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Development of core-shell nanocarrier system for augmenting piperine cytotoxic activity against human brain cancer cell line

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ABSTRACT

Brain tumor has a low prognosis with only 15% survival rate (5 years after diagnosis). Many of the current therapeutics have limited activity due to their inability to cross the blood brain barrier which retards drug accumulation in tumor site and causes drug resistance. Piperine, a phytochemical drug with poor solubility, could be an alternative to current therapeutics after evading its solubility and permeability limitations. Piperine micellization was optimized to improve drug solubility. Positively charged trimethyl-chitosan was synthesized then electrostatically adsorbed onto piperine nanomicelles forming core-shell nanoparticles. Physicochemical and morphological characterizations, and in-vitro release were performed. Cytotoxicity on human brain cancer cell line (Hs683) was evaluated using IC50 determination, cell cycle arrest analysis, apoptosis and enzyme-linked immunosorbent assay. Optimum piperine-loaded core-shell nanoparticles were successfully fabricated with double-phase release model. Significant improvement in cytotoxicity than free drug was noted with increasing in G2/M-phase and pre-GI-phase population, apoptotic/necrotic rates and inhibition of CDK2a.

1. Introduction

Cancer is ranked as the leading cause of death in the developed countries and the second leading cause of death in economically developing countries (Jemal et al., 1999). Brain tumor patients are around 3.5 per 100,000 people where about 650 people are diagnosed with a malignant brain tumor daily as reported by Ferlay et al. (2010). However, the main cause of brain tumor is still under investigation (Herholz et al., 2012; Kadam, 2013). Over the last few decades, phytomedicines demonstrated a pivotal role in drug discovery where 50% of FDA approved drugs are of natural origin (Newman and Cragg, 2012). Black pepper (*Piper Nigrum* L.) is a perennial vine grown for its berries, and it is usually used as a spice and medicine. Piperine (PIP) is the major alkaloid in *Piper Nigrum* L. which has a wide range of biological activities including, for instance, being anti-depressant, bioenhancer, antioxidant, apoptosis inhibitor, anti-inflammatory, antihypertensive and particularly anti-tumor, where PIP suppresses tumor growth and metastasis (Elnaggar et al., 2015a, 2015b; Lai et al., 2012). However, the therapeutic applications of PIP are limited because of its immunotoxicity, poor aqueous solubility, and high first pass metabolism. To overcome such limitations, some reported preliminary studies directed the efforts towards the use of nanotechnology (Elnaggar et al., 2015a, 2015b; Tyagi et al., 2011; Yusuf et al., 2013). These nanotechnology-based systems have been employed to overcome the main brain delivery limitations, particularly the blood brain barrier (BBB)-hindered penetration taking into consideration the effect of both particle size and surface charge. For instance, nanoparticles (NPs) with high zeta potential (high positive charge) have shown a toxic effect to the BBB, whereas most of the nanosystems used in the literature for brain delivery were either of moderate negative charge (−1 to −15 mV) or high negative charge (−15 to −45 mV) which shown the ability to cross the BBB (Saraiva et al., 2016).

The aim of this study was to develop new PIP-loaded optimized core-shell NPs to overcome poor drug solubility, enhance its permeability through BBB, and to improve its cytotoxic activity against human brain cancer. The first phase involved PIP nano-micellization using pluronic F127 to optimize solubility, particle size, surface charge and entrapment efficiency. Then, the second phase involved forming PIP-loaded core-shell NPs via coating the PIP-loaded nanomicelles (NMs) with a synthesized positively charged trimethyl chitosan via

Abbreviations: PIP, Piperine; PF127, Pluronic F-127; SDS, Sodium dodecyl sulfate; PVA, Polyvinyl alcohol; CS, Chitosan; TPP, Sodium tripolyphosphate; MTT, Dimethyl sulfate, 3-(4,5-dimethylthiazole-2-yl)-2,5-di-phenyl tetrazolium bromide; DMS, Dimethyl sulfate; DCM, Dichloromethane; NMR, Nuclear magnetic resonance spectroscopy; DSC, Differential scanning calorimetry; DLS, Dynamic light scattering; PS, Particle size; PDI, Polydispersity index; ZP, Zeta potential; SEM, Scanning electron microscopy; TEM, Transmission electron microscopy

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Table 1
Evaluation of uncoated and TMC-coated PF17 nanomicelles (NMs).

Code	Description	PIP:PF127	TMC:NMs	PS (nm) ± SD	PDI ± SD	Zeta potential (mV) ± SD
F1	Plain NMs	(0:5)		147.3 ± 14.8	0.28 ± 0.02	−20.8 ± 1.4
F2	PIP-loaded NMs	(1:5)		209.4 ± 4.6	0.22 ± 0.03	−13.6 ± 1.9
F3		(1:10)		216.8 ± 6.7	0.13 ± 0.03	−19.8 ± 0.4
F4		(1:15)		210.2 ± 13.4	0.43 ± 0.04	−20.5 ± 0.2
F5	TMC NPs		(2.5:0)	122.9 ± 14.6	0.30 ± 0.01	15.4 ± 1.4
F6			(5:0)	109.9 ± 14.4	0.38 ± 0.03	15.3 ± 0.8
F7			(7.5:0)	102.5 ± 2.4	0.29 ± 0.02	17.5 ± 0.2
F8	TMC-coated plain NMs	(0:15)	(2.5:1)	184.2 ± 23.9	0.30 ± 0.01	18.0 ± 1.5
F9		(0:15)	(5:1)	151.7 ± 32.8	0.39 ± 0.06	17.2 ± 1.4
F10		(0:15)	(7.5:1)	136.5 ± 8.2	0.26 ± 0.01	15.4 ± 1.2
F11	TMC-coated PIP-loaded NMs	(1:15)	(2.5:1)	269.5 ± 33.1	0.28 ± 0.02	12.6 ± 0.8
F12		(1:15)	(5:1)	308.6 ± 12.5	0.29 ± 0.02	14.1 ± 1.1
F13		(1:15)	(7.5:1)	361.2 ± 15.4	0.31 ± 0.02	16.4 ± 1.1

electrostatic interaction. The aim of trimethyl chitosan coating was to maintain a positive charge onto the nanocarrier over a wide pH range, confer mucoadhesive properties, and to enhance the permeability through BBB even at neutral pHs. Finally, the cytotoxic activity of the newly developed PIP-loaded core-shell nanosystems on human brain cancer cell line (Hs683) was evaluated via cell viability determination, estimation of IC50, cell cycle arrest analysis, apoptosis assay, and the enzyme-linked immunosorbent assay for CDK2a.

2. Materials and methods

2.1. Materials

Piperine (PIP, molecular weight of 285.34 Da and purity 98%) was purchased from Alpha Aesar (Ward Hill, MA, USA). Erlotinib was purchased from Sigma-Aldrich (Germany). Pluronic F-127 (PF127), sodium dodecyl sulfate (SDS), polyvinyl alcohol (PVA, Mw 13,000–20,000 Da), chitosan (CS) of Mw 260,000 Da, sodium tripolyphosphate (TPP), DMEM (Invitrogen/Life Technologies), FBS (Hyclone), insulin (Sigma), penicillin-streptomycin, trypsin, EDTA solution, ethanol, acetone, dimethyl sulfate,3-(4,5,-dimethylthiazole-2-yl)-2,5-di-phenyl tetrazolium bromide (MTT), dimethyl sulfate (DMS), dichloromethane (DCM) and acetic acid were purchased from Sigma-Aldrich in China and Germany. Brain cancer cell line, Hs683 (ATCC® HTB-138™) was obtained from American Type Culture Collection. Sodium hydroxide, sodium chloride, and dialysis membranes were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water purification was achieved using a Milli-Q system (Millipore).

2.2. Synthesis of trimethyl chitosan

Trimethyl chitosan (TMC) was synthesized according to Zarifpour et al. procedure (Zarifpour et al., 2013). Briefly, 1 g of CS was dissolved in a mixture of DMS and distilled water (4:1) with stirring for about 10 min. Then, a solution of NaOH and NaCl (1.5:1) was added to the CS solution to deprotonate the nitrogen atom which in turn interacts with the methyl groups from DMS. The mixture was left on a stirrer for 6–8 h till the reaction was completed. Afterwards, the final mixture was purified using a dialysis bag (MW cut-off 12 kDa, Severa) for 3–5 days to obtain pure TMC. The synthesized TMC was characterized by Fourier transform infrared (FTIR) spectroscopy and proton nuclear magnetic resonance spectroscopy (^{1}H NMR). The ^{1}H NMR spectrum was measured in D_2O on a 600 MHz spectrometer (Bruker-Biospin, Rheinstetten, Germany). The quaternization degree (DQ) of the synthesized TMC was determined using the standard ninhydrin assay. Briefly, an appropriate amount of CS or TMC was added to 1 mL of sodium acetate/acetic acid buffer solution and 2 mL of 3% ninhydrin agent. The mixture was then heated at 100 °C for 15 min. After cooling to room temperature, the volume of the samples was adjusted to 10 mL using 50% (v/v) ethanol/water. After centrifugation at 10,000 rpm for 15 min, absorbance of the supernatant was measured using UV–Vis spectrophotometry (Evolution UV 600, Thermo Scientific) at 570 nm against a blank reference. The concentration of the amino groups (mmol/mg) of CS and TMC was then determined by referring to a calibration curve that was determined using series of known concentrations of ethylene diamine. Degree of quaternization (DQ) was then calculated as follows:

$$DQ = \frac{[CS - NH2] - [TMC - NH2]}{[CS - NH2]} \times 100$$

2.3. Development of plain and PIP-loaded PF127 nanomicelles

Plain and PIP-loaded PF127 nanomicelles (NMs) were prepared using nanoprecipitation technique (Ali et al., 2016; Basak and Bandyopadhyay, 2013). Briefly, different ratios of PIP and PF127 were used to achieve the suitable size and homogeneity of the micelles, as presented in Table 1 (F1–F4). PIP and PF127 were dissolved in acetone as the organic phase, which was then gradually added to double its volume of distilled water as the aqueous phase. The obtained suspension was stirred till complete evaporation of solvent to produce PF127 NMs powder.

2.4. Development of core-shell nanoparticles via coating of PF127-based NMs

Plain and PIP-loaded core-shell NPs were developed via coating of PF127-based NMs with positively charged TMC (Chen et al., 2012; Sheng et al., 2015). Selected NMs were coated with TMC in such a way to optimize the particles overall size and zeta potential (Table 1: F8–F13). Briefly, NMs suspension was gradually added to TMC solution with stirring for 30 min to ensure the deposition of the TMC layer over the NMs followed by dropwise addition of TPP aqueous solution to the mixture while stirring for 30 min at TPP:TMC ratio of 1:10. The nanoparticles were then separated by centrifugation for 30 min at 20,000 rpm at 4 °C followed by lyophilization to obtain dried coated NMs (core-shell NPs). Similar procedures were repeated but without NMs to obtain crosslinked TMC NPs as control formulations (Table 1: F5–F7).

2.5. In-vitro characterization of the developed NMs

Particle-size, polydispersity-index and zeta-potential were measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25 °C. Morphological characterization was performed using SEM (Nona Nano SEM, FEI, USA). Also, HR-TEM (JEM-2100F; JEOL, USA) was used to visualize the shape and size of the particles. The specimen was viewed under the microscope at 10–100 k-fold enlargements at an accelerating voltage of 100 kV. Attenuated total reflectance (ATR) spectroscopy was