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Dual drug loaded chitosan nanoparticles—sugar-coated arsenal against pancreatic cancer

Karolyn Infanta David, Leela Raghav Jaidev, Swaminathan Sethuraman, Uma Maheswari Krishnan*

Centre for Nanotechnology & Advanced Biomaterials, School of Chemical and Biotechnology, SASTRA University, Thanjavur 613401, India

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ABSTRACT

Pancreatic cancer is an aggressive form of cancer with poor survival rates. The increased mortality due to pancreatic cancer arises due to many factors such as development of multidrug resistance, presence of cancer stem cells, development of a stromal barrier and a hypoxic environment due to hypo-perfusion. The present study aims to develop a nanocarrier for a combination of drugs that can address these multiple issues. Quercetin and 5-fluorouracil were loaded in chitosan nanoparticles, individually as well as in combination. The nanoparticles were characterized for morphology, size, zeta potential, percentage encapsulation of drugs as well as their release profiles in different media. The dual drug-loaded carrier exhibited good entrapment efficiency (quercetin 95% and 5-fluorouracil 75%) with chitosan: quercetin: 5-fluorouracil in the ratio 3:1:2. The release profiles suggest that 5-fluorouracil preferentially localized in the periphery while quercetin was located towards the core of chitosan nanoparticles. Both drugs exhibited considerable association with the chitosan matrix. The dual drug-loaded carrier system exhibited significant toxicity towards pancreatic cancer cells both in the 2D as well as in the 3D cultures. We believe that the results from these studies can open up interesting options in the treatment of pancreatic cancer.

1. Introduction

Pancreatic cancer is a deadly form of neoplasm with the highest level of mortality among cancers. Recent years have witnessed an increased incidence among the world population [1]. The lack of early symptoms and the detection of the disease at an advanced metastasis state are the prime reasons for this high mortality rate. In addition, the hypoxic environment of the pancreatic cancer tissues, highly invasive cancer cells and presence of a stromal barrier around the tumor that limits the entry of drugs, have all contributed to poor efficiency of therapeutic interventions [1]. The primary strategy currently employed by clinicians to treat pancreatic cancer is the removal of tumor by performing pancreaticoduodenectomy or Whipple's procedure followed by an aggressive chemotherapy regimen [1]. Commonly employed first line chemotherapeutic agents for treatment of pancreatic cancer are the nucleotide analogues, gemcitabine and 5-fluorouracil [1]. However, these drugs have been largely unsuccessful due to poor uptake in the cancer cells and the patient has a median survival of only 6.8 months [1]. Clinical studies are currently underway for a therapeutic formulation called FOLFIRINOX®, which is a combination of 5-fluorouracil, leucovorin, irinotecan and oxaliplatin. This new formulation exhibits a marginal improvement in the median survival of the patients at 11.6 months [2]. However, cancer resurrection is prevalent despite the therapeutic interventions attempted. Hence, there is an urgent need for the development of novel therapeutics in search of the Holy Grail for cancer treatment.

The advent of nanotechnology has opened up new vistas in medicine through introduction of smart nano-drug delivery systems that can selectively deliver appropriate levels of the therapeutic agent in a specific region [3]. Research is underway to develop nanocarrier for treatment of various diseases [4,5]. Several nanocarriers like liposomes have been commercialized and many more are under different phases of clinical trials [6,7]. Initially, single drug-loaded carriers were investigated extensively. However in the recent years, multi-drug loaded carriers are being developed to use the synergistic effect of the different molecules towards mitigating cancer [8]. In the context of pancreatic cancer treatment, the application of nanoparticles either for diagnosis or for treatment is still in a nascent stage. Very few reports are available in literature

* Corresponding author at: Departments of Chemistry, Bioengineering & Pharmacy, Centre for Nanotechnology & Advanced Biomaterials (CeNTAB), School of Chemical & Biotechnology, SASTRA University, Thanjavur, 613 401 Tamil Nadu, India. Fax: +91 4362 264120.
E-mail address: umakrishnan@sastra.edu (U.M. Krishnan).

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for the development of nanoparticle-mediated therapeutic strategies against pancreatic cancer. Poly(ethylene glycol)-coated iron oxide nanoparticles loaded with gemcitabine have been employed for treatment of pancreatic cancer [9,10]. The potential therapeutic activity of drug-loaded gold nanoparticles towards pancreatic cancer therapy has also been suggested [11]. A recent report had highlighted a curcumin-loaded magnetic nanoparticle system for the treatment of pancreatic cancer [12]. A L-fucose-bound liposomal delivery system containing Cy5.5 or cisplatin has been developed to treat CA19-9 expressing pancreatic cancer cells [13]. Thus, there exists further scope for development of other polymeric systems for nanocarrier-mediated drug delivery to pancreatic cancer cells.

Chitosan is a natural linear polysaccharide made up of repeating units of D-glucosamine and N-acetyl-D-glucosamine [14]. One of the major advantages of chitosan nanoparticles is its rapid uptake by cells due to its surface amine groups [15]. Several attempts to harness the property of chitosan nanoparticles towards addressing the challenges in pancreatic cancer have been reported [16]. A modified *N*, *N*-diethyl-*N*-methyl chitosan (DEMC) has been employed for gene delivery in ASPC-1 metastatic pancreatic cancer cells [17]. Enhanced anti-proliferative activity of herceptin (HER2)-conjugated gemcitabine-loaded chitosan nanoparticles has been investigated for potential pancreatic cancer therapy [15,18]. Gemcitabine-loaded chitosan and glycerol monooleate nanoparticles were evaluated using the pancreatic cell lines MiaPaCa2 and Bxpc-3. Snima et al., (2012) used *O*-carboxymethyl chitosan nanoparticles loaded with metformin for the treatment of pancreatic cancer [19]. These studies have employed single drug-loaded chitosan nano-formulations in the treatment of pancreatic cancer. Though dual drug combinations have been now explored for many types of cancer, not many reports on use of multiple drug combinations have been reported in literature for treatment of pancreatic cancer and hence the field remains wide open for the development of such combinations to address the challenges in pancreatic cancer therapy.

Different combinations of a cytotoxic agent with an anti-oxidant have been reported in literature to be effective against various forms of cancer [20]. Combination of 5-fluorouracil with the flavonoid quercetin has been demonstrated to exhibit synergistic activity in the treatment of colorectal cancer [21–23]. Xin et al. (2012) have also reported a synergistic activity between 5-fluorouracil and quercetin in the treatment of esophageal cancer [24]. However, this combination has not been investigated for pancreatic cancer treatment. The present study aims to investigate the potential of the dual drugs (5-fluorouracil and quercetin) encapsulated in chitosan nanoparticles to treat pancreatic cancer. As the tumor tissue is three-dimensional and hence restricts entry of drugs, the in vitro efficacy of the dual drug-loaded carrier was evaluated using both 2D and 3D culture models. It is pertinent to note here that the 3D culture model of pancreatic cancer has hitherto not been employed to evaluate nanocarrier systems.

2. Experimental

2.1. Materials

Chitosan, 5-fluorouracil (5-FU), quercetin dihydrate, sodium tripolyphosphate (STPP) were purchased from Sigma-Aldrich, USA. All other reagents used were of analytical grade.

2.2. Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by ionic gelation method [4]. Chitosan was dissolved in 10% (v/v) glacial acetic acid at a concentration of 5 mg/mL. To this solution, 0.1% sodium tripolyphosphate (STPP) solution was added drop-wise until an opalescent suspension was obtained. The resultant solution was continuously stirred for 2 h followed by centrifugation at 15,000 rpm for 25 min at 4 °C (Remi, C-24BL, India). The pellet formed was freeze-dried (Alpha 1-4S, Martin Christ GmbH, Germany) and used for further experiments.

2.3. Preparation of drug-loaded chitosan nanoparticles

For preparation of quercetin-loaded chitosan nanoparticles, an ethanolic solution of quercetin (1 mg/mL) was mixed with chitosan solution followed by drop-wise addition of STPP solution to obtain a suspension. This nanoparticle suspension was gently stirred for 2 h at room temperature to allow excess quercetin to adsorb on the nanoparticles and attain isothermal equilibrium. Quercetin-encapsulated chitosan nanoparticles were centrifuged at 15,000 rpm for 30 min,

washed repeatedly to remove any impurities and freeze-dried for further analyses. A similar procedure was adopted for encapsulation of 5-FU-loaded chitosan nanoparticles using an aqueous solution of 5-FU and also for dual drug-loaded chitosan nanoparticles, were quercetin and 5-FU solutions were mixed with chitosan solution followed by drop-wise addition of STPP. Dual drug-loaded chitosan nanoparticles were centrifuged at 15,000 rpm for 30 min, resuspended in water and freeze-dried for further experiments.

2.4. Characterization of the nanoparticles

The morphology of the nanoparticles was studied using field emission scanning electron microscopy (JSM 6701F, JEOL, Japan). The samples were placed in a brass stub using double sided carbon tape, sputter-coated with a thin layer of gold and imaged at an acceleration voltage of 3 kV. FTIR spectra for the samples were recorded by mixing a small quantity of the dried sample with potassium bromide (IR grade, Merck, Germany) using an agate mortar and compressed into thin pellets using a pelletizer. The spectra were recorded in an FTIR spectrometer (Spectrum 100, PerkinElmer, USA) between 4000 and 400 cm^{-1} and resolution of 4 cm^{-1} averaging 50 scans. Thermal analysis of the nanoparticles was carried out using differential scanning calorimetry (DSC, Q20, TA Instruments, USA). About 5 mg of the sample was taken in a standard aluminum pan and heated from 20 °C to 140 °C at a constant rate of 10 °C per minute under nitrogen atmosphere. The average particle size and zeta potential of the nanoparticles was measured using dynamic light scattering technique (NanoZS, Malvern Instruments, UK).

2.5. Determination of encapsulation efficiency (EE%)

Freshly prepared drug-loaded nanoparticles were centrifuged at 15,000 rpm for 30 min. The absorbance of the supernatant was determined using multi-mode reader (Infinite M200, Tecan, Austria). Quercetin was measured at 380 nm while 5-FU was measured at 265 nm. The absorbance values were converted in to quantity of drug using standard calibration plots. The encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulationefficiency}(\%) = \frac{(\text{Totaldrug} - \text{freedrug})}{\text{Totaldrug}} \times 100$$

2.6. Drug release studies

Drug-loaded chitosan nanoparticles were dispersed in 2 mL of phosphate buffered saline (PBS, 0.1 M, pH 7.4) and the entire sample was placed in a dialysis bag, which was then sealed at both the