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# Low Molecular Weight Gels Induced Differentiations of Mesenchymal Stem Cells

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**Abstract. Four low molecular weight gels (LMWGs) with different modulus were fabricated as scaffolds to investigate the differentiation of mesenchymal stem cells (MSCs). The MSCs differentiated to osteoblasts in rigid LMWGs while to chondrocytes in soft LMWGs. The critical modulus to induce the different differentiations was between 10 to 20 kPa.**

Stem cells have attracted great interest due to their diverse differentiations potentially applied in tissue engineering and cell therapy, however, the differentiation pathway control is concerned in the research of stem cells.[1-3] Addition of growth factors in medium was reported as an effective strategy to decide the differentiation of stem cells.[4] In tissue engineering, other than growth factors, the properties of scaffolds including topologies, compositions, morphology and porosity affected the differentiation of stem cells in different pathways.[5,6] For example, substrate rigidity acted a significant role in modulating the behaviors of stem cells.[7,8] Not only the attachment and proliferation but also the development and differentiation of stem cells were definitely changed. Modulus of scaffold, which is the parameter to characterize the rigidity of materials, has been extensively investigated to explore its effect on differentiation of stem cells.[9,10] Human adipose derived stem cells could be induced to chondrogenic and osteogenic or adipogenic and myogenic differentiations in scaffolds with different rigidity.[11] To bone marrow derived mesenchymal stem cells (MSCs), the myogenic or chondrogenic and adipogenic differentiations was also affected by the modulus of scaffolds.[12]

MSCs could differentiate to multiply cell lines. How to control the osteoblastic or chondrocytic differentiations is the focus in bone and cartilage tissue engineering.[13] It was clear that rigid scaffold such as hydroxyapatite (HA) induced the osteoblastic differentiation of MSCs, and soft material such as collagen hydrogel induced the chondrocytic differentiation pathway.[14,15] As HA and collagen are different scaffolds materials, other parameters such as compositions are involved in the inducing differentiation pathway. For the difficulty to adjust the modulus to simulate the rigidity from rigid HA to soft collagen in such a large magnitude within a scaffold substrate, it is hard to disclose the critical modulus for osteoblastic or chondrocytic differentiations of MSCs in a continuous process.

Low molecular weight gels (LMWGs) were self-assembled from gelators, the disassociation of LMWGs was considered as degradation, the homogeneity in bulk properties and biocompatibility of LMWGs were much better than classic covalent bonds cross-linked gels in biological applications,[16-18] the 3D network architecture of LMWGs was convenient to trap cells inside.[19,20] The introduction of biomimetic compounds to improve the cell affinity of LMWGs revealed exciting results, all kinds of cell lines including HepG2, AD293, NIH 3T3 and HeLa cells attached and proliferated well in the LMWGs.[16,21,22] Recent research showed that LMWGs could provide excellent environment for the growth of stem cells.[23] However, the effect of LMWGs on the differentiation of stem cells, especially to control the osteoblastic or chondrocytic differentiations of MSCs was not reported.

In this paper, the phenylboronic acid derivative based LMWGs were developed. The properties of LMWGs such as modulus could be regulated by the structures of hydrophobic and hydrophilic segments in the gelators.[24,25] The LMWGs with wide modulus ranged from rigid ($10^5$ Pa) to soft ($10^3$ Pa) were prepared, MSCs were seeded on the surface of gels without addition of growth factors to investigate their differentiation behaviours.

The synthetic route of gelator was presented in Scheme S1 in Electronic Supplementary Information (ESI). The received molecules in each step were characterized by $^1$H NMR as shown from Figure S1 to S7. As the gelator was not dissolved in water to form gel, biocompatible low molecular weight poly(ethylene glycol) (PEG200) was added in water to improve the solubility of gelator. Four transparent gels were obtained with dissolving the gelator in the mixture solvents of deionized water and PEG200 with different ratios, the concentrations of G1 to G4 were 40 mg/mL in the solvent

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† Footnotes relating to the title and/or authors should appear here.
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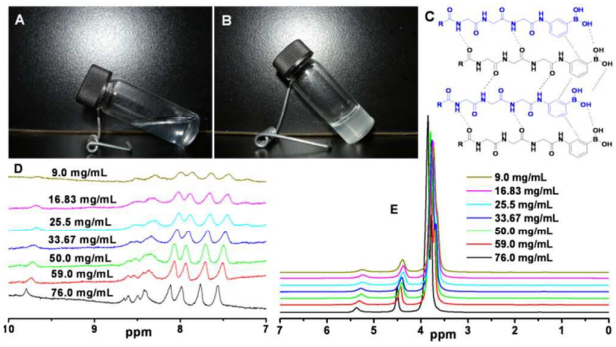


Figure 1. The interactions within LMWGs, A: the solvent of gelator in the mixture solvent of PEG200:$H_2O$=1:1 (30 mg/mL); B: the image of LMWGs (G2); C: the illustrated hydrogen bonding and π-π interaction within the gel; D and E: the $^1$H NMR spectra of the gel with different concentrations in the mixture solvent of PEG200:$D_2O$=1:1 to demonstrate the hydrogen bonding and the π-π interaction. The protons of different N-H (δ 8.02-δ 9.72) and Ar-H (δ 7.26 -δ 7.86) were shown in D, and the protons in O-H of PEG200 (δ 5.22-δ 5.37) were shown in E.

of PEG200:$H_2O$=4:1, 30 mg/mL in the solvent of PEG200:$H_2O$=4:1, 30 mg/mL in the solvent of PEG200:$H_2O$=1:1, and 20 mg/mL in the solvent of PEG200:$H_2O$=4:1. The images of the sol and gel were shown in Figure 1A and 1B. The mechanism of the self-assembly of gelators was attributed to the hydrophobic interaction as well as hydrogen bonding and π-π interaction between the gelators [26] which was illustrated in Figure 1C. The $^1$H NMR spectra of gelators with different concentrations were used to show the formation of hydrogen bonding and π-π interaction.[27] The protons of different N-H shifted downfield within 8.02 ppm to 9.72 ppm revealed the strengthened hydrogen bonding when the concentration of gelators increased from 9.0 mg/mL (gel) to 76.0 mg/mL (Figure 1D). The $^1$H NMR spectra of gelator in Figure 1E showed the gradual downfield shift of protons with increasing the concentration of gelator, the proton in O-H of PEG200 shifted downfield from 5.22 ppm to 5.37 ppm. The protons of PEG200 were involved in hydrogen bonding to accelerate the self-assembly of gelators.[28] The aromatic protons in the gelators were also gradually shifted downfield, and red shift was observed in the UV spectra of gelator with increasing concentrations (Figure S8), which indicated that the π-π stacking interaction was formed.[29] The gelators formed gel in isopropanol, the supramolecular self-assembly fibers were observed in the SEM image (Figure S9A), as PEG200 could not be eliminated in the SEM images, it filled in the porous architecture, thus the pores and fibers could not be observed in the SEM image of gel prepared in the mixture solvent of PEG200 and water (Figure S9B).

The rheological properties of the four gels were studied. The storage modulus (G') of the four gels was higher than their corresponding loss modulus (G"), it demonstrated that real gels were formed (Figure 2A).[30] The frequency dependence of the dynamic storage modulus (G') and loss modulus (G') decreased with the sequence of G1, G2, G3 and G4, it implied that the mechanical strength of the gels decreased from G1 to G4.[31] The strain dependence of the storage modulus of the four gels varied with the same rule. To G1, G2, and G4, the gelator concentrations were 40, 30 and 20 mg/mL in the same solvent. G1 showed the highest G', which was as high as 6x10$^4$ Pa when the strain was lower than 1%, and the G' of G4 was as low as 2x10$^3$ Pa with the same strain. The modulus of gels increased with increasing gelator concentration due to the strengthened hydrogen bonding and π-π interaction with increased concentration of gelators. Moreover, the rheological properties were influenced by the mixed solvent. G2 showed higher G' than that of G3 for more PEG200 was involved in hydrogen bonding to accelerate the self-assembly of gelators. When the strain was higher than 1%, the G' of all the four gels decreased dramatically, it revealed that the network structure of the gels were destroyed (Figure 2B). The complex viscosity of the four gels were tested and presented in Figure 2C, the complex viscosity of all the four gels decreased linearly with increasing frequency. G1 and G4 exhibited the highest and lowest complex viscosity under the circumstance of same frequency, respectively. Figure 2D gave the double-logarithmic plots of the relaxation modulus (G(t)) versus time(t) for the gels with different concentrations. For each gel, the plot exhibited a good linear relationship between relaxation modulus (G(t)) and time (t). We used dynamic time sweep to test the recovery property of the hydrogels (Figure S10). The initial storage modulus G' of the gel was about 5200 Pa, the G' was about 800 Pa after the stress was removed, and G' recovered to 4000 Pa within 1800 s. The G' recovery was due to the supramolecular architecture of the gel, which was physically cross-linked by hydrogen bonding, hydrophobic and π-π interactions to result in easy recombination to recover the 3D networks.[22] The results of rheological properties demonstrated that these LMWGs exhibited good gelation and recovery capability. The stimulus of the gels could be regulated by concentration and solvent, which was convenient to explore the differentiation of MSCs to osteoblasts or chondrocytes in homogeneous bulk materials.

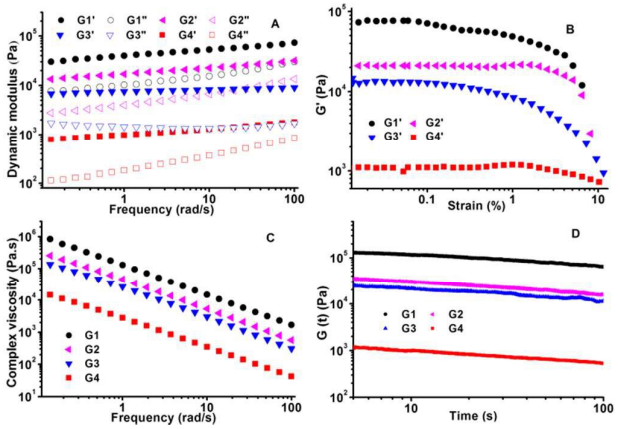


Figure 2. The rheological properties of gels, A: storage and loss modulus as a function of angular frequency for the gels; B: the strain dependence of storage modulus (G') for the gels; C: the complex viscosity as a function of angular frequency for the gels; D: the double-logarithmic plots of the relaxation modulus [G(t)] versus time (t) for the gels. The gels were prepared at 25 °C, the concentrations of gels were: G1: 40 mg/mL in PEG200:$H_2O$=4:1; G2: 30 mg/mL in PEG200:$H_2O$=4:1; G3: 30 mg/mL in PEG200:$H_2O$=1:1; G4: 20 mg/mL in PEG200:$H_2O$=4:1.

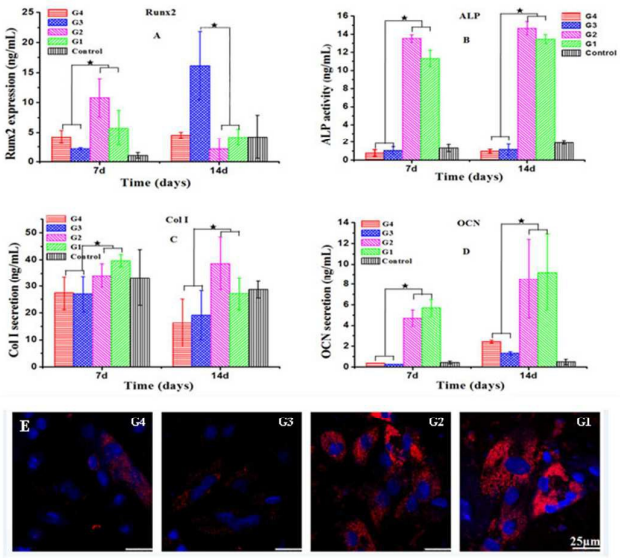


Figure 3. The Runx2 (A), ALP (B), Col I (C) and OCN (D) secretions of MSCs seeded in the four gels, E: OCN staining of MSCs in the four gels

MSCs were seeded on the surface of gels to evaluate the biocompatibility of gels. As shown in Figure S11, the constant increase of OD values over 7 days were seen in the four gels, indicating a steady cellular proliferation during the incubation. There were no significant differences among all the four gels. It revealed that the gels were non-toxic to MSCs. Confocal laser scanning microscopy was used to explore the alive cells and the migration of MSCs (Figure S12). The green fluorescence in different layers (focal planes) demonstrated that the MSCs could migrate from surface to bulk.[22] It was interesting to note that the MSCs in the gel with low modulus (G4) appeared oval cell shapes while the MSCs were well flattened and favorably spread within the gel with high modulus (G1).

Runx2, ALP, Col I and OCN secretions (Figure 3) were measured to analyze the osteogenic differentiation of MSCs.[32] The ALP activities of the gels with high modulus (G1 and G2) exhibited stabilized high levels, while the ALP activity of the gel with low modulus (G3 and G4) kept at stabilized levels with lower values. The ALP activity levels of the sample G2 in 7 and 14 days were 17.5 and 15.3 folds of those of sample G4, respectively. The Col I and OCN secretions of the MSCs on the gels with high modulus (G1 and G2) were significantly higher than those of the gels with low modulus (G3 and G4). Furthermore, the OCN secretions of the samples with high modulus increased with increasing incubation time, exhibiting a strong upward trend. The expressions of Runx2 in G2 and G1 exhibited an initial high expression level at 7 days and a downward trend afterwards. An opposite trend was found in the G3 sample, showing low expression at 7 days and a dramatic upward trend afterwards, likely due to the regulating role of Runx2 in the maturation from chondrocytes to hypertrophic chondrocytes in chondrocytic differentiation.[33] In addition, OCN staining of the four gels further supported the above conclusions (Figure 3E). After 14 days incubation, MSCs in the gels with high modulus showed obvious OCN production, whereas only a small signal was detected in the gels with low modulus. The results revealed that the gel with high modulus drove the MSCs to differentiate into the osteoblastic lineage with the significant up-regulation of ALP, Col I and OCN expressions.[34] When the elastic modulus increased from 20 kPa to 60 kPa (from G2 to G1), there was no remarkable difference in regulating MSC differentiation into osteoblastic lineage, indicating that the modulus of 20 kPa was sufficient for osteoblastic differentiation.

The Sox9, type II collagen, AGG and type X collagen, which indicated the early and latter chondrogenic differentiation,[35,36] were selected as markers to evaluate the chondrogenic potentials of the gels (Figure 4). For the early chondrogenic markers, the expression of Sox9 and Col II in the gels with low modulus (G4 and G3) was significantly higher than that in the gels with high modulus (G2 and G1). The expression of Sox9 and Col II in G4 and G3 increased with increasing cell culture time, while the expression of Sox9 and Col II in G2 and G1 kept at a relatively stable level, no large fluctuation was observed with the elongation of cell culture time. Similar trends were found for the latter chondrogenic markers of AGG and type X collagen, which were promoted in G4 and G3, indicating the enhancement of chondrogenic activities. Furthermore, the Sox9, Col II, AGG and Col X expressions of G3 were much higher than that of G4 all the time, while no significant difference was observed between G3 and G4 for AGG expression at 14 days. It indicated that the gel matrix with the modulus of ~10 kPa was more beneficial for chondrogenic linage. We then performed immunofluorescent staining for specific antibodies for type II collagen after 14 days incubation. There was appreciable secretion of collagen II matrix component in the G3 and G4 substrates, whereas almost no collagen II was detected in G1 and G2 with high moculus (Figure 4E). Overall, the rigid gel substrate stimulated the differentiation of MSCs into osteoblastic lineage, chondrocytic differentiation of MSCs was observed in soft gels. The matrix elasticity played an

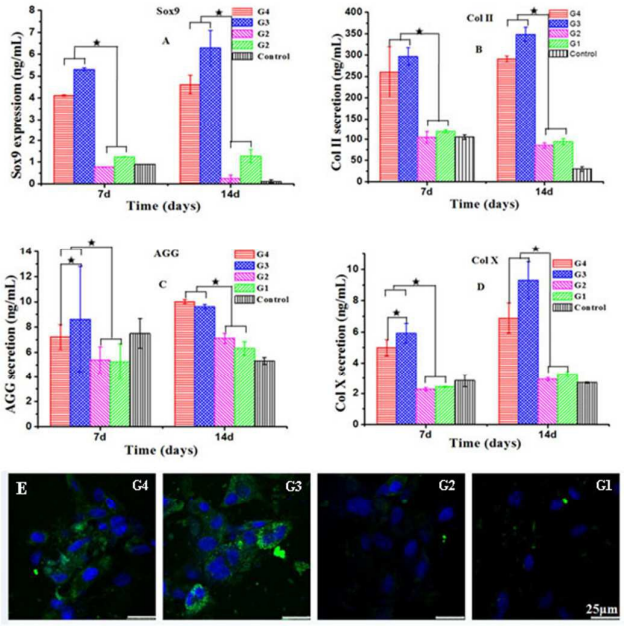


Figure 4. The Sox9 (A), Col II (B), AGG (C) and Col X (D) secretions of MSCs incubated in the four gels, E: immunofluorescent staining of MSCs in the four gels.s

important role in regulating the fate of MSCs. The matrix elasticity was reported to affect the structure of cytoskeleton in MSCs.[37] In return, the structural changes of cytoskeletal architecture affected the mechi-chemical signal pathways to affect the differentiation of MSCs into osteoblastic or chondrocytic lineage. MSCs on soft gel (G'~ 0.1-10 kPa) led to the MSCs into oval-shaped cells similar to chondrocytes. Rigid gels (G'~ 20-60 kPa) resulted in osteoid polygonal morphology to osteoblasts. The critical modulus to induce the differentiation of MSCs into osteoblasts or chondrocytes in LMWG substrates was between 10 to 20 kPa.

## Conclusions

We fabricated phenylboronic acid derivatives based LMWGs with different modulus as homogenous substrates to explore the osteoblastic or chondrocytic differentiation of MSCs in a continuous process, the gels was non-toxic to MSCs, the critical elastic modulus of the LMWGs for osteoblastic or chondrocytic differentiation of MSCs was between 10 to 20 kPa. Although the pathway of chondrogenis and osteogenesis of stem cells in gel substrates is still unknown, however, this research provides an effective strategy to regulate the differentiation of MSCs into osteoblasts or chondrocytes via elastic modulus mimicking bone or cartilage.

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