Journal of Controlled Release 232 (2016) 152–160

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Bone-targeted delivery of nanodiamond-based drug carriers conjugated with alendronate for potential osteoporosis treatment

Tae-Kyung Ryu [a], Rae-Hyoung Kang [a], Ki-Young Jeong [a], Dae-Ryong Jun [a], Jung-Min Koh [b], Doyun Kim [c], Soo Kyung Bae [c], Sung-Wook Choi [a,*]

[a] Department of Biotechnology, The Catholic University of Korea, 43 Jibong-ro Wonmi-gu, Bucheon-si, Gyeonggi-do 420-743, Republic of Korea
[b] Division of Endocrinology and Metabolism, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Republic of Korea
[c] College of Pharmacy, The Catholic University of Korea, 43 Jibong-ro Wonmi-gu, Bucheon-si, Gyeonggi-do 420-743, Republic of Korea

ARTICLE INFO

Article history:
Received 5 November 2015
Received in revised form 12 April 2016
Accepted 15 April 2016
Available online 17 April 2016

Keywords:
Nanodiamond
Alendronate
Bone targeting
Osteoporosis

ABSTRACT

This paper describes the design of alendronate-conjugated nanodiamonds (Alen-NDs) and evaluation of their feasibility for bone-targeted delivery. Alen-NDs exhibited a high affinity to hydroxyapatite (HAp, the mineral component of bone) due to the presence of Alen. Unlike NDs (without Alen), Alen-NDs were preferentially taken up by MC3T3-E1 osteoblast-like cells, compared to NIH3T3 and HepG2 cells, suggesting their cellular specificity. In addition, NDs itself increased ALP activity of MC3T3-E1 cells, compared to control group (osteogenic medium) and Alen-NDs exhibited more enhanced ALP activity. In addition, an *in vivo* study revealed that Alen-NDs effectively accumulated in bone tissues after intravenous tail vein injection. These results confirm the superior properties of Alen-NDs with advantages of high HAp affinity, specific uptake for MC3T3-E1 cells, positive synergistic effect for ALP activity, and *in vivo* bone targeting ability. The Alen-NDs can potentially be employed for osteoporosis treatment by delivering both NDs and Alen to bone tissue.

1. Introduction

Multi-functional nanoparticles have been of great importance in various biomedical applications, such as delivery systems [1,2], biosensors [3], bioimaging [4,5], and tissue engineering [6]. Among the many materials for nanoparticles, carbon-based materials have extended applications in biomedical engineering, including nanodiamonds (NDs), graphene, carbon nanotubes, fullerenes, carbon nanofibers, and nanohorns [7]. Recently, NDs, which are carbon-based allotrope nanoparticles of truncated octahedral composition, have attracted much attention as a promising nanomaterial due to their spherical morphology, high surface functionality, high biocompability, and strong hardness [8]. Chow et al. demonstrated inhibited tumor growth using doxorubicin-conjugated NDs by overcoming the drug efflux issue [9]. Guan et al. fabricated ND-based vehicles containing cisplatin and demonstrated pH-responsive release for cancer treatment [10]. So far, most studies related to drug delivery using NDs have been limited to cancer treatment.

Targeted drug delivery is considered a promising system capable of minimizing the side effects of therapeutic agents, particularly those of hormones. For bone targeted drug delivery, there are well-known functional ligands including bisphosphonate (BP), tetracycline [11], Alizarin Red S, and small peptide aspartic acid [12]. Among them, BPs (ex, alendronate (Alen), risedronate, etidronate) are often used for bone targeted drug delivery due to their high affinity to bone and therapeutic effects on bone diseases. Yokogawa et al. demonstrated specific delivery of estrogen to bone using a peptide with glutamic acid and aspartic acid [13]. Wang et al. found a slightly higher *in vivo* binding efficiency of Alen to bone, compared to that of an aspartic acid peptide [14]. BPs are often used as ligands due to their therapeutic effects in various bone diseases (osteoporosis, Paget's disease, and metastatic bone cancer), as well as their high affinity to bone tissue [15,16]. Choi et al. prepared poly(D,L-lactide-co-glycolide) nanoparticles modified with Alen and polyethylene glycol and demonstrated strong binding to hydroxyapatite (HAp) and *in vitro* release of estrogen [17].

Recently, Zhang et al. fabricated ND-composited biodegradable scaffolds and demonstrated their enhanced proliferation and differentiation of osteoblast, suggesting the positive effect of NDs on osteoblasts [6]. Inspired this report, we designed therapeutic carriers delivering both NDs and drug specifically into bone tissue for potential applications in bone disease treatment. In this work, Alen was chosen as a targeting ligand due to the advantage of easy conjugation to carboxyl groups at the surface of NDs. The rationale for selecting NDs as a carrier material was based on their high alkaline phosphatase (ALP) activity as well as the high surface functionality, biocompatibility, spherical morphology, and hardness. The advantages of NDs in targeted delivery include their spherical morphology and high surface functionality

* Corresponding author.
E-mail address: choisw@catholic.ac.kr (S.-W. Choi).

http://dx.doi.org/10.1016/j.jconrel.2016.04.025
0168-3659/

which facilitates rapid cellular uptake and employment of various and many types of functional ligands [8,18,19].

Herein, we designed Alen-conjugated NDs and evaluated their potential for bone targeted delivery by specifically delivering both biocompatible functional nanomaterials (NDs) and therapeutic agent (Alen) into bone tissue. We conjugated Alen and/or dye onto the ND surface and systematically evaluated their affinity to hydroxyapatite (HAp), cellular uptake behavior, and *in vivo* targeting ability, compared to dye-conjugated Alen. To our knowledge, this is the first *in vitro* and *in vivo* demonstration of bone-targeted delivery based on NDs.

2. Materials and methods

2.1. Materials

The NDs were supplied from Real-Derzinski, Co. (Russian Federation). Alen was kindly provided by Samjin Pharm. Co. Ltd (Seoul, Korea). Dimethyl sulfoxide (DMSO), *N*-hydroxysuccinimide (NHS), ethyl(dimethylaminopropyl)carbodiimide (EDC), poly(D,L-lactide-co-glycolide) (PLGA, L/G = 75/25, M_W = 66.000–107,000), pyrene, fluorescein 5(6)-isothiocyanate (FITC), and rhodamine B isothiocyanate (RITC) were purchased from Sigma-Aldrich (St Louis, MO, USA). The dialysis membranes with MWCO values of 3.5 and 12–14 kDa were purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). Alexa Fluor 546 phalloidin, phosphate buffered saline pH 7.4 (PBS), and culture media were purchased from Invitrogen (Grand Island, NY, USA). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (containing penicillin and streptomycin). The Cell Counting Kit-8 (CCK-8, Dojindo Co. Ltd., Tokyo, Japan) was used to quantitatively measure cell number. The Bio-Rad Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Conjugation of Alen to NDs

As reported previously [20], ND dispersion in acetone (0.1 wt.%) were obtained by treating with oleic acid. ND nanoparticles (NDs) with approximately 50 nm in size were fabricated by using a simple fluidic device [21]. For conjugation, amine-terminated FITC was synthesized by reacting FITC (3.2 mg) and ethylenediamine (5.5 μL) at a mole ratio of 1:10 in 1 mL of DMSO for 12 h at room temperature, followed by dialysis and then freeze-drying [22]. FITC and Alen were conjugated to the NDs by carbodiimide chemistry with EDC and NHS [23]. EDC (383.4 mg) and NHS (230.0 mg) were added to the ND dispersion (0.1 wt.%, 20 mL) in water and stirred at room temperature for 12 h to activate the carboxyl groups. The NDs in water (20 mL) were conjugated with amine-terminated FITC (2.0 mg) to obtain FITC-conjugated ND nanoparticles (NDs) and then with Alen (10 mg) to obtain FITC- and Alen-conjugated ND nanoparticles (Alen-NDs).

For comparison, FITC-conjugated Alen was prepared as a macromolecular conjugate by reacting Alen (10 mg) and FITC (2.0 mg) under the same conditions as those for the amine-terminated FITC. After 3 days of dialysis, Alen, NDs, and Alen-NDs were analyzed with a Zeta potential/size analyzer (Malvern Instruments Ltd., Worcestershire, UK), and a UV/vis spectrophotometer (Perkin Elmer, Norwalk, CT, USA). Surface morphology was characterized by a scanning electron microscopy (SEM; Hitachi, Tokyo, Japan). To evaluate the stability in serum, the NDs and Alen-NDs were dispersed in PBS (pH 7.4) containing 20% fetal bovine serum and their Zeta-potential and size were monitored for 14 days.

2.3. HAp affinity assay

To evaluate the affinity to HAp, Alen, NDs, and Alen-NDs (5 mL, 0.1 wt.%) were separately added to a HAp dispersion (10 mg/mL) in PBS and shaken for 1 h in the dark. After filtration through a syringe filter (0.22 μm pore size), absorbance of the filtrates was determined by a UV/vis spectrophotometer at 488 nm (characteristic FITC peak), corresponding to the unbound amount of sample to HAp. The binding ratio to HAp was defined as the reduction in percent absorbance at 488 nm.

2.4. Conjugation stability of Alen-NDs

To evaluate conjugation stability between Alen and NDs, the Alen-NDs (3 mg/mL) were incubated in PBS (pH 7.4) at 37 °C or aqueous HCl solution (6 M, control) at 90 °C. At the predetermined time points, the samples (0.2 mL) were withdrawn and then centrifuged at 5000 rpm for 10 min in ultrafiltration tubes (Amicon Ultra, MWCO 30 kDa, Merck Millipore, Billerica, MA, USA). The concentration of free Alen degraded from Alen-NDs in the ultrafiltrate was measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with API 5500 Q-Trap mass spectrometer (AB SCIEX, Foster City, CA, USA) and a 1260 HPLC system (Agilent Technologies, Wilmington, DE, USA) [24]. All experiments were carried out in triplicates.

Furthermore, the conjugation stability of Alen-NDs within cells was evaluated by analyzing the concentration of free Alen after cellular uptake of Alen-NDs. MC3T3-E1 cells (5×10^4 cells per well) were seeded on 24-well culture plates and incubated with Alen-NDs. At predetermined time points, the cells were washed three times with PBS to remove non-uptaken Alen-NDs and harvested from the culture plates using trypsin. The cell dispersions were centrifuged for 3 min at 1200 rpm, obtaining cell pellets. The cycle of freezing and thawing was repeated three times to destroy the cell membrane and then the concentration of free Alen was measured using the LC-MS/MS method after the centrifugation.

2.5. Cellular uptake

Human hepatoma (HepG2), mouse embryonic fibroblast (NIH3T3), and mouse calvaria-derived pre-osteoblast (MC3T3-E1) cells were purchased from the Korea Cell Line Bank (Seoul, Korea). Each cell line was seeded on 24-well culture plates at a concentration of 1×10^4 cells per well and cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin and streptomycin at 37 °C in 5% CO_2 and 95% air. After 24 h, each well was washed three times with PBS and replaced with new culture medium. NDs and Alen-NDs (0.1 mL, 0.1 wt.% in PBS) were added to the wells containing each cell line. At predetermined time (3 and 12 h), each well was washed three times with PBS to remove the NDs and Alen-NDs that were not taken up by the cells. To quantitatively measure the amount of cellular uptake, cells in each well were solubilized with 200 μL HCl for 24 h and transferred to a 96-well plate to measure the amounts of NDs and Alen-NDs using a microplate reader (Molecular Devices, Co. Ltd., Sunnyvale, CA, USA) at 488 nm. Cell number per well was determined using the CCK-8 assay according to the manufacturer's instructions. The amount of cellular uptake (pg/cell) was calculated as the amounts of NDs and Alen-NDs divided by the number of cells. In addition, MC3T3-E1 cells treated with NDs and Alen-NDs were washed three times with PBS and fixed in 4% paraformaldehyde (500 μL) at room temperature for 3 h, followed by washing three times with PBS. F-actin (red) and cell nuclei (blue) were stained with Alexa Fluor 546 phalloidin and 4-6-diamidino-2-phenylindole (DAPI), respectively. Cell morphology was observed under a confocal laser scanning microscope (Nikon, Tokyo, Japan).

2.6. ALP activity assay

After 1, 5, and 7 days of culture, alkaline phosphatase (ALP) activity was measured as described previously with some modifications. In brief, MC3T3-E1 cells were seeded at a density of 1×10^4 cells in 24-well plates and then incubated with Alen, NDs, and Alen-NDs