chemotherapeutic drugs in polymeric nanoparsecs, such as enhancing drug solubility, targeted drug delivery, shielding the drug from bloodstream degradation, low side effects, increasing the duration of drug exposure, and slowing the clearance time from the body⁽⁸⁾. Many

pharmaceutical grades or Food and Drug Administration (USFDA) certified polymers are available on the market that could provide the best drug delivery efficiency, i.e. polycaprolactone (PCL) poly lactic-co-glycolic acid (PLGA)⁽⁹⁾.

The toxicity of chemotherapeutic drugs may have been decreased by these polymers when encapsulated inside. In addition, proper engineering of these polymers may result in polymeric nanoparticles that may accumulate on the surface of the tumor's body⁽¹⁰⁾. The targeting of tumour cells may be carried out through active or passive targeting⁽¹¹⁾.

The tinny size of the nanoparticles helps them to accumulate in the leaky tumour vasculature environment so that in the presence of a poorly designed lymphatic drainage system they could easily penetrate within the tumour⁽¹²⁾. The reticuloendothelial system (RES) can easily destroy poorly engineered hydrophobic polymeric nanoparticles or particles that have a particle size below 200 nm, or else they can quickly take over by the liver, spleen, and lungs. This may sometimes contribute to the development of palmar-plantar erythema⁽¹³⁾.

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On the other hand, the bloodstream of those polymeric nanoparticles that have a hydrophilic surface showed an increased circulation time. Hydrophilic polymeric nanoparticles are therefore a possible candidate for targeting cancer cells⁽¹⁴⁾. However, nanoparticles must design with particle sizes below 200nm to avoid macrophagic uptake. In preclinical research, docetaxel, a BCS-IV medication, has been shown to have significant similarities compared to traditional formulations with rapid administration time⁽¹⁵⁾. There are many studies suggesting cell lines like, A549 can be targeted with PLGA nanoparticles' help when the PLGA surface was engineered with LFC131 peptide and doxorubicin⁽¹⁶⁾.

As far as NSCLC cells are concerned, it was often found that they produced resistance to chemotherapy and radiotherapy from the stem cells of the said cancer cells; hence, the recurrence rate of NSCLC is more⁽¹⁷⁾.

In lung cancer treatment, 5-fluorouracil (5-FU), a chemotherapeutic hydrophilic agent, is commonly used. 5-FU is an inhibitor of thymidylate synthesis that inhibits excess DNA proliferation⁽¹⁸⁾. It has, however, a low biological half-life and insufficient biodistribution, which derives its possible therapeutic significance form⁽¹⁹⁾. Usually, this drug is administered using iv route, but due to the inability to act on the target site, this cytotoxic drug produces a lack of site-specificity and adverse effects^(10, 20).

In the body, 5-FU can sometimes produce reactive oxygen species (ROS). The activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), which prevents ROS and ROS-induced cytotoxicity, will counterbalance the accumulation of drug-induced ROS⁽²¹⁾. But the main concern with 5-FU is that the, 5-FU is metabolized to fluorodeoxyridine monophosphate in the liver or intestinal mucosa by dihydrouracil dehydrogenase gets into the blood very easily. A drug delivery system that could safeguard 5-FU from bloodstream metabolism and target NSCLC would therefore be a successful translation research system⁽¹⁸⁾.

While preparing a stable nanoparticle, this study will examine the various forms of PLGA and certain process variables. In addition, the *in-vitro* cytotoxicity of the prepared formula against human A549 Isogenic lung cancer cell line (ATCC EML4-ALK fusion, Pune, India) will also be tested⁽²²⁾.

Material and Methods

Materials

Poly (Lactide-co-Glycolide) (PLGA) copolymers of different grades with molecular weight ratios, i.e., EXPANSORB®(lactide:glycolide75:25) Mw(100 kDa), EXPANSORB® (lactide:glycolide 50:50) Mw (50 kDa), EXPANSORB® (lactide:glycolide 50:50) Mw (20 kDa) was purchased from Sigma-Aldrich (Sigma Aldrich–Merck, Bengaluru, India),

5-FU was the gift sample form Neon Laboratories, Mumbai, India. Tween 80, methanol (HPLC grade), water (HPLC grade), polyvinyl alcohol (PVA) different molecular weight, sucrose and phosphate buffer saline (PBS pH 7.4), were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL), Mumbai, India

Preparation of 10% w/w 5-FU-Loaded PLGA nanoparticles

The solvent evaporation technique was used to make 10 w/w 5-FU loaded PLGA nanoparticles (This is equivalent to mixing 10 mg of FU with 90 mg of PLGA).

In this experiment, total of twelve formulations have been prepared. Among these, three were prepared by considering three different PLGA, i.e., (1) EXPANSORB® (lactide: glycolide 75:25) DLG 75-9E; which has molecular weight 100 kDa and end terminal ester group. (2)EXPANSORB® (lactide: glycolide 50:50) DLG 50-5A; which has a molecular weight of 50 kDa and end terminal -COOH group. (3) EXPANSORB® (lactide: glycolide 50:50) DLG 50-2A; which has a molecular weight of 20 kDa and end terminal -COOH group. Further, altering PVA loading (4%, 8% & 12%), sonication time (6 min, 12 min & 18 min) and PVA molecular weight (31 kDa, 47 kDa & 130 kDa) more nine formulations has been prepared considering PLGA (lactide:glycolide 50:50); DLG 50-2A as a constant class of PLGA. The 50 mg of 5-FU and 450 mg of PLGA were dissolved into 30 mL of methanol for this preparation⁽²³⁾. Transfer 120 mg of Polyvinyl alcohol(PVA) to another 50 mL beaker and dissolve it at 45°C in 30 mL of water. Thermodynamic stability of such isotropic system was improved by using PVA. In addition, the PLGA-5-FU solution was applied dropwise using an 18G needle to the PVA aqueous solution and kept for 60 minutes for stirring⁽²⁴⁾. The resulting solution was sonicated for 6 minutes at 60% power, then left overnight at 10,000 rpm for 480 minutes, with the supernatant removed. The pellets were resuspended in 30 mL distilled water and centrifuged three times for 30 minutes, with the supernatant removed after each centrifugation. By decantation, supernatant was removed. Finally, washed nanoparticles were suspended with 2.5 % d-Trehalose solution in 20mL and refrigerated using cryogenic freezer (Model no: MR-HF-IR-A3A10, Lab Freez Instruments, Chaina) for 24 hours at -85°C and further freeze-dried for 72 hours. Until necessary, the dried nanoparticles are stored in refrigeration conditions. Table 1 shown the prepared 5-FU-PLGA nanoparticles formulas.

Table 1. The formulation variables and sonication times of the 10% w/w 5-FU loaded poly lactic-co-glycolic acid nanoparticles. The shaded areas indicate the parameter that was varied for that particular group of formulations.

Variables	Formulations											
	PLGA			PVA Loading (%)			Sonication time (min)			PVA MW (KDa)		
	EXPANSORB® (lactide:glycolide 75:25) Mw(100 kDa)	EXPANSORB® (lactide:glycolide 50:50) Mw (50 kDa)	EXPANSORB® (lactide:glycolide 50:50) Mw (20 kDa)	4 % PVA	8 % PVA	12 % PVA	6 min	12 min	18 min	31 kDa	47 kDa	130 kDa
PLGA class	DLG 75-9E	DLG 50-5A	DLG 50-2A	DLG 50-2A	DLG 50-2A	DLG 50-2A	DLG 50-2A	DLG 50-2A	DLG 50-2A	DLG 50-2A	DLG 50-2A	DLG 50-2A
PVA loading (%)	5	5	5	4	8	12	5	5	5	5	5	5
Sonication time (min)	10	10	10	10	10	10	6	12	18	10	10	10
PVA Molecular weight (KDa)	75	75	75	75	75	75	75	75	75	31	47	130

Morphology and size of the 10% w/w 5-FU loaded PLGA nanoparticles

Using the JSM-IT800 Field Emission Scanning Electron Microscope, scanning electron microscopic images were taken of 10% w/w 5-FU loaded PLGA nanoparticles⁽²⁵⁾. During the operation process, a pinch amount of nanoparticles was added to the carbon tab and further coated with gold. The particle size and zeta potential were measured by the DelsaNano C instrument (Beckman Coulter, U.S.A.). During the determination of the particle size, 200 μ L of nanoparticles were suspended in 4mL of deionized water and further sonicated for 5 min to remove suspended air bubbles. The measurement was taken at 25°C.

The encapsulation efficiency of the 10 % w/w 5-FU-loaded PLGA nanoparticles

To evaluate the encapsulation efficacy of polymeric nanoparticles, 5 mg of lyophilized nanoparticles were dissolved in dichloromethane from each sample⁽²⁶⁾. The resulting mixture was kept overnight for drying at room temperature. Later, the residue is dissolved in methanol and filtered with a 0.22 μ l membrane filter (Millipore). The filtered samples were further analysed using the RP-HPLC process⁽²⁷⁾

In-vitro release of 10% w/w 5-FU loaded PLGA nanoparticles

The 5mg of lyophilized nanoparticles from each batch (n=5) were placed in the dialysis bag consisting of 1.5% of tween 80^(28, 29). The temperature was kept at 37°C throughout the experiment, and magnetic stirring at 50 rpm was used to keep the dissolution medium uniform. Subsequently, the bag was put in a beaker containing 40 mL of 1% w/v polysorbate 80 solutions. Each time a 400 μ L sample was extracted from the beaker at a fixed time interval and a fresh 400 μ L sample, 1% w/v polysorbate 80 was placed to preserve the sink condition. The experiment went on for fourteen days. The resulting liquid samples were further analysed using the RP-HPLC process.

Determinations of the serum half-life of 5-FU in the 10% w/w 5-FU loaded PLGA nanoparticles

The 5mg of 10% w/w 5-FU loaded PLGA nanoparticle formulations was drained in 500 mL of human serum albumin (HSA), and the mixture was gently shaken at 37°C and 13000 rpm for fifteen minutes to determine the half-life of the 5-FU⁽³⁰⁾. The supernatant was extracted and the specified particles were washed three times with 1mL of distilled water. In addition, the particles were dissolved in dichloromethane and left overnight. The resulting residue mixture was further combined with methanol and filtered using a 0.22 μ m membrane filter. The filtered samples were also analysed using the RP-HPLC process^(31, 32).

Estimation of 5-FU loaded PLGA nanoparticles stability by RP-HPLC method

5-FU 5 mg of each 10% w/w 5-FU loaded PLGA nanoparticle formulation (n = 5) was considered for the sample preparation. The HPLC analysis was performed on 1260 Infinity II Prime LC System (Agilent, CA) with Acclaim™ 120 C18 Reversed-Phase Analytical HPLC Columns, 3 μ m (Thermo Fisher Scientific, USA). The mobile phase comprises methanol:water (75:25%). The flow rate was 1.5 mL/min, where else, UV detection was performed at 266nm with an injection volume of 10 μ L. After necessary modifications to the Anitha, A., et al. procedure, the samples were prepared⁽³³⁾.

The cytotoxicity of the 10% w/w 5-FU-loaded PLGA nanoparticles against human A549 Isogenic cell line

For *in-vitro* cytotoxicity analysis, Human A549 Isogenic cell line was cultured for 48 hr.(5000/well in 96 well flat-bottomed microtiter plates). The cultured cells were exposed with different concentrations, i.e., 0.5nM, 1nM, 5nM, 15nM, 20nM, 25nM 50nM, 75nM, 100nM, 125nM, 150nM of 15% w/w 5-FU loaded PLGA nanoparticles and further 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium 236 Bromide (MTT) assay was performed⁽³⁴⁾. The native 5-FU was considered as the control group, and the experiment was repeated thrice, and further, IC₅₀ was calculated.

Statistical analysis

Statistical analysis was performed using ANOVA (GraphPad Prism version 8.0). Post-hoc comparisons of the mean were performed using Tukey's Honestly significance test. A significance level of p<0.05 was accepted to denote significance in all cases.

Results and Discussion

Surface morphology, particle size, and zeta potential of the 10% w/w 5-FU loaded PLGA nanoparticle

The surface morphology of the prepared 10% w/w 5-FU loaded PLGA nanoparticles were analyzed using SEM (Figure. 1).

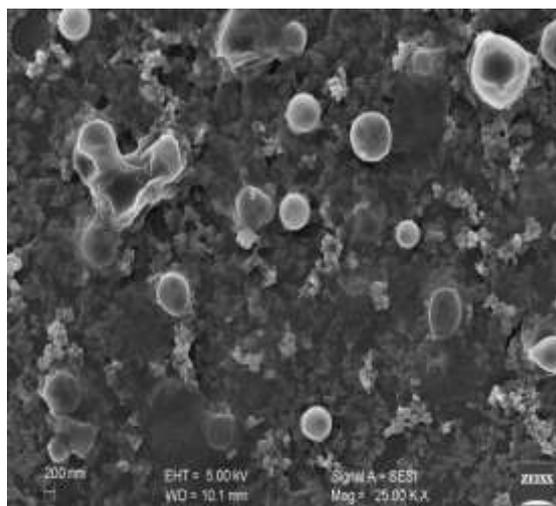


Figure.1. SEM image of 10% w/w 5-FU loaded PLGA nanoparticles

It was confirmed from the SEM image that the prepared nanoparticles are spherical in shape with a specific smooth surface area without any particular pitting and no characteristic agglomeration or mushroom effects have been observed. The smooth surface of the nanoparticles suggests that; there is little risk that the 5-FU drug leached out of the encapsulated nanoparticles; in other words, there was no such characteristic proof that the drug particles adhered to the outer surface of the nanoparticles. During the process of preparation, the untrapped 5-FU was washed out. If free 5-FU remains on the nanoparticles' surface, the initial burst effects may be impaired and the encapsulation efficacy subsequently slowed down. Pitting in nanoparticles, on the other hand, could promote the nanoparticles' low durability.

In addition, PLGA would become more soluble at altered pH conditions due to pitting, and thus could increase the release of the drug with improved degradation. Possible drug release at the target site will then become difficult. It is very difficult to disperse aggregated nanoparticles; often these aggregated nanoparticles become a cause for palmar-plantar erythrodysesthesia.

Depending on the sonication time and formulation variables, all the particles size of the prepared nanoparticles were obtained in the range of 156.36 nm to 198.34 nm (Table 2 & Figure. 2).

An attempt was made during preparation to prepare nanoparticles with a narrow distribution and smaller particle size (below 200 nm) during preparation, which will eventually help nanoparticles stay stable with less macrophagic absorption in systematic circulation. The reticuloendothelial opsonization process could be prevented by Bellow 200 nm particles⁽³⁵⁾⁽³⁶⁾

It was found that different forms of PLGA had no important effects on the size of the nanoparticles during particle size characterization ($p=0.114$).

However, the particle size of the nanoparticles is significantly reduced by increased PVA loading and sonication time ($P=0.012$). PVA molecular weight increases (Mowiol®-31 kDa, 47 kDa, 130 kDa) have not been reported to have a major effect on particle size ($p=0.136$). However, as the molecular weight of PVA increases to 130 kDa, the particle size decreases significantly ($p=0.0118$). Large increases in higher molecular PVA stabilise the nanoparticles' surface by preventing agglomerate from being collected. On the contrary, due to higher shearing stress, increasing sonication time decreases particle size. The increased molecular weight of PVA reduces particle size because PVP has less aqueous phase interactions with higher molecular weight. The reduced size of the particle was thus obtained.

As far as the surface charge of nanoparticles is concerned, almost all batches of nanoparticles have shown higher electrostatic charges, which means that cationic zeta potential has been observed (+25.56mV to +33.67mV). The nanoparticles' cationic zeta potential has a beneficial impact on binding to the extracellular environment that has been negatively charged⁽³⁷⁾. It was found that the outer surface of cancer cells has a negative charge; thus, cationic or positive nanoparticles can easily connect with cell membranes that are negatively charged. Nevertheless, in contrast to non-ionic or anionic nanoparticles, cationic nanoparticles have more penetrating properties with greater cytotoxicity^(38, 39). One crucial finding from this research outcome also established that, as such, there is no association between PLGA's altered molecular weight, and zeta potential.

Table 2. Particle size and zeta potential for the 10% w/w 5-FU loaded nanoparticles (mean \pm SD)

Variables	PLGA			PVA Loading (%)			Sonication time (min)				PVA MW (KDa)	
	DLG 75-9E	DLG 50-5A	DLG 50-2A	4	8	12	6	12	18	31	47	130
Particle Size (nm)	169.27 \pm	168.21 \pm	166.36 \pm	198.34 \pm	171.45 \pm	164.25 \pm	175.12 \pm	166.35 \pm	158.04 \pm	193.23 \pm	187.34 \pm	156.36 \pm
	7.08	6.21	7.34	9.34	6.34	8.26	6.62	5.56	8.23	8.34	8.35	7.21
Zeta potential (mV)	25.27 \pm	28.27 \pm	32.71 \pm	31.56 \pm	28.51 \pm	25.719 \pm	31.29 \pm	27.15 \pm	25.56 \pm	33.67 \pm	31.56 \pm	30.22 \pm
	6.36	3.78	3.11	4.35	2.11	8.35	2.35	4.11	2.26	2.56	2.36	1.36
Encapsulation efficiency (%)	85.45 \pm	90.34 \pm	84.11 \pm	98.25 \pm	87.17 \pm	75.67 \pm	96.27 \pm	84.12 \pm	74.28 \pm	95.34 \pm	86.25 \pm	72.56 \pm
	6.37	4.56	4.62	4.81	5.78	5.12	8.29	6.36	8.34	8.45	8.48	7.37

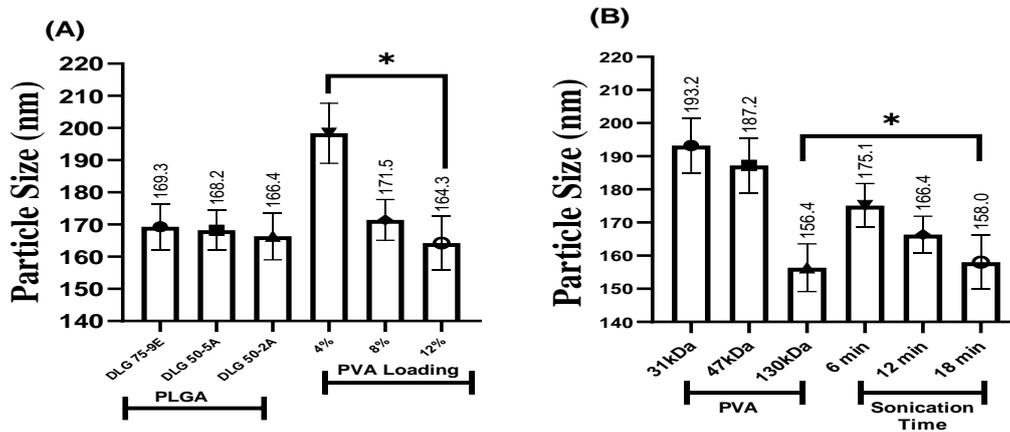


Figure 2. Particle size (A and B) 10% w/w 5-FU loaded PLGA nanoparticles. * indicates a significant difference

The encapsulation efficiency of the 10% w/w 5-FU loaded nanoparticles:

The nanoparticles encapsulation efficiency ranged from $72.56 \pm 7.37\%$ to $98.25 \pm 4.81\%$ on the basis of sonication period and formulation selection. Due to the presence of the same polymer ratio (50:50) and the same molecular weight, the selection of the PLGA polymer had no important effect ($P=0.212$) on the encapsulation efficacy of the nanoparticles. Other experimental results have also shown that PLGA can affect the product's encapsulation quality, but only when the lactide to glycolide ratio differs. Nevertheless, PLGA's lower molecular weight in an organic solvent would be highly soluble; thus, low encapsulation is probable. In this experiment, it was observed that the encapsulation efficiency of the nanoparticles reduces when PLGA loading increases ($p=0.0345$), because of decreased nanoparticle size with increased loading ability of PVA. Smaller nanoparticles will have less room to encapsulate 5-FU; low efficiency of encapsulation can be observed by chance. One hypothesis, however, can also be proposed, i.e. increased PVA concentration may bind with 5-FU; thus, the efficiency of 5-FU encapsulation decreases. From the current experiment, it was also observed that encapsulation efficiency ($p=0.0345$) of the nanoparticles decreases

significantly when the molecular weight of PVA is increased; due to the size decrease of the nanoparticles. The particle size also decreases with longer sonication time; thus, the efficiency of encapsulation decreases. One theory also indicates that increasing sonication time can assist degradation of PLGA within the polymeric solution to reduce the loading potential of PLGA for 5-FU, so that increased PLGA permeability can occur, allowing the drug to spread out during washing. Figure 3 shows the effect of PLGA, PVA loading, PVA molecular weight, and sonication time on drug entrapment efficacy (%).

Samira Khaledia et al. (2020)⁽⁴⁰⁾ recently developed polymeric nanoparticles for the delivery of 5-fluorouracil (5-FU) and Chrysin. The polymers used in this experiment were PLGA and PEG. In this study, 5-FU was encapsulated into the polymeric matrix using the double emulsions solvent evaporation method ($W_1/O/W_2$). The nanoparticles of 5-FU and Chrysin were determined using HPLC methods. The encapsulation efficacy of 5-FU was found to be 81.3 % in a dual drug delivery system. However, the highest encapsulation efficiency of the 10% w/w 5-FU loaded nanoparticles was found to be 98.254.81% in the current experiment. In this study, the encapsulation efficacy of 5-FU was improved.

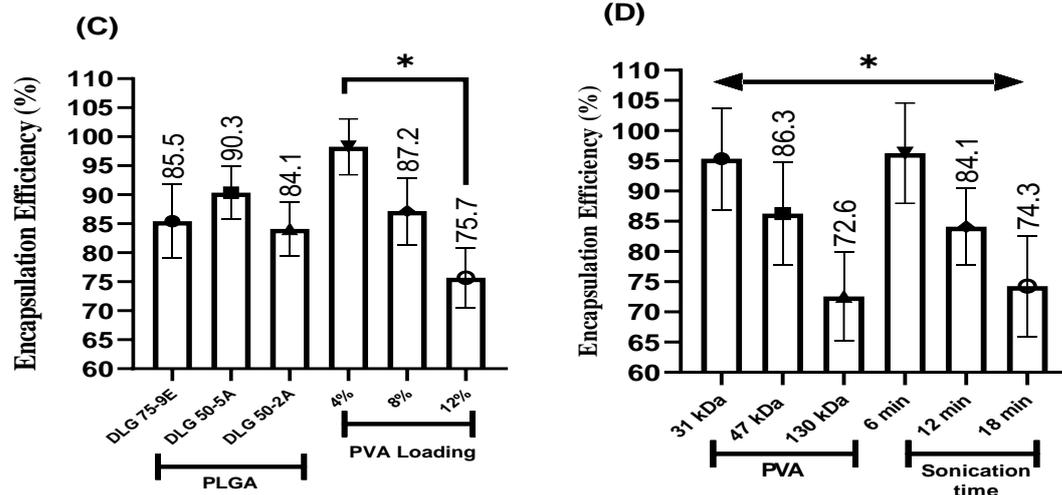


Figure 3. Encapsulation efficiency (C and D) of the 10% w/w 5-FU loaded PLGA nanoparticles. * indicates a significant difference

In-vitro 5-FU release from 10% w/w 5-FU-Loaded PLGA nanoparticles

Figure 4 (A-D) shows the *in vitro* release of 5-FU from PLGA nanoparticles over a period of 14 days. The standard diffusion-controlled drug release profile of all nanoparticles is due to the presence of PLGA. For the release of 5-FU from the nanoparticles, the selection of PLGA has paramount significance ($p=0.0234$). The degradation rate of the PLGA has been found to affect the release profile of nanoparticles. PLGA-DLG 75-9E has a degradation rate for two weeks, and PLGA-DLG 50-5A & PLGA-DLG 50-2A have a degradation rate for more than one month, according to the literature survey and recent research findings⁽⁴¹⁻⁴⁴⁾. From Figure 4(B) it can be concluded that; upon increases, the concentration of PVA (4-12%) would significantly ($p=0.0145$) increase overall surface area. The increased surface area of the nanoparticles has a significant burst effect for 8% PVA loaded nanoparticles at 2nd day. However, it was also observed that increasing PVA concentration up to 12% would significantly ($p=0.012$) decrease the release profiling of 5-FU from the nanoparticle surface; this is due to the formation of high coating over the polymeric nanoparticle surface and the construction of self-conjugation between 5-FU and PVA. From Figure 4(C), it was confirmed that as such, there were no significant defenses ($p=0.345$) reported in the release of 5-FU from the nanoparticles from day 1 to 7, containing PVA with a molecular weight of 31 kDa to 130 kDa. However, when the molecular weight of PVA increased up to 130 kDa, the nanoparticles' release patterns ($p=0.0145$) increased; this is due to the reduced particle size and higher surface area of the nanoparticles. Figure 3(C) shows that increasing sonication time would significantly ($p=0.0127$) increases 5-FU drug release from nanoparticles.

Higher sonication time (18min) reduces the size of the nanoparticles and increases overall surface area due to the degradation of PLGA within the solution.

Ana Cristina de Mattos et al.,(2016)(45) prepared prolonged-release PLA-PEG nanoparticles of 5-fluorouracil (5-FU). The *in-vitro* release of 5-FU was performed in the presence of pH 7.4 phosphate buffer solution and at 37°C. In 30 min, PLA nanoparticles release approximately 23% of 5-FU, and after 24 h, it removed 39% of 5-FU. After 320 h, it released about 52% of 5-FU from PLA-PEG nanoparticles. According to the researchers, the pronging of the 5-FU release could be caused by an anomalous mechanism of drug release as well as a slow erosion and diffusion process from the PLA-PEG surface of the nanoparticles.

However, an *in-vitro* drug release profile of twelve 10% w/w 5-FU loaded nanoparticles was found to sustain and control releases nature, which could achieve almost 70-90% cumulative drug releases within 14 days.

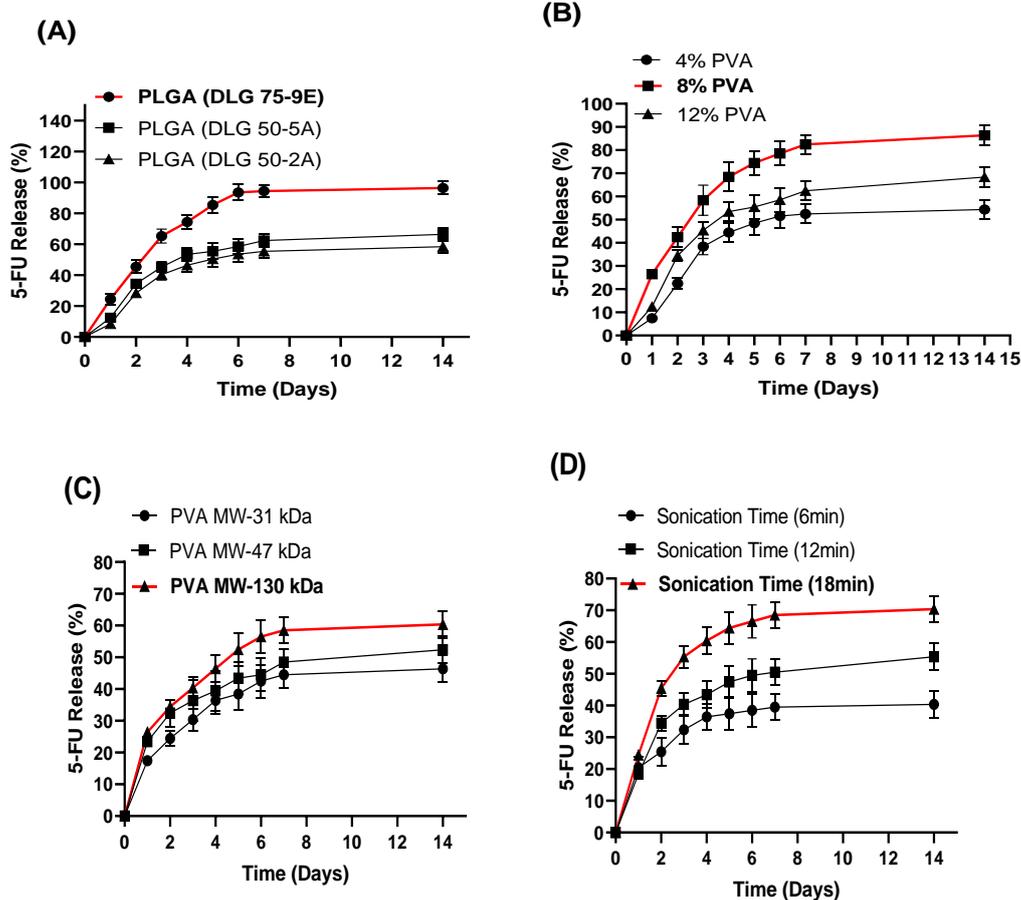


Figure 4. *In vitro* drug release for the 10 % w/w 5-FU loaded PLGA nanoparticles prepared using different PLGA polymers (A), PVA loadings (B), PVA molecular weights (C), and sonication times (D)

The ability of the 10% w/w 5-FU loaded PLGA nanoparticles to protect 5-FU from degradation in serum:

It is important to have extensive accumulation in the environment of cancer cells in an unaltered form for the successful anticancer effect of 5-FU. Since the half-life of 5-FU in the human body is only 10-20 minutes, generating an excellent anti-cancer effects on the site of action is a limiting step for 5-FU⁽⁴⁶⁾. Therefore, for the treatment of NSCLC, a drug delivery mechanism that prevents 5-FU from getting metabolised in the bloodstream is required. The literature review reported that PLGA alone could theoretically increase the half-life of the drug from 2 to 7 hrs^(47, 48). It is also essential to know how the choice of PLGA and sonication time can influence the degradation of nanoparticles within the bloodstream. Figure 5 indicating that 5-FU loaded nanoparticles significantly ($p=0.0121$) elevate the half-life of the 5-FU in serum environment, as compared to a native 5-FU drug, which was acting as a control. It has been confirmed from Figure 5(A) that the selection of PLGA has no effect on the increase in the half-life of 5-FU in the serum. More or less, as compared to the control group, the altered

PLGA grades increased the 5-FU half-life from 25 min to 180min. It was also found that approximately 56% of 5-FU remained in the blood serum after 180 minutes. Due to its $>200\text{nm}$ particle size, the retention of the nanoparticles in systematic circulation increases. The smaller size of nanoparticles makes the possibility of macrophagic absorption obsolete. The PLGA coating preserves 5-FU for a longer time in serum and increases the half-life of 5-FU. In addition, PLGA's lactide to glycolide ratio was the same with equal molecular weight, sonication time. Thus, the nanoparticles' level of permeability becomes identical and, therefore, the serum protection level of 5-FU being equivalent. On the contrary, the percentage of PVA used, and molecular weight has significant ($p=0.0118$) influence on the extension of 5-FU half-life in the blood serum. The 4%, 8%, and 12% PVA loaded nanoparticles enhance the half-life of the 5-FU up to 18 min (Figure 5B). Similarly, at the different molecular weight of PVA (31kDa, 47 kDa & 130 kDa) the half-life of 5-FU increases significantly ($p=0.0122$). In fact, the increased concentration and molecular weight of PVA reduces the size of the particles and thus increases the nanoparticles' surface area. Therefore, before it is exposed to 5-FU,

blood serum will have less time to migrate through the nanoparticles, and the risk of 5-FU degradation will be considerably less. The half-life of 5-FU in the nanoparticles decreases dramatically by increasing the sonication time ($p=0.0267$) (Figure 5C). This phenomenon is due to increasing

sonication time (18 min); degradation begins to disperse PLGA through the solution. Therefore, with greater serum exposure time, the resulting nanoparticles will have a higher diffusion rate, which eventually causes 5-FU to have a lower half-life.

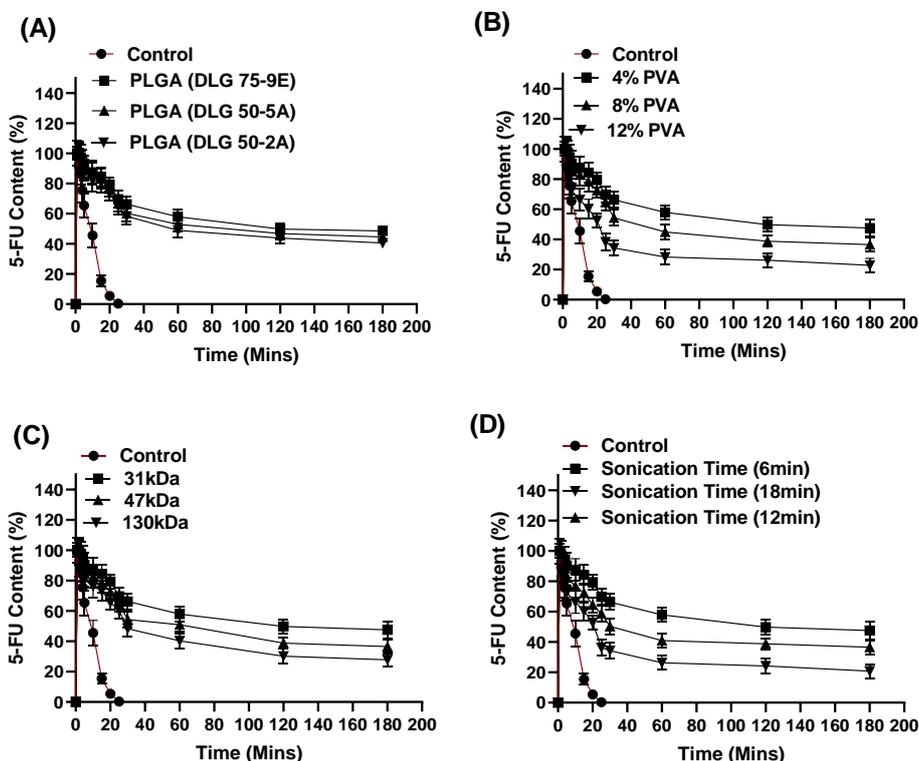


Figure 5. The influence of PLGA polymer (A), PVA loading (B), PVA molecular weight (C), and sonication time (D) on the 10% w/w 5-FU loaded nanoparticles to protect from degradation or engulfment from macrophages in blood serum

The cytotoxicity of the 10% w/w 5-FU loaded PLGA nanoparticles against human A549 Isogenic cell line

Figure 6 demonstrates the *in-vitro* cytotoxicity of the 10% w/w 5-FU loaded nanoparticles against the isogenic A549 cell. Figure 5 indicates that all formulations of nanoparticles exhibit cytotoxic effects against the A549 Isogenic cell line. However, the IC_{50} of all nanoparticles was significantly greater compared to the free 5-FU controlled group ($p=0.0145$) compared to the free 5-FU controlled group (8.34 nM). The PLGA selection, based on different grades, i.e. DLG75-9E, DLG50-5A, DLG50-2A, had an IC_{50} of 18.45 nM, 17.28 nM, and 17.11 nM, and had no important impact ($p=0.274$) on nanoparticles' cytotoxicity (Figure 6A). The rise in PVA loading, i.e. 4%, 8% & 12% with an IC_{50} value of 36.58 nM, 18.41 nM & 16.11 nM, significantly ($p=0.0156$) increases the cytotoxicity of the formulation of the nanoparticle

(Figure 6B). When the IC_{50} value for PVA MW-31 kDa, PVA MW-47 kDa, and PVA MW-130 kDa was 28.19 nM, 18.17 nM, and 14.33 nM, respectively, the increased molecular weight of the PVA increased the cytotoxicity of the nanoparticles marginally (Figure 6C). Increased sonication time (6min, 8min & 18min) partly increases nanoparticles' cytotoxicity (Figure 6D), with IC_{50} values of 34.62 nM, 18.44 nM, respectively, & 10.33 nM. Another main finding from the cytotoxicity studies was that for almost all formulations, the cell viability (%) of the A549 isogenic cell was found to be stabilised at 75 nM concentration.

The linear Relationship between particle size and IC_{50} value can be utilized to determine cytotoxicity. It was also observed that the particles which are having less particle size has low IC_{50} (Figure 6E).

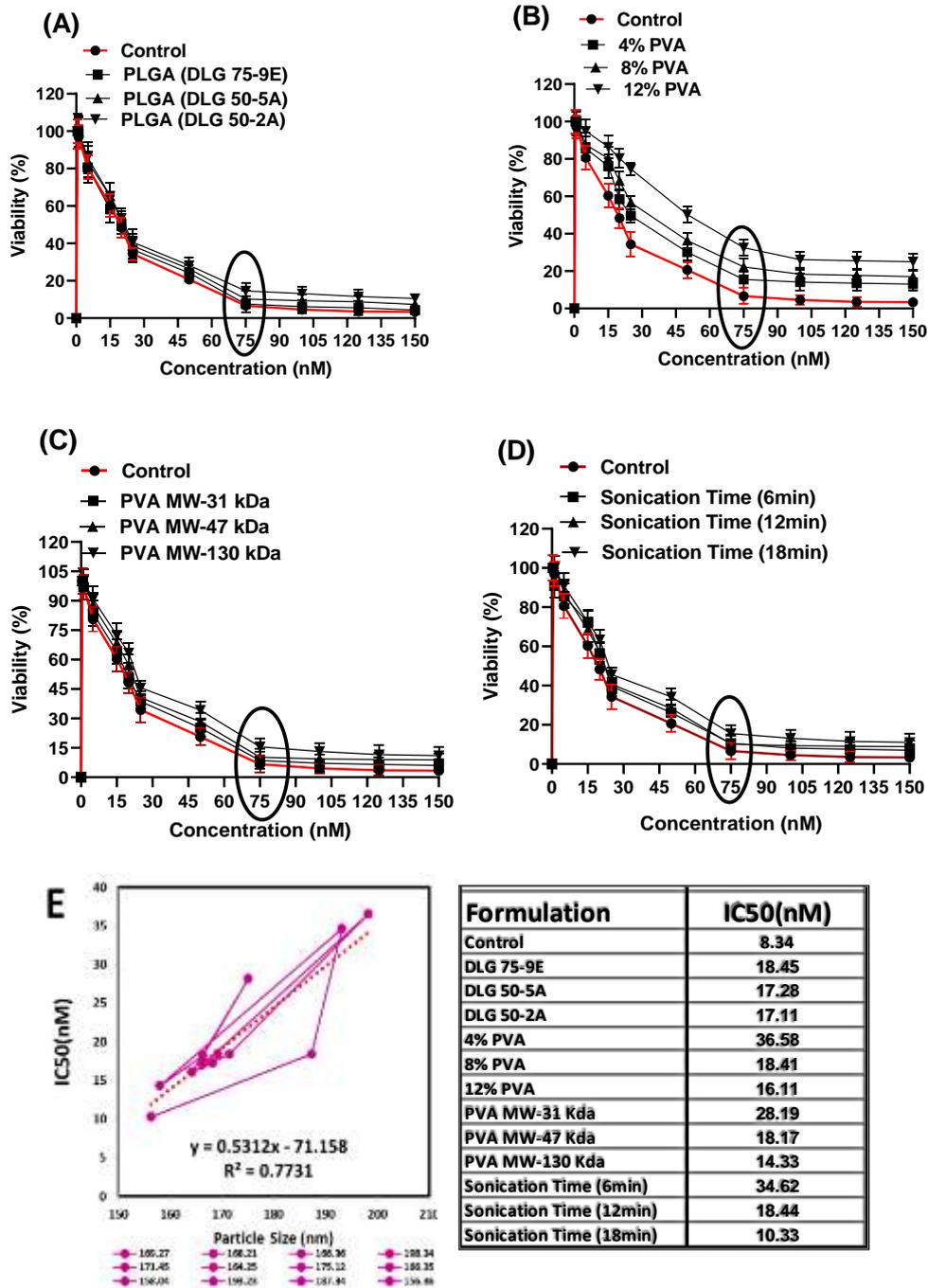


Figure 6. The influence of PLGA polymer (A), PVA loading (B), PVA molecular weight (C) sonication time (D) on the cytotoxicity of the 10% w/w 5-FU loaded nanoparticles with compared to native 5-FU controlled. The fundamental relationship between particle size and IC₅₀ value of the 10% w/w 5-FU loaded nanoparticles (E).

However, it was also understood that the nanoparticles' permeability could also play an important role, particularly for those prepared under 12 min and 18 min sonication time. At 18 min sonication time, with a 14.33 nM IC₅₀ value, the particle size was 156.36 nm. Whereas, the IC₅₀ value was significantly higher (p=0.0134) with nearby particle size (164.25 nm) at 12% PVA loading, the

IC₅₀ value was significantly higher (p=0.0134) (16.11 nM). The lower the value of IC₅₀, the greater the cytotoxicity would be⁽⁴⁹⁾. Therefore, as opposed to 12% PVA loaded nanoparticles, nanoparticles with 18 min sonication time will be more cytotoxic. For the nanoparticles, which were sonicated for 18 minutes, the same result was observed. IC₅₀ value was found to be 10.33 nM

during 18 min sonication time, while IC_{50} was around 14.33 nM with 156.34 nm particle size for those polymeric nanoparticles containing PVA with 130 kDa molecular weight. This result clearly shows that excess sonic time increases PLGA's degradation and infusibility in the solution. Ultimately, this results in an adequate amount of A549 Isogenic cell line drug (5-FU) diffusion and touch timings; thus, increasing cell death. It is evident from **Fig.4(D)** that the nanoparticles prepared with a sonication time of 18 min had a maximum cumulative drug release. As per Yves Marc Dupertuis et al. (2021), a combination of long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs) and 5-fluorouracil (5-FU) had significant anticancer effects on LS174T and HT-29 colorectal cell lines. This research suggests that 5-FU and n-3 PUFAs conjugated nanoparticles facilitate simultaneous drug transport to the cancerous site during tumor metastases⁽⁵⁰⁾. The findings of this study strongly suggest that 5-FU has significant anticancer effects against a variety of cell lines, which could explain the current research findings.

As per, the recent study conducted by Aditya Nath Pandey et al.,(2020)⁽⁵¹⁾ 5-FU can be encapsulated in PLGA nanoparticles, which could have potential anti-cancer effects against HT-29 and COLO-205 colorectal cancer cell lines. After 48 hours of in-vitro anti-cancer studies against HT-29 cell lines, 5-FU encapsulated PLGA nanoparticles showing 6.6% cell viability, whereas PLGA nanoparticles showing 16.6% cell viability against COLO-205 cell lines after 48 hr of studies.

There is a strong correlation between the conclusions reached by these two works and those reached by the current research study.

Conclusions

The research centred primarily on the multiple factors affecting the preparation of 10% 5-FU loaded poly lactic-co-glycolic acid nanoparticles. The various factors include different forms of PLGA (DLG 75-9E, DLG 50-5A & DLG 50-2A), PVA loading (4% 8% & 12%), PVA molecular weight (31 kDa, 47 kDa & 130 kDa) & sonication timings (6 min, 12 min & 18 min) had been involved in this experiment. It was evident from the research results that the impact of different types of PLGA had no major influence on different characteristics except the percentage of drug release of 5-FU loaded nanoparticles, obviously due to the nature of the PLGA's bio-degradation. The key finding of this experiment is that an increase in PLGA concentration, PVA loading, PLGA molecular weight, and sonication time will certainly reduce the size of the particles, eventually leading to surface area increases. Surprisingly, the particle size of all the formulations was less than 200 nm, eventually preventing reticuloendothelial opsonization and macrophagic uptake. In addition, this research was enriched with some tangible data,

suggesting how to generate 10% w/w 5-FU loaded PLGA nanoparticles from laboratory research to translational research on full-scale progenitor development.

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Conflict of Interest

The author declare that they have no conflict of interest.

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