Polymer 54 (2013) 565–571

Contents lists available at SciVerse ScienceDirect

Polymer

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

Synthesis and characterization of new poly(ethylene glycol)bisphosphonate vinylic monomer and non-fluorescent and NIR-fluorescent bisphosphonate micrometer-sized particles

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ARTICLE INFO

Article history:
Received 24 September 2012
Received in revised form
23 November 2012
Accepted 25 November 2012
Available online 7 December 2012

Keywords:
Bisphosphonate
Bisphosphonate vinylic monomer
NIR bisphosphonates particles

ABSTRACT

Bisphosphonates (BPs) are non-hydrolysable pyrophosphate analogs with high affinity to hydroxyapatite (HAP, the main inorganic ingredient in bones) and are mainly used for bone diseases treatments.

A new stable PEG-BP monomer and particles have been prepared for enhanced long term bone-targeted imaging and therapy applications. The new formed BP particles possess dual functionalities: chelation to the bone mineral, HAP, through the BP groups and covalent attachment of a dye or drug through primary amine groups.

The BP particles showed no cytotoxic effect on human osteosarcoma cell lines and minor toxicity on mouse macrophage cells, indicating that these BP particles are good candidates for *in vivo* testing. The BP monomer and particles exhibited inhibition of HAP formation and dissolution, similar to a commercial Alendronate. Near IR (NIR) fluorescent BP particles were obtained by conjugation of Cy7-NHS ester to the primary amine groups of the BP particles.

1. Introduction

Bisphosphonates (BPs) are currently the major class of drugs used for the treatment of bone diseases such as osteoporosis, Paget's disease, benign and malignant bone diseases, etc. [1–5]. BPs are used as potent bone resorption inhibitors [6,7]. Their unique structure, analogous to pyrophosphate, enables them to strongly chelate to bone minerals. Unlike pyrophosphate, which can be cleaved by enzymatic hydrolysis, the P–C–P bond in the BPs is resistant to hydrolysis [8].

The proposed biochemical mechanisms of the BPs, are extensively described in the literature [9–12], where a distinction is made between N-containing and non N-containing BPs. This distinction is also observed in clinical use.

As a rule, during bone disease treatment, BPs are administered orally or intravenously [13]. Orally-administered N-containing BPs show a very low absorption of about 0.7% with a small difference between the BPs, while non N-containing BPs show a slightly higher oral absorption of 2–2.5%. There have been many attempts to increase the low oral absorption with little success.

Several studies show that PEG derivatives pharmaceuticals exhibit better pharmacokinetics. PEG has been approved by the FDA for human intravenous, oral and dermal applications. PEG is non-toxic, biocompatible, non-immunogenic and soluble in a variety of solvents, including water. It is well known that PEG modifications on biomolecules may effectively reduce reticuloendothelial system (RES) clearance and afford a prolonged blood half-life time [14].

Optical imaging based on fluorescent probes is one of the most widely used tools for biological imaging, due to their strong visualization. Fluorescence labeled targeted molecules or particles can be detected by imaging instruments such as confocal microscopes and fluorescence scanners. The major advantage of fluorescence spectroscopy lies in the high signal-to-noise ratio, as compared to white-light technology. Materials exhibiting fluorescence in the near infrared (NIR) region (700–1000 nm), intended for use as imaging agents, are of great interest, as they result in a lower background signal and deeper penetration into biomatrices [15,16]. Other advantages are the low cost of the detection system, portability and real-time detection of multiple tissue parameters [17]. Among the various NIR fluorescent dyes, cyanine dyes have been used in a wide range of biological and chemical applications [15,18–22]. The cyanine dye family is characterized by polymethine chains linked to two heterocyclic rings containing nitrogen. Cyanine dyes are well-known for their water solubility, stability, high sensitivity and sharp fluorescence bands [23–25].

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0032-3861/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.polymer.2012.11.071

In this research, we focused on the design of a novel stable bone-targeted fluorescence micron-sized particles. For this purpose, a new stable PEG-BP monomer was synthesized (Fig. 1) and co-polymerized with a monomer containing a primary amine group (N-(3-aminopropyl) methacrylamide hydrochloride, APMA) and a cross-linker monomer (ethylene glycol dimethacrylate, EDMA). The formed crosslinked BP micrometer-sized particles possess therefore dual functionality: chelation to the bone mineral HAP, through the BP group, and covalent attachment to carboxylate (or carboxylate derivatives) compounds via the primary amine groups. For example, NIR fluorescent BP particles were prepared by conjugation of Cy7-NHS ester to the primary amine groups of the non-fluorescent BP particles.

2. Experimental part

2.1. Materials

The following analytical-grade chemicals were purchased from commercial sources and used without further purification: poly(-ethylene glycol)methacrylate (MA-PEG-OH, M_w 360), ethylene glycol dimethacrylate (EDMA), O-[(N-Succinimidyl)succinyl-aminoethyl]-O′-methylpolyethylene glycol (PEG-NHS, M_w 750), tris(-trimethylsilyl)phosphite, Alendronate, potassium persulfate (PPS), polyvinylpyrrolidone (PVP, M_w 360 K), chromium oxide, oxalyl chloride, Triton-x-100, activated charcoal, anhydrous methanol, anhydrous dichloromethane, anhydrous tetrahydrofuran, chloroform, acetone, anhydrous *N,N*-dimethylformamide (DMF), anhydrous dimethyl sulfoxide (DMSO), sulfuric acid 99%, hydroxyapatite (HAP) and Sephadex LH-20 from Sigma (Rehovot, Israel); N-(3-aminopropyl) methacrylamide hydrochloride (APMA) from Polysciences (Warrington, PA, USA); 2-morpholino ethanesulfonic acid (MES, pH 6) from Fisher Scientific; Dulbecco's modification of eagle's medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), 1% glutamine, 1% penicillin/streptomycin and mycoplasma detection kit from Biological Industries (Bet Haemek, Israel); cytotoxicity detection kit from Roche Diagnostics, USA; Cy7-NHS ester from Lumiprobe (Hallandale beach, FL, USA). Water was purified by passing deionized water through an Elgastat Spectrum reverse osmosis system (Elga Ltd., High Wycombe, UK).

2.2. Synthesis of the MA-PEG-BP monomer

The synthesis of the MA-PEG-BP monomer was carried out with MA-PEG-OH composed of PEG of M_w of 360 as the starting material. However, we expect larger PEG molecules to react in a similar way.

The MA-PEG-BP monomer was synthesized by three successive steps, as described in Fig. 2.

2.2.1. Synthesis of MA-PEG carboxylic acid (2) and MA-PEG carboxylic acid chloride (3)

MA-PEG carboxylic acid and carboxylic acid chloride were prepared according to the literature [26]. Detailed experimental procedures can be found in the Supplementary data.

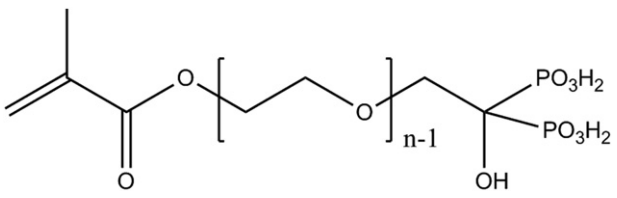

Fig. 1. Chemical structure of the MA-PEG-BP monomer.

2.2.2. Synthesis and characterization of the MA-PEG-BP monomer (4)

MA-PEG carboxylic acid chloride (**3**, 600 mg, 1.53 mmol) was dissolved in THF (5 mL). Tris(trimethylsilyl)phosphite (0.912 mL, 3.06 mmol, 2 eq.) was added and the mixture was stirred at rt for 1 h. The mixture was evaporated to dryness, followed by methanol (5 mL) addition and stirring of the dark mixture overnight at rt. The mixture was again evaporated. The residue was then eluted through LH-20 Sephadex column (20 eq) using water. The fractions were then analyzed by ^{31}P NMR. All fractions which showed a peak associated with the bisphosphonates at *ca.* δ 17 were combined and the solution was lyophilized. The solid residue was analyzed by ^{1}H, ^{31}P and ^{13}C NMR which showed the desired product (**4**).

^{1}H NMR (CD_3OD): δ 1.94 (s, 3H, Me), 3.64 (Brs, 22 H, (CH_2CH_2O)), 3.73 (m, 2H, CH_2CH_2OCO), 3.87 (s, 2H, $OCH_2C(PO_3H_2)_2$), 4.29 (m, 2H, CH_2OCO), 5.65 (s, 1H, vinyl), 6.12 (s, 1H, vinyl).

^{31}P NMR (CD_3OD): δ 17.94 (s, 2P, $C(PO_3H_2)$).

^{13}C NMR (CD_3OD): δ 17.8 (Me-acrylate), 53.4 (OCH_2CO_2), 65.66, 68.1 and 69.4 (OCH_2CH_2O), 126.4 (CH_2=C), 137.5 (C=CH_2), 167.2 (CO_2−acrylate), 103.4 ($COHP_2OH_4$).

TOF MS+: 780 (MH+, n = 12, 7.5%), 736 (MH+, n = 11, 13%), 691 (MH+, n = 10, 22%), 647 (MH+, n = 9, 37%), 603 (MH+, n = 8, 56.5%), 559 (MH+, n = 7, 82%), 515 (MH+, n = 6, 98%), 471 (MH+, n = 5, 100%),427 (MH+, n = 4, 82%),383 (MH+, n = 3, 51.5%).

2.3. Synthesis of the BP particles

In a typical experiment, BP particles of diameter of 280 ± 22 nm were prepared by a dispersion co-polymerization process [27] in an aqueous continuous phase, according to Fig. 3. For this purpose, 45 mg of MA-PEG-BP, 45 mg of APMA and 10 mg of EDMA were added to a vial containing 2 mL of 0.1 M MES buffer (pH = 6) containing 8 mg of PPS as initiator and 20 mg of PVP as stabilizer. For the polymerization, the vial containing the mixture was purged with nitrogen gas to exclude air and then was shaken at 83 °C for 24 h. The resulting particles were washed of excess reagents by extensive dialysis cycles (cut-off of 1000 k) with water.

2.4. In vitro characterization of the BP activity

In vitro study was performed in order to characterize the anti-calcification and anti-resorptive properties of the PEG-BP monomer and the BP particles. The BP activity was tested in two procedures and compared to alendronate: inhibition of HAP formation and inhibition of HAP dissolution, as follows:

2.4.1. Inhibition of HAP formation

Based on works by Francis [28] and Golomb [29], the inhibition of HAP formation was studied in a supersaturated calcium phosphate solution, and compared to the inhibition effect of Alendronate. The principle of this method relies on the ability of a BP group to create bidentate or tridentate chelation with free calcium ions in a supersaturated HAP solution.

2.4.2. Inhibition of HAP dissolution

Based on works by Golomb [30] and Van Gelder [31], inhibition of HAP dissolution by several BP compounds was examined by monitoring the calcium and phosphate released from the HAP into acetate buffer solution at pH 5.

2.5. Preparation of NIR fluorescent BP particles

NIR fluorescent BP micrometer-sized particles were prepared by reacting the primary amino groups of the BP particles with Cy7-NHS ester. Briefly, 1 mg of Cy7-NHS ester was dissolved in 1 mL