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# 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  $\mu\text{M}$  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.

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## 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.

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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  $\beta$ -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 tetramethylethylenediamine (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

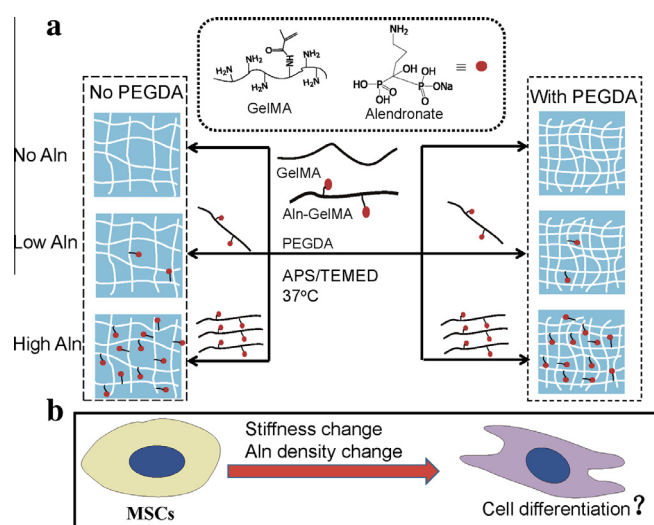
Methacrylated gelatin (GelMA) was synthesized according to the method reported previously [41,42]. Briefly, 4 g gelatin was dissolved in 40 mL phosphate buffer (pH = 8.0) at 70 °C. After being cooled to 45 °C, 40  $\mu$ L MA was added at a rate of 10  $\mu$ L/min under stirring, and the mixture was allowed 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 water solution, 0.25 mL 0.01% gelatin or GelMA solution, and 0.25 mL 4% NaHCO<sub>3</sub> 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-NH<sub>2</sub>) 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 <sup>31</sup>P nuclear magnetic resonance (<sup>31</sup>P 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% H<sub>2</sub>SO<sub>4</sub>, 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 K<sub>2</sub>HPO<sub>4</sub> at the same conditions.



**Fig. 1.** (a) Schematic illustration of the preparation of 6 hydrogels with different Aln contents and crosslinking degrees based on methacrylated gelatin (GelMA) and alendronate-grafted GelMA (Aln-GelMA). (b) Schematic illustration of the combinational effect of hydrogel stiffness and Aln density on the differentiation of MSCs.

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