

## Research Paper

# Enhancing Alendronate Release from a Novel PLGA/Hydroxyapatite Microspheric System for Bone Repairing Applications

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**Purpose.** The goal of this study was to exploit the multifunction of PLGA based microsphere as efficient alendronate delivery and also as potential injectable cell carrier for bone-repairing therapeutics.

**Materials and Methods.** Novel poly (lactic-co-glycolic acid) (PLGA)-hybridizing -hydroxyapatite (HA) microspheres loaded with bisphosphonate-based osteoporosis preventing drugs, alendronate (AL), are prepared with solid/oil/water (s/o/w) or water/oil/water (w/o/w) technique. Macrophage resistance was evaluated by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, DNA assay and Live/dead staining, and osteoblast proliferation and maturation was assessed by MTT assay, Alkaline phosphatase (ALP) activity assay and Real time-PCR.

**Results.** In such fabricated AL laden PLGA/HA microspheric composites (abbreviated "PLGA/HA-AL"), the introduction of HA component has been proven capable of largely enhancing drug encapsulation efficiency especially when the single emulsion protocol is adopted. The *in-vitro* drug (AL) releasing profile of PLGA/HA-AL system was plotted basing over 30 days' data collection. It indicates a sustained releasing tendency despite a minimal burst at the very beginning. The *in-vitro* bone-repairing efficacy of PLGA/HA-AL system was first tested with macrophages that are identified as precursors of osteoclasts and potentially responsible for osteoporosis. The results indicated that the AL release significantly inhibited the growth of macrophages. Additionally, as a central executor for osteogenesis, osteoblasts were also treated with PLGA/HA-AL system *in vitro*. The outcomes confirmed that this controlled release system functions to improve osteoblast proliferation and also enables upregulation of a key osteogenic enzyme ALP.

**Conclusions.** By pre-resisting osteoclastic commitment and promoting osteoblastic development *in vitro*, this newly designed PLGA/HA-AL controlled release system is promoting for bone-repairing therapeutics.

**KEY WORDS:** alendronate; bone repair; drug delivery; hydroxyapatite; microspheres; PLGA.

## INTRODUCTION

Bisphosphonates (BPs) are a class of drugs consisting of stable analogues of natural pyrophosphate compounds that inhibit bone resorption by osteoblasts, and are used in the clinical treatment of systemic metabolic bone diseases (1–3). Some BPs such as alendronate (AL), have already been used for clinical treatment of Paget's disease, osteoporosis, hypercalcemia of malignancy as well as inflammation-related bone loss (4,5). BPs are capable of strongly binding to bone

mineral calcium phosphate crystals and regulating their growth, aggregation and dissolution (6). The molecular structure of BP is characterized by their nonhydrolyzable P–C–P groups rather than P–O–P, which result in a poor metabolic absorption (7). Only 1% of BPs can be absorbed *via* oral administration and only 20% of the absorbed drug can be eventually incorporated in bones (7). Attempts have been performed to enhance the loading of BPs into materials such as poly (d-l-lactic-co-glycolic acid) (PLGA) microspheres or mesoporous silica for controlled release (8–11).

Hydroxyapatite (HA) inherently possesses high compressive and structural similarities to the minerals found in native bones or dentin (12). Therefore it has been widely adopted as biomaterials for bone regeneration making use of its bioactivity, biocompatibility, osteoconductivity and non-inflammatory property. HA particles or porous granules have been investigated as carriers for various drugs and proteins, such as antibiotics and growth factors (13–16). BPs have exceptional affinity to Ca<sup>2+</sup> ions of bone tissue. This property makes HA an excellent carrier for BPs delivery. Palazzo *et al.* developed biomimetic HA-blending-AL (HA-AL) nanocrystals as a potential antitumor drug delivery system

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(14). Boanini *et al.* optimized the BP content to 7 wt% in the nanocrystals (17).

PLGA microspheres have been widely used as vehicle materials for drug and gene delivery due to its excellent processability and controllable degradability (18,19). PLGA microsphere based materials are reported to have broader applications. Drug/protein laden microspheric materials could be utilized for bone defect repair and bone tissue regeneration. Laurencin *et al.* have developed microspheres based scaffolds in which polymers such as PLGA and Polyphosphazene were fabricated into microspheres *via* single emulsion techniques (20,21). The PLGA based microspheres could also be potentially utilized as cell carriers (22).

In this study, we take the advantages from both HA and PLGA by hybridizing them into composite microspheres in which AL is loaded to be delivered to fulfill bone repairing applications. Two fabricative methodologies respectively *via* water/oil/water emulsion (w/o/w) and solid/oil/water (s/o/w) emulsion techniques were adopted. This newly established controlled release system is abbreviated as "PLGA/HA-AL" system.

## MATERIALS AND METHODS

### Materials

PLGA (lactic/glycolic 1:1; Mw 31,000 Da; inherent viscosity 0.30 dl/g in chloroform at 30°C) was purchased from Daigang Biomaterials Inc. (Jinan, China). AL was purchased from Tianfeng Inc. (Henan, China). Poly(vinyl alcohol) (PVA) was obtained from Sigma-Aldrich (Singapore).

### Fabrication of HA-AL Particles

Plain HA particles were prepared following the method described in (23). HA-AL particles were prepared by adding 5 g plain HA particles (Fig. 1) into 100 ml AL solution (0.5 mg/ml).

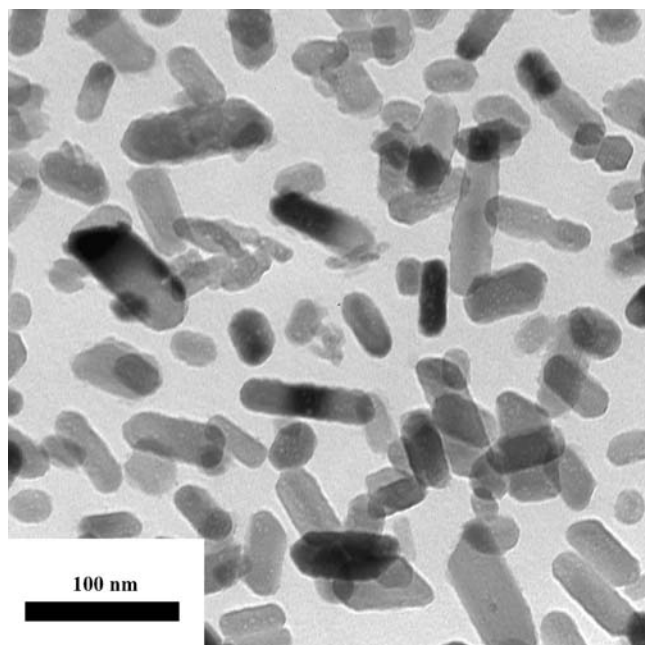


Fig. 1. TEM image of nano-HA particles.

The solution was kept at 37°C under stirring for 24 h. The deposited phase was isolated and washed in deionized water three times and dried. The achieved particles were sieved to select the ones smaller than 50  $\mu\text{m}$  diameter for further use.

### Fabrication of PLGA/HA-AL Microspheres

1. *By double emulsion method (DM)*: Fifty milligram AL powder was dissolved in 1 ml deionized water forming the first aqueous phase  $W_1$ . The organic phase was formed by dissolving 1 g PLGA into 5 ml methylene chloride. Plain HA particles (0.3 or 0.5 g) were added into the PLGA solution and distributed by ultrasonication. These HA-suspending solutions were then emulsified in  $W_1$  solution to form the primary  $W_1/O$ . The  $W_1/O$  emulsion was poured into 200 ml 1% PVA aqueous solution. The resultant  $W_1/O/W_2$  emulsion was stirred at 400 rpm for 4 h at room temperature to evaporate the solvent and solidify the PLGA/HA-AL-DM microspheres. They were finally washed in deionized water and dried. As a control, HA-null PLGA-AL-DM microspheres were similarly fabricated without the addition of HA particles into PLGA solution before emulsification in  $W_1$  solution.
2. *By single emulsion method (SM)*. One gram PLGA was dissolved in 5 ml methylene chloride in which HA-AL particles (0.3 or 0.5 g) were added. The mixture emulsion was poured into 200 ml 1% PVA aqueous solution and stirred at 400 rpm for 12 h. The resultant PLGA/HA-AL-SM microspheres were isolated, washed in deionized water and dried.

### Morphological Characterization

Morphological characterization was conducted using scanning electron microscopy (SEM, 30XLFEG, Philips, The Netherlands). The microspheres were immobilized on a cupreous stub and coated with gold. The microspheric size was measured in the range of  $10\times$  for all structures. Approximately 20 individual microspheres were randomly selected from each view. At least ten fields were randomly selected for measurements and at least 200 microspheres were analyzed for comparison. The morphology of HA particles was studied by Transmission Electron Microscopy (TEM, Philips CM300).

### Determination of Encapsulation Efficiency

1. *HA-AL particles*: The AL encapsulation efficiency of HA-AL particles was determined by suspending 50 mg HA-AL into 2 ml PBS buffer (pH=7.2) and incubating at 37°C during which the supernatant was periodically analyzed following the method reported in (24).
2. *PLGA/HA-AL microspheres*: PLGA/HA-AL microspheres were suspended and washed in methylene chloride to deposit the insoluble HA-AL particles out. The HA-AL particles and all supernatants were respec-

tively collected. The portion of encapsulated AL in HA-AL particles ( $C_{HA}$ ) was measured as described above (24). The collected supernatant, which is an AL suspension in PLGA/methylene chloride solution, was rinsed in an iron (III) chloride/perchloric acid solution so that the suspended AL was entirely extracted into the aqueous phase (24). The AL in the iron (III) chloride/perchloric acid solution extraction, which represents the portion of encapsulated AL in PLGA ( $C_{PLGA}$ ), was quantified using UV spectrophotometer also following the method as described above (24). Therefore, the total AL encapsulation efficiency in PLGA/HA-AL microspheres could be calculated by  $(C_{HA} + C_{PLGA})/C_{TID}$ , in which  $C_{TID}$  represents the total initial dosage of AL devoted for the whole process.

### In Vitro Release of AL from PLGA/HA-AL Microspheres

*In-vitro* AL release trials were performed in a shaking incubator at 60 rpm under 37°C. Fifty milligram AL-loaded microspheres were soaked in 20 ml PBS (pH=7.4). The sample media were collected at regular time intervals with equal amount of PBS makeup.

### Cell Culture

Human fetal osteoblasts (hFOBs) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified eagle's medium (DMEM)/Ham's F12 (1:1) cultural medium with supplements of 2.5 mM L-glutamine, 0.3 mg ml<sup>-1</sup> G418 and 10% (v/v) FBS. Macrophages were propagated in DMEM supplemented with 1.5 mg/ml sodium bicarbonate, 4.5 mg/ml glucose, 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. All reagents mentioned above were purchased from Gibco (Invitrogen, Singapore). Cell number was determined with a DNA assay using Sigma quantification kit (Sigma-Aldrich, Singapore) following the manufacturer's instruction.

### Cytotoxicity and Cell Viability

Cytotoxicity was evaluated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Roche Diagnostics, Germany] assay. Briefly, microspheres (PLGA/HA-AL-DM and PLGA/HA-DM) were placed in 24-well TCPS (tissue culture polystyrene) plates and cells ( $1 \times 10^4$  cells/ml) were seeded in the same wells and altogether incubated in a humid atmosphere under 37°C and 5% CO<sub>2</sub> for various periods of time before the MTT assay that was conducted following a standard protocol (25). Cells seeded on bare TCPS (no involvement of microspheres) with or without addition of soluble AL (4 mg/L) in the cultural media were respectively used as positive or negative control. Cell viability was also assessed using a Live/Dead assay kit (Invitrogen, Singapore) following the manufacturer's instruction.

### Alkaline Phosphatase (ALP) Assay

Osteoblasts that had been pre-washed with PBS were lysed in 0.5 ml PBS containing 0.1 M glycine, 1 mM MgCl<sub>2</sub>

**Table I.** Validated Primer Sequences for Real-time PCR

Gene	Direction	Sequence (5'-3')
ALP	Forward	GGGAACGAGGTCACCTCCAT
	Reverse	TGGTCACAATGCCACAGAT
OC	Forward	AGCAAAGGTGCAGCCTTTGT
	Reverse	GCGCTGGGTCTCTTCACT
Collagen I	Forward	CCTGCGTGTACCCCACTCA
	Reverse	ACCAGACATGCCTCTTGTCCCT
β-actin	Forward	CCTGGCACCCAGCACAAT
	Reverse	GGGCCGGACTCGTCATACT

and 0.05% Triton X-100. The lysate solution was incubated with *p*-nitrophenyl phosphate (pNPP) solution at 37°C for 30 min and then subjected to a spectrophotometer on which the absorbance at 405 nm was measured and recorded to indicate ALP concentration (26).

### Real Time Quantitative Reverse Transcription-Polymerase Chain Reaction

Osteoblasts was treated by PLGA/HA(50%)-AL-SM and PLGA/HA(50%)-SM for 7 days. Total RNA was isolated following TRIzol protocol (27) and subjected to RT with SuperScript™ First-Strand Synthesis System (Promega, USA). The yielded complementary DNA (cDNA) was then subjected to PCR examining gene expressions of osteocalcin (OC) and β-actin. The adopted primer sequences and PCR conditions are listed in Table I. The quantitative polymerase chain reaction (qPCR) was conducted with SYBR green assay (iQ supremix, Bio-rad). The gene expressions were quantified with a calculation of  $2^{-\Delta C_T}$ , where  $C_T$  represents the cycle number when an arbitrarily placed threshold was reached, and  $\Delta C_T = (C_{T,targetgene} - C_{T,\beta-actin})$ .

### Statistical Analysis

Experiments were repeated three times and results expressed as means ± standard deviations. MTT assay, DNA assay, ALP activity assay and Real time PCR results were evaluated by one way analysis of variance (ANOVA). A comparison between two means was analyzed using Tukey's test with statistical significance set at  $p < 0.05$ .

## RESULTS

### Morphology of PLGA/HA-AL Microspheres

The PLGA/HA-AL microspheres were fabricated as demonstrated in Fig. 2 and the morphologies of them are exhibited in Fig. 3. All types of drug-laden microspheres maintain in spherical shape among which only the DM ones present observable pores on the surfaces. It is believed that the pores are formed by a micro-foaming process due to the air bubbles and/or fluid drops involved during the homogenization step of fabrication (28). In contrast, the SM microspheres do not exhibit visible pores on surface but show even more wrinkling morphologies due to a greater HA enrichment that particularly happens to the ones blended with 50 wt% HA.

Data of particle size for all types of microspheres are listed in Table II, which indicates that the average sizes of DM



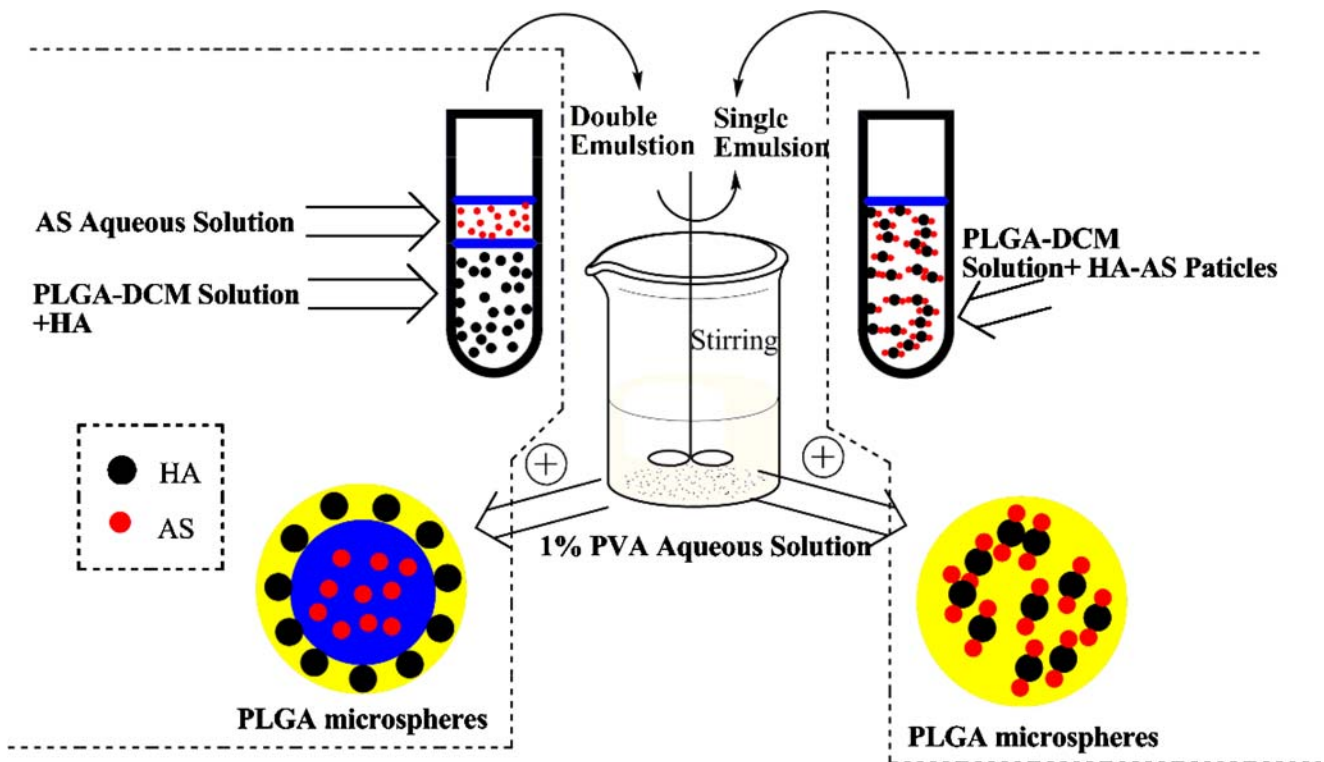


Fig. 2. A schematic diagram of PLGA/HA-AL microspheres fabricated by solid/oil/water (S/O/W) single emulsion technique and water/oil/water (W/O/W) double emulsion technique.

microspheres are generally larger than those of SM ones; while by the same emulsion technique, the average sizes monotonically decrease with the increase of HA content.

**Drug Encapsulation Efficiency**

Data of drug (AL) encapsulation efficiency for all types of PLGA/HA-AL(-SM and -DM) microspheres together with a control sample of HA-null PLGA-AL-DM are also listed in Table II. Without HA involvement, the AL encapsulation efficiency in PLGA-AL-DM was found significantly lower

than all the HA-added ones; while among the HA-added samples, the AL encapsulation efficiencies in SM samples were found further significantly higher than the DM ones.

**In-Vitro Drug Delivery**

The profiles of AL (cumulative *in vitro*) release over 30 days from both PLGA/HA-AL-SM and PLGA/HA-AL-DM are shown in Fig. 4, where all the curves indicate comparable shape with a common exponential tendency despite a minimal bursting initiation. Reflected by this

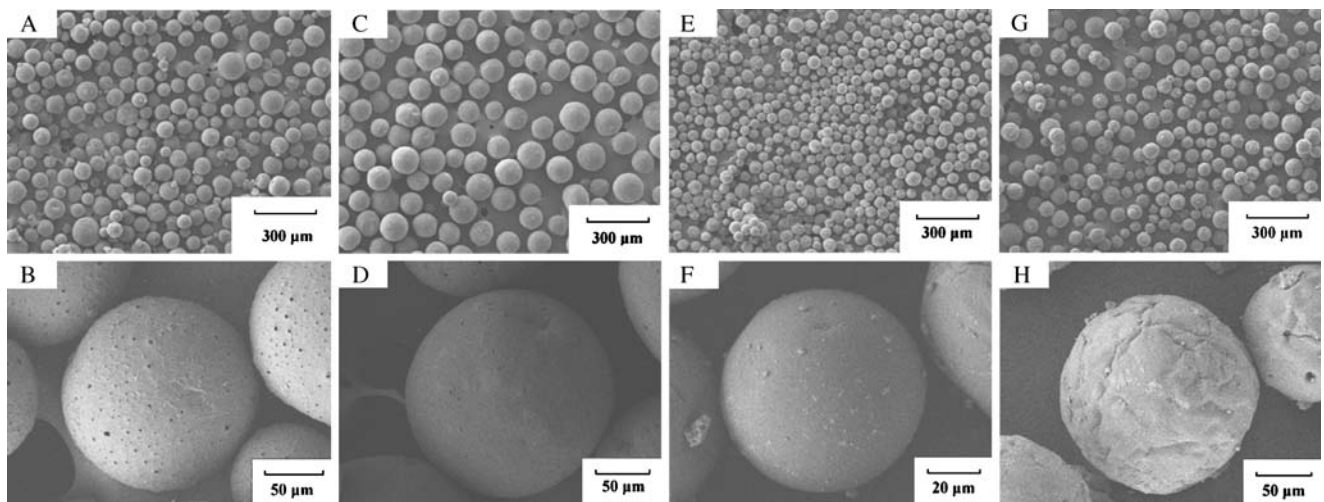


Fig. 3. SEM images of PLGA/HA-AL-SM and PLGA/HA-AL-DM. A, B PLGA/HA-AL-DM with 30% HA; C, D PLGA/HA-AL-DM with 50% HA; E, F PLGA/HA-AL-SM with 30% HA; G, H PLGA/HA-AL-SM with 50% HA.

**Table II.** Microspheres Size Distribution, HA Content and AL Encapsulation Efficiency Results of PLGA Based Microspheres

Batch	Size ( $\mu\text{m}$ )	HA content (T) (100%)	HA content (A) (100%)	Encapsulation efficiency (100%)
PLGA/HA(30%)-SM	89.20 $\pm$ 60.12	30%	22.14 $\pm$ 5.56	–
PLGA/HA(50%)-SM	92.36 $\pm$ 50.86	50%	41.28 $\pm$ 2.78	–
PLGA/HA(30%)-AL-SM	77.60 $\pm$ 58.27	30%	24.05 $\pm$ 2.02	92.51 $\pm$ 3.35
PLGA/HA(50%)-AL-SM	99.44 $\pm$ 36.25	50%	42.86 $\pm$ 8.55	89.36 $\pm$ 4.56
PLGA/HA(30%)-AL-DM	118.27 $\pm$ 82.56	30%	21.67 $\pm$ 6.76	33.27 $\pm$ 7.02
PLGA/HA(50%)-AL-DM	132.56 $\pm$ 67.33	50%	40.80 $\pm$ 3.88	36.18 $\pm$ 2.10
PLGA-AL-DM	136.85 $\pm$ 77.10	–	–	7.10 $\pm$ 1.55

T theoretical content, A actual content

tendency, the release proceeded in a remarkably high rate during first 4 days, accordingly 20~40% of total release had been accomplished by then; while after the curve inflexion at around day 4~5, the subsequent release rate lowered down so that it had taken another 25 days to fulfill another 40% of total release. Up to day 30, the total AL release had reached around 70~90% from each samples, among which the release rate appeared higher in DM samples (than in SM ones) or in lower-HA [30%] involved samples (than in higher-HA [50%] ones).

### Macrophage Resistance

Macrophage resistance by AL release from PLGA/HA-AL microspheres was studied by kinetic examinations of macrophage viability and proliferation, which were first generally visualized under fluorescent Live/Dead assay and then quantified with MTT assay and DNA assay. As shown in Fig. 5, the fluorescent microscopic images indicate the cell status on day 5 post-treatment: in contrast with a proliferative confluence in the AL-free negative control (Fig. 5F); the numbers of viable cells in PLGA/HA-AL-SM samples (Fig. 5A and B) and PLGA/HA-AL-DM samples (Fig. 5C and D) appeared comparably moderate as that in the soluble-AL-supplemented positive control (Fig. 5E). Besides the microsphere-free positive/negative controls, for further quantitative investigation, non-AL-laden PLGA/HA-SM microspheres were involved as additional controls to clarify non-AL-related background influence. The results indicated an AL release-induced macrophage resistance, as highlighted in Fig. 6. Ever since day 5 through day 7, the proliferation of macrophage was significantly hindered in AL-laden (PLGA/HA-AL-SM) system—the hindrance appeared comparable to that in the soluble-AL-supplemented positive control; contrastively, the growth of macrophage in non-AL-laden (PLGA/HA-SM) system remained as intact as that in the AL-free negative control.

### Osteoblastic Proliferation and Maturation

Promotion of osteoblastic proliferation and maturation by AL release from PLGA/HA-AL microspheres was respectively investigated *via* MTT assay and phenotype checking. The same groups of samples and controls as used in macrophage experiment were again employed for this purpose. As shown in Fig. 7, up to day 7, the enhancement of osteoblastic proliferation and ALP production with AL input (no matter by a microsphere-based release or a direct supplement) had appeared significant in contrast to the AL-

free controls. Besides the measurement of ALP secretion, osteoblastic maturation catalyzed by AL release was also confirmed by higher expression of the well acknowledged late bone marker OC, and type I collagen from PLGA/HA(50%)-AL-SM on day 7, as shown in Fig. 8.

### DISCUSSION

Oral administration is one of the most popular ways for medication (1). However, this most preferred administration of BPs is limited due to poor gastrointestinal (GI) absorption, which may even induce further side effects such as osteonecrosis of the jaws (29,30). Therefore, we developed HA and PLGA hybrid microspherical systems for (BP-based) AL release *in situ*, in which HA particles have been used as a vehicle for BP release because BPs exhibit strong affinity to Ca-P minerals in bones under physiological conditions; while PLGA plays an active role to encapsulate the dispersed HA particles (Fig. 2). By hybridization, it not only prolongs the release period but also prevents the nano-HA particles from being swept off by flowing body fluid from the implants.

Because of strong hydrophilicity, AL exhibits low encapsulation efficiency in traditional polymeric microspheres. Most of free drug molecules easily lapse at the beginning of release process. In this study, AL was loaded on HA nano-particles *via* strong BP-Ca chelation and the AL-laden HA particles were further encapsulated in PLGA. Therefore, the total encapsulation efficiency was significantly improved as compare with

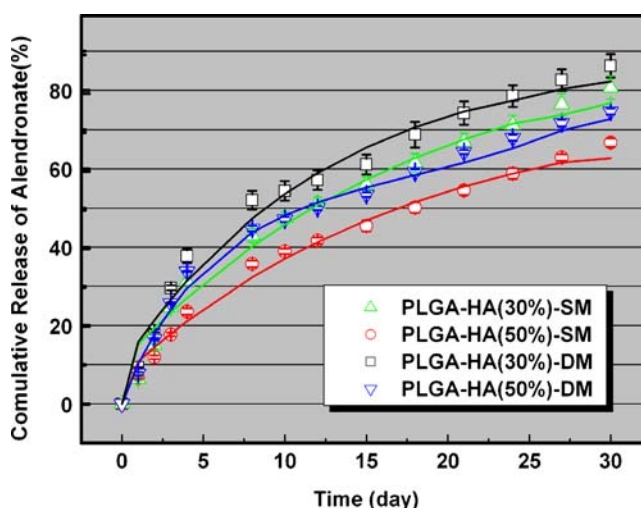
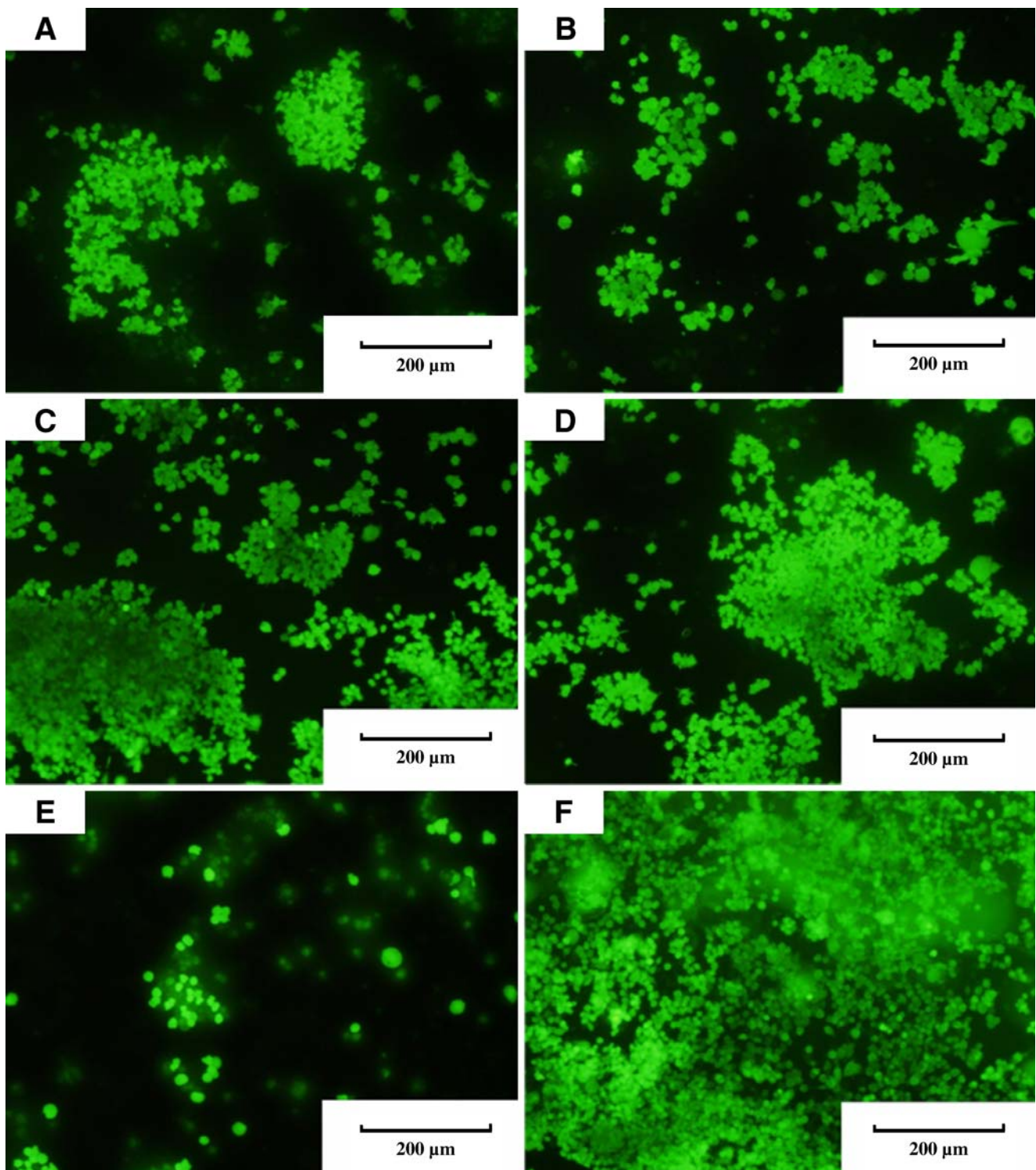


Fig. 4. Cumulative release of AL from PLGA/HA microspheres.



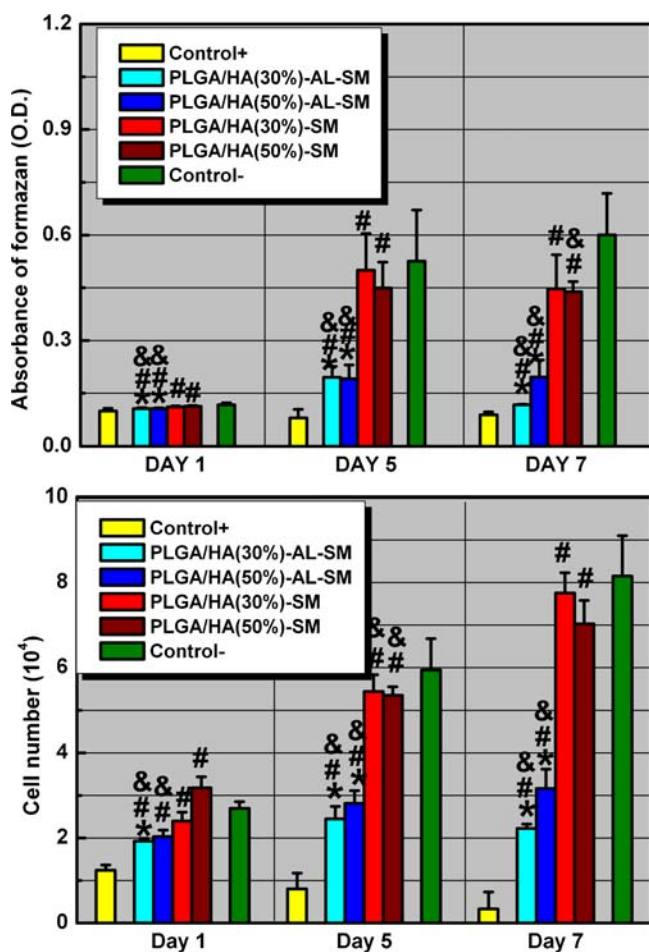
**Fig. 5.** Fluorescence photographs of macrophages after treated by PLGA/HA and PLGA/HA-AL microspheres, negative control (polystyrene of cell culture plate) and positive control (cell culture medium with 4 mg/L AL). PLGA/HA-AL-SM with 30% HA (A); PLGA/HA-AL-SM with 50% HA (B); PLGA/HA-AL-DM with 30% HA (C); PLGA/HA-AL-DM with 50% HA (D); positive control (E); negative control (F).

traditional strategies (Table I); furthermore, since the initial drug release burst can also be resisted due to the same reason, the release process was ultimately managed in control lasting over one month (Fig. 4).

In order to further enhance the drug encapsulation efficiency and simplify the preparation process, a single

emulsion technique was adopted. In single emulsion microspheres, AL was pre-attached to HA particles and then subjected to the emulsion process. The affinity between HA and AL is strong enough to prevent AL loss into aqueous phase of emulsion. In contrast with double emulsion method by which the HA exposure for AL loading is largely occupied

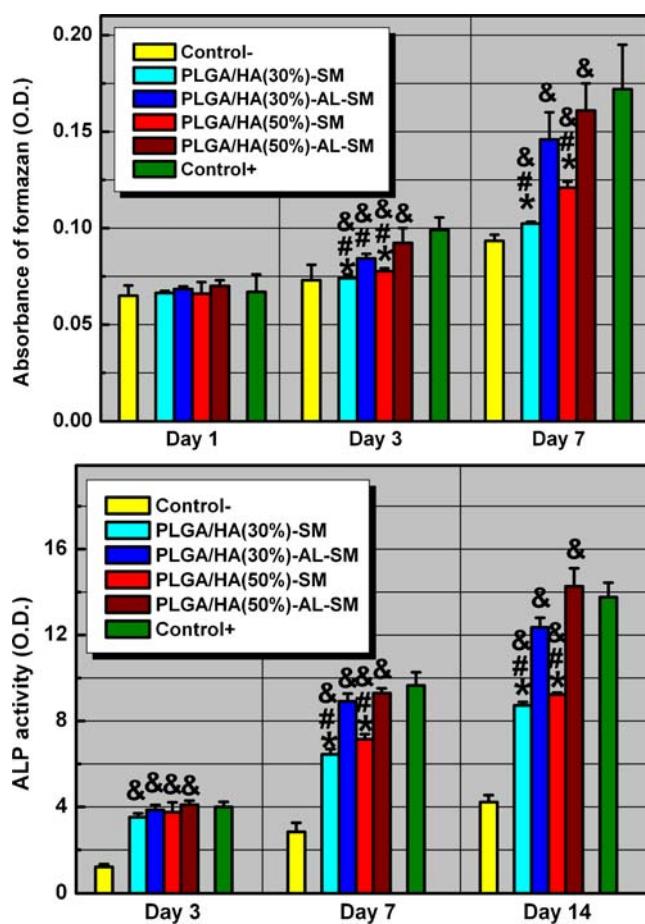




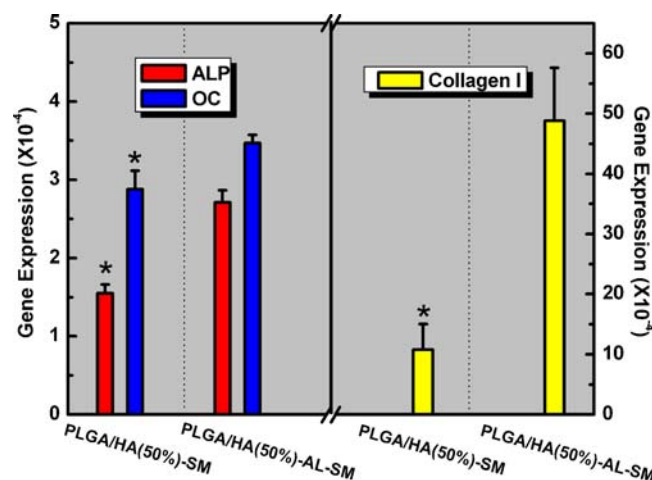
**Fig. 6.** Macrophages proliferation after 1, 5 and 7 days of culture on samples of PLGA/HA, PLGA/HA-AL-SM, negative control (polystyrene of cell culture plate) and positive control (cell culture medium with 4 mg/L AL). **A** Cell (macrophages) toxicity evaluation by MTT; **B** Relative cell proliferation ratios. Pound sign, asterisk and ampersand indicate statistical significance when compared with the PLGA/HA microspheres, positive control and negative control ( $p < 0.05$ ).

and blocked by the PLGA—containing organic phase, majority (almost 70%) of AL molecules failed to chelate with HA particles thus lapsed during the second emulsion. Hence, the microspheres produced by single emulsion technique (Table 1) gain much higher AL encapsulation efficiency (more than 85%) than those by double emulsion method (less than 40%).

The action of nitrogen-containing BPs, such as AL and Pamidronate, interferes with the mevalonate pathway of cholesterol synthesis by inhibiting farnesyl diphosphate synthase. This leads to reduction of geranylgeranyl diphosphate required for prenylation of guanosine triphosphate (GTP)-binding proteins, which are essential for osteoclast activity, so that AL is capable of effectively reducing bone absorption responsible by osteoclasts (31–37). Generally, there are three mainstream opinions about the origination of osteoclasts: 1) osteoclasts originate from the fusion of monocytes and/or macrophages; 2) monocytes, macrophages and osteoclasts all originate from common progenitor; and 3) osteoclasts are differentiated from macrophages, among which the third one is best acknowledged (38). The study by



**Fig. 7.** Osteoblasts proliferation and activity on samples of PLGA/HA, PLGA/HA-AL-SM, negative control (polystyrene of cell culture plate) and positive control (cell culture medium with 4 mg/L AL). **A** Cell (osteoblasts) toxicity evaluation by MTT after 1, 3, and 7 days culture; **B** ALP activity of osteoblasts after culturing for 3, day 7, and 14 days. Pound sign, asterisk and ampersand indicate statistical significance when compared with the PLGA/HA microspheres, positive control and negative control ( $p < 0.05$ ).



**Fig. 8.** Gene expression of osteocalcin (OC) and  $\beta$ -actin using real-time PCR. PLGA/HA microspheres without AL was used as control. Asterisk indicates statistical significance when compared with the PLGA/HA(50%)-AL-SM ( $p < 0.05$ ).

Quinn *et al.* (39,40) confirmed that mature monocytes and macrophages are capable of differentiating into osteoclasts when co-cultured with specific bone-derived stromal cell lines in the presence of 1.25 (OH)<sub>2</sub>D<sub>3</sub> and human M-CSF. Cohen-Sela *et al.* (41) reported that liposome-encapsulating BPs in polymeric nanoparticles transiently depleted of monocytes and macrophages. In this work, we specially investigated the response of macrophages to AL-laden microspheres. The results revealed that AL released from PLGA/HA scaffolds remarkably inhibited the growth and viability of macrophages (Figs. 5 and 6).

In contrast with osteoclastic resistance, AL is competent to promote the growth and commitment of osteoblasts, which were confirmed, respectively, by proliferative evaluation with MTT assay (Fig. 7) and phenotype checking with osteogenic markers such as ALP, OC and type I collagen (Fig. 8) (42). ALP is expressed mainly on cell surfaces or in matrix vesicles. It specifically degrades the organic phosphoesters in bone and cartilage, which inhibits cartilage mineralization and promotes the calcium deposition in bone. The gene expression of ALP increases before mineralization, and subsequently decreases after the initiation of mineralization (43,44). OC is an important late bone marker that regulates the formation and growth of bone minerals and therefore indicates the finalization of osteogenesis. Type I collagen holds overwhelming majority (over 90%) among the bony structural proteins and takes major responsibility for the tensile strength of the tissue and also provides templates for mineralization (43). The phenotype checking data indicated that osteoblasts treated by AL-laden microspheres exhibit significantly enhancement in expression of ALP, OC and type I collagen. Our findings agree with the studies by Im *et al.* (45) who demonstrated that AL prevents osteoblast apoptosis and indirectly contributes to the relative increase in cell number and activity, and also enhances the gene expression of osteogenic markers.

In summary, AL loaded PLGA/HA microspheres were fabricated by both single emulsion and double emulsion techniques in this study. The single emulsion microspheres showed good encapsulation efficiency (about 90%), which was much higher than the double emulsion microspheres. All groups of microspheres exhibited controlled release process without remarkable initial burst release. These *in situ* AL delivery systems could effectively inhibit the growth of macrophage while enhance the proliferation and commitment of osteoblasts. Therefore, as a favorable carrier of AL, PLGA/HA hybrid microsphere is a promising injectable and multifunctional vehicle for bone repair.

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