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Co-encapsulation of dual drug loaded in MLNPs: Implication on sustained drug release and effectively inducing apoptosis in oral carcinoma cells

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ABSTRACT

Combinations of natural bee wax flavones chrysin with a chemo drug have been exhibiting high potential with reduced adverse effect. To extend the synergistic effect of chrysin and improve the MLNPs (Multi Layer Nanoparticles) performance in drug release, layer-by-layer of poly [di(sodium carboxyphenoxy)phosphazene] (PDCPP) and poly (diallyldimethyl ammonium chloride) (PDADMAC) deposited on the $CaCO_3$ nanoparticles (CCNPs) surface. The results suggest spherical MLNPs of 237 nm are formed with high drug loading content with enhanced cellular uptake. Under acidic conditions, multi layer structure effectively controls burst release, providing sustained drug release for long period. The combined effect of chrysin and cisplatin improved the cytotoxic potential of MLNPs at 25 $\mu g.mL^{-1}$ concentration. Angiogenesis inhibitor chrysin activates reactive oxygen species (ROS) production and eventually leads to mitochondrial dysfunction. Furthermore, significant decreases of buccal pouch carcinoma in hamster model. Dual drug loaded MLNPs achieves 92% regressions of tumor volume as compared to cisplatin alone loaded MLNPs. In addition, Histopathology studies demonstrated the biocompatible effect of MLNPs on vital organs. This work provides a simple method to formulate multiple drugs in single nanosystem with high therapeutic efficacy on oral cancer.

1. Introduction

For cancer therapy, nanocarriers provide an opportunity to enhanced permeability and retention (EPR) effect; accumulate the drug at targeted sites, to sustain in blood circulation and reduce off-target delivery. In spite of these desirable features, certain drawback such as uncontrolled release, poor cellular uptake, inefficient tumor penetration and lack of perinuclear drug release need to be addressed. Initially, nanoparticles fabricate for functions like cross barriers to deliver the loaded drug at target sites. For instance, Multilayer nanoparticles (MLNPs) can maximize the efficacy by controlling properties like thickness, composition, roughness, porosity offering biomedical and pharmaceutics applications [1–5]. MLNPs were assembled via electrostatic interactions of anionic/cationic polyelectrolytes (PELs) [6–8]. Several strategies like seeded emulsion, dispersion polymerization, hetero-coagulation and layer-by-layer deposition are available for the development of multilayer structures [9–14]. High amount of drug loaded MLNPs response to the acidic stimuli, which could increase the release of drug at targeted site and biocompatibility nature [15]. Amphoteric MLNPs are eventually protonated at the acidic pH, which destabilizes the multilayer resulting in the release of the therapeutics at the targeted site. It is paramount fact to be considered for an ideal drug delivery system with polymer superior protection and minimize premature drug release using covalent links [16,17]. At present most of MLNPs are dominated by polymers like Poly (diallyl-dimethyl-ammonium chloride), poly (ethyleneimine), poly (allylamine) hydrochloride, poly(styrenesulfonate), poly(vinylsulfate) and poly(acrylic acid) or natural PELs like alginate or chitosan are used for layer-by-layer deposition techniques [18,19]. On the other hand, responsiveness of MLNPs with desirable size range was achieved by ionic strength, pH of the solution, polyelectrolytes concentration [20,21]. However, MLNPs was encapsulated with combinational drug were rarely reported up to now. This modular design enables the development of a core encapsulated with multiple drugs. In this, Cisplatin and Chrysin were incorporated in the porous mineralized $CaCO_3$ nanoparticles (CCNPs) core and polymeric shell. Calcium carbonate is the most abundant

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inorganic particle, which has been well utilized as a template core for the preparation of multi layer nanoparticles. Compared with other porous materials, $CaCO_3$ is cost effective with no addition of polymer additive, enabling it to be embedded into functional molecules [22]. CCNPs were able to controlled release of anticancer drugs, such as doxorubicin (DOX) and 5- fluorouracil, in response to acidic pH and also efficiently deliver therapeutic proteins to cancer cells. Bee-related natural products such as honey and wax have high therapeutic applications. Chrysin (5, 7- dihydroxyflavone) is a one of the natural bioactive dietary flavone abundantly found in bee wax and it has medicinal properties such as anticancer, anti-inflammatory, antioxidant, hepatoprotective, antimicrobial and anti-diabetic effects [23]. More evidence suggests that combination of natural compound with anticancer drug deliver via nanocarriers shows synergistic drug action and promising therapeutical approach [24,25].

The present study describes the detailed mechanistic aspects of assembly of MLNPs, and their physicochemical characterization to selectively target cancer cells and *in vivo* model. To achieve this, MLNPs were fabricated with poly[di(sodium carboxyphenoxy)phosphazene] (PDCPP) - poly (diallyl dimethyl ammonium chloride) (PDADMAC) alternative layer on CCNPs surface. Using this depiction, chrysin/cisplatin was encapsulated in the core shell layers, to form multi drug delivery system. In addition, comparison of single and dual drug loaded MLNPs in sustained drug release, cytotoxicity were performed in KB cells. Finally, *in vivo* hamster model showed tumor regression and biocompatibility efficiency of MLNPs.

2. Experimental

2.1. Materials

Cisplatin, chrysin and poly (diallyl dimethyl ammonium chloride), Hexachloro- cyclotriphosphate, Propyl-p-hydroxybenzoate, hydroxylamine, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), acridine orange (AO), ethidium bromide (EB), propidium iodide (PI), Hoechst 33342, Fluorescein isothiocyanate (FITC), Dichlorodihydrofluorescein-diacetate (DCHF-DA), and rhodamine-123 were supplied by Sigma-Aldrich. HClNP, $CaCl_2$, $NaHCO_3$ was obtained from Himedia Laboratories Ltd. All other chemicals were of reagent grade and used without further purification. Human oral cancer cell lines were procured from National Centre for Cell Science (NCCS), Pune, India. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution was used as the cell culture medium. Cells were grown in a humidified environment at 37 °C with 5% CO_2.

2.2. Synthesis of poly[di(sodium carboxyphenoxy)phosphazene] (PDCPP)

Poly(dichlorophosphazene) was prepared by thermal bulk polymerization of hexachloro cyclotriphosphate. Briefly, 0.1 g of hexachlorocyclotriphosphate was dissolved in dichloromethane (DCM) and chlorophosphoranimine was added to the mixture under magnetic stirring for 4 h. Then, DCM was removed under reduced pressure to form poly (dichlorophosphazene). Propyl-p- hydroxybenzoate (0.3 M) was prepared by dissolving in diglyme and heated at 110 °C for 30 min under the nitrogen atmosphere. The resulting solution was slowly added to sodium hydride suspension to form sodium salt of propyl-p-hydroxybenzoate. Finally, Poly(dichlorophosphazene) (1.12 g) was slowly added to the above mixture, temperature annealed at 120 °C for 10 h and reaction temperature was reduced to 85 °C and 0.3 L of potassium hydroxide was added and allowed to 2 h. The synthesized polymer was further precipitated by adding 0.6 L of sodium chloride (30%) solution. The molecular weight (Mw) and polydispersity index (PDI) of the synthesized polymer was measured by gel permeation chromatography (RID 20 A, Shimadzu, Japan).

2.3. Preparation of drug loaded $CaCO_3$ nanoparticles coated by multilayer polymer

Cisplatin and chrysin-loaded CCNPs were prepared at room temperature with some modification [21]. For encapsulation of drug cisplatin was dissolved in 0.9% saline solution and chrysin was dissolved in water: ethanol mixture of 1:0.5 ratios. This dissolved drug solutions of 5 $mg.mL^{-1}$ was mixed with 0.01 M $CaCl_2$ solution and the resulting mixture was added drop wise to 0.01 M Na_2CO_3 solution and vigorous mixing at different (800, 1400 and 2000) rpm for 12 and 24 h. After centrifugation, drug loaded CCNPs were suspended in 0.5 M NaCl solution and were then added to poly (diallyldimethylammonium chloride) (PDADMAC) (1 mM) solution for 30 min. The excess of polyelectrolyte solution was removed by two times centrifugation (12,000 rpm × 15 min) and washing with deionized water. Subsequently, CCNPs coated by a monolayer of polymers were re-suspended in poly[di(sodium carboxyphenoxy)phosphazene] (PDCPP) for 30 min, followed by two times centrifugation and washing.

2.4. Characterization

FTIR spectral analyses of the samples were carried out (FTIR, 27 series, Bruker, USA) by mixing MLNPs and KBr in the mass ratio of1:100 (w/w) was scanned from 400-4000 cm^{-1} with a resolution of 4 cm^{-1}. The average size, size distribution, and zeta potential of MLNPS in different pH were monitored by dynamic light scattering (Delsa TM Nano, Malvern, UK). Topography of the nanoparticles was recorded using Scanning Electron Microscope (VEGA3SB, TESCAN, Czech Republic). High-Resolution Transmission Electron Microscope (HR-TEM, Model Tecnai- Philips F30, FEI Co., Hillsboro) were measured for the samples diluted to 1:10000 ratios under the accelerating voltage of 220 kV in various magnifications.

2.5. Determination of drug loading and encapsulation efficiency

To determine drug loading, 10 mg of drug loaded MLNPs was dissolved in 5 mL of acetone; 10 mL of phosphate-buffered solution buffer (pH 7.4) was added, and the solution was gently heated to evaporate the solvent. The solution obtained was filtered and the concentration of cisplatin/chrysin was measured using an ultraviolet spectrophotometer (UV1800, Shimadzu, Japan) at 310 nm (for cisplatin) and 348 nm (for chrysin). For calculating the encapsulation efficiency, another 10 mg of MLNPs was dispersed in 10 mL of phosphate buffer solution under vigorous shaking on a vortex mixer (Neuation vortex mixer, India) at room temperature. The polymeric debris were removed by centrifugation (12,000 rpm, REMI, India). Then, the supernatant was taken and analyzed with a spectrophotometer (UV-1800, Shimadzu, Japan) at 310 nm (for cisplatin) and 348 nm (for chrysin). Drug loading and encapsulation efficiency were calculated using the following two equations

$$\text{Drug loading (\%)} = (\text{Wt. drug in nanoparticles/Wt. feeding polymer \& drug}) \times 100 \quad (1)$$

$$\text{Encapsulation efficiency (\%)} = (\text{Wt. drug in nanoparticles/Wt. feeding drug}) \times 100 \quad (2)$$

2.6. Drug release

Cisplatin/chrysin loaded MLNPs was placed in a dialysis membrane (MW: 10,000 Da) and dialyzed against 15 mL phosphate buffer solutions and sodium acetate solution. The release reservoir was kept at constant stirring, and at predetermined time intervals 4 mL of solution was taken for investigation. Simultaneously, 4 mL of fresh buffer solution was replaced to maintain the solution quantity and identical pH value. The amount of cisplatin/chrysin release was analyzed using a