Combinational effect of matrix elasticity and alendronate density on differentiation of rat mesenchymal stem cells

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Abstract

Differentiation of mesenchymal stem cells (MSCs) is regulated by multivariate physical and chemical signals in a complicated microenvironment. In this study, polymerizable double bonds (GelMA) and osteo-inductive alendronate (Aln) (Aln-GelMA) were sequentially grafted onto gelatin molecules. The biocompatible hydrogels with defined stiffness in the range of 4–40 kPa were prepared by using polyethylene glycol diacrylate (PEGDA) as additional crosslinker. The Aln density was adjusted from 0 to 4 μM by controlling the ratio between the GelMA and Aln-GelMA. The combinational effects of stiffness and Aln density on osteogenic differentiation of MSCs were then studied in terms of ALP activity, collagen type I and osteocalcin expression, and calcium deposition. The results indicated that the stiffness and Aln density could synergistically improve the expression of all these osteogenesis markers. Their osteo-inductive effects are comparable to some extent, and high Aln density could be more effective than the stiffness.

1. Introduction

The mesenchymal stem cells (MSCs) have the capacity to differentiate into bone, cartilage, muscle, fat and a variety of other connective tissues, leading to a great deal of interest in the field of regenerative medicine and tissue engineering [1–6]. Recently, growing evidence suggests that chemical, physical and mechanical signals from materials and neighboring cells have a profound impact on the differentiation of MSCs [7–10]. Therefore, it is of paramount importance to precisely understand the interaction between MSCs and the niche consisting of various chemical and physical signals [11–17].

It is known that physical properties such as elasticity and topography of the extracellular matrix are able to dominate the fate of stem cells. For instance, Engler et al. [18,19] demonstrated that MSCs display characteristics of neurogenic, myogenic, and osteogenic phenotypes after being cultured on hydrogel substrates mimicking the stiffness of neural, muscle, and bone tissues, respectively. McBeath et al. found that different sizes [20] of fibronectin ‘island’ can restrict MSC spreading and then dominate their differentiation. When the MSCs are allowed to adhere, flatten, and spread they shall undergo osteogenesis, whereas the unspread and round cells become adipocytes. Recently, it was found that different aspect ratio and subcellular curvature can modulate the differentiation of stem cells to adipocytes and osteoblasts [21]. Peng et al. [22–24] further made semi-quantitative investigation of the effects of cell shape on differentiation of MSCs, and revealed the optimal aspect ratios for adipogenic and osteogenic differentiation of MSCs. They found that the extents of both adipogenic and osteogenic differentiations are linearly related to the cell perimeter, which reflects the non-roundness or local anisotropy of cells.

Not only the physical properties, various small functional groups, peptides and proteins on both stiff substrates, i.e. silicon wafers, and soft substrates, i.e. poly(ethylene glycol) (PEG) hydrogels, can modulate MSC differentiation [25–30]. Moreover, Kilian and Mrksich demonstrated that the affinity and density of ligands at the cell-biomaterial interface also can be engineered to influence stem cell fate [31]. Among these molecules, alendronate sodium (Aln) is a kind of bisphosphonate drug, which is able to promote osteogenic differentiation of BMSCs via several mitogen-activated protein kinase (MAPK) pathways, such as extracellular signal-related kinases (ERKs) 1/2 and Jun amino-terminal kinases (JNK1/2/3) pathways, in a dose-dependent manner [32–34]. Besides, physically or chemically immobilized Aln also can induce osteogenic differentiation of MSCs. Zhu et al. [34] created a density gradient surface of Aln onto the polycaprolactone (PCL) membrane, and found that MSCs over-express osteogenic marker proteins on the surface, dependent on the local Aln density. Kim et al. [35] demonstrated that physical immobilization of Aln and bone morphogenic protein-2 on a titanium surface showed synergistic effect on improving osteoblast activity.
However, differentiation of stem cells usually happens in a complicated microenvironment which contains multivariate signals [7,13]. Therefore, it is of paramount importance to understand the impact of multivariate signals on stem cell fate, especially the interplay between different types of signals. Sometimes these signals have a synergistic effect on the differentiation of MSCs. For example, Jiang et al. [36] demonstrated the synergistic effect of nanofiber topography and released neuronal induction factor, retinoic acid, on enhancing MSC neural commitment. Sometimes the combinational effects became more complicated. Zouani et al. [37] studied the effect of mechanical properties and special growth factor in the same microenvironment on stem cell fate. Their results demonstrate that chemical grafting on relative stiff matrices (13–70 kPa) with an osteogenic factor (BMP-2 mimetic peptide) results only in osteogenic differentiation. When grafted on even softer hydrogel matrices (0.5–3.5 kPa), the BMP-2 mimetic peptide has no effect on the stem cell differentiation. Therefore, in order to predict the fate of MSCs in a complicated artificial environment, a more careful and case-sensitive study is required to understand the combinational effects of different types of signals.

In this work, the combinational effect of substrate stiffness and alendronate density is studied in terms of MSCs differentiation (Fig. 1b). Gelatin is chosen as the backbone of hydrogels due to its good biocompatibility and potential of modification [38–40]. In order to fabricate the hydrogels with controllable mechanical property, polymerizable double bonds are introduced onto the gelatin molecules via the reaction between methacrylic anhydride (MA) and amino groups of gelatin. Aln molecules are further grafted onto the gelatin backbone through aldehyde-activated reaction. Furthermore, polyethylene glycol diacrylate (PEGDA) is used as crosslinker to modulate the crosslinking density of the hydrogel and thus the stiffness (Fig. 1a). The combinational impact of the hydrogel stiffness and Aln density on MSCs’ neuronal, myogenic, and osteogenic differentiation is first evaluated in terms of expression of β-tubulin, MyoD, and calcium, respectively. Then the osteogenic differentiation of MSCs, which is significantly influenced by these two factors in the current study, is studied in terms of alkaline phosphatase (ALP) activity, expressions of collagen type I and osteocalcin, and calcium deposition.

2. Experiment section

2.1. Materials

Gelatin (type B), polyethylene glycol diacrylate (PEGDA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), bovine serum albumin (BSA), ascorbic acid, ammonium persulfate (APS), and tetramethylthelenediamine (TEMED) were purchased from Sigma–Aldrich, USA. Methacrylic anhydride (MA) was bought from Alfa Aesar, USA. Alendronate sodium (Aln) was obtained from Spectrum, USA. PicoGreen dsDNA kit was purchased from Life Technologies, USA. o-Cresolphthalein complexone (CPC), 8-hydroxyquinoline, and 2-amino-2-methyl-1-propanol (AMP) were obtained from TCI chemical, Japan. Other chemicals were of analytical grade and used as received. The water used in the experiments was purified by a Milli-Q water system (Millipore, USA).

2.2. Synthesis and characterization of methacrylated gelatin

Methacrylated gelatin (GelMA) was synthesized according to the method reported previously [41,42]. Briefly, 4 g gelatin was dissolved in 40 mL phosphate buffer (pH = 8.0) at 70 °C. After being cooled to 45 °C, 40 μL MA was added at a rate of 10 μL/min under stirring, and the mixture was allowed to react for 1 h. Into the solution 500 mL cold ethanol (−20 °C) was added to precipitate the methacrylated gelatin. After centrifugation, the precipitates were dissolved in water, sealed in a dialysis bag with a cut-off molecular weight of 3.5 kDa, and dialyzed against water for 3 d. The solution was lyophilized to obtain the white porous product, which was stored at −20 °C until use.

The substitution degree of MA was also quantified by measuring the contents of amino groups in gelatin before and after reaction by the Habeeb method using TNBS [43]. Briefly, 0.25 mL 0.01% (w/v) TNBS PBS water solution, 0.25 mL 0.01% gelatin or GelMA solution, and 0.25 mL 4% NaHCO3 solution were mixed in a centrifuge tube. After being incubated at 37 °C for 2 h, the absorbance at 420 nm was determined by UV–vis spectroscopy (UV-2550, Shimadzu, Japan). The concentration of amino groups in gelatin or GelMA was calculated by referring to a standard curve generated with a series of glycine solutions with different concentrations.

2.3. Synthesis and characterization of Aln-grafted GelMA (Aln-GelMA)

Alendronate sodium (Aln-NH2) was firstly reacted with excess glutaraldehyde in water overnight at 45 °C to obtain the aldehyde-modified Aln (Aln-CHO), which was then precipitated and washed with a large amount of cold acetone. After drying, 60 mg Aln-CHO was added into 10 mL GelMA PBS solution (100 mg/mL), and reacted overnight at room temperature. The product was dialyzed against water for 3 d. The solution was lyophilized to obtain yellow porous Aln-GelMA, which was stored at −20 °C until use. The chemical structure of the product was characterized by 31P nuclear magnetic resonance (31P NMR) (500 MHz, Cambridge). The Aln ratio in the Aln-GelMA was determined similarly by the aforementioned Habeeb method. Besides, the molybdate blue method was also used to determine the phosphorus content in Aln-GelMA [44]. Briefly, the Aln-GelMA was burned in a muffle furnace at 700 °C for 1 h. Residues were dissolved in 0.5 mL 16% H2SO4, and then mixed with 0.5 mL 2.5% (w/v) ammonium molybdate solution and 0.5 mL 10% (w/v) ascorbic acid solution. After being incubated at 37 °C for 2 h, the absorbance at 800 nm was determined by UV–vis spectroscopy. The phosphorus content in Aln-GelMA was obtained by referring to a standard curve created with K2HPO4 at the same conditions.
2.4. Hydrogel preparation and characterization

The GelMA macromonomers, APS, and TEMED were mixed to form a reaction solution. PEGDA was used as the crosslinker to modulate the cross-linking degree and thereby the stiffness of hydrogels. Aln-GelMA was added to adjust the Aln concentration in the hydrogels. The final concentration of each component is summarized in Table 1. 20 mM APS and TEMED were used as redox initiators. The reaction lasted for 6 h at 37 °C. The obtained hydrogels were washed with water to remove unreacted macromonomers and initiators.

The hydrogels were freeze-dried and characterized by FTIR spectroscopy. The incorporated Aln concentration in the hydrogels was determined by measuring the phosphorus content as described above.

The equilibrium swelling ratio of the hydrogels, which is correlated to the cross-linking density of the hydrogel network, was characterized. The mass of swollen hydrogel (Wsw) was measured after it was incubated in distilled water for 48 h at room temperature. The swelling ratio (SR) of the hydrogel was determined according to SR = (Wsw − Wdry)/Wdry, where Wdry is the original weight of the hydrogel.

The mechanical properties of the hydrogels (cylindrical shape, 15 mm in diameter and 5 mm in height) were measured by a mechanical tester (Instron 5543, USA) in a water tank containing PBS at 37 °C with a compression rate of 2 mm per min until failure occurred. The compressive modulus of the hydrogels was obtained from the linear region of the stress (2–3% strain). Each value was averaged from 4 parallel measurements.

2.5. Cell isolation and culture

Bone mesenchymal stem cells (BMSCs) were isolated from bone marrow of Sprague-Dawley rats (6–8 weeks old) according to the methods reported previously [45]. The procedures were performed in accordance with the “Guidelines for Animal Experimentation” by the Institutional Animal Care and Use Committee, Zhejiang University. Briefly, the BMSCs were obtained from the femoral shafts of rats by flushing out with 10 mL of culture medium (low glucose Dulbecco’s modified Eagle’s medium, LDMEM) supplemented with 10% fetal bovine serum (FBS, Life Technologies, New York, USA), 100 μg/mL penicillin and 100 U/mL streptomycin).

The released cells were collected in a 9 cm cell culture dish (Corning, USA) containing 10 mL culture medium and incubated at 37 °C with a compression rate of 2 mm per min until failure occurred. The compressive modulus of the hydrogels was obtained from the linear region of the stress (2–3% strain). Each value was averaged from 4 parallel measurements.

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<td><strong>Recipe of different hydrogels.</strong></td>
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2.6. Cell morphology and cell number

After culturing on the hydrogels for 21 d, the MSCs were washed with PBS 3 times, and fixed with 4% formaldehyde solution for 30 min at room temperature. They were further treated with 0.5% Triton/PBS solution at 4 °C for 10 min. After being washed with PBS 3 times, they were treated with 1% BSA/PBS solution to block nonspecific adsorptions for 2 h. The cells were finally stained with DAPI (100 ng/mL) for nucleus and rhodamine phalloidin solution (0.2 μM, Life Technologies) for cytoskeleton (F-actin) at 37 °C for 1 h. After washing with PBS 3 times, the cells were observed under a fluorescence microscope (IX81, Olympus).

After being cultured on the hydrogels for 21 d, the cell number was quantified by the PicoGreen® assay. In brief, the cells were lysed by repeated freeze–thaw cycles in the presence of 0.2% Triton X-100. Total double-stranded DNA was quantified after the samples were incubated with PicoGreen fluorescence dye in the assigned buffer, and the fluorescence emission intensity at 520 nm was measured according to the manufacturer’s instruction (PicoGreen® dsDNA Quantitation Kit, Invitrogen, USA). The cell number of each sample was calculated by referring to a standard curve recorded at the same conditions with known cell number of MSCs.

2.7. Alkaline phosphatase (ALP) quantification

Phenylphosphate can be hydrolyzed by ALP and form free phenol, which can react with 4-amino-antipyrine in the presence of alkaline potassium ferricyanide to form a red-colored complex, whose absorbance at 490 nm is directly proportional to the ALP activity in the specimen.

After being cultured on the hydrogels for 7 d, the MSCs were washed with PBS 3 times, and treated with 0.5% Triton/PBS at 4 °C for 24 h. After ALP was totally released, the solution was mixed with reagents from the colorimetric Kit (KeyGEN Biotech, China) according to the user’s manual. The absorbance at 490 nm was recorded by a microplate reader and the activity of ALP is calculated by referring to a standard curve. The ALP activity per 10^6 cells was reported.

2.8. Immunofluorescence staining

After being cultured on the hydrogels for 21 d, the MSCs were washed 3 times with PBS, and fixed in 4% paraformaldehyde for 30 min at 37 °C. After being washed 3 times in PBS, they were further treated in 0.5% (v/v) Triton X-100/PBS at 4 °C for 10 min to increase the permeability of the cell membrane. After 3 washes in PBS, they were incubated in 1% BSA/PBS at 37 °C for 30 min to block the non-specific interactions. Then the cells were incubated with a mouse monoclonal antibody against collagen type I and osteocalcin (Abcam, USA) for 1 h, respectively. After being washed twice in 1% BSA/PBS, they were further stained with fluorescent labeled IgG (Beyotime, China) and DAPI at room temperature for 1 h, and followed by 3 washes in PBS. The cells were observed under confocal laser scanning microscopy (CLSM, SP5, Leica, Germany). The images obtained were further analyzed by ImageJ software (National Institutes of Health, USA).

2.9. Western blotting

After culturing on 7 or 21 d on different hydrogels, the MSCs were washed with PBS three times and completely homogenized in radio immunoprecipitation assay buffer (RIPA) with protease...
inhibitors. The lysates were centrifuged at 12,000 rpm at 4 °C for 15 min, and separated on a SDS–PAGE. All the gels have been run under the same experimental conditions. After being transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA), the proteins were incubated overnight with antibodies (Abcam, USA) and detected using an enhanced chemiluminescence (ECL Western Blotting Substrate, Pierce, USA) system. The integral optical density (IOD) was determined using the software Bandscan 5.0.

2.10. Calcium deposition

The amount of deposited calcium by MSCs was measured by using the o-cresolphthalein complexone method [46,47]. Firstly, AMP buffer and staining solution were prepared, respectively. The AMP buffer was prepared by dissolving 7.06 g AMP in 35 mL water, whose pH values was adjusted to 10.7 using 6 M HCl, and the final volume was adjusted to 50 mL by water. The staining solution was prepared by dissolving 5 mg CPC and 50 mg 8-hydroxyquinoline into 3 mL 12 M HCl solution, whose volume was adjusted to 50 mL water. After being cultured for 21 d, the cells were incubated in 0.5 M HCl for 8 h on an ice/water bath under shaking. After centrifugation at 1000 g for 5 min, the supernatant (5 µL) was mixed with 100 µL staining solution and 100 µL AMP buffer. 5 min later, the absorbance at 570 nm was determined by a microplate reader (BioRad 680, USA). The calcium content of each sample was determined by referring to a standard curve generated by calcium chloride at the same conditions. The calcium content per 10^5 cells was reported.

2.11. Statistical analysis

At least three independent experiments were carried out if not otherwise stated. Results are reported as mean ± standard deviation, and are analyzed using a paired student’s t-test. The significant difference level was set at p < 0.05.

3. Results and discussion

3.1. Characterization of gelatin-based macromonomers (GelMA and Aln-GelMA)

The polymerizable carbon double bonds were grafted to gelatin molecules to obtain GelMA by the reaction with methacrylic anhydride (MA) molecules under alkaline environment. Compared to the 1H NMR spectrum of gelatin, in the spectrum of GelMA new resonance peaks appeared at 5.60 and 5.36 ppm which are assigned to the protons of H2C=CH(CH2)–, confirming the success of MA grafting (Fig. S1a). The degree of MA substitution (SDMA) was determined to be 12.0 ± 0.3% and 14.6% by Habeeb assay and 1H NMR spectroscopy [41], respectively.

Aldehyde molecules were further grafted onto GelMA via coupling between the amine groups of Aln and GelMA [34,48]. Compared with the FTIR spectrum of Aln (Fig. S1b), the absorbance at 1714, and 2934, 2865 cm⁻¹ appeared, which are assigned to the stretching vibration of C=O, and the Fermi resonance between stretching vibration and bending vibration of C–H, respectively. This result confirms the successful introduction of the aldehyde group onto Aln molecule. The Aln-CHO was grafted onto the backbone of GelMA by a simple incubation. Although no apparent difference can be found between the 1H NMR spectra of Aln-GelMA and GelMA (Fig. S1a), the 31P NMR spectra (Fig. 2) reveal that only the Aln-GelMA had an obvious resonance peak at 17.6 ppm. The total substitution degree (SDMA+Aln) of Aln-GelMA was determined to be 22.0 ± 0.4%, implying that the SDAln was about 10%, and the Aln concentration was 31 µmol in 1 g Aln-GelMA. The Aln content in the Aln-GelMA was also determined by the molybdate blue method [44]. The phosphorus element content in 1 g Aln-GelMA was found to be 58.4 µmol, suggesting that the Aln content in 1 g Aln-GelMA was 29.2 µmol since one Aln molecule has two phosphonate groups. These results are consistent with each other, and reveal the successful synthesis of Aln-GelMA.

3.2. Preparation and characterization of hydrogels

In order to maximize the adjustable range of mechanical properties of the hydrogels [49], herein the highest concentration of gelatin-based macromonomer, i.e. 20%, was used in this study. By using 20 mM APS and TEMED as initiators, the hydrogels were formed within 5 min at 37 °C. After gelation, the hydrogels were kept under 37 °C for another 6 h for complete polymerization. Demonstrated by Engler et al., hydrogels with low modulus (0.1–10 kPa) are feasible for neurogenesis, and hydrogels with relatively high modulus (25–40 kPa) are able to promote osteogenesis [19]. In this study, the stiffness of hydrogels was further modulated by the addition of 20 wt.% PEGDA, which improved the compressive modulus of the resulting hydrogels from 4 to 40 kPa. Moreover, the Aln density in the hydrogels was varied by adjusting the ratio between GelMA and Aln-GelMA. Totally 6 types of hydrogels with variable stiffness and Aln density were prepared as shown in Table 1.

Compared to the FTIR spectra of GelMA and Aln-GelMA hydrogels (Fig. 3a), in the spectra of the GelMA/PEGDA and Aln-GelMA/PEGDA hydrogels, new peaks appeared at 1101 and 1730 cm⁻¹, which are assigned to the C–O asymmetric stretching vibration of –C–O–C and the C=O stretching of the acetate group respectively. This result suggests the successful incorporation of PEGDA into the hydrogel. However, due to the rather low concentration of Aln in the hydrogels, no obvious difference was found in the FTIR spectra of GelMA/PEGDA, Aln-GelMA-1/PEGDA, and Aln-GelMA-2/PEGDA hydrogels.

Hydrogel swelling is related with the hydrogel network and mechanical properties [50,51]. The swelling ratio of the hydrogels decreased significantly after PEGDA incorporation (Fig. 3b) as a result of improvement of crosslinking density. By contrast, the compressive moduli of the hydrogels (Fig. 3c) increased from ~4 to ~40 kPa. The minor difference in Aln concentration did not have significant influence on the swelling and mechanical properties of the hydrogels. By variation of the feeding ratio of Aln-GelMA, the Aln concentration in the hydrogels was adjusted from 0 to 0.2 ± 0.03 µM (~0.2 µM), and further to 3.9 ± 0.1 µM (~4 µM), respectively (Fig. 3d). Therefore, the gelatin-based
hydrogels with two different stiffness (denoted as 4 and 40 kPa) and three different densities of Aln (denoted as 0, 0.2 Aln, and 4 Aln) have been successfully prepared.

3.3. Morphology and proliferation of MSCs

After culturing for 21 d on all hydrogels, the MSCs showed similar spindle morphology because of the high cell density, suggesting all hydrogels had good cytocompatibility (Fig. 4a–f). As shown in Fig. 4g, in general a similar number of MSCs was found on the hydrogels without or with a lower Aln concentration, likely due to the good biocompatibility of the hydrogels. However, about 20% decrease ($p < 0.05$) of the MSC number was found on the hydrogels containing the highest density of Aln. This phenomenon is likely attributed to the differentiation of MSCs which suppresses cell proliferation, as revealed in the following results.

3.4. Osteogenic differentiation of MSCs

The stemness of obtained MSCs was first verified by differentiation tests. The MSCs were incubated in adipogenic and osteogenic medium for 14 d, and then stained with Oil red and Alizarin Red S, respectively. Results (Fig. S2) indicate that the MSCs could undergo adipogenic differentiation and osteogenic differentiation in the corresponding differentiation mediums, as evidenced by the lipid droplet formation and calcium deposition, respectively. In order to reveal the potential combinational impact of hydrogel stiffness and Aln density on the MSCs’ multilineage differentiation, the expressions of myogenic marker MyoD1 and neurogenic marker β-tubulin were firstly evaluated (Figs. S3 and S4). The MSCs did not show an obvious expression of MyoD1 and β-tubulin on all types of hydrogels, indicating they did not undergo myogenic or neurogenic differentiation under current stimuli. Since the well spread MSCs are inclined to undergo osteogenesis [34], several kinds of osteogenesis-related markers were then investigated.

Alkaline phosphatase (ALP) is an important osteogenic marker in earlier stage of osteogenesis [34]. The expressions of several differentiation hallmarks at relative later stage were also studied at protein level. Collagen type I (COL) and osteocalcin (OCN) are both important for osteogenic differentiation. It has been proved that COL is the most abundant protein in the organic/inorganic composite matrix of bone tissue [52]. OCN is the most abundant noncollagenous protein in the bone matrix, which plays an essential role in bone formation and remodeling [53]. An osteogenic tissue is capable of forming an extracellular matrix that can regulate mineralization, which represents its ultimate phenotypic expression [54]. Therefore, after being cultured for 21 d, the calcium deposition was measured and used as a hallmark for osteogenic differentiation [55,56].

After 7 d culture, the expressions of ALP (Figs. 5 and 7a) by the MSCs were significantly enhanced along with the increase of Aln concentration in the hydrogels regardless of their stiffness, revealing that the osteo-inductive effect of Aln is dose-dependent [32,34]. At the same Aln density, the expressions of ALP by the MSCs were significantly enhanced on the stiffer hydrogels. The highest expressions of ALP were observed on the stiffest hydrogel with the highest Aln density (40 kPa/4 Aln), suggesting the synergistical effect of stiffness and Aln on the osteogenesis of MSCs. In contrast, the expressions of COL and OCN (Fig. 7a) by the MSCs were only slightly enhanced in the hydrogels with higher Aln concentrations and stiffness. This might be attributed to the fact that COL and OCN were only actively expressed at the later differentiation stage.

After 21 d culture, the expressions of ALP (Fig. 7b) by the MSCs on the softer hydrogels regardless of the Aln concentration and the stiffer hydrogel without Aln were significantly reduced, compared...
to the counterparts after 7 d culture, respectively. This might be attributed to the fact that ALP is mainly expressed at the earlier differentiation stage. However, the expressions of ALP by the MSCs on the stiffer hydrogels with Aln (40 kPa/0.2 Aln and 40 kPa/4 Aln) were still quite high, suggesting that the synergistical effect of stiffness and Aln can prolong the expression of ALP, and thereby might be feasible for osteogenesis of MSCs. The expressions of COL and OCN at 21 d were significantly enhanced than their counterparts after 7 d culture (Fig. 7), and also were enhanced along with the increase of Aln concentration in the hydrogels regardless of their stiffness (Figs. 6 and 7b). Similar to the ALP expressions at 7 d, at the same Aln density, the expressions of COL and OCN by the MSCs at 21 d were significantly enhanced on the stiffer hydrogels. The highest expressions of COL and OCN were observed on the stiffest hydrogel with the highest Aln density (40 kPa/4 Aln), proving again the synergistical effect of stiffness and Aln on the osteogenesis of MSCs. As shown in Fig. 8, the calcium deposition...
by the MSCs was also enhanced along with the increase of Aln density and hydrogel stiffness.

Besides the synergistical effect of hydrogel stiffness and Aln density, it might be more important to find out which one is more effective for osteogenesis between the stiffness and Aln density. Since the process of mineralization is most significant for dictating osteogenesis [54], it is used to figure out the importance of these two factors. Fig. 8 shows that the calcium deposition increased to 1.3–2.6 folds on the stiffer hydrogels compared to the softer ones with the same Aln density. Meanwhile, the calcium deposition increased to 1.4 or 2.2 fold along with the increase of Aln density from 0 to 0.2 μM on the softer and stiffer hydrogels, respectively. This means the effect of stiffness and low density of Aln (0.2 μM) is more or less comparable. When the Aln density increased from 0 to 4 μM, the calcium deposition on the softer and stiffer hydrogels increased to 2.0 or 5.0 folds, respectively. This result implies the osteogenic effect of higher density of Aln (4 μM) is much more effective than the stiffness (40 kPa). Therefore, one can conclude that (i) higher stiffness and Aln incorporation can synergistically

Fig. 7. Western blotting analysis of ALP, OCN and COL expressed by MSCs on various hydrogels after (a) 7 d and (b) 21 d culture, respectively. The left panel shows the photo of gels and the right panel shows the relative integral optical density of ALP, COL and OCN calculated from the images by Bandscan software. The protein expression level was normalized to that of the respective expression of β-actin, which was used as a reference standard. * and ** indicate significant difference at p < 0.05 and p < 0.01 levels, respectively. All gels have been run under the same experimental conditions.

Fig. 8. Calcium contents determined by the o-cresolphthalein complexone method in MSCs being cultured on hydrogels for 21 d, respectively. * and ** indicate significant difference at p < 0.05 and p < 0.01 levels, respectively.
promote the osteogenesis, and (ii) the osteo-inductive effect of Aln is more effective than the stiffness if its density is high enough.

Several previous studies have demonstrated the synergistic effect of different signals on stem cell differentiation. For example, Kim et al. [17] found that nanotopography and co-culture with endothelial cells synergistically promote osteogenesis, and the nanotopography seemed to take a more crucial role. Kaur et al. [57] has also demonstrated the synergistic effects of nanotopography provided by tobacco mosaic virus and phosphate on the osteogenic differentiation of MSCs. Zhao et al. [58] built titanium nanotubes of different diameters loaded with strontium. They found that 10 μm diameter of nanotubes with long-lasting strontium release showed the best osteogenic properties of MSCs. However, there are several less reports to compare the impact of two competing factors on stem cell differentiation. For example, Zouani et al. demonstrated that chemical grafting of BMP-2 mimetic peptide on relative stiff matrices (13–70 kPa) results in osteogenic differentiation, while the similar grafting on very soft hydrogels has no effect on the stem cell differentiation [37]. Besides, Banks et al. [59] demonstrated that the stiffness substrates direct osteogenic lineage commitment of adipose-derived mesenchymal stem cells (ASCs) regardless of the presence or absence of growth factors (BMP-2 or PDGF), while softer substrates require biochemical cues to direct cell fate. Furthermore, Engler et al. [60] demonstrated that differentiation of MSCs is unaffected by the collagen density tethering on hydrogels with different stiffness. All these results suggest that the mechanical property of the substrate usually has a stronger impact on the differentiation of stem cells over chemical cues, such as extracellular protein and growth factors. However, in this study we found that the osteo-inductive effect of higher Aln density (4 μM) is more effective than the high stiffness (40 kPa). This might be attributed to the different impact of chemical cues, suggesting more careful case by case study is required to reveal the impact of stem cell differentiation in a complicated microenvironment.

4. Conclusion

The gelatin-based hydrogels with defined stiffness (4 and 40 kPa) and Aln density (0, 0.2 and 4 μM) were successfully prepared. Enhancing the stiffness and Aln density were found to improve osteogenesis of mesenchymal stem cells synergistically in terms of ALP, COL, OCN and calcium expressions. Furthermore, the osteo-inductive effect of Aln molecules and higher modulus of the substrate is comparable to some extent. The insight understanding of the interplay between the chemical cues and substrate stiffness is useful to unveil the differentiation behaviors of MSCs within a complicated microenvironment containing multivariate signals, and subsequently guide the design of biomaterials for controlling stem cell fate.

Acknowledgments

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1, 4, 6 and 7 are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2015.03.018.

Appendix B. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.03.018.

References

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