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In situ DOX-calcium phosphate mineralized CPT-amphiphilic gelatin nanoparticle for intracellular controlled sequential release of multiple drugs

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ABSTRACT

A co-delivery strategy has been developed to achieve the synergistic effect of a hydrophobic drug (camptothecin, CPT) and a hydrophilic drug (doxorubicin, DOX) by utilizing the unique structure of amphiphilic gelatin/camptothecin @calcium phosphate–doxorubicin (AG/CPT@CaP–DOX) nanoparticles as a carriers in order to replace double emulsions while preserving the advantages of inorganic materials. The hydrophobic agent (CPT) was encapsulated via emulsion with an amphiphilic gelatin core, and subsequently mineralized by CaP–hydrophilic drug (DOX) through precipitation to form a CaP shell on the CPT–AG amphiphilic gelatin core so that drug molecules with different characteristics (i.e. hydrophobic and hydrophilic) can be encapsulated in different regions to avoid their interaction. The existence of the CaP shell can protect the DOX against free release and cause an increased transfer of DOX across membranes, overcoming multidrug resistance. Release studies from core–shell carriers showed the possibility of achieving sequential release of more than one type of drug by controlling the pH-sensitive CaP shell and degradable AG core. The highly pH-responsive behavior of the carrier can modulate the dual-drug-release of DOX/CPT, specifically in acidic intracellular pH environments. The AG/CPT@CaP-DOX nanoparticles also exhibited higher drug efficiencies against MCF-7/ADR cells than MCF-7 cells, thanks to a synergistic cell cycle arrest/apoptosis-inducing effect between CPT and DOX. As such, this core–shell system can serve as a general platform for the localized, controlled, sequential delivery of multiple drugs to treat several diseases, especially for multidrug-resistant cancer cells.

1. Introduction

Although several drug-delivery systems have been used to improve the efficacy of cancer therapy, this disease is still one of the deadliest known to humans. Recently, synergistic combinations of different drugs with different physiochemical properties have been recognized as a realistic route toward overcoming multidrug resistance (MDR) and other undesired effects [1]. Cancer's MDR is responsible for the high recurrence rate and the ultimate failure of chemotherapy. The most frequent causes of MDR include the overexpression of the ATP-binding cassette (ABC) superfamily of transporters, transmembrane proteins that act as drug-efflux pumps by actively removing drugs from cells and decreasing intracellular drugs to levels below the concentrations required for cytotoxicity [2]. Co-delivery systems for cancer therapy have been the subject of numerous investigations over the past decades, including organic frameworks such as liposomes [3–5], polymer–drug conjugates [6,7], organic polymer carriers [8–10] and inorganic mesoporous nanoparticles [11,12]. Unlike single-therapeutic-agent therapy, multiple-agent therapy can modulate different signaling pathways in cancer cells, maximizing the therapeutic effect in order to overcome the mechanisms of MDR [13,14]. For example, a carrier containing paclitaxel and carboplatin has been widely used for the treatment of ovarian and lung cancer [15]. The versatility and flexibility of these delivery vehicles allows the simultaneous encapsulation of multiple types of drugs for use in cocktail therapy, particularly when both different chemotherapeutic drugs and biologics are involved. Although the application of combinational drug treatments has demonstrated efficacy in the treatment of tumor cells, many of the single-carrier scaffolds are not readily amenable to control ratiometric delivery and synchronized release of multiple drugs. It is also noted that the problems raised by the molecular complexity of drugs (in particular for cancer, cardiovascular diseases, neurological disorders, malaria and AIDS) need to be

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overcome [1,16–18]. In the hope of overcoming these drawbacks, inorganic material systems have garnered increasing attention in nanomedicine due to their highly stable physicochemistry and biochemistry [19]. In particular, calcium phosphate (CaP) has a number of attractive properties, such as biocompatibility, the capacity to carry a variety of therapeutic agents and pH-dependent biodegradable character, which is advantageous for controlled drug release and intracellular delivery [20]. A capable carrier equipped with both the characteristics of organic and inorganic materials has the potential to act as a multiple functional co-delivery system, and may effectively inhibit MDR.

In this study, a biodegradable organic/inorganic core–shell co-delivery system with sequential release was proposed for the controlled release of multiple drugs (co-delivery of doxorubicin (DOX) and camptothecin (CPT)) in the hope of enhancing anti-tumor efficacy. We employed a degradable amphiphilic gelatin (AG), developed in this laboratory, to encapsulate the hydrophobic agent, CPT [21]. The inorganic CaP shell acts as a reservoir for the hydrophilic drug and was deposited by the coprecipitation of CaP on the core [22]. This layer will enable efficient encapsulation and intracellular delivery of hydrophilic anticancer drugs, depending on the surface charge of the individual drug molecules. CPT and DOX were used as model drugs due to their different property and non-overlapping cytotoxic profiles. DOX interferes with the religation reaction of topoisomerase II, whereas CPT acts as an inhibitor of topoisomerase I. In both cases, CPT and DOX have been identified to interfere with DNA topoisomerases thereby causing DNA damage [18]. Specific elements of the design of the AG/CPT@CaP-DOX nanoparticles enable the co-delivery of multiple drugs, and offer the following advantages: (i) DOX was encapsulated by CaP to avoid drug-efflux pumping by transmembrane proteins; (ii) co-precipitation with CaP protects the DOX against free release; (iii) drug molecules with different characteristics (i.e. hydrophobic CPT and hydrophilic DOX) can be encapsulated in different regions to avoid their interaction; and (iv) the pH-sensitive CaP shell and degradable AG core can release both drugs in a controlled sequential fashion. The enhanced therapeutic effects of co-loaded drug combinations in AG/CPT@CaP-DOX nanoparticles were demonstrated by their cytotoxicity against drug-resistant cells.

2. Materials and methods

2.1. Materials

Gelatin type A (Bloom 300), hexanol anhydride, calcium chloride, ammonium dihydrogen phosphate and sodium hydroxide were supplied by Sigma. Fluorescein isothiocyanate (FITC, Sigma) was used to label the nanoparticles for visualization under a fluorescence microscope. DOX hydrochloride was used as the hydrophilic drug and was obtained from Sigma. CPT was used as the hydrophobic drug and was also obtained from Sigma.

2.2. Synthesis of amphiphilic gelatin molecules

AG was previously developed in this laboratory [11]. Briefly, 1.25 g of gelatin was taken up in 20 ml of water, gently mixed with 2 ml of a 0.1 N NaOH solution, and stirred for 0.5 h at 70 °C. Subsequently, 4 ml of hexanol anhydride was added to 20 ml of gelatin hydrolyzate with stirring at 70 °C. After 5 h, the mixture was cooled to room temperature and adjusted with dilute NaOH to a pH value of 7.4. The resulting solutions were collected by dialysis tubing cellulose membrane, after dialysis with an ethanol solution (25% v/v) for 24 h. The gel was then dried in an oven at 60 °C to yield the powdered product.

2.3. Synthesis of AG/CPT nanoparticles

To prepare the AG/CPT nanoparticles, 1.5 mg CPT was dispersed in 0.5 ml chloroform to form a uniform organic phase. Then, 100 mg AG (a polymer binder) was dissolved in 2 ml of deionized (DI) water. After the AG was completely dissolved, the organic phase was added into the reaction solution. The mixture was emulsified for 1 min with an ultrasonicator at 50 W. During ultrasonication, the mixture was heated to evaporate the organic solvent. Afterwards, the mixture was stirred and heated again at 50 °C on a hot plate to ensure complete removal of the organic phase. The final products were washed three times with DI water and centrifuged at 13,281*g* for collection of the precipitate.

2.4. Synthesis of AG/CPT@CaP nanoparticles

DI water (5 ml) was mixed with 60 μl of 0.1 M $(NH_4)H_2PO_4$ and 100 μl of 0.1 M NaOH (pH 10). To this mixture, 100 μl of 0.1 M $CaCl_2$ and 2 mg of AG/CPT nanoparticles, prepared as described above, were added consecutively, while the suspension was stirred at room temperature (typically 25 °C). The suspension was stirred for an additional 30 min before analysis and storage. The final product was collected via centrifugation at 13,281*g* and decantation of the supernatant liquid. The sample was redissolved in DI water and stored at 4 °C.

2.5. Characterization of nanoparticles

The morphologies of the different nanoparticles were examined by transmission electron microscopy (TEM, JEM-2100, Japan) and scanning electron microscopy (S6500, JEOL, Japan). Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter (d, nm), and laser Doppler anemometry (LDA) was used to determine the zeta potential (mV). The DLS and LDA analyses were performed using a Zetasizer 3000ES (Malvern Instruments, Malvern, UK). The X-ray diffraction (XRD) patterns of the different nanoparticles were collected with an X'Pert Powder X-ray diffractometer (PANanalytical, the Netherlands) equipped with a Cu K_α radiation source (wavelength = 1.5405 Å) from 20° to 70°. Energy-dispersive X-ray spectroscopy (EDX) was used to calculate the Ca/P molar ratio of the AG/CPT@CaP nanoparticles.

2.6. Synthesis of AG/CPT@CaP-DOX nanoparticles

DI water (5 ml) was mixed with 60 μl of 0.1 M $(NH_4)H_2PO_4$ and 100 μl of 0.1 M NaOH (pH 10). To this solution was added 100 μl of 0.1 M $CaCl_2$, 2 mg of AG/CPT nanoparticles and 2 ml of a 1.5 mg ml^{-1} DOX solution while the suspension was stirred at 400 rpm at room temperature (typically 25 °C). After 1 h, the suspension was washed three times with DI water and centrifuged at 13,281*g* to collect the products. Free DOX was removed by centrifugation at 13,281*g* at 4 °C for 10 min. The drug concentration in the supernatant was analyzed with reference to a calibration curve using ultraviolet (UV) absorption at 490 nm, a strong absorption band of DOX, on an Agilent 84531 UV–vis spectrometer. High-performance liquid chromatography (HPLC) was used to determine amount of CPT released. An aliquot of 10 μl of the supernatant solution was injected into the HPLC for CPT analysis. The mobile phase consisted of acetonitrile and 1% citric acid solution (pH 3 adjusted with 1 M NaOH solution) (50:50, v/v) and was delivered at 1 ml min^{-1} on an Agilent Zorbax column (C18, particle size 5 μm, 4.6 mm × 150 mm). CPT was quantified by UV detection at 366 nm.

The encapsulation efficiency (EE) was obtained using the following equation:

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