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

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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.

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] 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]. 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].

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]. 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]. 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], solid lipid NP [6], biodegradable NP [7] and nano-emulsions [8]. Other examples of nano-scaled delivery systems for cancer treatment and cancer theranostics include metallic NP [9–13] and nanocomposites [14]. These formulations are of particular interest as they can be easily adjusted to improve pharmacokinetic profile and drug-carrying properties [15].

Targeting of anti-cancer chemotherapeutic agents down to the level

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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 [2,3]. 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]. 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] 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 min 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.

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

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]. 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]. 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 2×10^6 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 [20,21].

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