

Synthesis, characterization and osteoconductivity properties of bone fillers based on alendronate-loaded poly(ϵ -caprolactone)/hydroxyapatite microspheres

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Abstract A superior drug controlled release system capable of achieving efficient osteogenesis is in imperative demand because of limited bone substitute tissue for the treatment of bone defect. In the present study, we investigated the potential of using poly(ϵ -caprolactone)–hydroxyapatite (PCL–HA) composite microspheres as an injectable bone repair vehicle by controlled release of alendronate (AL), a medicine that belongs to the bisphosphonates family. The PCL/HA–AL microspheres were prepared with solid/oil/water emulsion technique, which included two processes: (1) AL was loaded on the hydroxyapatite nanoparticles; (2) the HA–AL complex was built in the PCL matrix. The spherical PCL/HA–AL microspheres were characterized with its significantly improved encapsulation efficiency of hydrophilic AL and better sustained release. Human bone

mesenchymal stem cells (hMSCs) were cultured on the surface of these microspheres and exhibited high proliferative profile. Specifically, in osteogenic medium, hMSCs on the surface of PCL/HA–AL microspheres displayed superior osteogenic differentiation which was verified by alkaline phosphatase activity assay. In conclusion, by presenting strong osteogenic commitment of hMSCs in vitro, the PCL/HA–AL microspheres have the potential to be used as an injectable vehicle for local therapy of bone defect.

1 Introduction

The treatment of bone defect originated from trauma, tumor metastasis and skeletal abnormalities compromises limited host source of bone repair. In the past three decades, researchers have witnessed the emergence of controlled release technology as an important field of pharmaceutical sciences [1]. Currently, drug-loaded microparticles have been proposed as injectable vehicle for the treatment of some bone or cartilage defects [2, 3]. To fabricate the suitable carriers that can support cell growth, proliferation and differentiation remains challenging. The development and design of biodegradable microspheres carrying drugs or bioactive factors for the therapeutic application also require a fundamental understanding of how the cells and tissues react to the microspheres.

Both polymers and inorganic substances have been tested for their usage as carriers of many drugs. A variety of inorganic materials such as hydroxyapatite (HA) [4], bioactive glasses [5], and bone cement [6] have been employed as the controlled release drug delivery system in humans with limited success. Among them, HA is the principal inorganic constituent of human bones, which has been widely adopted as biomaterials for bone repair and bone tissue regeneration

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owe to its biocompatibility, non-immunogenicity, non-inflammatory behavior, high osteoconductivity property [7, 8]. However, its poor mechanical strength and slow degradation rate limited the direct application as a drug carrier for local therapy. It is desirable to combine the HA with other biomaterials to overcome these defects [9]. Given that biodegradable polymers such as poly(ϵ -caprolactone) (PCL), polylactide (PLA), and poly(lactide-co-glycolide) (PLGA) have been used in biomedical field for a long time, they have attracted many researchers to examine the possibility of using them as carriers for controlled release. Specifically, PCL, a commercially available product approved for human application, has been extensively studied as an implantable or injectable biodegradable carrier making use of its excellent processability, good mechanical property and controllable degradability [10–12]. The clinical applications of PCL have demonstrated that it is compatible with physiological environment since it can be hydrolyzed into metabolite byproducts which are innocuous to the human body [13]. The combination of HA with PCL can be expected to combine the advantages of both biomaterials to obtain the optimal vehicles for local therapy.

Prostate cancer is the most commonly diagnosed malignancy in men. A majority of patients suffer from skeletal metastasis after developing androgen-independent, hormone refractory prostate cancer [14, 15]. During the past three decades, bisphosphonates (BPs), such as pamidronate, alendronate (AL), ibandronate, zoledronate, are indispensable for the treatment of both benign and malignant bone diseases such as osteoporosis and other bone defects induced by the metastasis of prostate cancer [16]. Bisphosphonates are a family of analogous pyrophosphate compounds that affect cellular processes involved in bone formation and resorption activity [17]. Recently, numerous studies have described the ability of BPs to inhibit tumor cell adhesion and invasion [18]. For instance, AL has been extensively used in clinical cases including malignant cancers, which have showed no severe side effects. It has been proved to be capable of inhibiting the activity of mature osteoclasts and promoting the proliferation of osteoblasts [19, 20]. However, AL is easily lapsed in aqueous conditions during fabrication process because of its high hydrophilicity [21]. In addition, the cytotoxicity of high AL concentration can induce side effect. In order to improve the AL loading amount and avoid its side effect, many researchers resort to HA to prolong the release of AL [22, 23]. Bigi et al. [24] made HA–AL thin films by assisted pulsed laser evaporation, and they found that the deposition of AL modified HA thin films were able to promote osteoblasts differentiation and restrain osteoclast proliferation. Usually, the strong hydrophilic AL exhibits low encapsulation efficiency in polymeric microspheres and processes the rapid release [25]. The AL-laden HA particles encapsulated in polymer

not only improve the encapsulation efficiency but also prolong the release period. Shi et al. induced the synovium mesenchymal stem cells (SMSCs) to osteogenesis by controlled release of AL from a mesoporous silica (MS)–HA composite that was embedded in PLGA microspheres. The total encapsulation efficiency of AL was significantly improved, and the hybrid microspheres showed significant controlled release profile of AL [25]. In this study, the AL release system was explored by packaging the AL laden HA–AL construct into the PCL microspheres.

Recently, human bone mesenchymal stem cells (hMSCs) have drawn more attention to the clinical application in the treatment of bone defect because they can self-renew and differentiate into various types of cells such as osteoblasts [26]. Osteogenic differentiation of hMSCs is a mainstream tissue engineering strategy for bone repair [27]. AL possesses higher chemical and physical stability to be utilized in hMSCs osteogenic commitment [28]. It has been reported that AL has a positive effect on the osteogenic differentiation of hMSCs [28]. However, the bioactivity of AL is very low via oral administration due to poor gastrointestinal (GI) absorption and only a small portion of the absorbed drug can be eventually incorporated in bone [29]. In order to pursue efficient osteogenesis of hMSCs by AL treatment, the controlled release system with high AL loading is required.

This study focused on fabricating and characterizing a novel injectable delivery system taking advantage of both PCL and HA, which could be potential for the treatment of bone defect induced by malignant cancer. Double emulsion (D/M) and solid/oil/water (S/O/W) emulsion technique were employed to elaborate the drug encapsulation systems, and the AL encapsulated PCL microspheres were characterized and compared. Furthermore, the cell viability and alkaline phosphatase (ALP) activity of hMSCs on AL loaded PCL microspheres were also examined.

2 Materials and methods

2.1 Materials

PCL with an average molecular weight of 40,000 g/mol was purchased from Daigang Biomaterials. HA was synthesized using the process described in Ref. [30]. AL, re-purified before usage, was obtained from Tianfeng Inc., Henan, China. Methyl cellulose M20 (MC) was purchased from Guoyao Chemical Reagents Limited, Shanghai, China. hMSCs were a gift from Research Center of Stem Cell of Sun Yat-sen University. Bone marrows were donated from sixteen healthy volunteers aged at 25–35 in accordance with guidelines of the Institutional Human Care and Ethics Committee at the University of Sun Yat-sen University. Cell related reagents would be listed exclusively.

2.2 Preparation of HA–AL particles

HA–AL particles were prepared by dispersing 1 g HA particles in 10 ml AL aqueous solution (1 mg/ml) and then stirred continuously for 24 h under 37°C. The resulted suspension was centrifuged and washed with deionized water for several times before vacuum drying. The obtained powders were stored in the desiccator for further use.

2.3 Fabrication of PCL/HA–AL and PCL/AL microspheres

- (1) *Solid/oil/water emulsion (S/O/W)*: 5 g PCL was dissolved in 40 ml methylene chloride before HA–AL particles were added. Subsequently, the amounts of HA–AL particles accounted for 15% or 30% of PCL mass were added to the above PCL methylene chloride solution. The emulsion was poured into 600 ml 0.4% MC aqueous solution and stirred at 600 rpm for 12 h. The resultant PCL/HA–AL microspheres were washed with deionized water and vacuum dried.
- (2) *Double emulsion (DM)*: 5 g PCL was dissolved in 40 ml methylene chloride. 1 ml AL (2 mg/ml) aqueous solution was dropped into 8 ml PCL methylene chloride solution. After that, the mixture was homogenized under 10,000 rpm for 30 s. The resultant mixture was poured into 600 ml 0.4% MC aqueous solution and stirred at 300 rpm for 4 h. The ultimate PCL/AL microspheres were isolated, washed with deionized water and vacuum dried.
- (3) *Oil/water (O/W)*: The control group of pure PCL microspheres was prepared by O/W method. 5 g PCL was dissolved in 40 ml methylene chloride. After that, the solution was poured into 600 ml 0.4% MC aqueous solution and stirred at 600 rpm for 12 h. The resultant microspheres were treated as the same way as PCL/HA–AL and PCL/AL microspheres did.

2.4 General characterizations of PCL based microspheres

Size distribution of the microspheres were determined by laser light scattering (Malvern, MS2000, USA). The lyophilized particles (1 g) were suspended in large amount of distilled water under stirring and analyzed. The morphology of three kinds of PCL based microspheres was conducted by scanning electron microscopy (SEM, FEI Quanta 200, The Netherlands). The microspheres were immobilized on a cupreous stub and coated with gold for two times before observation. The morphology of HA–AL particles was performed by Transmission Electron Microscopy (TEM, Philips CM300). Three individual experiments were performed for each batch.

2.5 Fourier transform infrared spectroscopy

Fourier-transformed infrared spectroscopy (Icolet Nexus FT-IR, Thermo Electron Corporation) was used in the wave number range of 4,000–400 cm^{-1} . Experimental spectra of solid samples were taken as about 1 mg samples suspended in about 100 mg of KBr. Three parallel samples were analyzed for each batch.

2.6 Encapsulation efficiency of D/M and S/O/W made microspheres

For the S/O/W made microspheres, the content of HA in the PCL/HA–AL microspheres was determined by the following method: 50 mg of vacuum dried PCL/HA–AL microspheres were dropped in 20 ml acetic acid with a pH of 4.5 for 6 h. Weighed the microspheres together with the vessel, the microspheres' weight loss after the immersion could be calculated and considered as the HA content. Five parallel samples were repeated for each batch.

1. *AL in HA–AL particles*: The AL encapsulation efficiency of HA–AL chelate was measured by pouring 50 mg HA–AL into 2 ml PBS buffer (pH 7.2) and keeping at 37°C during which the supernatant was periodically analyzed until stable following the method recorded in Ref. [31].
2. *AL in PCL/HA–AL microspheres*: PCL/HA–AL microspheres (200 mg) were dissolved in methylene chloride (30 ml) to deposit the insoluble HA–AL particles. The deposited HA–AL particles and the supernatant were collected respectively. AL was measured by two parts: first, the part ($C_{\text{part 1}}$) encapsulated in HA–AL; second, the part ($C_{\text{part 2}}$) encapsulated in the collected supernatant which was composed of PCL/methylene chloride and AL. AL in the HA–AL particles was measured using the method described above. The second part was measured according to Ref. [21]. Briefly, the collected supernatant was rinsed in iron(III) chloride/perchloric acid solution to extract the AL into the aqueous phase. The AL in the iron(III) chloride/perchloric acid solution extraction was quantified using UV spectrophotometer. The above two parts together represented the whole AL encapsulated in PCL/HA–AL microspheres. The encapsulation efficiency was calculated as follow: $C_{\text{part 1+part 2}}/C_{\text{theory}}$. Five parallel samples were repeated for each batch.

2.7 In vitro drug release evaluation

AL release experiments were performed in a shaking incubator at 87 rpm under 37°C. 50 mg AL-loaded microspheres were soaked in 20 ml PBS (pH 7.2). 2 ml

supernatant of the sample media was collected at regular time intervals and the same volume of fresh PBS was added. The AL concentration in the supernatant was evaluated using the quantification method described in Ref. [20]. Briefly, the obtained supernatant was rinsed in an iron (III) chloride/perchloric acid solution so that the AL can be entirely extracted into the aqueous phase. The AL concentration was examined using UV spectrophotometer according to the method described in Ref. [20]. Five parallel samples were examined for each batch.

2.8 Cell culture

hMSCs were propagated in Dulbecco's modified eagle's medium (DMEM) with supplements of 1.5 mg ml⁻¹ sodium bicarbonate, 10% (v/v) fetal bovine serum (FBS), 3 mg ml⁻¹ 4-(2-hydroxyethyl)piperazine-1-erhanesulfonic acid (HEPES). The cells were incubated in a humidified incubator at 37°C with 5% CO₂. The medium was changed every 3 days. All the reagents in this section were purchased from Gibco (Invitrogen, USA).

2.9 Cells seeding on the microspheres

The PCL based microspheres were first sterilized. Each kind of microspheres (containing 100 µg AL) was dispersed in 48-well TCPS (tissue culture polystyrene) plates with the bottom pre-coated with 2% sterile agarose gels. 20 µl passage 5 hMSCs in suspension (5 × 10⁴ cells/well) were seeded on each microspheres. The cells/microspheres construct was incubated at 37°C with 5% CO₂ in a humidified incubator. After 3 days, the medium was changed to remove non-adherent cells, and the medium was subsequently changed every 3 days. To observe the hMSCs adhering on the surface of the microspheres, the hMSCs and the microsphere samples were harvested, washed with PBS, fixed with 2.5% glutaraldehyde for 20 min, dehydrated in a series of graded concentrations of ethanol, vacuum-dried, and then imaged by SEM. Meanwhile, hMSCs cultured on the surface of the various types of microspheres were analyzed by a Live/Dead assay kit (Invitrogen) according to the manufacture's instruction using inverted fluorescence microscope as described previously [32]. Before cell staining, the microspheres were washed three times by PBS. Three parallel samples were analyzed for each batch.

2.10 Cell viability on the drug carriers

Cell viability on the microspheres was measured after 3, 7 and 14 days of culture using a broadly used method named MTT (Sigma) assay. The cells/microspheres constructs were washed three times with PBS and then incubated at

37°C for 4 h in 200 µl 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution. The following assay was conducted following the recommendatory protocol. Cells seeded on the pure PCL microspheres were used as control. The experiment was performed in triplicate and three independent experiments were conducted.

2.11 ALP activity

Osteogenic differentiation was induced by incubating cell laden microspheres in osteogenic medium (DMEM supplemented with 10% FBS, 100 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate; OGM) for 7 days and 14 days. The medium was changed every 3 days. The experiment was performed in triplicate. On day 7 and 14, the ALP activities were measured. At the time points, the microspheres with cells were pre-washed with PBS and the adherent cells were removed from the microspheres. 300 µl of a lysis buffer was added, incubated on ice for 10 min and the lysates were centrifugated at 4°C for 5 min at 13,000 rpm using an ultracentrifuge (Thermo, CA, USA). Each sample (20 µl supernatant) was incubated with 200 µl 5 mM p-nitrophenyl phosphate liquid substrate (pNPP, Sigma) at 37°C for 15 min. The reaction was terminated by adding 200 µl 0.1 M NaOH aqueous solution. The absorbance of the samples was detected at 405 nm using a Thermo plate reader (Thermo, CA, USA). Each ALP activity measurement was normalized by the protein content, which was measured by the BCA protein assay reagent (Invitrogen, USA). Pure PCL microspheres was used as control. Three completely individual experiments were conducted.

2.12 Statistical analysis

Data were expressed as mean ± standard deviations. The statistical significance was determined using one way analysis of variance (ANOVA). A comparison between two means was analyzed by Tukey's test. *P* value <0.05 were considered statistically significant.

3 Results and discussion

3.1 Particle size distribution of microspheres

The particle size distribution (PSD) of four kinds of PCL based microspheres were shown in Fig. 1. The diameters of most PCL/15%HA-AL, PCL/30%HA-AL and pure PCL microspheres were about 100 µm, and the most DM-PCL/AL microspheres were smaller than 100 µm. The diameters of PCL/15%HA-AL, PCL/30%HA-AL and pure PCL

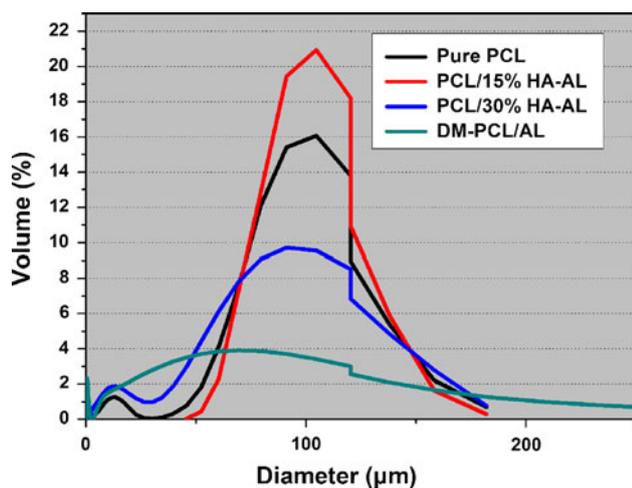


Fig. 1 Particle size distribution of pure PCL, DM-PCL/AL, PCL/15%HA-AL and PCL/30%HA-AL microspheres. Representative image was from three individual experiments

microspheres were distributed in relative narrow span while the diameters of DM-PCL/AL microspheres displayed a wide distribution. A wave was observed in the PSD of both PCL/30%HA-AL and pure PCL microspheres shown in Fig. 1, indicating that some microspheres with smaller diameters appeared. The sizes of microspheres made by S/O/W, O/W and DM method were determined by several factors such as stirring speed, solution concentration, concentration of surfactant etc. We speculated that the DM-PCL/AL microspheres with relatively smaller diameter may be attributed to the high homogenization speed, which might be beneficial to longer release of the drug [33].

3.2 Morphology of four kinds of microspheres

The SEM images of four kinds of microsphere and TEM image of HA-AL particles were shown in Fig. 2. All of the

microspheres maintained a spherical shape, but apparently, the pure PCL and DM-PCL/AL microspheres possessed a smoother surface than the microspheres loaded with HA-AL particles. Some solid particles could be obviously observed on the surface of PCL/HA-AL microspheres. On the basis of the images, a conclusion could be drawn that the microspheres' surface became rougher with the enrichment of HA-AL particles. PCL/15%HA-AL showed comparable particle size with PCL/30%HA-AL, suggesting that the enrichment of HA-AL particles had little influence on the particle size of PCL/HA-AL microspheres. No significant difference was observed between the morphology of pure PCL and DM-PCL/AL microspheres. TEM image of HA-AL particles demonstrated that the HA-AL particles presented a polygon shape with a diameter of about 50 nm.

3.3 Fourier transform infrared spectra of the microspheres

The Fourier transform infrared (FT-IR) spectrum of microspheres was shown in Fig. 3. In the spectrum of pure PCL microspheres (Fig. 3a), bands at $1,727\text{ cm}^{-1}$ was due to $\nu(\text{C}=\text{O})$ in the polymer carbon main chain. Broad peaks ranging from $1,000$ to $1,330\text{ cm}^{-1}$ were attributed to $\nu(\text{C}-\text{O}-\text{C})$, and the methane stretching vibration $\nu(\text{C}-\text{H})$ were observed at $2,946$ and $2,867\text{ cm}^{-1}$. Figure 3b illustrated the FT-IR spectrum of the DM-PCL/AL microspheres. All the peaks, which were assigned to PCL, could be observed. However, the signals of AL were hardly to be obtained by FT-IR spectra probably because the ratio of AL in DM-PCL/AL microspheres was very low. In the FT-IR spectra of both PCL/15%HA-AL microspheres (Fig. 3c) and 30%HA-AL microspheres (Fig. 3d), the PO_4^{3-} bands belong to HA were located at 602 , 961 , $1,040$ and $1,097\text{ cm}^{-1}$, and the low intensity O-H derived vibration was observed at $3,569\text{ cm}^{-1}$. Meanwhile, the typical peaks

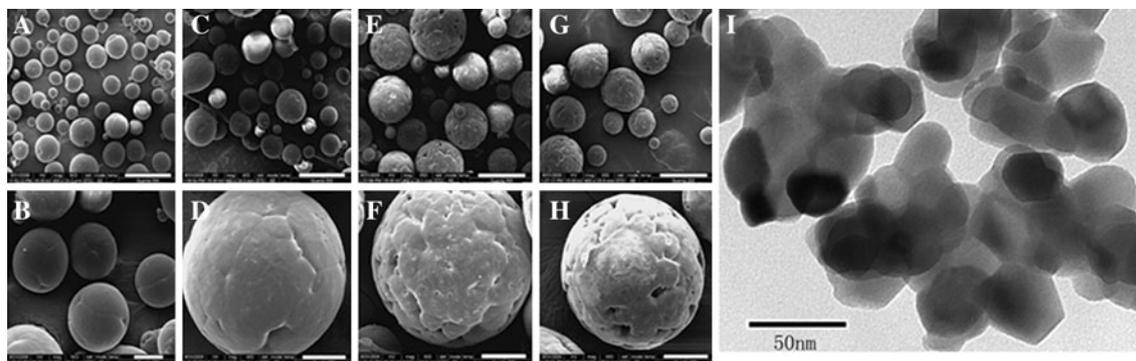


Fig. 2 SEM images of pure PCL (a, b), DM-PCL/AL (c, d), PCL/15%HA-AL (e, f) and PCL/30%HA-AL (g, h) microspheres, scale bar of the upper group $200\text{ }\mu\text{m}$; scale bar of the lower group $50\text{ }\mu\text{m}$;

TEM image of HA-AL nanoparticles (i), scale bar of 50 nm . Representative images were from three individual experiments

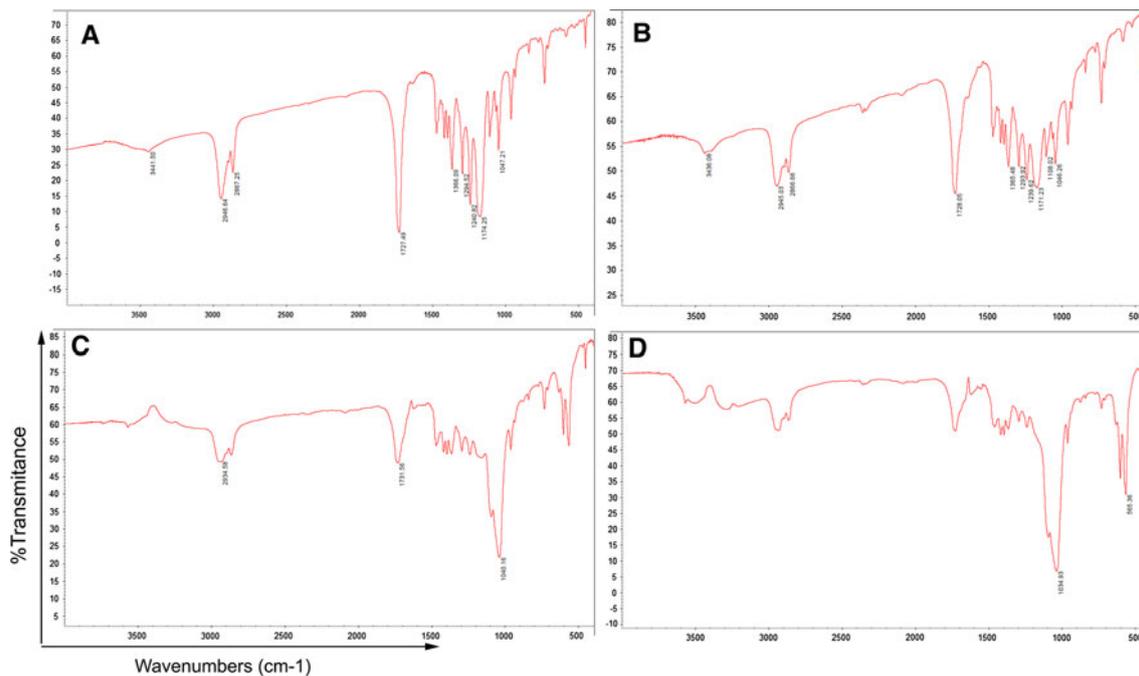


Fig. 3 FR-IR spectra of pure PCL (a), DM-PCL/AL (b), PCL/15%HA-AL (c) and PCL/30%HA-AL (d). Representative images were from three individual experiments

caused by PCL could be found in the spectra. The strong intensities of the P–O stretching vibration peaks, especially at around $1,040\text{ cm}^{-1}$, suggested that HA was successfully blended with PCL matrix. An additional feature should be mentioned that compared with the spectrum of PCL/15%HA-AL microspheres, the peaks' areas corresponding to the absorption of HA increased in PCL/30%HA-AL microspheres, indicating that the content of HA in PCL/30%HA-AL microspheres was obviously increased.

3.4 Encapsulation efficiency of DM-PCL/AL, PCL/HA-AL microspheres

In our study, AL was firstly loaded onto the HA particles and furtherly encapsulated in the PCL matrix, which facilitated the higher encapsulation efficiency of PCL/HA-AL microspheres. The AL encapsulation efficiency of DM-PCL/AL and PCL/HA-AL microspheres was shown in Table 1. The PCL/15%HA-AL microspheres enwrapped $11.05 \pm 1.02\%$ (HA mass/PCL mass) HA particles with an drug encapsulation efficiency of $77.31 \pm 3.35\%$, which showed higher encapsulation efficiency than that of PCL/30%HA-AL microspheres. The drug encapsulation efficiency of PCL/HA-AL microspheres was significantly higher than that of DM-PCL/AL, which was only $4.75 \pm 0.91\%$. The high drug encapsulation efficiency of PCL/HA-AL microspheres should be ascribed to HA-AL complexes while the very low encapsulation efficiency of

DM-PCL/AL microspheres may be because of the strong hydrophilicity of AL.

3.5 In vitro release of AL

The in vitro cumulative release profiles of AL within 21 days from three kinds of PCL related microspheres were illustrated in Fig. 4. Just as shown in Fig. 4, the profiles of AL release from the PCL/15%HA-AL and PCL/30%HA-AL microspheres exhibited the comparable curve, which were very different from that of the DM-PCL/AL microspheres. All kinds of microspheres exhibited a burst AL release with remarkably high release rate during the first 4 days. For the PCL/HA-AL microspheres, about 30% of the total loading AL was released during these days. However, nearly 70% of the AL was released in the DM-PCL/AL microspheres within the first 4 days, indicating that this release system had little effect on sustained release of AL. In the next 3 days, almost all of AL release had been accomplished from the DM-PCL/AL microspheres while after the curve inflexion at around day 4–6, the PCL/HA-AL microspheres significantly reduced AL release rate so that it took another 17 days to fulfill another 40% of total AL release. Up to day 21, the cumulative AL release reached around 70–80% from the PCL/HA-AL microspheres. The initial burst release was probably caused by the rapid diffusion of drug adsorbed on the microsphere surface while the subsequent durative release might be

Table 1 HA content and AL encapsulation efficiency of PCL/HA–AL and DM-PCL/AL microspheres

Batch	HA content (T) (100%)	HA content (A) (100%)	AL encapsulation efficiency (100%)
PCL/HA(15%)–AL	15%	11.05 ± 1.02	77.31 ± 3.35
PCL/HA(30%)–AL	30%	20.86 ± 2.55	72.36 ± 4.25
DM-PCL/AL	–	–	4.75 ± 0.91

T theoretical content, A actual content

attributed to the HA–AL complexes and the degradation of microspheres. The release profile of PCL/HA–AL system may involved the following two processes [34]: first, the AL release from HA–AL was partly controlled by the solubility of HA; second, the HA–AL particles were enwrapped in the bulk of PCL microspheres, which could further retard AL release.

3.6 Cell viability on the DM-PCL/AL and PCL/HA–AL microspheres

Attachment and adhesion occurred in the first phase of material/cells construct interactions, which will influence the cell’s capacity to proliferate and differentiate on the materials [35]. As shown in Fig. 5, the green fluorescence (Fig. 5a–d) and long, spindle-shaped cells (Fig. 5e–h) were respectively evident on the surface of microspheres, indicating hMSCs attached to the microsphere surface. A little amount of hMSCs were observed on the surface of pure PCL (Fig. 5a) and DM-PCL/AL (Fig. 5b) microspheres after 5 days of culture. In comparison, the PCL/HA–AL microspheres exhibited the more viable cells (green fluorescence) on the surface (Fig. 5c, d). The results were

consistent with that of SEM (Fig. 5e–h), suggesting that the PCL/HA–AL microspheres could promote the attachment of hMSCs compared with the pure PCL and DM-PCL/AL microspheres. It may stem from the possibility that introducing HA to the PCL microsphere system, which resulted in a rough surface, facilitated the attachment of hMSC. The cell viability on the PCL based microspheres was analyzed by measuring the activity of the mitochondrial dehydrogenases, which is referred to the MTT assay. hMSCs were incubated with DM-PCL/AL and PCL/HA–AL microspheres to evaluate the cytotoxicity of the three carriers. Figure 6 showed the results of MTT tests. All of the four microspheres showed an increasing trend of absorbance of formazan as the culture day went longer, suggesting a trend of increasing cell proliferation for all the microspheres. According to cell proliferation on the microspheres, all of the microspheres presented good biocompatibility. Cells on the DM-PCL/AL microspheres exhibited the highest viability at day 3 while at 7th and 14th day cells on PCL/15%HA–AL microspheres proliferated rapidly and showed the highest viability, indicating that PCL/15%HA–AL microspheres exhibited best biocompatibility. Compared with the pure PCL and DM-PCL/AL microspheres, the cells on PCL/15%HA–AL microspheres showed better viability at 7th and 14th day, which suggested that appropriate amount of HA had no cytotoxicity and maybe promoted the proliferation of hMSCs.

3.7 ALP activity

hMSCs underwent osteogenic differentiation on the microspheres, which was assessed by pNPP assay. ALP is an important enzyme of osteogenic differentiation that specifically degrades the organic phosphoesters and promotes the calcium deposition in bone [36, 37]. The increase of ALP activity indicates the occurrence of osteogenesis [36]. Figure 7 showed the ALP activities of hMSCs cultured on pure PCL, DM-PCL/AL, PCL/15%HA–AL and PCL/30%HA–AL microspheres in OGM. The ALP activity levels of hMSCs cultured on all the microspheres upregulated during 14 days of culture, respectively. However, hMSCs cultured on the PCL/15%HA–AL microspheres presented higher ALP activities than the pure PCL microspheres (control) and displayed the highest ALP activity at both 7th and 14th day after inducement, indicating the strongest osteogenic capacity of hMSCs cultured on the PCL/15%HA–AL microspheres. It was worthwhile to mention that hMSCs on both the PCL/15%HA–AL and PCL/30%HA–AL microspheres exhibited much higher ALP activities in contrast to hMSCs on the DM-PCL/AL microspheres at both 7th and 14th day, which was probably attributed to the rapid release of AL encapsulated in DM-PCL/AL microspheres, suggesting that the sustained

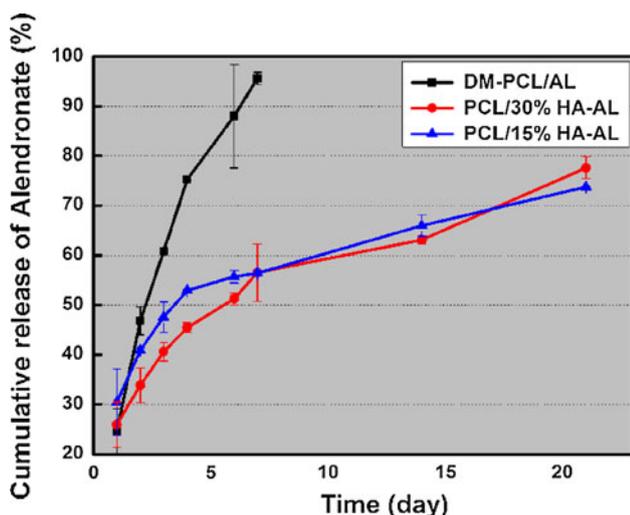


Fig. 4 Cumulative release of alendronate from DM-PCL/AL, PCL/15%HA–AL and PCL/30%HA–AL microspheres. Points mean of five experiments (n = 5); Bars ISE

Fig. 5 The fluorescence images and SEM micrographs of hMSCs adhering to the surface of the pure PCL (a, e), DM-PCL/AL (b, f), PCL/15%HA-AL (c, g) and PCL/30%HA-AL (d, h) microspheres after 5 days of in vitro culture. The fluorescence images were determined by Live/Dead staining. Representative images were from three individual experiments

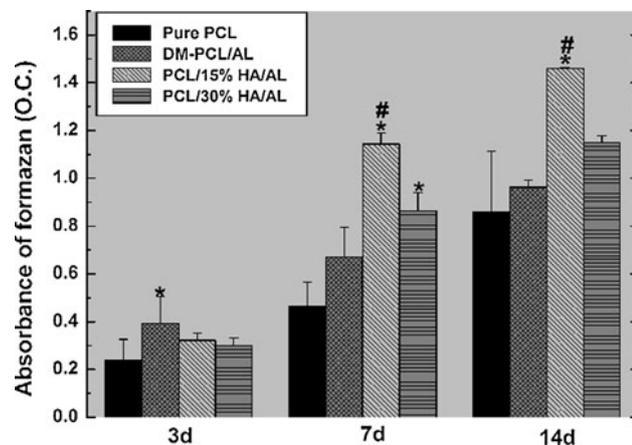
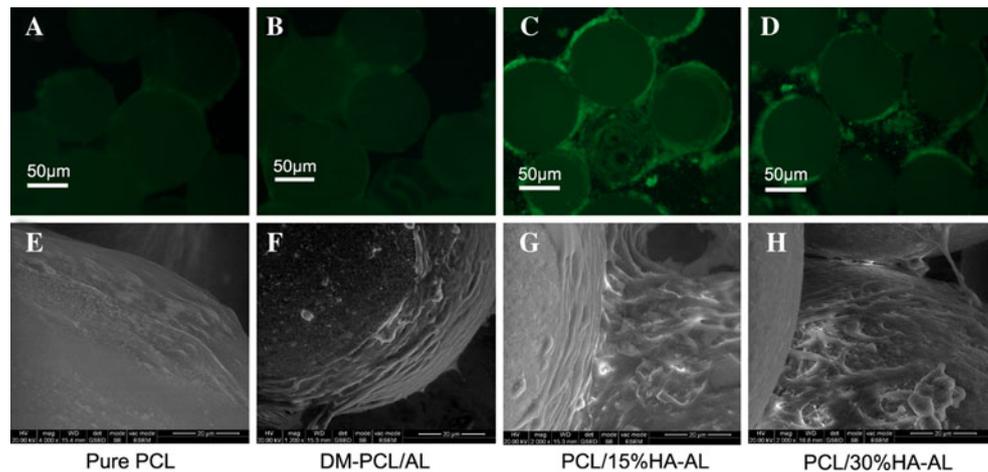


Fig. 6 Viability of hMSCs after 3, 7 and 14 days of culture on the samples of pure PCL, DM-PCL/AL, PCL/15%HA-AL and PCL/30%HA-AL microspheres. Pure PCL microspheres without AL was used as control. Columns mean of three individual experiments ($n = 3$); Bars ISE. Asterisk and Pound sign indicate statistical significant when compared with the pure PCL microspheres and DM-PCL/AL microspheres, respectively ($p < 0.05$)

release of AL significantly facilitated the osteogenic differentiation of hMSCs in OGM [38]. The results indicated that the PCL/15%HA-AL microspheres might be promising as an injectable bone repair vehicle for the treatment of bone defect.

4 Conclusion

In this study, AL loaded PCL/HA-AL microspheres were fabricated by a solid/oil/water (S/O/W) emulsion method for the treatment of bone defect. The PCL/HA-AL microspheres presented a narrow size distribution which could guarantee a high production. The FT-IR spectra of

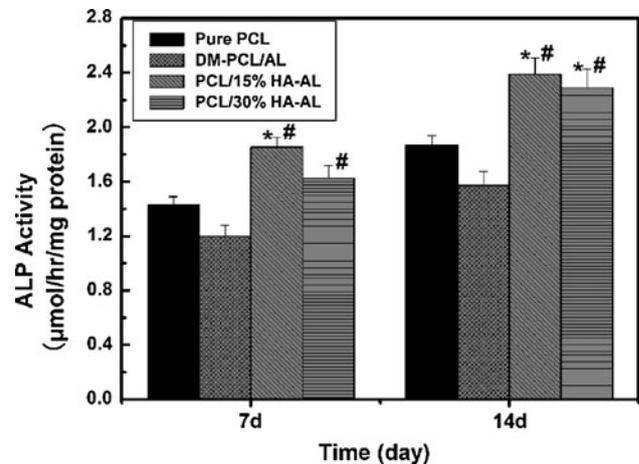


Fig. 7 ALP activity of hMSCs after 7 and 14 days of culture on the pure PCL, DM-PCL/AL, PCL/15%HA-AL and PCL/30%HA-AL microspheres in OGM. Pure PCL microspheres was used as control. Columns mean of three individual experiments ($n = 3$); Bars ISE. Asterisk and Pound sign indicate statistical significant when compared with the pure PCL microspheres and DM-PCL/AL microspheres, respectively ($p < 0.05$)

the PCL/HA-AL microspheres verified that the HA-AL particles were successfully hybridized into PCL microspheres. PCL/HA-AL microspheres exhibited sustained release profile after a slight burst release for more than 21 days. The spherical PCL/HA-AL microspheres, especially PCL/15%HA-AL microspheres, showed little cytotoxicity. Comparison between DM-PCL/AL and PCL/HA-AL microspheres confirmed the advantage of latter microspheres. The results of ALP activity of hMSCs cultured on these microspheres in OGM indicated that the PCL/HA-AL microspheres could facilitate the differentiation of hMSCs. To sum up, the PCL/HA-AL microspheres have the potential to be used as an injectable vehicle for the treatment of bone defect.

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