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Effects of pH and thermally sensitive hybrid gels on osteogenic differentiation of mesenchymal stem cells

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Abstract

Osteoblastic differentiation of mesenchymal stem cells from bone marrow is an essential step for bone formation. The osteogenesis is normally induced by chemical mediators. Recent laboratory studies have revealed that mechanical properties of an extracellular matrix, typically hydrogels with different modules, also affect the fate of stem cells. The question is how to adjust their mechanical properties inside the body in biomedical applications. In this study, we designed/used a novel extracellular matrix, namely, a hybrid gel made of billions of injectable small thermally and pH-sensitive poly(*N*-isopropylacrylamide-*co*-acrylic acid) microgels whose swelling at the body pH and temperature physically jammed them and mesenchymal stem cells together, which enabled us to *in situ* apply an adjustable mechanical stress on those embedded stem cells. By treating the cell layer with the microgels, we found that an earlier incorporation of the microgels significantly increases the alkaline phosphatase activity, while a later addition of the microgels after the primary calcium deposition enhances the extracellular matrix mineralization in the mesenchymal stem cells cultures accompanied by up-regulation of osteogenic marker genes expression, presumably due to the calcium fixation by the carboxyl groups inside the microgels and the physical contact between the microgels and mesenchymal stem cells layers. These microgels provide an extracellular matrix microenvironment to affect the fate and biological behavior of mesenchymal stem cells, facilitating their potential applications in regenerative therapies.

Keywords

Microgels, p(*N*-isopropylacrylamide-*co*-acrylic acid), mesenchymal stem cells, extracellular matrix, osteogenesis

Introduction

The tissue engineering is about creating functional replacements for tissues damaged by injury or disease via a combination of cells, scaffolds, and soluble mediators.[1–3] However, impaired tissues or organs fail to provide sufficient cell numbers for tissue repair and regeneration, in the meantime the repair process takes weeks with raised risk of inflammation or infection.[4] To address this clinical need, stem cells' unique advantage of self-renewal and the potential of differentiating into multiple cell types lead them to be a promising cell source for regenerative medicine therapies.[2,5,6] Particularly, mesenchymal stem cells (MSCs) have emerged as a clinically relevant cell source due to its abundance and multipotent capabilities.[7–10]

Traditionally, the fate of stem cells is regulated either *in vivo* or *in vitro* by genetic and molecular mediators (e.g. growth and transcription factors).[11,12] However, more and more recent evidences have demonstrated that physical interaction between stem cells and extracellular matrix (ECM) can also influence the cell fate.[13–16] In a landmark work, Discher and coworkers[17,18] planted MSCs on polymer gels with different elasticity and showed that MSCs could differentiate into multiple cell types responding to matrix stiffness.

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Their study revealed that in the microenvironment, the mechanical properties of the ECM also affect the cell fate besides the stimulus from chemical mediators, secreted factors, and neighboring cells. Later, experimental results from different laboratories have confirmed such an ECM effect.[15,19]

At the molecular level, it is still unclear whether such mechanical properties of ECM affect the transcription, the translation, or both. However, the lack of a detailed mechanism has not prevented people to seek its potential biomedical applications. Obviously, it is rather difficult to apply a controllable and *in situ* mechanical stress on the implanted stem cells inside a body. Recently, Yang et al found that hMSCs retain mechanical information from past physical environments and this mechanical dosing influences future cell fate decision.[20] Therefore, it opens a door to use *in vitro* conditions outside the body to guide the stem cell differentiation inside.

In order to use mechanical properties of ECM to *in vivo* affect the stem cell fate, we designed injectable and dually pH and thermally sensitive microgels, a very tiny three-dimensional (3D) network made of polymer chains, to affect the fate of the implanted stem cells. The idea is to mix the microgels together with stem cells during the injection and use the *in situ* jamming of individual microgels swollen at the body temperature and pH to generate a hybrid bulk gel that applies mechanical stress on each surrounded stem cells (Figure 1).

Here the "hybrid" means a combination of chemical and physical gels; namely, each microgel itself contains a chemically cross-linked polymer network, while their jamming together to form the bulk gel is due to physical interaction. In addition, using small microgels has other advantages, including (1) fixation of stem cells inside the body and (2) encapsulating and slowly releasing all the necessary mediators to stem cells to direct their differentiation. The microgels typically have a size of 10^2–10^3 nm and exist as a stable dispersion.[21] Due to their small size, individual microgels can swell more quickly when they are brought to the body temperature and pH in comparison with their bulk gel counterparts that are used as ECM in most of previous stem cell differentiation studies.

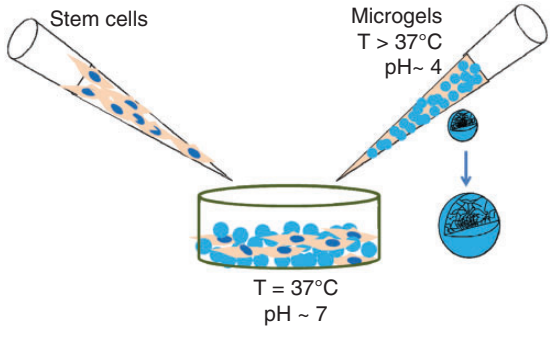

Figure 1. Illustration of encapsulation of MSCs by hybrid gel made of microgels.

Among environmentally sensitive polymers, thermally sensitive poly(*N*-isopropylacrylamide) (PNIPAM) with a lower critical solution temperature (LCST ~32°C) is the mostly studied.[22–24] Namely, above 32°C, PNIPAM becomes insoluble in water so that the gel network made of such a polymer expels water and collapses. Upon lowering the temperature, individual microgels can swell up to 10 times in their size. The LCST of PNIPAM microgels can be tuned by incorporating another comonomer. In the current study, using hydrophilic acrylic acid (AA), we are able to increase its LCST from its original 32°C to the body temperature. In addition, using such a weak electrolytic comonomer also makes the microgels pH and ionic strength sensitive. In the current study, after preparing a series of thermally/pH sensitive P(NIPAM-AA) microgels with a diameter of ~2 μm and a similar cross-linking density but different comonomer ratios, we studied their effects on osteogenesis by introducing them at different stages in 2D MSCs culture.

Materials and methods

Materials

N-isopropylacrylamide (NIPAM, Fluka) was recrystallized from toluene/n-hexane. Acrylic acid (AA, Adrich), *N,N'*-methylenebis acrylamide (MBA, Fluka), and potassium persulfate (KPS. Merck) were used as received without further purification. Deionized water was used in all the experiments.

Synthesis of P(NIPAM-AA) microgel particles

In precipitation polymerization, 3 g monomer in different NIPMA/AA ratios and 0.03 g *N*, *N'*-methylenebisacrylamide (MBA) was dissolved in 140 mL D.I. water. The mixture was stirred and purged with nitrogen to get rid of oxygen for 30 min. After heating the system to 70°, 0.24 g potassium persulfate (KPS) solution (dissolved in 10 mL D.I. water, purged with nitrogen for 30 min) was added to initialize the reaction. The reaction was carried on for 6 h under the protection of nitrogen flow. The product was purified by centrifugation at 8500 g and resuspended.

Characterization of P(NIPAM-AA) microgel particles

The AA content in microgels was determined by indirect titration. Namely microgel solution was firstly supplemented with sufficient NaOH for 3 h to ensure total neutralization. The excess NaOH was then titrated with HCl (0.1 mM). For scanning electron microscopy (SEM),

0.2 mg/mL microgel solution was loaded on to a silica wafer, dried under room temperature overnight, and imaged on a FEI Quanta 400 FEG microscope operating at 15 kV. The ζ potential of microgel particles was measured at 25°C by using the ZetaPlus (Brookhaven Instruments Co., Holtsville, New York). The microgel particles were dispersed in different pH buffers and characterized by using a laser light scattering (LLS) spectrometer (ALV/DLS/SLS-5022F) equipped with a multi-τ digital time correlator (ALV5000) and a cylindrical 22 mW He-Ne laser ($\lambda_0 = 632$ nm, UNIPHASE). The details of the LLS instrumentation and theory can be found elsewhere.[25,26] Firstly 1.5 mL PBS buffer was clarified by filtrating through 200 nm filter (Millipore) at room temperature to remove dust particles and 10 μL microgel solution (20 mg/mL) was added in before the temperature-dependent LLS measurement.

Isolation and culture of MSC

Bone marrow-derived MSCs were isolated from 4-week-old C57BL6 mice using the established protocol. Briefly, the femora of mice were taken and connective tissue was removed. Then both ends of the femora were cut and the bone marrow was flushed out with PBS from the femoral cavity. The cells were purified by controlling the digestion time. MSCs were maintained in α-MEM supplemented with 10% fetal bovine serum (Gibco) containing 1% penicillin/streptomycin and 1% glutamine (Gibco). The cells were incubated under a humidified atmosphere of 5% CO_2 in air at 37°C and passaged every 5–6 days using 0.25% (w/v) trypsin-EDTA solution (Gibco).

MTT assay of cytotoxicity of microgels on MSCs

The cytotoxicity was evaluated by MTT assay. The MSCs were seeded in a 96-well plate at an initial density of 5000 cells per well. After 24 h, the dispersions with different microgel concentrations (in plain α-MEM) were added. Such treated MSCs were incubated in a humidified environment with 5% CO_2 at 37°C for 48 h. The MTT reagent (20 μL, 5 mg/mL) was then added to each well. The cells were further incubated for 4 h at 37°C. The medium in each well was then removed and replaced by 100 μL DMSO. The plate was gently agitated for 15 min before the absorbance (A) at 490 nm was recorded by a microplate reader (Bio-rad, USA). The cell viability was calculated by $(A_{treated}/A_{control}) \times 100\%$, where $A_{treated}$ and $A_{control}$ are the absorbance of the cells cultured with medium containing microgels and fresh culture medium, respectively. Each experiment condition was done in quadruple. The data were shown as the mean value plus a standard deviation (±SD).

BrdU incorporation assay of microgels effect on MSCs proliferation

In our experiment, BrdU Kit (Roche Life science) was used. MSCs cells were seeded in a 96-well plate at an initial density of 5000 cells per well. After 24 h, the dispersions with different microgel concentrations were added (in plain α-MEM). After the treatment, the BrdU labeling reagent was added into each well and the cells were further incubated for 24 h in a humidified environment with 5% CO_2 at 37°C. Then, MSCs were fixed and denatured with the FixDenat reagent for 30 min at room temperature, followed by incubation with Anti-BrdU-POD for 2 h at RT. The cells were then washed with PBS three times and incubated with the substrate solution for 30 min at room temperature. The optical density of 96-well plates was measured with a microplate reader (Bio-rad, USA) at a wavelength of 370 nm and a reference wavelength of 492 nm. The cell proliferation capacity was calculated by $(A_{treated}/A_{control}) \times 100\%$, where $A_{treated}$ and $A_{control}$ are the absorbance of the cells cultured with medium containing microgels and fresh culture medium, respectively. Each experiment condition was done in quadruple. The mean value plus a standard deviation (±SD) is shown.

Osteogenic differentiation of MSCs in vitro

For the osteogenic differentiation, $\sim 3 \times 10^4$ cells were placed in 24-wells plate. When the cells reached confluence, the osteogenic medium (α-MEM composed of 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, 50 μg/mL L-ascorbic acid (Sigma), 10 mM β-Glycerophosphate (Sigma), and 10^{-7} M dexamethasone (Sigma-Aldrich)) was supplemented and changed every 3 days. The cells were harvested at Day 14 or 21.

Treating MSCs by medium containing microgels

Medium containing microgels were prepared by diluting concentrated microgel dispersion (75 mg/mL, PBS) to a required concentration in control (α-MEM), osteogenic medium or other specially conditioning medium. The cells were plated in 24- or 6-well plate. The medium with the microgels was supplemented after the confluence and replaced every 3 days before the cells were harvested at Day 14 or 21.

Alkaline phosphatase and Alizarin Red S staining

For the cytochemical staining, the medium was first removed. The cell layers were rinsed with PBS and then fixed by 10% formalin for 15 min at room

temperature. After removing the fix solution, the cells were washed by D.I. water. The 1-Step NBT/BCIP Solution (Thermo Scientific) or the Alizarin Red S staining solution (2% aqueous solution, pH = 4.1 ~ 4.3) was added. The staining process was monitored and run for up to 1 h. Afterwards the staining solution was aspirated and the cell layer was washed by D.I. water three times followed by air dry.

Real time reverse transcriptase-polymerase chain reaction

RNA was extracted using the RNAiso Plus (TaKaRa); 1 μg RNA was reversibly transcribed to cDNA using the 10 μL PrimeScript RT Master Mix (TaKaRa) by the recommended protocol. The product (cDNA) was diluted to 30 μL. One μL of the cDNA solution was further used as a template for real time PCR in an ABI7900HT Real Time PCR system. The expression of mRNA was determined using the standard SYBR Premix Ex Taq kit (Takara).

Statistical analysis

The results of triplicated experiments were reported as mean ± standard deviation. Student's *t*-test and analysis of variance (ANOVA) are used to determine the statistical significance, and values of $p < 0.05$ are considered significant.

Results and discussions

pH sensitivity of microgels with different AA content

We prepared P(NIPAM-AA) microgels with W_{AA}: $W_{AA+NIPAM}$ (mass ratio) ranging from 10% to 30%. The dispersion becomes unstable and undergoes a phase separation when W_{AA}:$W_{AA+NIPAM}$ is higher than 25%. Therefore, only those stable dispersions were used in the current study. Table 1 summarizes the initial feeding and actual AA contents determined by titration in different microgel dispersions, which shows that most of AA was copolymerized inside. Hereafter, we use the feeding ratio to denote the microgels with different AA contents.

Table 1. Feed ratio and actual amount (M_{AA}: $M_{(AA+NIPAM)}$, mass ratio) of AA incorporated into microgels.

Sample ID	1	2	3	4
Feed ratio (AA)	10%	15%	20%	25%
Incorporation amount	8.8%	13.1%	19.0%	23.4%

Figure 2(a) shows the pH-dependent ζ potential of the microgels with different AA contents. With its pKa around 4.35, the carboxyl groups ionize when pH increases so that AA changes from hydrophobic to hydrophilic. The ionization also makes individual microgels more swollen due to the osmotic pressure generated by counter ions (Figure 2b). At pH = 7, the ζ potential decreases as the AA content increases because more carboxyl groups get charged; while at pH ~4, most of $-COO^-$ are protonated so that the ζ potential becomes independent on the AA content. With a sufficient concentration, the swelling of the microgels at pH ~7 can change a dispersion into a hybrid bulk gel made of billions of jammed swollen microgels (Figure 2b inserts).

Temperature sensitivity of microgels with different AA content

Figure 3(a) shows that the hydrodynamic radius of microgels in solution are narrowly distributed after polymerization (T = 25°C pH = 4). Figure 3(b) presents that microgels gradually swell as the temperature decreases. In comparison with pure PNIPAM microgels, the change of $<R_h>$ at the LCST is less sharp because the electrostatic repulsion among the charged $-COO^-$ groups partially cancels the hydrophobic attraction at higher temperatures. Note that at ~37°C, all the microgels used in the current study have a size of 1.5–2 μm. Table 2 summarizes the pH dependence of four microgels with different AA contents. A combination of pH and temperature effects enables us to increase the size of individual microgels by a factor of ~2, i.e. a volume increase of ~8 times (in reference of T = 60°C, pH = 4). Namely, if the microgels in the initial dispersion have a volume fraction of 12.5%, the swelling will lead to a hybrid gel (T = 37°C, pH = 7).

Cytotoxicity and effect on proliferation capacity of MSCs by microgels

After characterizing the pH and temperature dependence of the microgels, we checked their cytotoxicity on MSCs and effect on the MSCs proliferation, respectively, using the MTT and BrdU incorporation assays, as shown in Figures 4 and 5. In the low concentration range (<20 mg/mL), the microgels are nearly non-cytotoxic to the MSCs. As the microgel concentration increases, the cell viability gradually decreases. Note that in our current study the microgel concentration was within the limit of <20 mg/mL. Figure 5 shows that in the low-concentration range the inclusion of the microgels are nearly harmless in the MSCs proliferation in comparison with the control.

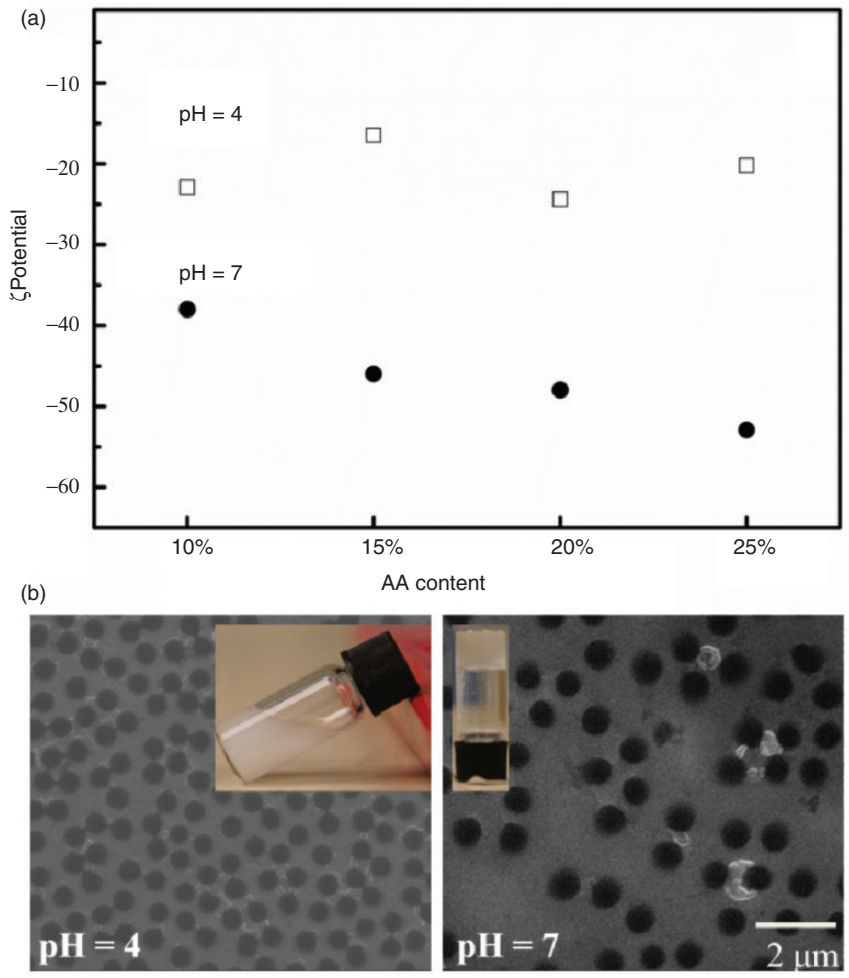

Figure 2. AA content dependence of microgels at differnet pH enviorments: (a) presents AA content dependence of ζ potential at different pH enviorments and (b) shows SEM imagines of microgels (20% AA) at different pH. Inserts in (b) reveal how a microgel dispersion (20% AA, 40 mg/mL) changes into a hybrid gel when pH changes from 4 to 7. The scale bar is 2 μm for both pH = 4 and pH = 7 SEM images.

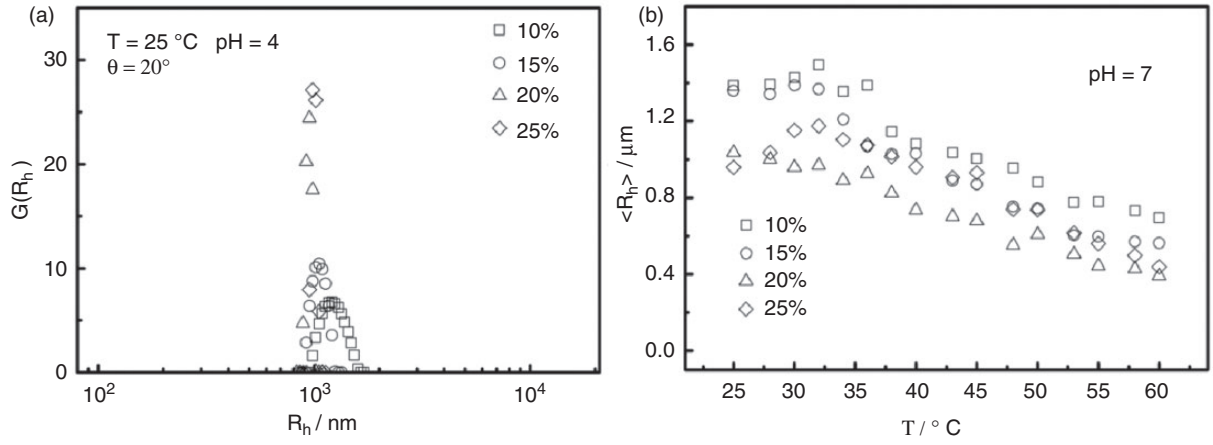

Figure 3. Distribution and temperature dependence of size for four microgels with different AA contents. (a) hydrodynamic radius distribution of microgels with different AA content at pH = 4. The scattering angle is 20° and temperature is controlled at 25°C. (b) temperature dependence of average hydrodynamic radius of four microgels with different AA contents.

Table 2. pH dependence of average hydrodynamic radius ($<R_h>$) of microgels with different amounts of comonomer AA at 37°C.

AA content	10%	15%	20%	25%
pH = 4	1.17 μm	0.99 μm	0.96 μm	0.98 μm
pH = 7	1.26 μm	1.05 μm	0.89 μm	1.05 μm

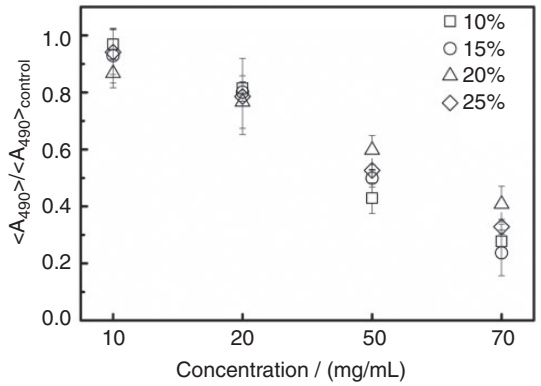

Figure 4. Concentration dependence of cytotoxicity of microgels on MSCs. MTT assay was performed to examine the cytotoxicity of microgels on MSCs with indicated concentrations. The control was set to 1.0.

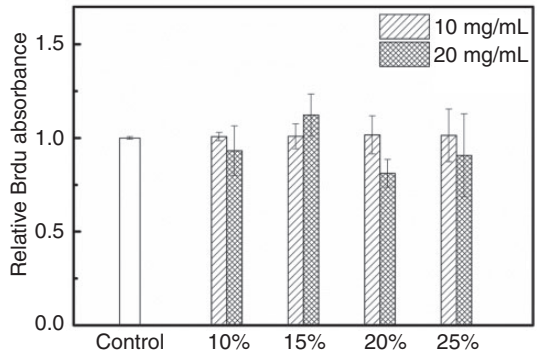

Figure 5. AA content dependence of effect of microgels on MSCs proliferation. BrdU incorporation assay was performed to determine the effect of AA content on the proliferation rate of MSCs. The control was set to 1.0.

Osteogenic differentiation of MSCs with incorporated microgels through all stages

We supplement MSCs with a control medium (α-MEM) that contains different types of the microgels with a concentration of 10 mg/mL when the cells become confluent. The insert of Figure 6 shows that after being cultured for 14 days, the alkaline

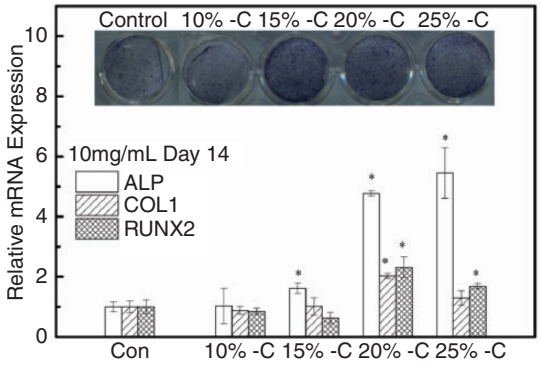

Figure 6. Microgels dependence of relative mRNA expression with three different marker genes (ALP, COL1, and RUNX2) after 14 days, where insert shows ALP staining of MSCs treated with control medium (α-MEM) with and without different types of microgels (10 mg/mL); $*p < 0.05$ and $n = 3$.

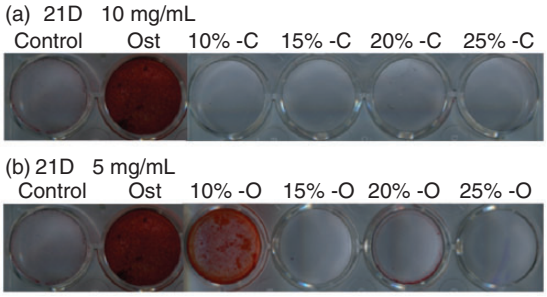

Figure 7. Microgel type and concentration dependence of mineralization of MSCs cultures stained with Alizarin Red S after 21 days, where (a) in controlled α-MEM medium (-C) at 10 mg/mL; and (b) in full osteogenic medium (-O) at 5 mg/mL.

phosphatase (ALP) activity of the treated cells was higher than that of the control. Figure 6 also shows the up-regulation of two more osteogenic marker genes, COL1 and RUNX2. Our results indicate that the microgels may have a positive effect on the early osteogenic differentiation.

In order to determine whether the ECM mineralization, an index of mature osteoblast differentiation, can be triggered by treating MSCs with the microgels alone, we cultured MSCs up to 21 days by feeding the cells with a control medium containing different types of the microgels (10 mg/mL). No ECM mineralization was observed after the Alizarin Red S staining. In contrast, Figure 7(a) shows that the MSCs cultured with the full osteogenic medium have a bright red color, revealing a full response of the mineralization after the osteoblastic differentiation.

It has been known that the differentiation of MSCs *in vitro* depends mostly on culture conditions. Osteogenic differentiation of MSCs *in vitro* is induced by the presence of dexamethasone, ascorbic acid, and

β-glycerophosphate. Considering this, we dissolved microgels into full osteogenic medium and cultured the cells up to 21 days. However, only cells treated with low AA content (10% AA) and microgel concentration (5 mg/mL) present positive signal for Alizarin Red S stains, which is much weaker than MSCs cultured under full osteogenic medium (Figure 7b).

The above results show that besides an important role of chemical mediators, the microgels also have some effects, such as promoting the ALP activity without any added chemical mediators (Day 14). However, the incorporation of the microgels in either the control or the osteogenic medium failed to induce the ECM mineralization after 21 days, comparing with the full osteogenic medium treatment. Since the full osteogenic medium is very well conditioned and rather difficult to prevail, we wonder whether a similar phenomenon is also observable in a diluted osteogenic medium.

To prepare the conditioned medium with different concentrations of inducing factors, we mixed the full osteogenic medium with the control medium at a volume ratio of 3:1, 1:1, or 1:3; namely, to attain three-quarters, half, or one-quarter osteogenic medium. The 15% microgels incorporated (10 mg/mL) in the various conditioned mediums are also simultaneously prepared. On both Days 14 and 21, we found that under medium treatment alone, the ALP activity of the cells was diminished as the concentration of inducing factor increased from zero to full, while the ECM mineralization displayed a reversed phenotype. We also note that the ECM mineralization state of the cells on Day 21 was stronger than that on Day 14 as shown in the Alizarin Red S staining. However, for the cells treated by the microgels-incorporated medium, in spite that the ALP activity was higher than that under the correlated medium with an identical concentration of inducing factors, all the Alizarin Red S staining signals were completely eliminated (Figure 8a and b). The osteogenic marker genes expression of the cells under the above conditions revealed a similar tendency. ALP mRNA level of the cells gradually became lower when the concentration of inducing factor increases (Figure 8c). In addition, the mRNA level of

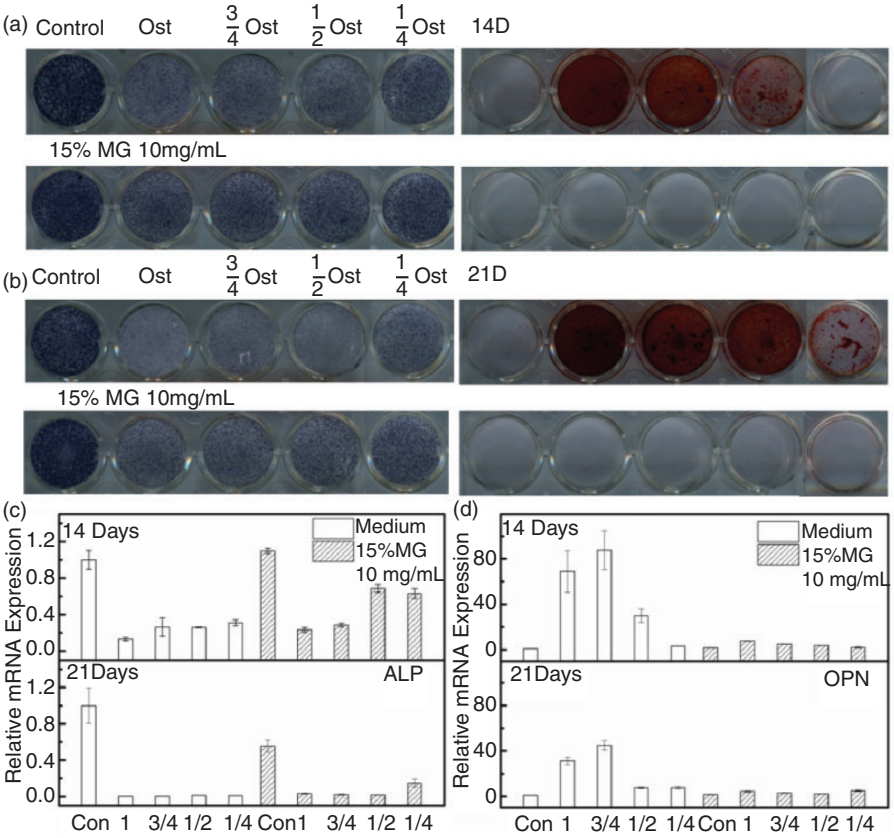

Figure 8. Osteogenic differentiation of MSCs treated with different conditioned mediums on Days 14 and 21, where left and right panels of (a) and (b) respectively show ALP activity and Alizarin Red S staining; first and second rows of (a) and (b) respectively show results without and with incorporated microgels; (c) and (d) respectively show ALP and OPN mRNA expressions of the cells treated with different conditioned mediums without (hollow) and with (patterned) microgels on Days 14 and 21.

the mature osteoblastic marker osteopontin (OPN) was remarkably up-regulated when the concentration of inducing factors increase. The incorporation of the microgels restored the ALP activity notably but dramatically eliminated the OPN expression, as shown in Figure 8(d). Our results clearly show that the incorporation of the microgels through all the stages elevates the ALP activity but eventually restrain the ECM mineralization.

It has been known that the *in vitro* osteogenesis closely resembles the *in vivo* bone formation, passing through three different stages as discussed in details as follows.[8,27] In the first stage (Days 1–4), there is a remarkable increase in the cell number. The second early osteoblastic differentiation stage (Days 5–14) is characterized by the transcription and protein expression of ALP and COL1. In the third late stage (Days 14–28), the expression levels of ALP and COL1 gradually decrease and the mature osteoblast markers, osteopontin (OPN) and osteocalcin (OCN) are highly expressed together with the depositions of calcium and phosphate. Note that each microgel contains a large amount of anionic carboxyl groups. Once the deposition starts, the microgels will function as a sponge to absorb calcium ions so that they will not be able to anchor to the MSCs layer. On the other hand, the adsorption also lowers calcium ions free in the medium. Since the medium was replaced every 3 days, the mineralization process is continuously disrupted. This is why the osteogenesis of MSCs is suppressed but the proliferation capacity is maintained in a low-calcium environment, only resulting in a prominent increase in the cell numbers.[28,29] Considering that the squeezing of MSCs in a limited surface area forces them to interact with each other, we would expect that it is such interaction that might lead to the primary osteogenic differentiation with an elevated expression of ALP, COL1, and RUNX2.

After knowing that the added microgels deplete calcium ions, which inhibits the mineralization and prevents MSCs to reach their full differentiation state, we decided to incorporate the microgels at some specific time points because the ALP activity was lifted up in the presence of the microgels. In one way, we introduced the microgels only in the early culture stage and replaced them with the full osteogenic medium afterwards. In another way, we first cultured MSCs in the full osteogenic medium and replaced it with the medium incorporated with the microgels after calcium phosphate is primarily formed to avoid the adsorption of free calcium ions. In such a way, we allow calcium ions to anchor on the cells first so that the interaction between the anchored cationic calcium ions and anionic carboxylic groups on the microgels will bring them together.

Osteogenic differentiation of microgel-treated MSCs at an early culture stage

To study the effects of treating MSCs with the microgel-incorporated medium at an early culture stage, we divided our studies into groups. In the first group, we kept the cells in the control or the osteogenic medium with the microgels (5 mg/mL) for 7 or 14 days before changing to a pure osteogenic medium for another 14 (MG7 + MD(Ost)14) or 7 (MG14 + MD(Ost)7) days. In the second group, the cells were treated in an identical protocol but with a doubled microgel concentration (10 mg/mL).

Figure 9 shows that for a given microgels concentration, MG7 + MD(Ost)14 leads to a higher mineralization degree than MG14 + MD(Ost)7, presumably due to a sufficient contacting time between the cells and the osteogenic inducing factors since the third step in osteogenesis is time-consuming and requires ~14 days. This might also explain why MG14 + MD(Ost)7 leads to a higher ALP activity but much less differentiation into osteoblasts. Also note that in all the cases studied, both the Alizarin Red S staining and the OPN mRNA expression level reveal that the mineralization degree decreases as the AA content increases. Moreover, we found that a lower microgel concentration led to a higher mineralization degree, especially when MG14 + MD(Ost)7 was used, supporting the above absorption argument; namely, more anionic AA groups (a higher AA content or microgel concentration) in the medium will result in the adsorption of more cationic calcium ions so that the osteogenesis will be hindered more profoundly. Using MG7 + MD(Ost)14, we found that the control medium with the microgels leads to a higher mineralization degree than the osteogenic medium with the microgels, further supporting the absorption argument; namely, introducing the microgels at an early stage lifts the ALP activity, especially in the control medium, and supplementing the full osteogenic medium later led to more substantial differentiation of MSCs into osteoblast.

Osteogenic differentiation of MSCs treated with microgels after calcium deposit

In the osteogenic differentiation, MSCs undergo the osteoblast lineage specification, matrix maturation, and mineralization. Hydroxyapatite (HA) deposition occurs before the maturation of collagen matrix in the bone formation. Therefore, we added the microgels onto the MSCs layer as soon as a trace amount of calcium phosphate appeared and then examined its effect on the osteogenesis.

It is expected that due to the entropy gaining of counter ions, the interaction between calcium ions

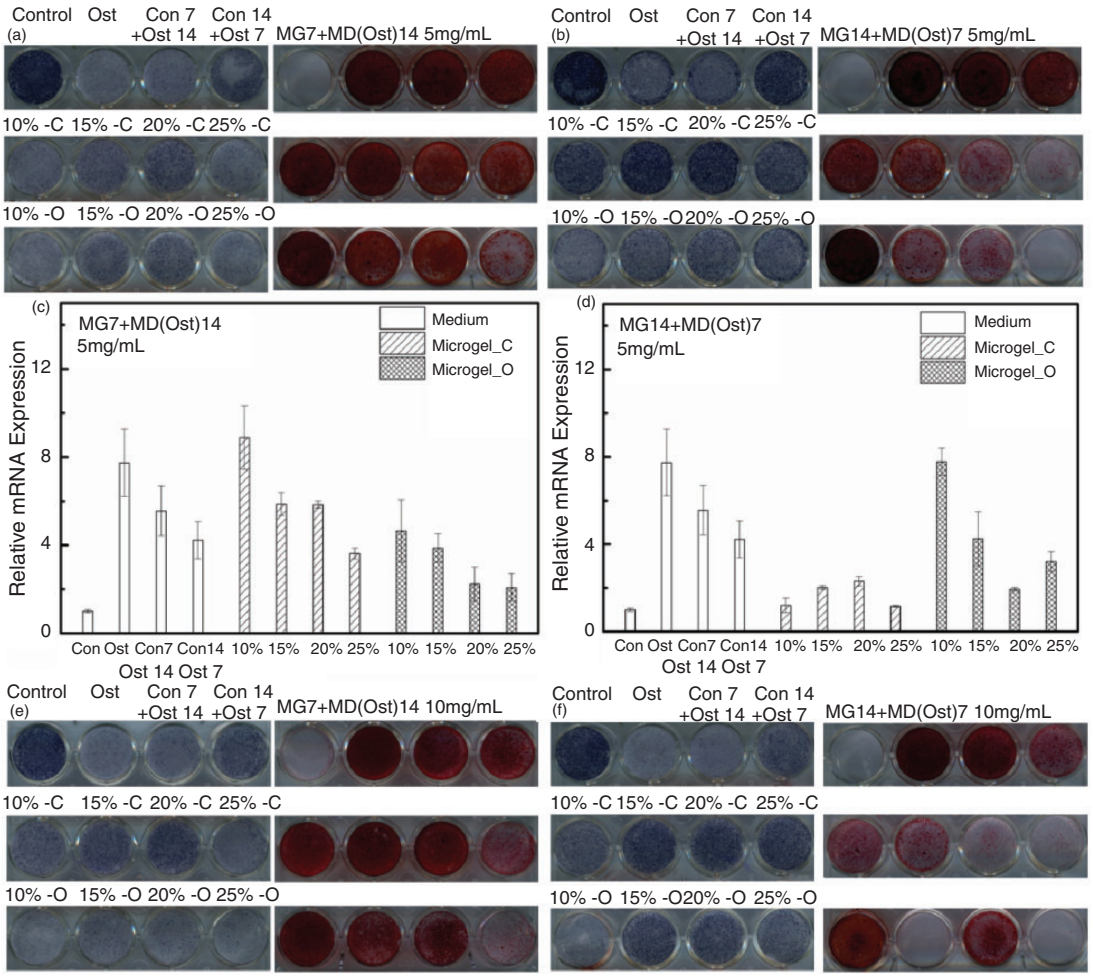

Figure 9. Effects of adding microgels at different early culture stages on osteogenic differentiation of MSCs, where left and right panels show ALP and Alizarin Red S staining in a, b, e and f; MG and MD are denoted to microgel and medium, respectively; (a) first row displays medium treatment. They are marked as Control (normal medium 21 days), Ost (osteogenic medium 21 days), Con7 + Ost14 (normal medium 7 days followed by osteogenic medium 14 days), and Con14 + Ost7 (normal medium 14 days followed by osteogenic medium 7 days); the second and third rows show that MSCs treated first with Con or Ost containing different types of microgels (5 mg/mL) for 7 days and then with full osteogenic medium for another 14 days before harvested; (b) MSCs treated first with Con or Ost containing different types of microgels (5 mg/mL) for 14 days and then with full osteogenic medium for another 7 days; (c) and (d) show levels of OPN mRNA of MSCs cultured under MG7 + MD(Ost)14 and MG14 + MD(Ost)7 with a microgel concentration of 5 mg/mL, respectively, where Microgels_C and Microgels_O denote control and osteogenic mediums with microgels, respectively; (e) and (f) are similar to (a) and (b) but with a doubled microgel concentration.

anchored on the cells and carboxyl groups on the microgels periphery would bind the cells and the microgels together, subsequently affecting further mineral deposition on ECM. To test it, we kept the cells in the osteogenic medium for 7 days before replacing it with either the control or the osteogenic medium with the microgels (5 or 10 mg/mL) for 14 days, which is denoted as MD(Ost)7 + MG14 hereafter.

Figure 10(a) and (b) reveal that after 7 days in osteogenic medium, (1) MSCs cultured in the control medium with the microgels for 14 days (second row) have a higher mineralization degree than that in the osteogenic medium for 7 days before replacing it with the pure normal medium for 14 days (Ost7 + Con14) and (2) MSCs cultured in the osteogenic medium with the microgels for 14 days (third row) have a comparable or even higher mineralization degree than that in the osteogenic medium for 21 days (Ost21). The results in Figure 10(a) and (b) are in accord to the mRNA expression of OPN in the cases of MD(Ost)7 + MG14 with two different microgel concentrations, as shown in Figure 10(c) and (d). The Alizarin Red S staining (Figure 10a and b) shows that the increase of the AA content enhances the ECM mineralization. In addition, a higher microgels concentration leads to more ECM mineralization. These results are just reverse to those

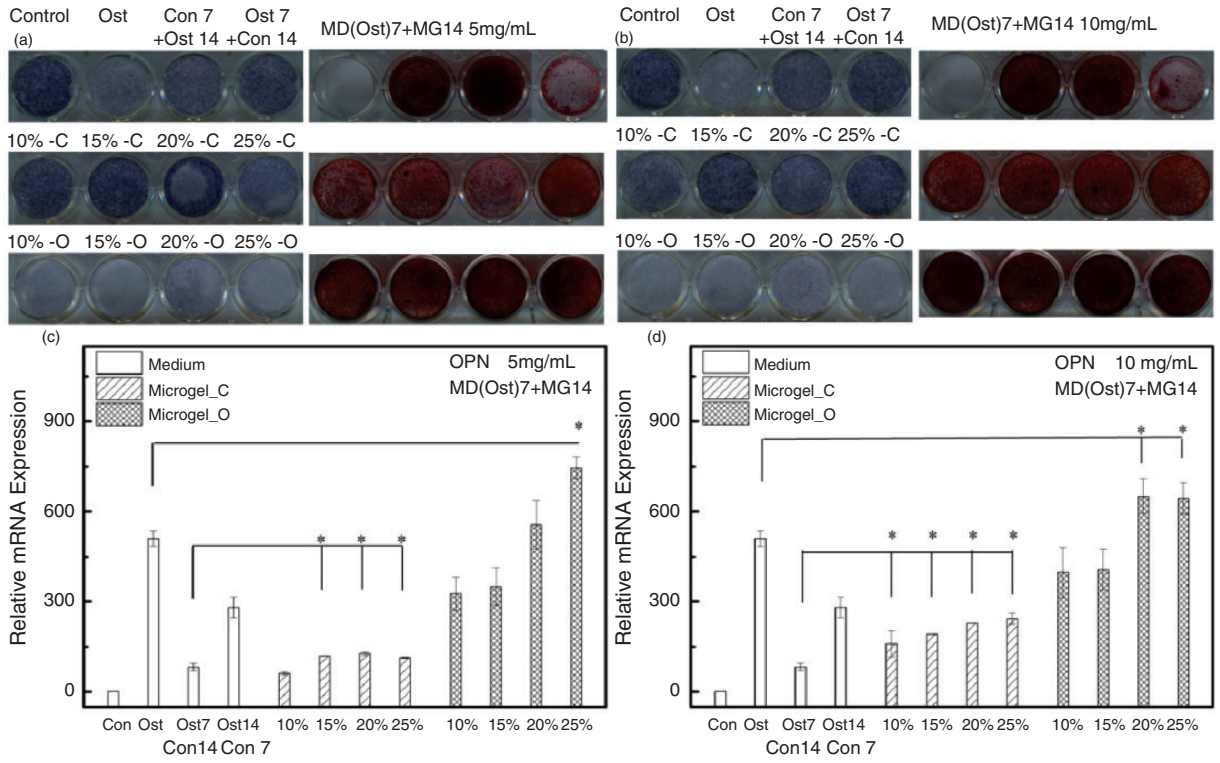

Figure 10. Effect of microgels on osteogenic differentiation of MSCs at late stage of culture, where left and right panels are ALP and Alizarin Red S stainings, respectively; (a) first row shows cells after different medium treatments, while second and third rows are cells treated first with full osteogenic medium for 7 days and then with control or osteogenic medium containing different types of microgels (5 mg/mL) for another 14 days before harvest; (b): similar to (a) except a higher microgel concentration (10 mg/mL); (c) and (d): microgel concentration dependence of OPN expression level using MD(Ost)7 + MG14, where Microgels_C and Microgels_O denote control and osteogenic mediums with microgels, respectively; $^*p < 0.05$ and $n = 3$.

observed in the culture medium with the microgels incorporated at an early stage as described before.

It is expected that after the primary ECM mineralization, the gain of translational entropy of all the counter ions on cationic calcium ions, and anionic carboxyl groups would drive the microgels toward and attaching to the cell surface so that a higher microgel concentration or AA content should result in a stronger binding of the microgels onto the cell surface. The addition of the microgels after most of calcium ions are fixed on the cell surface reduces the adsorption of calcium ions free in the medium. Moreover, the adsorbed microgel layer not only stabilizes calcium ions on the cell surface but also applies a physical stress on the cells, which affects the differentiation and function of MSCs, reflecting in the mRNA expression of osteoblastic marker OPN.

To further examine the role of the microgel layers in the osteogenesis, we purposely cultured the MSCs layer first in a normal or an osteogenic medium for 7 days and then in a replaced osteogenic medium with four different types of the microgels (10 mg/mL) for another 14 days. Figure 11 shows that only the cells cultured first with the osteogenic medium for 7 days have a significantly positive Alizarin Red S signal, which reveals

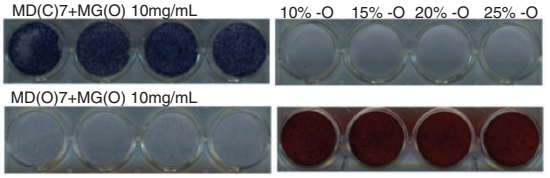

Figure 11. Comparison of ALP activity (left) and Alizarin Red S signal (right) of cells, where microgels were added in late stage without or with primary calcium deposition; first and second rows are cells treated by normal (MD(C)) and full osteogenic mediums (MD(O)) for 7 days, respectively, before replaced by osteogenic medium with four different types of microgels (10 mg/mL) for another 14 days. Samples are harvested on Day 21.

that it is critically important to anchor calcium ions on the cell surface before the adsorption of the microgels and the generation of the ECM effect.

In comparison with those commonly used bulk hydrogels, using small microgels enables us to inject and mix them with stem cells in biomedical applications. Their smaller size also makes them to respond to the temperature and pH stimulus much faster. A mentioned before, using our currently engineered

pH and thermally sensitive microgels, we are able to effectively and conveniently induce different interactions between ECM and MSCs to encapsulate the cells inside a 3D hybrid gel matrix, plant the cells on top of a 2D hybrid gel layer, or cover the cells with the microgels. Originally, we thought to use a controllable swelling of individual microgels to apply an adjustable mechanical stress on their encapsulated MSCs to affect their differentiation. Unexpectedly, our results reveal that the copolymerization of those carboxyl groups into the microgels not only shifts their swelling temperature to 37°C but also affects the fate of calcium ions generated in ECM during osteogenic differentiation of MSCs.

It should be noted that the correlation between the calcium ion concentration and different cellular behaviors, including the cell growth, differentiation, apoptosis, and motility, has been studied by actively adding or removing calcium ions to or from the culture medium.[28,29] It is known that a higher calcium concentration inhibits the ALP activity but stimulates the expression of OPN associated with the osteogenesis differentiation.[28] In the current study, the calcium concentration in the culture medium is passively adjusted by the addition of the microgels at different osteogenic stages because of the interaction between the carboxylic groups on the microgels and calcium ions.

Conclusion

Using thermally sensitive poly(*N*-isopropylacrylamide) (PNIPAM)-based microgels that shrink at higher temperatures but swell at the body temperature, we designed a system that can change from a dispersion to a hybrid bulk hydrogel made of billions of small jammed swollen microgels around 37°C. The copolymerization with AA with PNIPAM makes the phase transition temperature adjustable and the microgels sensitive to pH. Mixing such microgels with stem cells enables us to actively adjust the mechanical stress on the cells via the microgel swelling and the calcium ion concentration via the Ca^{2+}/COO^{-} interaction. This is why the cells cultured in the full osteogenic medium with the microgels from the very beginning have a relatively higher ALP activity but show no mineralization because cationic calcium ions required for the osteogenesis are removed by the anionic microgels. To promote the osteogenesis, the microgels have to be added in the late stage of osteogenic differentiation after most of calcium ions are deposited on the cells. It is those cell surface-deposited calcium ions that attract the microgels and induce the mechanical stress that remarkably promotes the mineralization to a level higher than that even under the full osteogenic environment. Therefore, the dual functions of the microgels make them either as an inhibitor to prevent the osteogenesis or as a promoter in the differentiation of MSCs, depending on at which osteogenic stage the microgels are added in the culture medium. Our results reveal that the hybrid gels made of thermally/pH sensitive P(NIPAM-AA) microgels as an ECM niche affect the stem cell fate and biological behavior in a context-dependent mode, which should be valuable for stem cell-based therapies.

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