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Calcium phosphate-phosphorylated adenosine hybrid microspheres for anti-osteosarcoma drug delivery and osteogenic differentiation

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ABSTRACT

Biocompatibility, biodegradability and bioactivity are significantly important in practical applications of various biomaterials for bone tissue engineering. Herein, we develop a functional inorganic-organic hybrid system of calcium phosphate-phosphorylated adenosine (CPPA). Both calcium phosphate and phosphorylated adenosine molecules in CPPA are fundamental components in mammalians and play important roles in biological metabolism. In this work, we report our three leading research qualities: (1) CPPA hybrid microspheres with hollow and porous structure are synthesized by a facile one-step microwave-assisted solvothermal method; (2) CPPA hybrid microspheres show high doxorubicin loading capacity and pH-responsive drug release properties, and demonstrate positive therapeutic effects on six osteosarcoma cell lines *in vitro* and a mouse model of 143B osteosarcoma subcutaneous tumor *in vivo*; (3) CPPA hybrid microspheres are favorable to promote osteogenic differentiation of human bone mesenchymal stem cells (hBMSCs) by activating the AMPK pathway, with satisfactory evidences from cellular alkaline phosphatase staining, alizarin red staining, real time PCR and western analysis. The as-prepared CPPA hybrid microspheres are promising in anti-osteosarcoma and bone regeneration, which simultaneously display excellent properties on drug delivery and osteogenic differentiation of hBMSCs.

1. Introduction

Osteosarcoma is one of the most common aggressive neoplasms which arise from mesenchymal cells. This malignant tumor is prevalent in children and adolescents, occurring around the joints, such as the knee, hip, and shoulder [1–3]. A complete radical resection is a prior choice for the treatment of this cancer [2]. However, it was difficult to attain the en bloc resection in most cases during the surgery. Thus, postoperative chemotherapy is critical for the destruction of the remaining cancer cells and prevention of cancer recurrence. Moreover, chemotherapy with greater precision, focusing on intra-tumor administration, could boost local drug concentration and in turn enhance the therapeutic effect, while reducing the risks of systemic side effects of systemic chemotherapy administration. Meanwhile, the surgical therapy of osteosarcoma usually leads to a bone defect which needs necessary repair or reconstruction. Although autologous bone graft is the preferred choice for restoring bone defects, it is also limited by the donor sites and the long-time bone absorption before repairing [4]. Thus, synthetic biomaterial, like calcium phosphate ceramics and cement [5,6], may provide good alternatives for the treatment of the bone defects.

Biomaterials with high biocompatibility, biodegradability and bioactivity are promising in practical applications of bone tissue engineering [7]. Calcium phosphate based materials are popular nonmetal inorganic biomaterials with good biocompatibility and bioactivity, which could enhance osteoinduction and bone formation by improving alkaline phosphatase and enhancing the osteopontin synthesis without obvious cytotoxicity [8,9]. Amorphous calcium phosphate (ACP) with its unique structure can be

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biodegraded by cells, making it an ideal biomaterial in its use as an additive to orthodontic cement to promote bone defect repair [10,11]. Also, the ACP could effectively restrain a weak acid environment and aseptic inflammation caused by the absorption of the biodegradable polyester. So it could work as a good component for tissue engineering scaffold and coating for screw and steel used for bone surgery [12]. Furthermore, the ACP particles can also be used in drug delivery, due to its adjustable degradation rate and high biocompatibility [13,14]. Therefore, ACP could be an ideal candidate as a multifunctional delivery system for postoperative chemotherapy and bone defect reconstruction.

Adenosine, in forms of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) play an important role in energy transfer in various processes of metabolism. Adenosine also displays a significant influence on bone metabolism, and modulates osteoblast and osteoclast differentiation of human bone marrow cells, making a possibility in the potential application for treatment and prevention of multiple myeloma-associated bone disease [15,16]. Considering the merits of adenosines, we suppose that the adenosine-based hybrid materials may lead to a functional system in drug delivery and bone tissue engineering.

Herein, we developed a microwave-assisted solvothermal strategy for a one-step synthesis of calcium phosphate-phosphorylated adenosine (CPPA) hybrid microspheres. The CPPA hybrid microspheres showed a high doxorubicin (DOX) loading capacity and pH-sensitive drug release properties, which performed enhanced *in vitro/vivo* therapeutic efficiency for various osteosarcoma cell lines and subcutaneous tumors in nude mice. Furthermore, we found that the CPPA shows excellent properties for promoting osteogenic differentiation of human bone mesenchymal stem cells (hBMSCs). The CPPA hybrid hollow microsphere could work as a promising biomaterial with dual functions, drug delivery system as DOX-loaded CPPA/DOX microsphere for *in-situ* anti-osteosarcoma therapy and osteogenic induction system as CPPA microsphere itself for subsequent bone regeneration.

2. Experimental section

2.1. Synthesis and characterization of CPPA hybrid microspheres

2.1.1. Synthesis of CPPA hybrid microspheres

For the synthesis of CPPA hybrid microspheres, 1 mL of NaOH solution (1 M, Sinopharm, China) was added into a mixture of $CaCl_2$ solution (50 mM, 20 mL, Sinopharm, China) and ethanol (2 mL, Sinopharm, China) under magnetic stirring, then 18 mL of adenosine triphosphate disodium (33 mM, Sigma, USA) solution was added to the above mixture. After stirring for 10 min, the above solution was transferred into an autoclave (60 mL) and heated at 110 °C for 10 min in a microwave oven (MDS-6, Sineo, China). After cooling to the room temperature, the product was collected by centrifugation, washed three times with deionized water, and freeze-dried at −50 °C. The control sample synthesized without using ethanol was also prepared and labeled as CPPA-C.

2.1.2. Synthesis of hydroxyapatite nanorods

The hydroxyapatite nanorods (HANRs) can be easily prepared by a coprecipitation method and have been widely studied in biomedical studies. Therefore, the HANRs were chosen in this study as a control sample. For the synthesis of the control sample of HANRs, 200 mL of $Na_2HPO_4{\cdot}12H_2O$ (0.12 M, Sinopharm, China) solution was added dropwise to 500 mL of $CaCl_2$ (0.08 M, Sinopharm, China) solution under mechanical stirring at room temperature. Meanwhile, the pH value of the above solution was maintained at pH 11 by slowly adding the NaOH solution (1 M, Sinopharm, China). Then the above solution was continued to stir for 10 h under mechanical stirring at 70 °C water bath. The product was collected, washed with deionized water, and freeze-dried at −50 °C.

2.1.3. Characterization

The measurements of the dynamic light scattering (DLS) and zeta potential of the as-prepared products were taken on a zeta potential analyzer (ZetaPlus, Brookhaven Instruments). The morphology of the products was characterized by field-emission scanning electron microscope (SEM, FEI Magellan 400, USA) and transmission electron microscope (TEM, Hitachi H-800, Japan). The phases and compositions of the products were characterized by X-ray diffractometer (Rigaku D/max 2550 V, Cu Kα radiation, $\lambda = 1.54178$ Å), FTIR spectrometer (FTIR-7600, Lambda Scientific, Australia) and STA 409/PC simultaneous thermogravimetric analyzer (TG, STA 409/PC, Netzsch, Germany, heating rate 10 °C min^{-1}, air atmosphere). The Brunauer–Emmett–Teller (BET) specific surface areas (SBET) and pore size distribution of the products were analyzed by a specific surface area and pore size analyzer (V-sorb 2800P, Gold APP, China). The UV–vis spectrophotometer (UV-2300, Techcomp) was used to measure the concentration of doxorubicin hydrochloride at a wavelength of 480 nm. An inductively coupled plasma (ICP) optical emission spectrometer (JY 2000-2, Horiba, France) was used to measure the Ca and P elements released from the products.

2.2. In vitro DOX drug loading/release and degradation

2.2.1. In vitro DOX drug loading and release

CPPA hybrid microspheres (200 mg) were immersed in 30 mL of doxorubicin hydrochloride (3.5 mM, Shanghai Aladdin Reagent Co Ltd, China) solution, followed by ultrasonic treating for 5 min and shaking in a sealed vessel at a constant rate (120 rpm) at 37 °C for 24 h. Then, the DOX-loaded CPPA hybrid microspheres (CPPA/DOX) were collected by centrifugation and freeze-dried at −50 °C. The DOX encapsulation efficiency and loading capacity of the CPPA hybrid microspheres was determined by the DOX concentrations of the DOX solution (diluted 40 times) before and after DOX loading. Encapsulation efficiency $= (M_0 - M_1)/M_0 \times 100\%$, and loading capacity $= (M_0 - M_1)/M_S$, where M_0 is the total mass of DOX, M_1 is the mass of DOX in the supernatant and M_S is the mass of the CPPA sample. For the drug release experiments, 5 mg of the CPPA/DOX powder was soaked in the phosphate buffer solutions (PBS, 8 mL) with different pH values of 7.4, 6.0 and 4.5, respectively, and shaken with constant shaking speed (120 rpm) at 37 °C. 0.5 mL of the supernatant was extracted at given time intervals and replaced with the same volume of fresh PBS with the same pH value. The released DOX in the supernatant was measured by a UV–vis spectrophotometer at a wavelength of 480 nm.

2.2.2. Degradation of CPPA

CPPA hybrid microspheres (18 mg) were immersed in 30 mL of normal saline and acetic acid-sodium acetate buffer solutions with different pH values (pH 6.0 and 4.5) and shaken with constant shaking speed (140 rpm) at 37 °C in a desk-type constant-temperature oscillator (THI-92A, China). At given time intervals, 0.5 mL of the supernatant was extracted for ICP analysis and replaced with the same volume of fresh solutions.

2.3. In vitro cellular studies

2.3.1. Cell culture

Human osteosarcoma cells, 143B, MG63, U2Os and Saos2, and the osteoblast cells, hFOB 1.19, were purchased from American Type