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PNIPAAm modified mesoporous hydroxyapatite for sustained osteogenic drug release and promoting cell attachment

Tao Wu^{a,1}, Lei Tan^{b,1}, Ning Cheng^a, Qi Yan^a, Yu-Feng Zhang^a, Chuan-Jun Liu^{b,*}, and Bin Shi^{a,*}

^aThe State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, 237 Luoyu Road, Wuhan 430079, PR China

^bKey Laboratory of Biomedical Polymers of Ministry of Education, College of Chemistry and Molecular Science, Wuhan University, Wuhan 430072, PR China

Abstract

This work presented a sustained release system of simvastatin (SIM) based on the mesoporous hydroxyapatite (MHA) capped with poly(N-isopropylacrylamide) (PNIPAAm). The MHA was prepared by using cetyltrimethylammonium bromide (CTAB) as a template and the modified PNIPAAm layer on the surface of MHA was fabricated through surface-initiated atom transfer radical polymerization (SI-ATRP). The SIM loaded MHA-PNIPAAm showed a sustained release of SIM at 37 °C over 16 days. The bone marrow mesenchymal stem cell (BMSC) proliferation was assessed by cell counting kit-8 (CCK-8) assay, and the osteogenic differentiation was evaluated by alkaline phosphatase (ALP) activity and Alizarin Red staining. The release profile showed that the release of SIM from MHA-SIM-PNIPAAm lasted 16 days and the cumulative amount of released SIM was almost seven-fold than MHA-SIM. Besides, SIM loaded MHA-PNIPAAm exhibited better performance on cell proliferation, ALP activity, and calcium deposition than pure MHA due to the sustained release of SIM. The quantity of ALP in MHA-SIM-PNIPAAm group was more than two fold than pure MHA group at 7 days. Compared to pure MHA, better BMSC attachment on PNIPAAm modified MHA was observed using fluorescent microscopy, indicating the better biocompatibility of MHA-PNIPAAm.

Keywords

BMSCs; Bone; Mesoporous hydroxyapatite; PNIPAAm; Simvastatin

1. Introduction

Since the clinical application of autogenic and allogenic bone grafts are limited, synthetic bone substitutes provide a promising solution for patients suffering from bone defects. In the past few decades, much efforts have been made to improve the feature of the bone substitutes, such as plugging in osteogenic elements [1], adjusting the pore volume or size

*Corresponding authors: cjliu@whu.edu.cn (C.-J. Liu), shibin_dentist@126.com (B. Shi).

¹These authors contributed equally to this work.

[2], and encapsulating osteogenic drug [3]. For bone substitute, the added osteogenic drug in materials greatly contributes to the treatment of bone defects. An ideal osteogenic drug-delivery system should possess the following properties: (1) biocompatibility and bioactivity; (2) osteoconductivity; (3) noninflammatory property; (4) proper particle size; (5) nontoxicity; (6) sustained drug release ability; and (7) good mechanical properties [4–7]. Simvastatin (SIM), a derivative of statin, is widely used to treat hyperlipidemia. Recently, SIM has been administered to promote bone regeneration [8]. It is involved in suppressing the TNF- α -to-Ras/Rho/MAPK pathway while enhancing BMP-Smad signaling pathway during osteogenesis [9]. As to the toxic concentration of SIM, 1.0×10^{-2} mM SIM presented obvious cytotoxicity to human epithelial cell line KB, but 1.0×10^{-5} to 1.0×10^{-3} mM SIM had no appreciable cytotoxicity [10]. For the effective concentration of SIM to promote osteogenesis in local administration, 1.0×10^{-4} mM and 1.0×10^{-3} mM SIM increased alkaline phosphatase (ALP) activity for MC3T3-E1 cells and 2.5×10^{-3} mM SIM increased MG-63 cells' BMP-2 generation [11,12]. Thus, it is a key issue to control SIM in an effective range of concentrations.

To control the release of SIM, many delivery systems have been studied, including hydrogels, microspheres and inorganic porous materials. Gelatin [8,13], alginate [14], or hyaluronic acid [15] based hydrogels are extensively applied in the release of SIM. Hydrogels are easily loaded with drugs and capable of being injected, but the weak mechanical properties limit its application as bone substitutes. For microspheres, PLGA is considered to be a good drug carrier owing to its excellent biocompatibility and biodegradability [16,17]. However, PLGA microspheres provide a large initial burst release [18]. Therefore, to overcome this problem, polymers such as poly(β -amino ester) (PBAE) [18], polyethylene glycol (PEG) [19], and poly(L-lactic acid) (PLLA) [20] were added to adjust the release profile of the drug. Inorganic porous materials were also used as bone substitutes, such as α -tricalcium phosphate (α -TCP) [21], β -tricalcium phosphate (β -TCP) [22–24], and hydroxyapatite (HA) [18]. SIM was also mixed with these materials to be drug delivery scaffolds. However, SIM from the simply mixed HA/SIM system would release completely in a short time, which limited its application for promoting bone regeneration.

HA crystals in bone are plate-like with width of 15–30 nm and length of 30–50 nm [25]. As an alternative, mesoporous hydroxyapatite (MHA) particles elicit more advantages than HA for drug delivery, such as the ordered mesoporous structure, controllable pore size and improved surface area, allowing it to be a potential candidate in bone regeneration [4,26,27]. Besides, MHA possesses hydroxyl group, which could be chemically modified as an ideal drug carrier. However, the pure MHA nanoparticles showed uncontrollable drug releasing kinetics, which lead to a burst release of ibuprofen of over 50% within 1 h [28]. Therefore, it is necessary to modify the surface of MHA with capping agents to avoid the burst release of the drug.

The polymer capping agents endow mesoporous nanoparticles sustaining release properties [29–31]. Poly(N-isopropylacrylamide) (PNIPAAm), a thermal-responsive polymer, has been well studied in the field of cell harvesting and widely used as an injectable hydrogel. Below the lower critical solution temperature (LCST, 32 °C), PNIPAAm chains become hydrophilic and show extended conformations in water so that cells will detach mildly from

the PNIPAAm modified surface. Above the LCST, the PNIPAAm chains become hydrophobic and collapse on the material surface. The proteins and cells attached on the hydrophobic surface can be improved at 37 °C. Moreover, the cells adhered to the surface of PNIPAAm modified HA grow better than on HA [32,33]. The polymer chains on the surface of mesoporous particles would collapse when the chains are in hydrophobic state at physiological temperature (37 °C). Then the loaded drug would release slowly and realize long-term release [34].

Herein, we synthesized a SIM delivery system combining both the osteogenic potential of MHA and the sustained release properties of PNIPAAm together. A certain amount of SIM was loaded into the MHA-PNIPAAm and encapsulated below 32 °C. The sustained release of SIM from MHA-SIM-PNIPAAm at the effective concentration lasted over 16 days at 37 °C. Compared to MHA-SIM, improved cell proliferation and better osteogenic differentiation of MHA-SIM-PNIPAAm was observed due to the sustained release of SIM. The cell attachment on the surface of hydrophobic PNIPAAm modified MHA was also improved at physiological temperature.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (PNIPAAm), (3-aminopropyl) triethoxysilane (APTES, 99%), 2-Bromoisobutyryl bromide (BIBB), triethylamine, N, N, N', N', N''-pentamethyldiethylenetriamine (PMDETA, 99%), FITC-phalloidin, β -glycerol phosphate, L-ascorbic acid, and dexamethasone were purchased from Sigma-Aldrich. $K_2HPO_4 \cdot 3H_2O$, NaOH, $CaCl_2$, and cetyltrimethylammonium bromide (CTAB) were obtained from Sinopharm Chemical Reagent Co. Ltd. All solvents were purified with calcium hydride before use. CuBr (Shanghai Chemical Reagent) was purified with acetic acid at 80 °C overnight before use. Simvastatin (98%) was purchased from Calbiochem®. α -MEM and penicillin/streptomycin (P/S) solution were purchased from HyClone. Fetal bovine serum (FBS) was purchased from Gibco. Triton X-100 was purchased from Amresco. DAPI was purchased from Beyotime. CCK-8 was purchased from Dojindo Molecular Technologies.

2.2. Synthesis of MHA and MHA-PNIPAAm

MHA was synthesized using hydrothermal method in the presence of CTAB (Fig. 1). To obtain MHA-NH₂, MHA was modified with APTES. Then the initiator was introduced to the MHA-NH₂ by using BIBB. After that, The MHA-PNIPAAm was prepared through SI-ATRP method on the surface of MHA.

In detail, K_2HPO_4 (5.5 g) and cetyltrimethylammonium bromide (CTAB, 8.7 g) were dissolved in 100 mL distilled water, and the pH of solution was adjusted to 12.0 using NaOH aqueous solution (1.1 M, 30 mL). Subsequently, $CaCl_2$ aqueous solution (0.4 M, 60 mL) was added dropwise to the solution above and stirred at 120 °C for 24 h. The white precipitation was collected and washed with excess distilled water and dried in vacuum. The product was calcined at 600 °C for 6 h to remove the residual CTAB [35].

MHA (2.0 g) was suspended in anhydrous toluene (60 mL), and then 3-aminopropyl triethoxysilane (APTES, 3 mL) was added to the mixture and the solution was heated to 116 °C for 24 h. The particles were filtered and washed with toluene and ethanol for three times, respectively. The product was dried in vacuum overnight to be MHA-NH₂.

Then MHA-NH₂ (1.7 g) and anhydrous triethylamine (1.5 mL) were dispersed in anhydrous dichloromethane and placed in ice bath. After that, 2-Bromoisobutryl bromide (BIBB, 1.5 mL) was added dropwise and the solution was stirred for 1 h. Then the solution was stirred at ambient temperature for 24 h. The particles were washed with dichloromethane, ethanol and distilled water, respectively. The product was dried in vacuum overnight to be MHA-Br.

MHA-Br (190.0 mg), N-isopropylacrylamide (NIPAAm, 1.1 g), N, N, N', N', N''-pentamethyldiethylenetriamine (PMDETA, 65 µL), and distilled water/methanol (1/1, 8 mL) were added to polymerization pipe. The mixture was degassed by two freeze-pump-thaw cycles under argon. CuBr (15 mg) was added to the pipe and degassed two cycles. The solution was stirred under 28 °C for 24 h. The particles were filtered and washed with excess DMF and DI water to remove the absorbed polymer. The product was dried in vacuum to obtain MHA-PNIPAAm.

2.3. Characterizations

The morphology of MHA and MHA-SIM-PNIPAAm nanoparticles was observed by scanning electron microscopy (SEM, QUANTA 200, The Netherlands) and transmission electron microscopy (TEM, HRTEM, JEOL, JEM-2010FEF, Japan). For SEM analysis, the samples were dried in vacuum and coated with gold to reduce charging effect before performed at 30 kV. Spectra from Fourier transform infrared spectroscopy (FT-IR) were determined by Perkin–Elmer spectrometer. The surface areas and pore size distributions were obtained by Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) analysis (Micromeritics ASAP 2020). X-ray photoelectron spectroscopy (XPS) was operated by KRATOS XSAM800 spectrometer. The structure of samples were analyzed by X-ray diffraction (XRD) (Bruker D8 ADVANCE, Germany), using Cu K α radiation ($\lambda = 1.54056$ Å). UV–vis spectra of SIM were examined by UV-2401 PC of Shimadzu.

2.4. Drug loading and release study

MHA-PNIPAAm (270.0 mg) was dispersed in 10 mL distilled water, then 10 mL of 0.5 mM SIM ethanol solution was added and stirred in 10 °C for 24 h. The solvent was evaporated and the dried product was collected, which was MHA-SIM-PNIPAAm. As a control, the unmodified drug loading of MHA was operated similarly.

To evaluate the release of SIM from capped MHA, MHA-SIM-PNIPAAm (10.0 mg) and MHA (10.0 mg) were first soaked in phosphate buffered saline (PBS, pH = 7.4) at 37 °C for 24 h to remove absorbed SIM and then dispersed in 40 mL 0.2% 1-hexadecanesulfonic acid sodium PBS solution (pH = 7.4) at 37 °C, respectively. At desired time intervals, 1 mL supernatant of solution was withdrawn and 1 mL fresh solution was made up. The release profile of SIM from supernatant was determined by UV–vis spectrophotometer at 238 nm wavelength.

2.5. BMSC isolation and in vitro culture

Bone marrow mesenchymal stem cells (BMSCs) were chosen as a candidate as the seed cells because they had the potential to differentiate into mesodermal lineages including osteoblast, chondrocyte, and adipocyte [36–38]. BMSCs were obtained from three randomly selected 8-week-old Wistar female rats. After euthanasia by sodium pentobarbital and cervical dislocation, bone marrow was flushed out of femur and tibia using α -MEM, containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) solution in standard sterile condition. The isolated BMSCs were cultured in plastic culture dish with 10% FBS and 1% P/S solution in a 5% CO₂ incubator at 37 °C. After 48 h of incubation, the non-adherent hemocytes were washed away. Then the culture medium was changed every three days. After two passages, cells were used in the whole study [39].

2.6. Preparation of SIM dissolution extracts

Before soaking in α -MEM without serum at 37 °C for 24 h, 10 mg MHA and MHA-SIM-PNIPAAm nanoparticles were sterilized in advance. Then the soaked solution was discarded to remove unabsorbed SIM. Every 10 mg of MHA or MHA-SIM-PNIPAAm was dipped in 40 mL fresh α -MEM with 10% FBS and 1% P/S at 37 °C for 24 h. After transient centrifugation at 1000 rpm for 1 min and then filtration by 10 mL syringe with a 0.22 μ m bacterial filter, the supernatant liquid was collected. Subsequently, two kinds of extracts and a blank control were used for the following cell culture [1].

2.7. Fluorescence microscopy analysis

MHA and MHA-PNIPAAm nanoparticles were pressed as a 1 cm diameter and 2 mm width mode. After ultraviolet disinfection, the plates were soaking in α -MEM at 37 °C in 5% CO₂ for 2 h. BMSCs were seeded at 5×10^5 cells per well on MHA or MHA-SIM-PNIPAAm plates in 12-well plate. After 8 h of incubation, cells were washed with PBS two times and fixed with 4% paraformaldehyde for 15 min at room temperature. The samples were washed with PBS three times and permeabilized with 0.1% Triton X-100 for 2 min and then washed with PBS two times again. Cells were stained for 30 min by FITC-phalloidin (5 μ g/mL) at room temperature. After washed with PBS for two times, samples were incubated with DAPI (10 μ g/mL) for 5 min at room temperature. The stained cells were photoed by a fluorescent microscopy to record their adhesion and morphology [40].

2.8. Cell counting kit-8 (CCK-8) assay

The proliferation of BMSCs in material extracts was measured by the CCK-8 according to the manufacturer's protocol. In briefly, BMSCs were seeded into 48-well plates at a density of 7×10^3 per well. After 24 h of cell adhesion, the culture medium was replaced by 500 μ L per well material extracts medium of MHA, MHA-SIM-PNIPAAm or blank culture medium for 1, 3, 5, 7, 9, 11 days. Then the CCK-8 assay was performed by incubating in 10% CCK-8 solution at 37 °C in 5% CO₂ incubator for 1 h. For CCK-8 measurement, 100 μ L of incubated cell suspension was transferred to a 96-well plate. The absorbance of the culture medium was measured at 490 nm by microplate reader [36].

2.9. Quantitative alkaline phosphatase activity

BMSCs were seeded in a 12-well plate at a density of 3×10^5 cells per well. After 24 h of incubation, culture medium was changed to material osteogenic extracts medium containing α -MEM supplemented with 10% FBS, 10 mM sodium β -glycerol phosphate, 50 μ g/mL L-ascorbic acids and 1.0×10^{-8} M dexamethasone. Quantitative ALP activity was measured at 7 and 14 days. At each time point, the culture media was removed and washed with PBS three times before treated with 200 μ L per well of 0.1% Triton X-100 and 2 mM MgCl_2 to dissolve the cells. Then, cell suspensions were transferred to 1.5 mL tubes and were centrifuged at 14,000 rcf at 4 °C for 10 min. Afterwards, 50 μ L of cell lysates at each well was transferred to new 96-well plates and the ALP activity and total amount of protein were determined by *p*-nitrophenyl phosphate method. After 1 h of incubation at 37 °C, the reaction was stopped by the addition of 50 μ L of 1 N NaOH. The production of *p*-nitrophenol was determined by the absorbance at 405 nm in microplate reader. Using *p*-nitrophenol as a standard, the ALP activity was calculated based on standards curves and then normalized to the total protein content, which was determined by BCA protein assay kit [41].

2.10. Alizarin Red staining

BMSCs were seeded at 3.0×10^5 cells per well into 12-well plate and changed to material osteogenic extracts medium after 24 h of incubation. The medium was changed every 4 days. 1.0 g Alizarin powder was dissolved into 50 mL distilled water, and pH = 4.2 was adjusted. At 14 days, cells were fixed with 4% paraformaldehyde for 15 min at 37 °C and rinsed in distilled water for 3 times. Then the fixed cells were stained in Alizarin Red solution for 15 min and washed in distilled water to remove excess stain. The calcium nodule staining was photoed by Canon DSLR camera [41]. The mineralized nodules were dissolved by hexadecylpyridinium chloride and the OD values were obtained at 562 nm for quantitative data [42,43].

2.11. Statistical analysis

For quantitative analysis, all results are expressed as mean $\pm$ standard deviations (SD) of three independent experiments. The data were submitted to analysis of variance, and means were compared by the Student's test. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Characterization of MHA and MHA-PNIPAAM

The morphology and structure of MHA (Fig. 2a) and MHA-PNIPAAM (Fig. 2c) were observed by SEM and TEM, which showed that the prepared nanoparticles were uniformly distributed in rod-like shapes. The shape and size of nanoparticles remained consistent after PNIPAAM coating, which indicating the stability of nanoparticles structure after chemical modification. The mesoporous structure of nanoparticles could be clearly observed in TEM images (Fig. 2b, d). Compared with unmodified MHA, around 5 nm polymer layer was

shown in MHA-PNIPAAm group, which indicated the successful modification of PNIPAAm on the surface of MHAs [33].

BET analysis illustrated the nitrogen adsorption–desorption isotherms of unmodified MHA and MHA-PNIPAAm and mesopore size of pure MHA. Both of the N₂ adsorption/desorption isotherms proved the mesoporous structure of particles. (Fig. 3). However, the isotherm became flat due to the covering of PNIPAAm. The pore size of MHA was around 4 nm (as shown in the insert).

X-ray diffraction (XRD) patterns of particles were shown in Fig. 4. The small-angle diffraction ($2\theta = 0-8^\circ$) revealed the characteristic peak occur at (100) Bragg peak, resulting from the existence of pores structure. After the modification of PNIPAAm, the peak was deduced, indicating the coating of polymer on the pores surface (Fig. 4a). No differences in XRD patterns was shown between MHA and MHA-PNIPAAm at wide-angle ($2\theta = 10-60^\circ$), which could be explained by that PNIPAAm modification on MHA did not alter the crystallization of other phases (Fig. 4b).

The surface functionalization of MHA, MHA-Br and MHA-PNIPAAm was monitored by FT-IR spectrometer (Fig. 5). The typical broad peaks observed at 638 and 3572 cm⁻¹ were ascribed to the stretching vibrations of hydroxyl group. Additionally, the absorption band at 964 and 1043 cm⁻¹ were raised from the phosphate stretching modes. The weak absorption band of amide group (secondary amide CO stretching) at 1560 cm⁻¹ indicated the immobilization of initiators on the surface of MHA (Fig. 5b). In addition, the increased intensity of 1560 cm⁻¹ and appearance of 2974 and 2924 cm⁻¹ (–CH₂) suggested that the successful modification of PNIPAAm onto MHA (Fig. 5c).

To further confirm the chemical modification, XPS was applied to evaluate the detail chemical elements of the nanoparticles (Fig. 6). The signals of Ca, P and O elements proved the formation of MHA (Fig. 6a). The N and Br elements appeared at 400 and 69 eV in XPS spectra indicated the successful introduction of initiators (Fig. 6b). Compared with MHA-Br, the C and N element percentages of MHA-PNIPAAm increased, while the percentage of P element decreased due to the covering of the PNIPAAm layer on the surface (Fig. 6a).

3.2. Drug release profile

To investigate the release behavior of SIM in vitro, 10.0 mg MHA-SIM or MHA-SIM-PNIPAAm was placed in 40 mL 0.2% 1-hexadecanesulfonic acid sodium PBS (pH = 7.4) solution for 16 days. Before the measurement, both MHA-SIM and MHA-SIM-PNIPAAm were soaked in PBS solution at 37 °C for 24 h to remove unabsorbed SIM. A large amount of unabsorbed SIM in was washed away during the soaking in PBS while PNIPAAm modified MHA could retain SIM inside. At desired time intervals, the supernatant solution was sucked to measure the absorbance of SIM and calculated the cumulative release concentration via UV–Vis spectra. The release curve of MHA-SIM showed that the release only lasted three days, and amount of the released SIM did not increase with the following days (Fig. 7). Moreover, only 10×10^{-4} mM of SIM was released from MHA-SIM in total. The release profile of drug from MHA-SIM demonstrated the low capacity of drug loading of bare MHA. However, from the release curve of MHA-SIM-PNIPAAm, the release of

SIM lasted 16 days, and almost sevenfold amount of SIM was released than MHA-SIM. Furthermore, the daily SIM release was nearly 10×10^{-4} mM in MHA-SIM-PNIPAAm group during the first week, which is in an effective range of concentrations. The high capacity of drug loading can be explained by the capping PNIPAAm covered on the surface of MHA, and the sustained release of SIM from MHA-SIM-PNIPAAm is due to the existence of the collapsed PNIPAAm coating. The polymer layer coating acts as a barrier to control the release of SIM.

Since 1.0×10^{-2} mM SIM showed cytotoxicity, but 1.0×10^{-5} to 2.5×10^{-3} mM SIM promoted osteogenesis obviously [10], 10 mg of MHA or MHA-SIM-PNIPAAm was dipped in 40 mL fresh α -MEM to get a effective concentration SIM dissolution extracts. According to the release profile, SIM would maintain an effective concentration in half month to enhance bone regeneration.

3.3. Cell attachment and viability

It was reported that higher initial cell adhesion could reduce the recombination time between material and cells [44]. BMSC attachment on MHA and MHA-PNIPAAm plates was observed under fluorescent microscopy. After 8 h of incubation, the number of BMSCs attached on MHA-PNIPAAm plate (Fig. 8c) was higher than those on MHA plate (Fig. 8a), which was consistent with the results measured by cell density (Fig. 8e). In high magnification ($\times 40$), BMSCs spread over on the MHA-PNIPAAm plate (Fig. 8d), while BMSCs on the MHA plate, they tended to distribute in elongated shapes (Fig. 8b). Sufficient cell spreading is an important indicator of the improved adhesion. The staining of actin filaments demonstrated the shape and spreading of cellular skeleton and rigidity during attachment [45]. Thus, the above results showed an increased cell spreading and attachment on the MHA-PNIPAAm at the early stage at 37 °C [32,33]. Previous studies showed that nanotopography and nanoscale roughness were positively correlated to cell interaction [33]. According to the SEM images, MHA modified with PNIPAAm did not exhibit a significant change in surface topology. However, the polymer brushes formed on the surface of MHA increased the contact area between MHA and Ca ion. After their binding, anionic HPO_4^{2-} migrate to the Ca ion anchor point, which subsequently enhance BMSC adhesion and spreading over the surface [46].

The cellular biocompatibility of MHA-SIM-PNIPAAm was tested by CCK-8 assay (Fig. 9). All four of the groups displayed that the cell proliferation was increased during 11 days in culture. Significant statistical differences were shown between MHA-SIM-PNIPAAm group and MHA or the blank group at days 3, 5 and 7, indicating that MHA-SIM-PNIPAAm extracts could promote BMSC proliferation. However, the difference between MHA-SIM and MHA-SIM-PNIPAAm group was only presented difference on day 5, which could be explained by the residual effect of SIM on BMSC proliferation. Compared to blank control, MHA and MHA-SIM group also showed higher levels of cell viability at day 3 and day 5. Owing to the Ca ion and P ion released from MHA nanoparticles [47]. However, no difference in cell viability was detected between MHA and MHA-PNIPAAm group, indicating that PNIPAAm had no effect on BMSCs. At day 5, these four groups showed statistically difference during BMSC logarithmic growth phase, except for MHA and MHA-

SIM group [40]. Thus, from the results of cell morphology, surface attachment as well as BMSC proliferation curves proved that MHA-SIM-PNIPAAm possesses better biocompatibility than pure MHA.

3.4. Osteogenic differentiation

ALP activity is a significant index of osteogenic differentiation especially in early stage [48]. ALP activity of BMSCs was determined after 7 and 14 days culture in material extracts during osteogenic induction (Fig. 10h). At day 7, ALP activity of MHA-SIM-PNIPAAm group was obviously higher than the other two groups. At day 14, ALP activity was continuously increasing in MHA-SIM-PNIPAAm group compared to the blank group. However, there was no statistically difference between MHA-SIM-PNIPAAm and MHA group. The higher ALP activity was detected in MHA group than blank group at 14 days, which was consistent with previous studies showing the enhanced ALP activity of BMSCs growing on the MHA [49,50]. Thus, both MHA-SIM-PNIPAAm and MHA were capable of enhancing osteogenesis, while MHA-SIM-PNIPAAm exhibited a better performance. The potential mechanism might be attributed to the released Ca ion and P ion from MHA nanoparticles [47]. High Ca ion concentrations stimulated pre-osteoblast chemotaxis to the certain site and their maturation produce new bone [51]. Similarly, the release of P ion also activated related bone forming signaling molecule in process of bone formation such as bone morphogenetic protein-2(BMP-2). BMP-2 stimulates P ion transport by osteogenic cells primarily via the sodium-dependent phosphate transporters [52]. In addition, SIM released from nanoparticles could synergize osteogenic differentiation via suppressing TNF- α -to-Ras/Rho/mitogen activated protein kinase and enhancing BMP-Smad signaling, resulting in the enhanced expression of type-I collagen, osteopontin, osteocalcin and bone sialoprotein [9,53]. Moreover, SIM stimulated the expression of vascular endothelial growth factor (VEGF) in osteoblasts by reducing protein prenylation and down-regulating the phosphatidylinositol-3 kinase pathway, which promoted ALP activity and enhances the responsiveness to PTH [54].

To further analyze osteogenic differentiation, the Alizarin Red staining was performed after 14 days of culture in osteogenic medium extracts (Fig. 10). Mineralized nodules were formed in all groups, while the amount of matrix mineralization in MHA-SIM-PNIPAAm group was highest (Fig. 10c, f). Quantitative measurement of Alizarin Red staining further confirmed the results (Fig. 10g). In conclusion, MHA-SIM-PNIPAAm presents as a good osteogenic potential contributing to the bifarious effect of the Ca ion and P ion release and the controlled SIM release.

4. Conclusion

A sustained release of SIM based on MHA capped with PNIPAAm was presented. The capping agent PNIPAAm realized the sustained release of SIM from MHA-PNIPAAm-SIM and the hydrophobic surface could promote the cell attachment at 37 °C. This delivery system could maintain a therapeutic concentration of SIM at the implantation site. Cellular studies revealed that MHA-SIM-PNIPAAm nanoparticles improved BMSC proliferation and

osteogenic differentiation. These results indicated that MHA-SIM-PNIPAAM could be used as a bone substitute with sustained osteogenic drug release.

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Fig. 1.
Schematic illustration of the preparation of MHA-SIM-PNIPAAAM.

Fig. 2.
SEM and TEM images of MHA (a, b) and MHA-PNIPAAm (c, d).

Fig. 3.
The nitrogen adsorption–desorption isotherms of MHA, MHA-PNIPAAm and pore size distribution of MHA (insert).

Fig. 4. Small-angle (a) and wide-angle (b) XRD pattern of MHA and MHA-PNIPAAm.

Fig. 5.
FT-IR spectra of (a) MHA, (b) MHA-Br and (c) MHA-PNIPAAM.

Fig. 6.
XPS spectra of (a) MHA and MHA-PNIPAAm, and (b) MHA-Br.

Fig. 7. Simvastatin release curve of MHA-SIM and MHA-SIM-PNIPAAAM nanoparticles at 37 °C in PBS (pH= 7.4).

Fig. 8. Fluorescence microscopy images of BMSCs attached on MHA (a, b) or MHA-PNIPAAm (c, d) for 8 h. (a and c, $\times 20$; b and d, $\times 40$). (e) Density of BMSCs attached on MHA or MHA-PNIPAAm surface for 8 h ($n = 3$ per group; $*P < 0.05$).

Fig. 9. BMSC proliferation in blank, MHA, MHA-PNIPAAM, or MHA-SIM-PNIPAAM extraction medium for 1, 3, 5, 7, 9, 11 days, as determined by CCK-8 assay. (n = 4 per group; *P < 0.05).

Fig. 10.

Alizarin Red staining of BMSCs cultured in blank (a, d), MHA (b, e), or MHA-SIM-PNIPAAM (c, f) in osteogenic medium for 14 days. Quantitative analysis of Alizarin Red staining (g). Quantitative ALP activity of BMSCs cultured in blank, MHA, MHA-SIM-PNIPAAM in osteogenic medium extracts for 7 and 14 days ($n = 3$ per group; $*P < 0.05$) (h).