# Polymeric nano-encapsulation of 5-fluorouracil enhances anti-cancer activity and ameliorates side effects in solid Ehrlich Carcinoma-bearing mice

Yusuf A. Haggaga,b\*, Mohammed A. Osmana ,Sanaa A. El-Gizawya, Ahmed E. Godac, Maha M. Shamloulad, Ahmed M. Faheeme and Paul A. McCarronb

*aDepartment of Pharmaceutical Technology, Faculty of Pharmacy, University of Tanta, Tanta, Egypt. bSchool of Pharmacy and Pharmaceutical Sciences, Saad Centre for Pharmacy and Diabetes, Ulster University, Cromore Road, Coleraine, Co. Londonderry, BT52 1SA, UK. cDepartment of Pharmacology and toxicology, Faculty of Pharmacy, University of Tanta, Tanta, Egypt. dPathology Department, Faculty of Medicine, University of Tanta, Tanta, Egypt. eSunderland Pharmacy School, Department of Pharmacy, Health and Well Being, University of Sunderland, Sunderland SR1 3SD, UK.*

\*Corresponding author

Yusuf A. Haggag a,b\*

Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Tanta, Tanta, Egypt, and School of Pharmacy and Pharmaceutical Sciences, Saad Centre for Pharmacy and Diabetes, Ulster University, Cromore Road, Coleraine, Co. Londonderry, BT52 1SA, UK.

E-mail: [Y.haggag@ulster.ac.uk](mailto:Y.haggag@ulster.ac.uk) and [jo\_pharm85@yahoo.com](mailto:jo_pharm85@yahoo.com).

Graphical Abstract

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# Abstract

Biodegradable PLGA nanoparticles, loaded with 5-fluorouracil (5FU), were prepared using a double emulsion method and characterised in terms of mean diameter, zeta potential, entrapment efficiency and *in vitro* release. Poly (vinyl alcohol) was used to modify both internal and external aqueous phases and shown have a significant effect on nanoparticulate size, encapsulation efficiency and the initial burst release. Addition of poly (ethylene glycol) to the particle matrix, as part of the polymeric backbone, improved significantly the encapsulation efficiency. 5FU-loaded NPs were spherical in shape and negatively charged with a size range of 185-350 nm. Biological evaluation was performed *in vivo* using a solid Ehrlich carcinoma (SEC) murine model. An optimised 5FU-loaded formulation containing PEG as part of a block copolymer induced a pronounced reduction in tumour volume and tumour weight, together with an improved percentage tumour growth inhibition. Drug-loaded nanoparticles showed no significant toxicity or associated changes on liver and kidney function in tested animals, whereas increased alanine aminotransferase, aspartate aminotransferase and serum creatinine were observed in animals treated with free 5FU. Histopathological examination demonstrated enhanced cytotoxic action of 5FU-loaded nanoparticles when compared to the free drug. Based on these findings, it was concluded that nano-encapsulation of 5FU using PEGylated PLGA improved encapsulation and sustained *in vitro* release. This leads to increased anti-tumour efficacy against SEC, with a reduction in adverse effects.

**Key words:** Nanoparticles, PEG-PLGA, 5-Fluorouracil, sustained action, Solid Ehrlich Carcinoma.

# 1. Introduction

Breast cancer is the fifth leading cause of morbidity and mortality in the developed World. Annually, more than 1 million women worldwide will receive a positive diagnosis [[1](#_ENREF_1)] and significant challenges still exist that hinder a recognised cure. Most currently applied treatments for breast cancer adopt approaches based on chemotherapy, surgery, radiation and biological therapies [[2](#_ENREF_2)]. Chemotherapy, in particular, is an established therapeutic approach for treatment of localised and metastatic breast cancer, but toxicity and adverse side effects afflicting normal tissue function remain problematic. Non-selective drug distribution is often the cause and this exacerbates the challenges associated with drug-based therapies [[3](#_ENREF_3)].

5-Fluorouracil (5FU) is a pyrimidine analogue and a first-line chemotherapeutic agent employed in the treatment of several solid tumours, such as breast, colorectal, and head and neck cancers. It has a broad spectrum of activity against various types of cancer and has a mode of action based on interfering with thymidylate synthesis. This leads to apoptosis in cancerous cells [[4](#_ENREF_4)]. A short biological half-life, non-selective distribution, variable oral bioavailability and toxicity, however, limit its therapeutic applicability. Several attempts are described that attempt to overcome these limitations, whilst preserving therapeutic effect [[2](#_ENREF_2)]. Many are based on developing novel delivery strategies, the designs of which use nanotechnology to formulate of a sub-micrometre nanoparticle (NP). These colloidal, carrier-mediated drug delivery systems include liposomes [[5](#_ENREF_5)], solid lipid NP [[6](#_ENREF_6)], biodegradable NP [[7](#_ENREF_7)] and nano-emulsions [[8](#_ENREF_8)]. Other examples of nano-scaled delivery systems for cancer treatment and cancer theranostics include metallic NP [[9-13](#_ENREF_9)] and nanocomposites [[14](#_ENREF_14)]. These formulations are of particular interest as they can be easily adjusted to improve pharmacokinetic profile and drug-carrying properties [[15](#_ENREF_15)].

Targeting of anti-cancer chemotherapeutic agents down to the level of the specific tumour cell is desirable for a number of obvious reasons. Effective targeting maximises the anticancer effect, whilst protecting surrounding healthy tissue from exposure to collateral cytotoxic damage [[2](#_ENREF_2), [3](#_ENREF_3" \o "Nair K, 2011 #503)]. Although individual strategies to achieve targeting are numerous, exploitation of the enhanced permeation and retention (EPR) mechanism is a frequently described approach. Nano-scaled carriers accumulate preferentially in tumour tissue as a result of the EPR effect, enabling formation of a local drug depot and providing continuous supply of encapsulated drugs into the microenvironment [[16](#_ENREF_16)]. Therefore, an aim of this study was to exploit the EPR effect and develop, characterise and evaluate *in vivo* 5FU-loaded biodegradable NP prepared using the double emulsion solvent evaporation method. The polymers chosen for the study were based on the poly (lactide-*co*-glycolide) (PLGA) backbone. A second aim of the work was to investigate the effect of increasing poly (ethylene glycol) (PEG) functionality on key NP properties, such as encapsulation efficiency and release rate. This was done using a block copolymer (PLGA-PEG) in either pure form or diluted (1:1) with PLGA. An optimised formulation was considered as one with size ˂ 200 nm, a narrow size distribution (PDI ˂ 0.2) and no particle aggregation. Passive tumour targeting of 5FU to enhance anticancer activity and diminish side effects was studied using an optimised NP formulation *in vivo* using a solid Ehrlich carcinoma tumour model in mice.

# 2. Materials and Methods

## 2.1. Materials

PLGA with a 50:50 lactic:glycolic ratio (Resomer® RG 503H, MW 34 kDa) and poly(ethylene glycol) methyl ether-block-poly(lactide-co-glycolide) (PEG average Mn 5,000, PLGA Mn 55,000) were purchased from Sigma Chemical Co. (St. Louis, USA). 5-Fluorouracil (HPLC powder), poly(vinyl alcohol) (PVA, 87-89% hydrolysed, molecular weight 31,000-50,000) and phosphate-buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, USA). Dichloromethane and acetonitrile were of HPLC grade and all other reagents were of analytical grade. Water used in the work was produced to Type 1 standard (Milli-Q®, 18.2 mΩ cm at 25 °C).

## 2.2. Preparation of 5FU-loaded NP

A modified, double emulsion, solvent evaporation method [[17](#_ENREF_17)] was employed in this study. 5FU was dissolved in 0.2 ml of aqueous solvent (either water or 3% w/v PVA) to form the internal water phase and mixed with 2.0 ml of dichloromethane (DCM) containing 50 mg of polymer. The primary emulsion was then dispersed into a 1% w/v PVA solution (20 ml) and both emulsion phases were emulsified using an ultrasonic homogeniser equipped with a 3.2 mm probe (Cole-Parmer, 4710 series, United States). Overnight stirring under vacuum was used to remove DCM and prevent pore formation on the surface of the NP. After formation, NP were collected by centrifugation at 10,000 *g* for 30 minutes at 4 °C (Sigma Laborzentrifugen GmbH., Germany), washed three times with ultrapure water and 2% w/v sucrose solution and lyophilised using freeze drying (Labconco., Kansas City, MO). The freeze-dried NP were stored in a desiccator at ambient temperature. The formulation variables and identifier codes are listed in Table 1.

## 2.3. Physicochemical characterization of 5FU-loaded NP

The particle size and distribution of 5FU-loaded NP were determined using dynamic light scattering (Zetasizer 5000, Malvern Instruments, Malvern, UK). An aliquot from the NP suspension was diluted in ultrapure water and measurements taken in triplicate. Laser Doppler Electrophoresis (Zetasizer 5000, Malvern Instruments, Malvern, UK) was used to measure the zeta potential of 5FU-loaded NP. Nanoparticulate suspensions were diluted in aqueous 0.001 M KCl solutions to adjust conductivity, with the average of three measurements recorded. Finally, NP surface morphology was characterised using transmission electron microscopy (JOEL JEM 2000 EX200) operating at an accelerating voltage of 80 kV. A sample of NP suspension was positioned on a Formvar-coated grid with addition of evaporated carbon and allowed to air-dry.

## 2.4. Determination of 5FU encapsulation efficiency

5FU content was determined by an indirect procedure. The concentration of non-encapsulated 5FU in the supernatant was measured using high pressure liquid chromatography (Waters® C18-5 column mm, 5 µm) at a flow rate of 0.7 ml min‑1 with UV detection (265 nm) [[18](#_ENREF_18)]. Isocratic elution was used, comprising a mobile phase of water: acetonitrile of 97: 3 (% v/v), respectively, and 4-amino-benzoic acid as internal standard. 5FU encapsulation in the NP was calculated from the difference between the initial amount of 5FU added and the non-entrapped drug remaining in the supernatant after NP fabrication. Each sample was assayed in triplicate and the mean percentage 5FU encapsulation efficiency was calculated.

## 2.5. In vitro release studies

5FU-loaded NP (5.0 mg) were suspended in 1.0 ml of PBS (pH 7.4) and put into a dialysis tube (MWCO 2000 Da). The sealed tube was placed into 50 ml of aqueous receiver phase (PBS, pH 7.4) and stirred at 100 rpm at 37 ± 2°C. At specific time intervals, an aliquot of receiver phase (1.0 mL) was taken and replaced with the same volume of fresh PBS [[19](#_ENREF_19)]. The samples were analysed in triplicate to determine 5FU concentration using HPLC.

## 2.6. In vivo study

The antitumour activity of 5FU-loaded NP was evaluated *in vivo* on mice, bearing a solid tumour of mammary origin. An Ehrlich Ascites Carcinoma (EAC) cell line was obtained from the Experimental Oncology Unit of the National Cancer Institute (NCI), Cairo University, Egypt. The cancer cell viability was evaluated at 98%, as judged by the trypan blue exclusion assay. A xenograft model of Solid Ehrlich Carcinoma (SEC) was induced in female Swiss albino mice by implanting 2x106 viable EAC cells suspended in 0.2 ml isotonic saline. EAC cells were aspirated from the peritoneal cavity of mice, washed with saline and implanted subcutaneously in the back of each mouse. The tumour developed in 100% of mice with a palpable solid tumour mass achieved within 12 days post-implantation [[20](#_ENREF_20), [21](#_ENREF_21" \o "Awara, 2004 #313)].

## 2.6.1. Animals groups and treatment protocol

Thirty adult female Swiss albino mice (18–20 g) were fed water and standard pellet chow (EL-Nasr Chemical Company, Cairo, Egypt) *ad libitum* for the duration of the *in vivo* experiment. Mice were housed and allowed to acclimatise to laboratory conditions for 7 days prior to the beginning of the experiment. The *in vivo* experimental work was conducted in accordance to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) approved by Animal Ethical Committee of Tanta University, Egypt. All mice were rendered tumour bearing and divided randomly into 3 equal groups, each comprising 10 animals. The control group received an injection of isotonic saline. Tumour-bearing mice in the first treated group were given an intraperitoneal administration of free 5FU at a dose of 10 mg 5FU kg-1. The second treated group received an intraperitoneal administration of 5FU-loaded NP (F6) at a dose of 10 mg 5FU kg-1 [[22](#_ENREF_22)]. The treatment protocol for all groups was started on day 12 and extended to day 28 post-implantation.

## 2.6.2. Tumour volume (V), percentage tumour growth inhibition (% TGI) and tumour weight.

Tumour volumes were recorded from the start point at day 12 post-implantation and thereafter every 2 days till the last measurement taken at day 28 post-implantation and just prior to sacrifice of surviving aminals. A vernier calliper was used to record dimensions (mm) and the following formula applied to calculate the volume of the developed tumour mass [[23](#_ENREF_23)];

Drug efficacy was expressed as the percentage tumour growth inhibition calculated as;

where *T* is the mean relative tumour volume (RTV) of the treated groups and *C* is the mean RTV in the control group. RTV is defined as *Vx/V1*, where *Vx* is the tumour volume at each point of the experiment before mice scarification and *V1* is the tumour volume at the starting point of the treatment [[24](#_ENREF_24)]. After termination of the experiment, all animals were sacrificed and tumours were excised and weighted. The changes in tumour weights of SEC samples were recorded.

## 2.6.3. Processing of tumour tissue samples

At the end point of the experiment (day 28 post-implantation), all surviving mice were sacrificed. The tumour was excised, washed immediately with ice-cold saline and the specimen preserved in 10% formalin solution. After treatment with xylene, the specimens were embedded in paraffin blocks. Sections (5 µm) were cut and stained with hematoxylin and eosin (H and E) prior to examination and histological characterisation.

## 2.6.4. Biochemical analysis

An examination of specific blood biochemistry parameters was used to assess the effects of 5FU at the cellular level. Serum samples were analysed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT), to assess hepatic damage. Serum creatinine level was evaluated and used as an indicator for possible renal damage. Blood biochemistry was examined in free drug treated and 5FU-loaded NP treated groups and compared with data from the control group. Blood samples were collected from the orbital sinus of each animal in a plain glass centrifuge tube without anticoagulant. Samples were left to clot at room temperature and centrifuged at 3000 *g* for 15 minutes. Sera were then separated and stored at -80 °C prior to analysis. All clinical chemistry analysis was carried out using a SmartLab Batch Analyzer (Biodiagnostic Ltd., Gizza, Egypt).

## 2.7. Statistical analysis

Results are presented as mean ± SD for *in vitro* studies and mean ± SEM for *in vivo* studies, respectively. Comparative analyses between groups were carried out using one-way analysis of variance followed by Tukey’s *post hoc* test. A value of p<0.05 was considered statistically significant.

# 3. Results

## 3.1. Effect of PEG content of the polymer

The physicochemical properties of three different formulations of 5FU-loaded NP (F1, F3 and F5) made from polymer blends of different PEG content are seen in Fig. 1. 5FU-loaded PLGA NP (F1) were significantly bigger in size than 5FU-loaded NP prepared from the 1:1 polymer mix and PEG-PLGA copolymer (F3 and F5, respectively). Increasing the concentration of PEG in the polymer matrix of the NP caused a decrease in size, with the smallest observed with F5 prepared from PEG-PLGA (Fig. 1A). All NP formulations had a low polydispersity index (PDI) ranging from 0.119 to 0.285. Increasing PEG in the NP matrix reduced the overall negative surface charge, with PLGA NP exhibiting the highest negative zeta potential (-20.11 mV) compared to the polymer mix (F3) and the PEGylated PLGA NP (F5). 5FU loading and encapsulation efficiency were significantly increased in the polymer mix and PEGylated PLGA NP (F3 and F5) compared to PLGA (F1). Increasing the PEG concentrations resulted in a significant increase in 5FU encapsulation efficiency (Fig. 1C). The i*n vitro* release profile of (F1, F3 and F5) showed that the 5FU burst release was faster and significantly higher form the F5 which released approximately 47.79% of 5FU within the first 24 hours, compared to 30.97% and 32.47% of 5FU released from the F1 and F3, respectively (Fig. 1D).

## 3.2. Effect of PVA addition to the internal aqueous phase

In this study, PVA was used as a stabiliser at 3% w/v in the internal aqueous phase. The physicochemical properties of different 5FU-loaded NP (F2, F4 and F6) prepared using this modification to the primary emulsion is shown in Fig. 2. Particle size was observed to fall following PVA addition, as can be seen when Fig 1(A) is compared to Fig 2(A). Addition of PVA to the internal phase showed a significant decrease in PDI values of (F2, F4 and F6) compared to (F1, F3 and F5), respectively. The distribution was monodisperse (Fig. 3). However, a negligible effect on the surface charge of 5FU-loaded NP was observed when Fig. 1B is compared to Fig. 2B. Furthermore, use of internal phase stabiliser showed a significant increase in the entrapment efficiency in all NP types (Fig. 2C). The highest encapsulation efficiency (80.37%) was observed in F6 using the PEG-PLGA copolymer.

The effect of internal aqueous phase stabiliser on the 5FU release is seen in Fig. 2D. A significant increase in the release rate of 5FU from PLGA NP was observed. The burst release was also seen to decrease following addition of PVA to the primary emulsion. From these results, it is feasible that incorporation of PVA into the internal aqueous phase of PLGA NP has promoted diffusion of 5FU molecules out from NP matrix [[25](#_ENREF_25), [26](#_ENREF_26)]. This can be compared to the *in vitro* release profile from F4 and F6 when 3% w/v of PVA is added to the internal phase. The burst release was significantly lower than that from similar NP without PVA (F3 and F5). An initial burst of 19.41% and 26.04% was observed from (F4 and F6) compared to 32.47% and 47.79 % of (F3 and F5), respectively.

## 3.3. Transmission electron microscopy

Fig. 4 shows a representative transmission electron micrograph of F6. These 5FU-loaded NP had a smooth spherical shape with a narrow size distribution. The NP surface was free from visible pores and the average size obtained from TEM was comparable to that obtained by dynamic light scattering.

## 3.4. In vivo anti-tumour activity

All animal groups (control and treated) appeared healthy throughout the study and no substantial loss in body weight was detected. There were no signs of decreased activity, which would have indicated general toxicity from the free drug or 5FU-loaded NP formulations.

The *in vivo* tumour growth inhibition study in mice with established SEC tumours after 5FU treatment showed a time dependent anti-tumour effect. Initially, from the start of the treatment to the 8th day after treatment, the tumour size of treated groups (Group II and III) was not significantly different from the control group (Group I) (Fig. 5). After 10 days of 5FU treatment, the size of tumour mass in Group III treated with 5FU-loaded PEG-PLGA NP was significantly lower compared to the free drug-treated group and the control group. In Group II, the 5FU dose was efficient in restraining further tumour growth and a significant increase in tumour size was not observed. The average size of the tumour mass was found to be 1410 mm3 at the end of the treatment and the percentage tumour growth inhibition (% TGI) was 18.63 %. A more pronounced effect was recorded in Group III, treated with 5FU-loaded PEG-PLGA NP. The volume of tumour mass was significantly decreased at each time point from the 10th day after treatment till the end of the experiment. The final mean size of tumour mass was found to have reduced to 678 mm3 and the % TGI was 60.35 % (Fig. 6). It is clear that the 5FU-loaded PEG-PLGA NP possessed a greater anti-tumour activity when compared to 5FU in solution and lead to a significant reduction in tumour size.

At the end of the treatment, all animals were sacrificed and SEC excised and weighed. Fig. 7 shows the change in tumour weights in control and treated groups. The average tumour weight in the control group after 16 days of treatment was 3.41±0.46 g. Treatment of mice with free drug reduced the mean of tumour weight to 2.06 ±0.17 g with a percentage reduction of 39.58%. The percentage reduction in tumour weights was 70.67% in mice treated by 5FU-loaded PEG-PLGA NP, which is statically significant compared to free drug treated mice. The average tumour weight of Group III was 1.00±0.29 g.

Histopathological examination of SEC from the three groups revealed the typical picture of this type of tumour (Fig. 8A-8D). Examination of sections prepared from the tumour tissue of the control group (Fig. 8A) showed malignant cells with hyperchromatic nuclei, increased nucleo/cytoplasmic ratio, bizarre forms with pleomorphic changes, multinucleated tumour with giant cells, massive necrosis and spread in solid sheets (H and E. x400). Sections prepared from Group II (treated with free 5FU) (Fig. 8B) showed well-circumscribed tumour sections surrounded by oedema and inflammatory cells. The presence of some viable tumour cells and a collar of inflammatory cellular filtrate and fibroblastic proliferation were observed. These findings support previous results recorded after tumour volume measurements that showed plain 5FU was efficient in restraining further tumour growth. Histopathological examination of Group III (treated by 5FU-loaded NP) revealed significantly different profiles (Fig 8C and 8D). Specimens showed no viable tumour cells, together with necrotic malignant cells with dystrophic calcification. Sections revealed mononuclear cellular infiltrate as well as macrophage infiltration of the necrotic tumour tissue.

## 3.5. Biochemical analysis

In the final part of the study, an assessment of the effect of 5FU-loaded PEG-PLGA NP on liver and kidney functions was compared to that following exposure to free 5FU. ALT and AST are released into the blood after extensive tissue injury. Specifically, elevated levels of ALT are associated with liver injury. Biochemical analysis of ALT and AST levels in the serum samples did not show any significant change between control group and 5FU-loaded NP treated group. However, statistically significant elevations in the levels of these enzymes were observed in the animals treated with free drug when compared to controls (Table 2). With regards to kidney function, serum creatinine levels from the group treated with free 5FU were almost doubled. However, 5FU-loaded PEG-PLGA NP showed a non-significant increase (Table 2). It can be concluded from these results that 5FU-loaded PEG-PLGA NP are better tolerated when compared to the free drug.

# 4. Discussion

Polymeric NPs are of particular interest in novel drug delivery strategies. They offer enhanced chemical and physical stability, together with improved stability of sensitive pharmaceutical actives, especially following lyophilisation [[27](#_ENREF_27)]. They can be loaded with a wide range of therapeutically relevant compounds, such as small molecular drugs, proteins, peptides and oligonucleotides, using a multitude of fabrication methodologies [[17](#_ENREF_17), [28-30](#_ENREF_28)]. Encapsulation sustains and controls drug release over a protracted period in vivo [[31](#_ENREF_31)] and NP enable multiple ligand coupling to form multifunctional colloidal formulations. Unsurprisingly, these sophisticated particulate systems provide many opportunities for advanced tumour targeting [[32](#_ENREF_32)].

PLGA was used in this study as it possesses numerous favourable characteristics. It is a ubiquitous synesthetic polymer used in the development of sub-micrometer particulate systems, with acceptable biocompatibility, biodegradability and versatility [[33](#_ENREF_33)]. It is pharmaceutically acceptable from a regulatory standpoint and can be adapted readily by addition of functionality to the polymeric backbone. As a result, PLGA-PEG copolymers are widely investigated for both fundamental research and product development because PEGylated polymeric NP can significantly reduce systemic clearance when compared to the PEG-free NP [[16](#_ENREF_16)].

Polymeric NPs formulated from PLGA have shown promising applications in the delivery of 5FU. This is especially true for colon [[34](#_ENREF_34), [35](#_ENREF_35)] and breast cancers [[2](#_ENREF_2), [7](#_ENREF_7), [36](#_ENREF_36)]. Modification of PLGA by addition of hydrophilic polar polymeric sections, such as PEG, improves both antitumour efficiency and targeting characteristics [[19](#_ENREF_19), [37](#_ENREF_37)]. This approach is also associated with further advantages, such as improved aqueous solubility, reduced aggregation, improved stability, low immunogenicity, low opsonisation and prolonged *in vivo* half-life. PEG-PLGA polymeric NP, loaded with 5FU, have been shown to sustain the release over 5 days, whilst improving anticancer action *in vivo* by inhibiting peritoneal dissemination of colon cancer in mice [[37](#_ENREF_37)].

The effect of formulation parameters on the properties of PEG-PLGA NP was investigated in this work and has been widely investigated by many groups [[38](#_ENREF_38), [39](#_ENREF_39)]. Because of the hydrophilic nature of 5FU, the double emulsion formation technique is extensively used for the preparation of 5FU-loaded NP. However, low encapsulation efficiency [[2](#_ENREF_2), [35](#_ENREF_35), [40](#_ENREF_40)] and a high initial burst effect [[2](#_ENREF_2), [19](#_ENREF_19)] are often observed. Perhaps the most important parameter in the double emulsion procedure is the nature of the matrix polymer used [[41](#_ENREF_41)]. Therefore, in this work, the addition of PEG was used to overcome these difficulties associated with ineffective loading. The PEG content had a significant impact on NP size, encapsulation efficiency and initial burst release. Higher PEG content resulted in a smaller nanoparticle size, attributable to the short chain length of PEG-*b*-PLGA compared to PLGA and polymer mix. The NP size is often increased by an increase in the hydrophobic segment [[42](#_ENREF_42)], which is a similar finding to that reported in other studies [[31](#_ENREF_31)]. The presence of PEG in the NP as a non-ionic hydrophilic polyether decreased their zeta potential by shielding polymeric anionic charge [[43](#_ENREF_43)]. Furthermore, PEG content was shown to increase drug entrapment attributable to the amphiphilic property of the PEG-PLGA polymer with the hydrophobic PLGA block and hydrophilic PEG blocks.

*In vitro* release data showed that a burst release phenomenon was occurring with all NP formulations used in this work. This is attributed to drug attached primarily to the surface of the NP [[2](#_ENREF_2)]. Therefore, it was feasible that higher amounts of 5FU were associated with the surface of PEGylated NP surface when compared to PLGA NP. Moreover, PEG chains are hydrophilic and expected to hydrate more effectively in an aqueous release medium, thereby disrupting the integrity of the polymer matrix [[16](#_ENREF_16), [44](#_ENREF_44)].

In this work, an optimised formulation (F6) was selected for further evaluation. Optimisation was defined as a size ˂ 200 nm, a narrow size distribution (PDI ˂ 0.2) with no aggregated or large (>1 μm) particulate structures. Optimisation of 5FU-loaded NP by adding PVA as an internal aqueous phase stabiliser showed a significant effect on NP size, encapsulation efficiency and initial burst release. PVA reduces the dynamic interfacial tension and increases the stability of the primary emulsion against premature emulsion coalescence [[45](#_ENREF_45), [46](#_ENREF_46)]. It is also known that PVA has a similar effect when present in the secondary emulsion phase and it is reported as having a more dominant effect in the double emulsion [[47](#_ENREF_47)]. Therefore, PVA stabilises and decreases the size of the water-in-oil emulsion droplets, especially when PEGylated polymers are used [[29](#_ENREF_29)]. In this work, a significant increase in the encapsulation efficiency was observed in all types of NP. Interestingly, other work has shown that decreasing particle size has resulted in a large mass transfer area leading to poor encapsulation [[26](#_ENREF_26), [29](#_ENREF_29)]. The hydrophilic nature of 5FU leads to leakage into the external aqueous phase during the initial phases of particle formation resulting in low encapsulation efficiencies [[2](#_ENREF_2), [35](#_ENREF_35), [40](#_ENREF_40)]. In this work, a high concentration of PVA in the internal aqueous phase will modify its viscosity, minimising the leaching of 5FU into external aqueous phase [[48](#_ENREF_48)].These results were in good agreement with the findings of others [[46](#_ENREF_46), [49](#_ENREF_49)].

The *in vitro* release profile of F6 showed the lowest initial burst and an extended release profile over a 7-day period. PVA in the primary emulsion phase is expected to congregate at interfacial sites and influence drug release [[50](#_ENREF_50), [51](#_ENREF_51)]. 5FU-loaded NP prepared using PEG-PLGA polymer and PVA addition to the internal phase were significantly lower in size and higher in encapsulation efficiency compared to formulations fabricated by other types of polymers at the same conditions. Formulation F6 exhibited the lowest NP size (185 nm), the best encapsulation efficiency (80.37%) and sustained 5FU release for seven days. The experimental release data of F6 were fitted to different release models. The results of these manipulations, expressed in terms of the correlation factor (r2), showed that the best model describing the 5FU release from PEG-PLGA polymer was the zero order model. These results are different from our previous results, which showed Higuchi release kinetics from the same polymer [[29](#_ENREF_29)]. This can be attributed to presence of the PVA layer.

*In vivo* administration of PEGylated NP has some important considerations. It has been previously described that a higher PEG density on the surface of the NP can decrease the mobility of the PEG molecules and minimise the steric hindrance effect of the PEG layer [[52](#_ENREF_52)]. If the PEG content is too low, opsonins will bind to the NP surface and the stealth effect will be decreased [[53](#_ENREF_53)]. Therefore, in order to achieve an intermediate PEG chain concentration between the mushroom and the brush conformation (low and high PEG content), PLGA, PEG-PLGA and a ratio composition of 1:1 (w/w) of polymers PLGA: PEG-PLGA were finally selected for this study.

A tumour is a pathological state characterised by uncontrolled proliferation [[54](#_ENREF_54)]. In the present study, we investigated the *in vivo* effects of 5FU and 5FU-loaded PEG-PLGA NP on the proliferation of SEC cells in tumour bearing mice. Previous investigations have indicated the potential of colloidal delivery of anticancer agents [[33](#_ENREF_33)], with 5FU being of particular interest to our group. As in keeping with other such therapeutic agents, 5FU possesses a range of serious side effects [[22](#_ENREF_22)]. In this work, the SEC xenograft model was induced in mice. It is an established model commonly used to investigate different chemotherapeutic treatment strategies for treating breast cancer [[55](#_ENREF_55)]. This model reflects a high-grade malignancy due to its virulence, quick development and infiltrative nature [[56](#_ENREF_56)]. Therefore, it can be used as a potential model to study the curative effect of 5FU *in vivo*.

Delivery of 5FU using PEG-PLGA NP suppressed the tumour growth significantly (3.2 fold reduction) and was more pronounced than the regression observed in animals treated with free drug. This difference in clinical effect may relate to the sustained release observed using the NP formulation and contrasts with the short duration of action of the free drug. A significant decrease in tumour weight observed at the end of the treatment confirmed the enhanced ant-cancer activity of 5FU-loaded NP when compared to free drug-treated group. Histopathological examination revealed destruction of tumour tissue following 5FU PEG-PLGA NP administration, with the appearance of dead and necrotic cells in the tumour tissue.

One of the most common side effects associated with 5FU use is liver and kidney dysfunction, *inter alia*, which leads to a reduction in therapeutic activity and patient survival time [[22](#_ENREF_22)]. In this work, 5FU-loaded PEG-PLGA NP showed a non-significant increase in the levels of AST, ALT and serum creatinine in tumour-bearing mice compared with the control group. The levels of these biochemical parameters were significantly lower than those in the free drug-treated group. Therefore, it was concluded that PEG-PLGA nanoparticles prevented damage to liver and kidney function caused by 5FU and PEG-PLGA nanoparticles may be judged to be a safe carrier for 5FU. The rapid uptake of PLGA nanoparticles by the macrophages of the reticulo-endothelial system (RES), primarily in the liver and spleen could be significantly reduced by modifying their surface with polyethylene glycol (PEG). The presence of PEG chains on the surface can protect NP from capture by macrophages, improves its cytoplasmic transport and reduces possible enzymatic degradation [[57](#_ENREF_57)].

# 5. Conclusions

5FU-loaded PLGA NPs were prepared using a modified double emulsion technique comprising different PEG content and PVA concentration in the internal aqueous phase. An optimum PEG-PLGA NP was selected, which had the highest drug loading, the lowest particle size of 185 nm, whilst sustaining the drug release for 7 days. *In vitro* release studies of 5FU-loaded NP showed that addition of PVA as internal stabiliser played a role in determining the sustained release profile. An important part of this study was that *in vivo* results confirmed the enhanced anti-cancer activity of 5FU-loaded NP by achieving 60.35% tumour growth inhibition. Histopathological examinations showed destruction of tumour tissue after NP treatment and the NP drug delivery system was found to be less toxic to liver and kidney tissues when compared to the free drug. The emergence of sustained release formulations of 5FU is of clinical significance because it will improve the therapeutic response by providing effective tumour regression along with causing minimal side effects compared to regular 5FU administration.

**Conflict of interest:** No financial or personal relationships with other people or organizations that could inappropriately control this study. There are no competing interests.

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# Figure legends

Figure 1. Effects of PEG content on (A) NP size, (B) zeta potential, (C) encapsulation efficiency and (D) *in vitro* release when the internal aqueous phase is water (F1, F3 and F5). Values are mean ± SD with n = 3. For 1A-1D, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared with PLGA NP. Δp < 0.05, ΔΔp < 0.01, ΔΔΔ p< 0.001 when compared to the 1:1 PLGA:PEG-PLGA.

Figure 2. Effects of 3% w/v PVA as internal aqueous (F2, F4 and F6) phase on (A) NP size, (B) zeta potential, (C) encapsulation efficiency and (D) *in vitro* release. Values are mean ± SD with n = 3. For 2A-2D, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared with PLGA NP. Δp < 0.05, ΔΔp < 0.01, ΔΔΔ p< 0.001 when compared to the 1:1 PLGA:PEG-PLGA.

Figure 3. Representative graphs showing particle size distribution of 5FU-loaded PEG-PLGA NP prepared with (A) no PVA (F5) and (B) PVA 3% w/v (F6) in the internal phase. Size analysis showed evidence of aggregation (blue arrow) in 5FU-loaded PEG-PLGA NP prepared with no PVA in the internal phase, which was absent in those prepared with the PVA stabiliser.

Figure 4. TEM images of 5FU-loaded PEG-PLGA NP (F6).

Figure 5. Tumour volume of the studied groups at recording points every 2 days from the 1st day (start point of treatment) to the last record at the 16th day (end point of experiment). Values are mean ± SEM with n=10. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared with Group I (control group). ΔP<0.05, ΔΔ P<0.01, ΔΔΔP<0.001 compared with Group II (treated by free 5FU 10 mg kg-1).

Figure 6. Percentage tumour growth inhibition (% TGI) in Group III (treated with 5FU-loaded PEG-PLGA NP 10 mg kg-1) relative to Group I (control group).

Figure 7. Tumour weight of studied groups after the end of treatment. Values are mean ± SEM with n=10. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared with Group I (control group). ΔP<0.05, ΔΔ P<0.01, ΔΔΔP<0.001 compared with Group II (treated by free 5FU 10 mg kg-1).

Figure 8. Histopathological findings of SEC sections stained with H&E. Group I (A) showing cellular details of the tumour; the cells are polymorphic in shape, containing relatively large, highly chromatophilic nuclei with one or more prominent nucleoli; giant tumour cells are also seen (H and E. x400). Group II (b) showing viable tumour cells surrounded by a layer of oedema and inflammatory cells (H and E. x400). Group III (C) showing necrotic tumour cells with dystrophic calcifications surrounded by a layer of inflammatory cells (H and E. x400), (D) showing sections with macrophages and mononuclear cellular infiltrate surrounding necrotic tumour cells (H and E. x100).

### Table 1. Formulation variables used in the preparation of 5FU-loaded nanoparticles

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Formulation ID | Polymer type | Polymer  Amount (mg) | Drug loading (% w/w) | Internal phase stabiliser |
| F1 | PLGA | 50 | 10 | water |
| F2 | PLGA | 50 | 10 | 3% w/v PVA |
| F3 | PLGA:PEG-PLGA (1:1 w/w) | 50 | 10 | water |
| F4 | PLGA:PEG-PLGA (1:1 w/w) | 50 | 10 | 3% w/v PVA |
| F5 | PEG-PLGA | 50 | 10 | water |
| F6 | PEG-PLGA | 50 | 10 | 3% w/v PVA |

### Table 2. Effect of 5FU and 5FU-loaded NP on serum level of blood biochemical parameters

|  |  |  |  |
| --- | --- | --- | --- |
| Test | Control | 5FU | 5FU PEG-PLGA NP |
| serum creatinine)  (mg dl-1) | 0.58 ±0.25 | 1.25 ± 0.15\*\*Δ | 0.73 ± 0.218 |
| ALT  (units L-1) | 15.94 ± 3.45 | 51.45 ± 8.83\*\*\*ΔΔ | 28.27 ± 6.98 |
| AST  (units L-1) | 34.59 ± 4.65 | 67.96 ± 7.45\*\*Δ | 43.56 ± 4.28 |

Values are represented as mean ± SD with n=10. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared with control. Δp < 0.05, ΔΔp < 0.01, ΔΔΔ p< 0.001 compared with 5FU PEG-PLGA NP .