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Calcium phosphate nanoparticles functionalized with alendronate-conjugated polyethylene glycol (PEG) for the treatment of bone metastasis

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ABSTRACT

Because of the peculiarity of the bone microstructure, the uptake of chemotherapeutics often happens at non-targeted sites, which induces side effects. In order to solve this problem, we designed a bone-targeting drug delivery system that can release drug exclusively in the nidus of the bone. Alendronate (ALN), which has a high ability to target to hydroxyapatite, was used to fabricate double ALN-conjugated poly (ethylene glycol) 2000 material (ALN-PEG_{2k}-ALN). The ALN-PEG_{2k}-ALN was characterized using ^{1}H NMR and ^{31}P NMR and FTIR. ALN-PEG_{2k}-ALN-modified calcium phosphate nanoparticles (APA-CPNPs) with an ALN targeting moiety and hydrophilic poly (ethylene glycol) arms tiled on the surface was prepared for bone-targeted drug delivery. The distribution of ALN-PEG_{2k}-ALN was tested by X-ray photoelectron spectroscopy. Isothermal titration calorimetry data indicated that similar to free ALN, both ALN-PEG_{2k}-ALN and APA-CPNPs can bind to calcium ions. The bone-binding ability of APA-CPNPs was verified via ex vivo imaging of bone fragments. An in vitro release experiment demonstrated that APA-CPNPs can release drug faster in an acid environment than a neutral environment. Cell viability experiments indicated that blank APA-CPNPs possessed excellent biocompatibility with normal cells. Methotrexate (MTX) loaded APA-CPNPs have the same ability to inhibit cancer cells as free drug at high concentrations, while they are slightly weaker at low concentrations. All of these experiments verified the prospective application of APA-CPNPs as a bone-targeting drug delivery system.

1. Introduction

Because of the pecularity of the physiological environment, the skeleton is the most common organ to be affected by metastatic cancer (Breuksch et al., 2016; Mundy, 2002; Roodman, 2004; Suva et al., 2011). According to the 2011 statistic data by the American Academy of Orthopedic Surgeons (AAOS), about half of the 1.2 million cancer cases that occur every year come with bone metastasis (H. and MD., 2011). Among these cancers, breast and prostate cancers have the highest prevalence in bone metastasis (Miller et al., 2013). The clinical therapies for bone metastasis concentrate mainly on surgical resection, radiotherapy and chemotherapy (Chen et al., 2012). However, these therapies are not being fully utilized because of the uptake of chemotherapy agents in non-targeted sites and their related side effects (Clines and Guise, 2008). Therefore, designing a bone-targeting drug delivery system that can release drug exclusively in the nidus of the bone would be significant.

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is specific to the skeleton (Choi and Kim, 2007b; Uskokovic and Uskokovic, 2011). Bone diseases such as cancer metastasis result in the exposure of hydroxyapatite to the blood. The hydroxyapatite that is exposed around the metastasis site can be regarded as an ideal target for drug carriers. Because of their unique affinity for the inorganic component hydroxyapatite, molecules such as tetracyclines, bisphosphonates, and acidic oligopeptides have been used as a targeted group to target bone tissue (Fu et al., 2014). When compared to acidic oligopeptides, Wang et al. found that

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bisphosphonates, with a hydroxyl group and two phosphate groups, can bind to all types of hydroxyapatite, while acidic oligopeptides binds preferentially to a higher crystalline hydroxyapatite (Wang et al., 2006). Meanwhile, bisphosphonates are widely used for their binding capability and for their profound effect on osteoporosis. Nitrogen-containing bisphosphonates, such as alendronate (ALN) and zoledronate, inhibit farnesyl pyrophosphate synthase activity and disrupt the mevalonic acid pathway. Osteoclasts lose their function and undergo apoptosis without the mevalonic acid pathway (Low and Kopeček, 2012). The anti-osteoporosis property makes bisphosphonates important drugs for the clinical therapy of cancer metastasis-induced bone osteoporosis or facture. Additionally, bisphosphonates are in demand in the design of targeted drug carriers. Kiran et al. built zoledronate-anchored PLGA-PEG nanoparticles with docetaxel inside. The PLGA-PEG-zoledronate NPs showed enhanced apoptotic activity and higher retention at the bone site (Ramanlal Chaudhari et al., 2012). Laura et al. synthesized poly(γ-benzyl-L-glutamate)-PEG_{6k}-alendronate ($PBLG_{10k}$-PEG_{6k}-ALN), which can form nanoparticles spontaneously and has a strong affinity for hydroxyapatite (de Miguel et al., 2014b).

For clinical applications, amorphous calcium phosphate sediments exhibit similar properties to hydroxyapatite. With good biocompatibility, biodegradability and osteoconductivity, it can stimulate tissue regeneration and has been widely used as a bone repairing material in the areas of orthopedics and dentistry (Boskey, 1997; Zhao et al., 2011). Peter et al. found that the peri-implant bone density was increased after grafting zoledronate to a hydroxyapatite coating on titanium implants (Peter et al., 2005). Grover et al. similarly concluded that the presence of pyrophosphate in calcium phosphate cements appeared to stimulate the mineralization of the healing bone around the implant (Grover et al., 2013). Because of its excellent biocompatibility and unique physical properties, today it is used as drug carrier for DNA (Tobin et al., 2013; Zhang et al., 2010), siRNA (Xu et al., 2014), anti-bacterial drugs (Chen et al., 2014) and anti-cancer drugs (Iafisco et al., 2009; Mukesh et al., 2009).

Inspired by these ideas, a novel bone-targeting nanoparticle with high biocompatibility and stability was designed and prepared in this study. We conjugated ALN to each side of polyethylene glycol 2000 (PEG_{2k}) via an amide bond to obtain the novel hydrophilic material ALN-PEG_{2k}-ALN. This material, with good biocompatibility (Pedraza et al., 2008), was designed to anchor on the surface of calcium phosphate nanoparticles in order to control the diameter (less than 40 nm) and give the nanoparticles the ability to target bone as well as prolong the circulation time of NPs in the vascular system. These ALN-PEG_{2k}-ALN (APA)-modified calcium phosphate (CP) nanoparticles (NPs) were designated APA-CPNPs. With ALN-PEG_{2k}-ALN anchored on the surface, this nanoparticle showed obvious binding ability to bone tissue, which made it a promising drug delivery system. An in vitro release experiment showed that APA-CPNPs slowly release drug in normal physiological conditions while releasing drug quickly in cancer metastasis sites due to the acidic environment induced by cancer cells. The anticancer effect of APA-CPNPs was similar to free chemotherapeutics.

2. Experiment

2.1. Materials

Polyethylene glycol 2000 (PEG_{2k}, Mw = 2000, BioUltra), hydroxyapatite (powder, Sigma), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and 2-morpholinoethanesulfonic acid (MES, BioUltra) were purchased from Sigma-Aldrich (St. Louis, USA). Alendronate (ALN) sodium was purchased from TCI (Tokyo, Japan). Methotrexate (MTX) was provided by Amresco, LLC (Solon, OH, USA). Succinic anhydride (SA, 99%), 4-dimethylaminopyridine (DMAP, 99%), triethylamine (TEA, 99%), 1-*E*thyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 98%) and N-hydroxysuccinimide (NHS, 98%) were purchased from Aladdin (Shanghai, China). Sephadex® G25 was purchased from GE (Fairfield, USA). Dulbecco's Modified Eagle Medium (DMEM), pancreatic enzymes and newborn calf serum (NCS) were purchased from Gibco. Ultrapure water (18.2 Ω) was provided by PURELAB (High Wycombe, UK). MCF-7 cells and HeLa cells were purchased from ATCC (Manassas, VA). All other reagents were purchased as analytical reagent grade and used as received.

Sprague–Dawley rats (200 ± 5 g) and Kunming mice (20 ± 2 g) were supplied by the Laboratory Animal Center of Sun Yat-sen University. All experimental procedures were approved and supervised by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

2.2. Synthesis of ALN-PEG_{2k}-ALN

2.2.1. COOH-PEG_{2k}-COOH

COOH-PEG_{2k}-COOH was synthesized by reacting PEG_{2k} with succinic anhydride. Briefly, PEG_{2k} (10 mmol, 20.66 g), succinic anhydride (25 mmol, 2.50 g), 4-dimethylaminopyridine (20 mmol, 2.44 g) and triethylamine (20 mmol, 2.75 mL) were dissolved in anhydrous dioxane. The reaction was stirred at room temperature overnight under inert atmospheric conditions. Next, the mixture was dropped into CCl_4 and filtered to isolate triethylamine hydrochloride from the product. After redundant CCl_4 was removed, the mixture was dropped into cold diethyl ether. After being stored at −4 °C overnight, the precipitation of crude COOH-PEG_{2k}-COOH was recovered by filtration and dried under vacuum. Two grams of crude COOH-PEG_{2k}-COOH was placed into a $NaHCO_3$ saturated solution and filtered. Upon cooling, the pH of the filtrate was adjusted to 1 using concentrated HCl. Extraction with $CHCl_3$ (3 × 5 mL) was performed, and the white waxy solid of pure COOH-PEG_{2k}-COOH was obtained by precipitation in cold diethyl ether. The structure was confirmed via NMR with the following parameters: COOH-PEG_{2k}-COOH ^{1}H NMR (400 MHz, $CDCl_3$) δH: 4.21–4.24 ppm (m, 4H), 3.40–3.90 ppm (m, 176H), 2.60–2.62 ppm (m, 8H).

2.2.2. ALN-PEG_{2k}-ALN

The synthesis of ALN-PEG_{2k}-ALN was achieved based on a slightly modified carbodiimide chemistry approach (Chung et al., 2006; de Miguel et al., 2014b).

Solution A: To pre-activate the carboxylic group and obtain the NHS-PEG_{2k}-NHS solution, a mixture of COOH-PEG_{2k}-COOH (0.063 mmol), EDCI (1.25 mmol) and NHS (1.25 mmol) was dissolved in 2-morpholinoethanesulfonic acid buffer (0.1 M, pH 5.5), and stirred first on an ice bath for 30 min and then at room temperature for another 30 min. Solution B: ALN (300 mg) was dissolved in 1 mL of 2 M NaOH and 800 μL of H_2O. Upon cooling, the pH was adjusted to 7–8 using 1 M HCl. Solution A was added slowly into solution B under vigorous stirring, and the pH of the reaction was maintained at 7–8 using 1 M NaOH. The mixture was stirred for 1 h on ice and the 24 h on a water bath (37 °C). Next, the water was evaporated in vacuo.

Purification of ALN-PEG_{2k}-ALN from free ALN was performed via size exclusion chromatography using Sephadex® G25. The PEG-containing fraction was identified using cobalt thiocyanate and was collected (Giger et al., 2013). The white cottony solid of pure ALN-PEG_{2k}-ALN was obtained by lyophilization of the collected fractions. The structure was confirmed via NMR with the following parameters: ALN-PEG_{2k}-ALN ^{1}H NMR (400 MHz, D_2O) δH: 4.31 ppm (m, 4H), 3.50–4.00 ppm (m, 176H), 1.70–2.10 ppm (m, 8H); ^{31}P NMR (400 MHz, D_2O) δP: 18.11 ppm.