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# High-activity chitosan/nano hydroxyapatite/zoledronic acid scaffolds for simultaneous tumor inhibition, bone repair and infection eradication

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ABSTRACT

Implanted biomaterials combined tumor inhibition and bone repair property are urgently needed to address the huge bone destruction and the high local recurrence following primary surgery in bone tumor therapy. In this work, a high-activity chitosan/nano hydroxyapatite (CS/nHA) scaffold containing zoledronic acid (CS/nHA/Zol) was prepared with a facile method. The prepared CS/nHA/Zol scaffolds exhibited excellent tumor inhibition property towards giant cell tumor of bone (GCT) *in vitro* through inducing cells apoptosis by up-regulating pro-apoptosis genes expression and reducing the osteoclastic activity of tumor cells by down-regulating osteoclastic genes. Meanwhile, the prepared scaffolds possessed well biocompatibility and osteoinductivity as compared to pure CS/nHA scaffolds. Furthermore, the prepared scaffolds also presented outstanding antibacterial activity against clinical pathogenic *S. aureus* and *E. coli*. These overall findings successfully demonstrated the prepared CS/nHA/Zol scaffolds had a multifunction of tumor therapy, bone repair, and antibacterium, which provides a new approach possessed promising advantages in bone tumor therapy.

## 1. Introduction

Giant cell tumor of bone (GCT) occupies approximately 5% of primary bone tumors and 20% of benign bone tumors, which mainly occurs between the ages of 20 and 40 [1,2]. Although approximately 80% GCTs are benign, they represent highly osteolytic destruction, local aggression, and a recurrence rate ranged from 20% to 50% [3,4]. Further, nearly 6% GCTs develop pulmonary metastases with poor outcomes [5]. To effectively eradicate GCTs, surgery is currently the best treatment option, for example intralesional curettage. However, the huge bone destruction and the high local recurrence or metastasis rate following primary surgery still remain a formidable clinical challenge [6]. In spite of numerous techniques having been developed for bone defect reconstruction after tumor curettage, including using allografts, iliac bone grafts, irradiated bone grafts and so on, those techniques still have disadvantages. For instance, allograft, one of the most commonly used materials in tumor-induced bone defect, has no antitumor effect and relates to infectious diseases transmission, immunological reactions [7,8]. Thus, new therapy strategies and biomaterials combining antitumor and bone repair activity are urgently needed.

Composite materials, made from two or more constituent materials with different physical or chemical properties, can acquire different characteristics from the individual components. Recently, the combine of organic materials with inorganic materials especially the nanomaterials in one entity have received ever growing concern in composite biomaterials and varied techniques are applied for their fabrication [9,10]. The nanocomposite materials formed by incorporated nanomaterials into the main matrix can not only enhance the mechanical strength but also render new functionalities to the materials. Inorganic nanomaterials are studied in different areas and developed with promising characters for various applications [11–13], such as gene and drug carrying [14,15], photodynamic and photothermal effect [16,17], fluorescence capability [18], as well as antimicrobial property [18,19]. Take advantage of these multiple highly desired features, multifunction composite materials are currently used in the fields of catalysts, gene and drug delivery, biosensors, bioimaging, optogenetics, infection treatment, and tumor therapy [20,21]. In the other hand, modified the

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inorganic nanomaterials with organic polymers is also appealing in improving biocompatibility, escaping mononuclear phagocyte system, and intensifying therapy effect [14]. These researches indicate that composite materials are promising approaches for biomedical application and suggest they can extensively expand in many different fields of science in future.

In bone regeneration, inorganic salts, carbohydrates, and proteins play crucial roles in the biomineralization [22,23]. Chitosan (CS), as a natural polycationic linear polysaccharide deacetylated from chitin, has been used in various tissue engineering biomaterials because of its numerous advantages like biocompatibility, biodegradability, antimicrobial, and antioxidant activities, but CS alone in scaffold shows poor mechanical strength and fast degradation rate [24,25]. Some inorganic substances are added into CS scaffolds to enhance the mechanical strength. Nano hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, nHA), a principal inorganic constituent, constitutes 50–70% of human bone, has been extensively studied in bone repair biomaterials owning to its excellent biocompatibility, bioactivity as well as osteoinductivity [26]. The addition of nHA in CS scaffolds increases the mechanical properties, mimics the natural structure of bone, keeps the good porosity and biocompatibility simultaneously [27]. Moreover, CS/nHA scaffolds promote mesenchymal stem cells differentiation and mineralization by up-regulating osteogenic genes and possess better bone regeneration *in vivo* [28,29]. Although many CS/nHA scaffolds have been developed for promoting bone formation as bone engineering materials, however to our best knowledge, there are very few biomaterials with the multifunction of effectively killing the residual tumor cells as well as healing of the large bone defect after surgical resection of GCT.

Among current medicines for bone tumor therapy, zoledronic acid (Zol), a third-generation nitrogen-containing bisphosphonate, has been widely used due to its efficacy in reduction of primary bone tumor growth as well as bone metastasis of lung, breast, prostate, and other solid tumors [30,31]. However, intravenous injection with Zol, which is the most common route of medication at present, may lead to adverse effects such as osteonecrosis of the jaws, hypocalcemia, hypophosphatemia, and so on [32]. Previous study has reported that local adjuvant therapy is useful in controlling recurrence rates of GCT [33]. Therefore, local application of Zol can be more attractive and some materials like Zol loaded cements have been developed and proved to be effective in GCT therapy [34,35].

In the present study, we developed a novel chitosan/nano hydroxyapatite/zoledronic acid scaffold with a facile method. Notably, the well-defined porous scaffold showed superb biocompatibility as well as osteoinductivity while a significant inhibition on the proliferation of giant cell tumor cells. Further experiments proved the inhibition was through inducing cells apoptosis by up-regulating pro-apoptosis genes expression. Moreover, the scaffold reduced the osteoclastic activity of tumor cells by down-regulating osteoclastic genes, and exhibited a strong antibacterial property towards clinical bacterium, which showed promising potential for *in situ* treatment of giant cell tumor and repair of tumor-induced bone defect.

## 2. Materials and methods

### 2.1. Preparation of CS/nHA/Zol scaffolds and CS/nHA scaffolds

The CS/nHA/Zol scaffolds were prepared using *in situ* precipitation method. Briefly, Chitosan (Sigma, 2% *w/v*) was dissolved in 2% acetic acid. $Ca(NO_3)_2$ (2 M) solution in ethanol was added into CS solution and then $K_2HPO_4$ (1.2 M) solution in distilled water was added dropwise. The mixture with a Ca/P ratio of 1.67 was stirred until homogenous. Then the mixture poured into 48-well plates, frozen overnight at − 80 °C, and freeze dried in a lyophilizer (Alpha1-4 LSC Plus, Christ) overnight. The scaffolds were soaked in 0.5 M NaOH for 8 h room temperature for the *in situ* process, followed by washing with distilled water repeatedly until neutrality. 500 μL USP reference standard zoledronic acid (1 mM) was added into 10 mg scaffold to combine with the nHA overnight, and then lyophilized again. The CS/nHA scaffolds were fabricated with the same procedure without addition of Zol.

### 2.2. Characterization of the scaffolds

The surface morphology and pore size of the scaffolds were examined using scanning electron microscopy (SEM). Scaffold samples were dried under vacuum, gold coated, and examined with SEM (S-3000N, Hitachi).

The Fourier transform infrared spectroscopy (FTIR) was used to examine intermolecular interaction between the components in the scaffolds. The spectra of CS, Zol and the spectra of CS/nHA/Zol and CS/nHA scaffolds were recorded using the KBr pellet method in an FTIR spectrophotometer (VERTEX 70, Bruker) with the range of 4000 to 500 $cm^{-1}$.

The swelling behavior of the scaffolds was evaluated by a classical method [36]. Briefly, the dry weight of scaffolds was recorded and then scaffolds were immersed in phosphate buffer saline (PBS) at 37 °C with different times. The wet weight was recorded after scaffolds were taken out and removed surface water by filter paper. The swelling ratio was determined by using the following formula:

$$\text{Swelling ratio} = (\text{Wet weight} - \text{Dry weight})/\text{Dry weight} \quad (1)$$

### 2.3. Cell proliferation

Cell proliferation of bone giant cell tumor cells (GCTBs, established as our previous reported [37]) and human bone marrow mesenchymal stem cells (hBMSCs, Cyagen Biosciences) co-cultured with different Zol concentrations (0, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 mM) was determined by standard Cell Counting Kit-8 (CCK-8) assay. Briefly, $5 \times 10^4$ GCTBs were seeded and incubated with 500 μL Zol containing medium at 37 °C. hBMSCs cultured with or without Zol were also studied. The 0 mM/L Zol in above experiments was set as control group. After 24 h co-culture, H-Dulbecco's Modified Eagle Medium (H-DMEM, Gibco) or L-Dulbecco's Modified Eagle Medium (L-DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) was discarded, CCK-8 solution (10%) was added to each well, and incubated for 2 h, and optical densities (OD) were determined at 450 nm using microplate reader (Multiskan GO, Thermo Scientific). Then the relative cell viability was calculated by the following equation:

$$\text{Cell viability}\ (\%) = OD_{sample}/OD_{control} \times 100 \quad (2)$$

Cell proliferation in the scaffolds (10 mg) with different times (1, 4, 7 days) was also determined by CCK-8 assay with the same procedure as above.

### 2.4. Cell morphology in the scaffolds

SEM was used to observe cell morphology in the scaffolds. After cultured for 4 days, GTCBs or hBMSCs loaded scaffolds were washed with PBS for 3 times, and then fixed with 3% glutaraldehyde for 4 h in 4 °C. Then the scaffolds were dehydrated in a graded ethanol series (30, 50, 70, 90, and 100%, respectively). The cells loaded scaffolds were dried under vacuum, gold coated, and examined with SEM (S-3000N, Hitachi).

### 2.5. Live/dead cell staining

The GTCBs were co-cultured with different scaffolds for 4 days, then washed with PBS, and stained with Calcein-AM and propidium iodide (PI, Sigma) at room temperature for 30 min. After washed with PBS to remove the free dyes, the cells were examined by confocal laser scanning microscopy (CLSM, LSM 700, ZEISS).