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# Alendronate coated poly-lactic-co-glycolic acid (PLGA) nanoparticles for active targeting of metastatic breast cancer

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ABSTRACT

Delivery of therapeutic agents to bone is crucial in several diseases such as osteoporosis, Paget's disease, myeloproliferative diseases, multiple myeloma as well as skeletal metastasizing cancers. Prevention of cancer growth and lowering the cancer induced bone resorption is important in the treatment of bone metastasizing cancers. Keeping in mind the low diffusivity and availability of cell surface targets on cancer cells, we designed a targeted system to deliver chemotherapeutic agents to the bone microenvironment as an approach to tissue targeting using alendronate (Aln). We co-encapsulated curcumin and bortezomib in the PLGA nanoparticles to further enhance the therapeutic efficiency and overall clinical outcome. These multifunctional nanoparticles were characterized for particle size, morphology and drug encapsulation. The particles were spherical with smooth surface and had particle size of 235 ± 70.30 nm. We validated the bone targeting ability of these nanoparticles *in vitro*. Curcumin and bortezomib are known to have synergistic effect in inhibition of growth of cancer; however there was no synergism in the anti-osteoclastogenic activity of these agents. Surprisingly, curcumin by itself had significant inhibition of osteclastogenic activity. *In vivo* non-invasive bioimaging showed higher localization of Aln-coated nanoparticles to the bone compared to control groups, which was further confirmed by histological analysis. Aln-coated nanoparticles protected bone resorption and decreased the rate of tumor growth as compared to control groups in an intraosseous model of bone metastasis. Our data show efficient attachment of Aln on the surface of nanoparticles which could be used as a drug carrier for preferential delivery of multiple therapeutic agents to bone microenvironment.



## 1. Introduction

Preferential delivery of therapeutic agents to the bone has potential to significantly improve the clinical outcomes of several bone related diseases such as osteoporosis, Paget's disease, multiple myeloma, myeloproliferative disease and skeletal metastasis of several epithelial cancers [1–6]. Altered balance of anabolic and catabolic pathways in bone marrow microenvironment leads to enhanced activity of osteoclasts leading to decreased bone strength [4–6]. In addition, cancer cells residing in bone further enhance the osteoclastic activity by secretion of several cytokines such as parathyroid hormone-related protein (PTHrP), receptor activator of nuclear factor kappa-B ligand (RANKL), interleukin-6 (IL-6) [6]. The release of calcium and other cytokines by osteoclast cells during bone resorption, in turn, activates growth of cancer cells residing in bone marrow *via* vicious cycle [6,7]. The abundance of cytokines and constitutive activation of oncogenic pathways in cancer cells leads to environment mediated drug resistance (EMDR) in bone metastasizing cancers [5]. The current treatment modality of bone metastatic cancer includes radiation and systemic chemotherapy [2,3,8]. However, these modalities are greatly limited due to presence of multiple bone metastatic nodules which are difficult to treat by localized radiation therapy and systemic side effects [3,8]. EMDR is most commonly managed by a combination of different chemotherapeutic agents [8], however achieving desired payload of drugs at the site of metastasis is still a major hurdle [2].

Collectively, novel approaches to deliver multiple chemotherapeutic agents to the site of cancer to overcome drug resistance and

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cancer induced bone resorption, decrease cancer growth and increase in overall quality of life (QOL) of cancer patients by reduction of painful mobility compromising fractures is of utmost importance [2,4,8]. Nanoparticle mediated targeted delivery of chemotherapeutic agents to the bone is an attractive approach to overcome most of the above mentioned problems [1,2,9,10]. Bisphosphonates, commonly used as palliative therapy, are bone seeking molecules which bind to active remodeling site of bone [11,12]. The nitrogen containing bisphosphonates, such as Aln, are very efficient in binding to the bone and reducing bone resorption [12,13]. Previous studies have successfully conjugated Aln to polymers such as PLGA [14] or surface functionalized different nanoparticles such as liposomes [15], to deliver the cargo to the bone marrow microenvironment. Cenni et al have demonstrated that Aln conjugated PLGA are non-toxic and safe to use in humans [16]. Others have also conjugated biomolecules directly to Aln to demonstrate its targeting efficiency [17]. Recent evidence also suggests that combination of Aln with chemotherapeutic agents such as doxorubicin enhances its anti-resorptive activity [18]. However, delivery of a single agent is not sufficient to overcome the EMDR induced by fertile surroundings of the bone marrow [5]. Several studies have demonstrated that combination of chemotherapeutic agents is very efficient in inhibition of such EMDR [3,19]. In this study, we investigated the feasibility of encapsulating bortezomib and curcumin in nanoparticles to further increase their therapeutic efficiency.

We took the approach of conjugating bisphosphonates having high affinity towards the hydroxyapatite to target nanoparticles to the bone, where metastasis is most commonly seen [12]. Moreover, we combined two drugs, curcumin [20] (a non-toxic multi-target chemopreventive/chemotherapeutic agent) and bortezomib [21] (a clinically tested proteasome inhibitor), which have been shown to have synergistic effect in inhibition of cancer cell growth [22,23]. Furthermore, curcumin is also known to inhibit the RANKL induced bone resorption and inhibit cytokine signaling [24]. Combining these two chemotherapeutic agents will not only efficiently prevent the growth of cancer, but also it may decrease the dose and dosing frequency due to the preferential delivery using nanoparticles. We have used a novel method of non-covalent insertion of a chemical cross linker in the PLGA matrix, which is capable of ligand functionalization by carbodiimide chemistry [25]. This method is of particular advantage as the activated nanoparticles are formulated in a single step and ligand functionalization could be carried out at physiological pH in an aqueous system [25].

In this study, we formulated multifunctional bone targeted nanoparticles encapsulating two chemotherapeutic agents and studied their physical characteristics. We validated the retention of bone seeking ability of Aln, as well as anti-cancer and anti-osteoclastogenesis ability of curcumin and bortezomib. We have monitored the bone targeting ability of the nanoparticles *in vitro* and *in vivo*. We extended our studies in an intraosseous model of bone metastasis of breast cancer, to establish the therapeutic efficiency of the nanoparticles.

## 2. Materials and methods

### 2.1. Materials

Poly(D,L-lactide-co-glycolide) 50:50; inherent viscosity 1.13 dl/g; mw 50,000 was purchased from Lakeshore Biomaterials (AL, USA). Polyvinyl alcohol (PVA; mw 30,000–70,000), curcumin, ethyl acetate, methanol, ethanol and Nile red were purchased from Sigma–Aldrich (MO, USA). Bis(Sulfosuccinimidyl) suberate (BS3) was purchased form ProteoChem Inc. (CO, USA). DMEM low glucose media, RPMI media, antibiotic solution, fetal bovine serum and fluorophore conjugated anti-mouse antibodies were obtained from Invitrogen (Carlsbad, CA, USA). The 4T1 and MDA-MB-231 breast cancer cells were purchased from ATCC and RAW 264.7 cells were kindly provided by Dr. Bharat Aggarwal from MD Anderson Cancer Center, Houston, TX. RANKL and Aln sodium was purchased from Santa Cruz Biotechnology (CA, USA).

### 2.2. Preparation and characterization of activated and drug loaded PLGA nanoparticles

The activated nanoparticles were prepared using solid/oil/water (s/o/w) emulsion solvent evaporation method reported elsewhere with slight modification [25–27]. Briefly, 70 mg of PLGA dissolved in 1 ml of ethyl acetate was added to 1% aqueous solution of PVA containing 0.5 mg/ml of BS3. This mixture was sonicated at room temperature using ultrasonic processor UP200H system (Hielscher Ultrasonics GmbH, Germany) at 40% amplitude for 2 min in continuous mode. The excess solvent was evaporated under continuous stirring for 45 min to 1 h followed by centrifugation. The separated nanoparticles were washed thrice and resuspended in water followed by lyophilization on ATR FD 3.0 system (ATR Inc., MO, USA) and stored until further use at 4 °C. To make curcumin and bortezomib loaded nanoparticles, similar procedure was followed by adding required amount of curcumin and bortezomib in 1 ml of ethyl acetate along with PLGA.

### 2.3. Physical characterization of nanoparticles

Particle size was measured by using Nanotrac system (Mircotrac, Inc., Montgomeryville, PA, USA) by suspending nanoparticles in PBS. Scanning electron microscopy (SEM) and FTIR was performed using Hitachi S-3000N Variable Pressure SEM and Nicolet 6700 FTIR spectrometer instruments, respectively.

### 2.4. Conjugation of alendronate on nanoparticles

The desired amount of lyophilized nanoparticles and Aln (1:1 w/w ratio) was weighted and suspended separately in 500 μl of PBS at room temperature for 15 min. The two solutions were mixed and incubated for 1hr at room temperature for Aln conjugation. The reaction was stopped by addition of 100 μl of Tris Buffer (pH 7.4) for 15 min at room temperature and centrifuged to remove unconjugated Aln. The pellet was resuspended in either PBS or medium for further use. The amount of Aln attached on the surface of nanoparticles was determined by measuring the quantity of Aln in reaction supernatant using TNBS assay for detection of primary amino group using protocol described in literature [28]

### 2.5. Measurement of curcumin and bortezomib loading in nanoparticles

To measure drug loading, 2 mg of nanoparticles were incubated in 1 ml of 50% ethanol for 14 days. The resulting solution was centrifuged and the supernatant was analyzed using Cary Eclipse UV–Vis spectrophotometer (Varian Inc., Australia). The total amount of curcumin and bortezomib in the solution was back calculated from absorption intensity using a calibration curve.

### 2.6. In vitro drug release from nanoparticles

The release of curcumin and bortezomib from nanoparticles was performed using a previously established method with slight modifications [25,29]. A known amount of nanoparticles was suspended in PBS and kept in dialysis unit (3 kDa cut off) at 37 °C in 30 ml of incubation media containing 50% v/v ethanol. Two milliliter of sample was taken at each time point and replaced with same amount of fresh incubation media. The absorption intensity of curcumin and bortezomib in the collected samples was measured and the amount of drugs released was back calculated using a calibration curve obtained in incubation media as mentioned before.

### 2.7. In vivo biodistribution of Aln-coated and non-coated nanoparticles

Athymic female nude mice (5–6 weeks) were purchased from Harlan Laboratories Inc. (IN, USA) and acclimatized for one week at UNTHSC animal housing facility. The mice were then injected with 5 mg of Nile red stained Aln-coated or non-coated nanoparticles, suspended in 150 μl of PBS *via* lateral tail vein. The mice were then imaged using IVIS Lumina XR (Caliper Life Sciences Inc., MA, USA) at specified time points. Images of same animals before injecting the nanoparticles were taken and used to set the background while analyzing the data. Similar experiment was repeated for histological analysis and the mice were sacrificed after 6 h to harvest the organs.

### 2.8. Preparation of iron oxide encapsulated PLGA nanoparticles

The oleic acid iron oxide nanoparticles were prepared as specified in literature [30,31]. These nanoparticles were precipitated by mixing the suspension with 100% ethanol (1:3) and vortexed at maximum speed for 1 min. The pellet of nanoparticles obtained after centrifugation at 14000rpm for 15 min, was resuspended in PLGA containing ethyl acetate solution. The iron oxide encapsulated nanoparticles were then obtained by sonication as explained above. The Aln-coated and non-coated,