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Fractal Structure of Hydrogels Modulates Stem Cell Behavior

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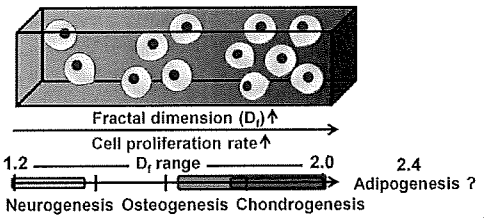
Supporting Information

ABSTRACT: Fractal dimension (D_f) is an index to describe the irregular continuous structure by quantifying the complexity. The concept of fractals has been employed to describe the complicated structure of polymer gel and human tissue. This study examined the effect of D_f on cell proliferation and stem cell differentiation in six polymer hydrogels with D_f ranging from 1.2 to 2.1. It was observed that fibroblasts and mesenchymal stem cells (MSCs) grew faster in hydrogels with higher D_f. Moreover, hydrogels with a fractal structure of $D_f \leq 1.4$, ≥ 1.6, and ≥ 1.8 promoted the neural, osteogenic, and chondrogenic differentiation of MSCs, respectively. The fractal structure of gel can modulate cell proliferation and fate, which provides an insight into designing the appropriate fractal and molecular structure of polymer hydrogel for biomedical applications.



A fractal is an infinitely and repeating geometric pattern, which displays self-similar at every scale.[1] Fractal dimension (D_f) is an index for quantifying the complexity of structure, which is a continuous analogue of geometric dimension. The traditional definition of dimension is an integer value, but D_f can be a fractional number.[2] The concept of fractals has been employed to describe the complicated structure of human tissues such as the vascular tree, bronchial tree, and cranial nerves.[3−5]

In the world of soft matters, polymeric chains, and gels also possess fractal structure.[6−8] In a polymer gel, D_f has been used to describe the network structure, where the formula linking D_f and the critical scaling (n) is $n = 3(5 - 2D_f)/2(5 - D_f)$,[9] where the value of n can be obtained by small-angle X-ray scattering (SAXS)[10] or by rheological measurement on critical gel at the gel point.[11] According to theories, polymer gels formed by diffusion-limited cluster aggregation (DLCA) have a D_f of 1.78, while those formed by reaction-limited cluster aggregation have a D_f of 2.1.[12] SAXS experiments showed that agarose, a natural polysaccharide, had D_f values between 1.4 and 1.8.[13] Poly(*N*-isopropylacrylamide) had a D_f of 1.49 by rheological measurement and light scattering experiment.[8]

Substrate stiffness or rigidity plays a significant role in modulating cell behavior, in particular for stem cells. Surface rigidity not only affects cell attachment and proliferation,[14] but also has a profound influence on stem cell development and fate.[15] Hydrogels are hydrophilic polymer gels with high water retention and mass transport.[16,17] Because hydrogels provide a more physiological three-dimensional environment, culturing cells in hydrogels is considered better than the conventional monolayer culture on a plastic dish.[18,19] Specifically, hydrogels of physical network formed under certain conditions do not contain toxic chemical cross-linkers and are convenient research tools. When cells are cultured within a hydrogel, the gel modulus is important and its effect on cell function has been studied quite extensively.[20,21] Human adipose derived stem cells cultured in stiffer gels (~33 kPa) are prone to chondrogenic and osteogenic differentiation, while those in softer gels (~10 kPa) are prone to adipogenic and myogenic differentiation.[22] Stiffer gels (~15 kPa) promote the myogenic differentiation of bone marrow derived mesenchymal stem cells (MSCs), while softer gels (~1 kPa) promote the chondrogenic and adipogenic differentiation.[23] The modulus values suitable for stem cell differentiation into a specific cell lineage vary among studies. In spite of the tremendous amount of work on cell−matrix interaction, no study has ever performed to examine the possible influence of fractal structure and fractal dimension of a three-dimensional polymer gel on cell proliferation or differentiation.

In this study, we established six polymer gels with physical networks of similar rigidities. We then evaluated the D_f of each polymer gel to show that they had different D_f values ranging from 1.2 to 2.1. Fibroblasts and MSCs were seeded in various polymer gels where their growth and differentiation were analyzed. We demonstrated that, even when the chemistry and rigidity of the gels were identical, a difference in fractal structure as manifested by different D_f could change cell growth and differentiation within such polymer gels.

The six gels included gelatin of type A (porcine skin, 300 Bloom, Sigma), agarose-1 (Agarose-LE, USB Affymetrix, U.S.A.), agarose-2 (type IB, Sigma), agarose-3 (type IX, Sigma), agarose-4 (Difco, BD Biosciences, U.S.A.), and soy protein isolate (type 974, Archer Daniels Midland, U.S.A.). Gelatin, agarose, and soy protein are the commonly used

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natural hydrogels for tissue engineering applications. The physical and biochemical properties of these hydrogels, including stiffness and biological modifications, have been shown to influence cell behavior. Rheological measurements (G' vs ω and G'' vs ω) were performed continuously to determine the sol–gel transition (gel point). Gelation of various hydrogels was conducted in the appropriate temperature so the transition point could be clearly observed. Sol–gel transition point was reached when the two log–log curves became parallel to each other (slope $n' = n'' = n$), as shown in Figure 1. The scaling (n) was related to the fractal dimension

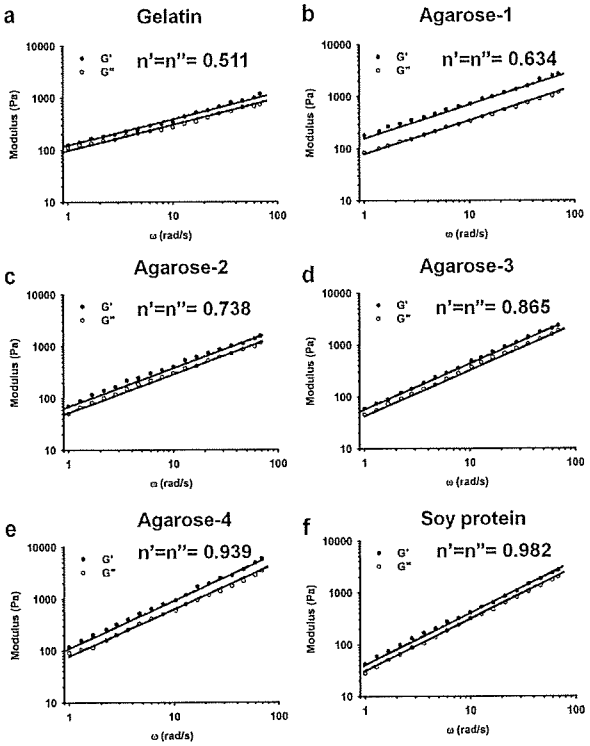

Figure 1. Determination of fractal dimension (D_f) by rheological experiments. The log–log plots of shear storage modulus (G') and shear loss modulus (G'') vs frequency (ω) for each hydrogel at the gel point showed parallel power law behavior (with equal slopes $n' = n''$). The value of D_f for each hydrogel (presented in Table 1) could be obtained from the slope of the hydrogel at the gel point (critical scaling); (a) gelatin, (b) agarose-1, (c) agarose-2, (d) agarose-3, (e) agarose-4, and (f) soy protein.

(D_f) by $n = 3(5 - 2D_f)/2(5 - D_f)$. The D_f values of gelatin, agarose-1, agarose-2, agarose-3, agarose-4, and soy protein based on this method were 1.99, 1.82, 1.68, 1.48, 1.36, and 1.28, respectively (Table 1).

The D_f values of various hydrogels were confirmed by SAXS experiments conducted at the beamline 23A of National Synchrotron Radiation Research Center (Hsinchu, Taiwan). The photon energy was at about 10 keV. Results are demonstrated in Figure 2. The slope of the log intensity vs log scattering vector (q) curves at the low q range was obtained. D_f was defined as the negative of the slope (Table 1). The D_f values obtained by rheology and SAXS agreed with each other with a deviation of only 2–3%. Interestingly, when $D_f < 1.7$, the values obtained by rheology were slightly higher; when

Table 1. Fractal Dimensions of Hydrogels and the Average Proliferation Rate for Cells Grown in Each Hydrogel (~2.5 kPa)

hydrogels	D_f (rheology)	D_f (SAXS)	growth rate[a] (fibroblasts)	growth rate[a] (MSCs)
gelatin	1.99	2.05	131.0 ± 3.7	112.6 ± 6.1
agarose-1	1.82	1.87	122.5 ± 2.4	100.4 ± 2.0
agarose-2	1.68	1.64	120.3 ± 4.0	94.4 ± 6.9
agarose-3	1.48	1.44	111.9 ± 3.8	80.5 ± 4.2
agarose-4	1.36	1.34	94.2 ± 6.7	63.9 ± 6.1
soy protein	1.28	1.25	90.3 ± 6.6	60.7 ± 4.7

[a]Unit: 10^3 cells/cm^3 per day in average.

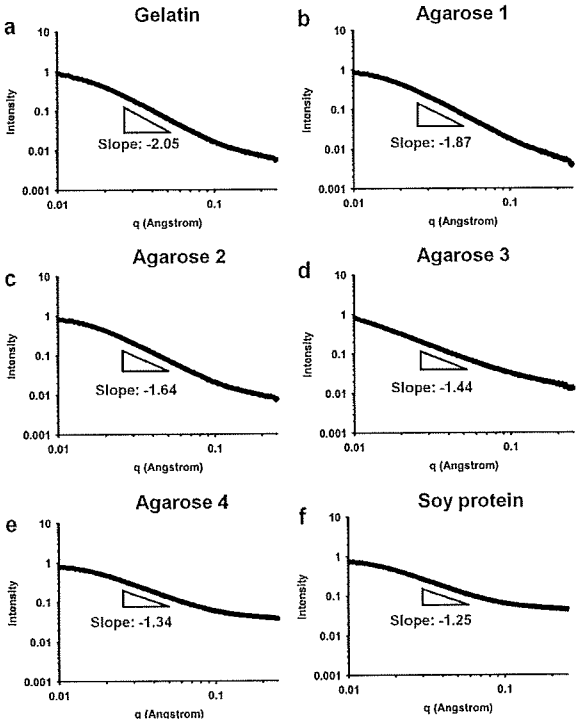

Figure 2. Determination of D_f by SAXS experiments. The slope of the log–log plot of scattering intensity vs scattering vector (q) at the low q range showed power law behavior for each hydrogel. The value of D_f was the negative of the slope (presented in Table 1); (a) gelatin, (b) agarose-1, (c) agarose-2, (d) agarose-3, (e) agarose-4, and (f) soy protein.

$D_f > 1.7$, the values obtained by rheology were slightly lower. In either case, the six hydrogels studied had different fractal dimensions in the range of $D_f \sim 1.2$–2.1. The scanning electron microscopic (SEM) images of the six gels showed no significant difference in the structure (Figure S1, Supporting Information). It was thus difficult to visualize the difference in fractal structure of gels by imaging techniques.[6,24]

To investigate the effect of fractal structure on cell growth in the hydrogel, the concentration of each hydrogel was first adjusted so the stiffness (G' at 37 °C) was similar in the range 2.50 ± 0.05 kPa (Figure S2a, Supporting Information). The proliferation of fibroblasts (L929) and human umbilical cord derived MSCs in the hydrogels is shown in Figure S3 (Supporting Information), with the average cell growth rate summarized in Table 1. Fibroblasts grew faster than MSCs

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when seeded at the same number (10^6 cells/cm^3). A larger gel D_f was correlated with a higher cell proliferation rate. Compared to fibroblasts, MSCs were more sensitive to D_f, where an 87% increase in proliferation was observed as D_f (SAXS) increased from 1.25 to 2.05. For fibroblasts, a 45% increase was observed as D_f increased from 1.25 to 2.05. Among the different D_f values, an increase from 1.32 to 1.44 resulted in the most significant changes in cell proliferation. The D_f values of stiffer hydrogels (in the range 25.0 ± 0.5 kPa, Figure S2b, Supporting Information) were similar to those of softer hydrogels (~2.5 kPa; Figure S4, Supporting Information). In hydrogels with greater stiffness (~25 kPa), MSCs proliferated more slowly than those in hydrogels with smaller stiffness (~2.5 kPa). Nevertheless, MSCs remained to grow faster in hydrogels with a larger D_f value (Figure S5, Supporting Information). Spearman's rank correlation coefficient analysis (Figure S6, Supporting Information) showed that the correlation coefficient of cell proliferation and stiffness (correlation coefficient was 0.850 for agarose-1 and was 0.900 for agarose-3, $p < 0.001$) was higher than that of cell proliferation and D_f value (correlation coefficient was 0.700 for both 2.5 and 25 kPa, $p < 0.005$). Although stiffness has a higher effect on cell proliferation, the D_f value still showed a statistically significant impact on cell proliferation.

The impact of D_f on cell differentiation of MSCs in hydrogels (~2.5 kPa) is shown in Figure 3. In all hydrogels after 3 weeks of adipogenic induction, the expression of adipogenic marker genes PPARγ2 and LPL was upregulated but not significantly different among each group (Figure 3a). The result was confirmed by Oil O staining and quantification of lipid droplets (Figure S7a, Supporting Information). After osteogenic induction, hydrogels with higher D_f values ($D_f \geq 1.6$) revealed a 1.7–2.4× greater expression level of osteogenic marker genes Runx2 and OCN (Figure 3b). The Alizarin red S staining and quantification of calcium deposition are shown in Figure S7b (Supporting Information). Higher calcium contents were observed in hydrogels with higher D_f. After chondrogenic induction, hydrogels with higher D_f ($D_f \geq 1.8$) showed a 1.8–2.9× higher level of chondrogenic marker genes Sox9, aggrecan, and type II collagen (Col II) than the other hydrogels (Figure 3c). Safranin O staining revealed the presence of more cartilage matrix for hydrogels with larger D_f values (Figure S7c, Supporting Information). The staining for soy protein was not performed because of the opaqueness of the gel. Quantification of glycosaminoglycan (GAG, a component of aggrecan) showed that MSCs secreted more extracellular matrix in hydrogels with large D_f values. Conversely, the expressions of neurogenic marker genes (nestin, GFAP, and β-tubulin; shown in Figure 3d) and immunostaining of β-tubulin (Figure S7d, Supporting Information) were not significantly different among the groups after neurogenic induction.

The influence of D_f on the differentiation of MSCs in stiffer hydrogels (~25 kPa) is shown in Figures 4 and S8 (Supporting

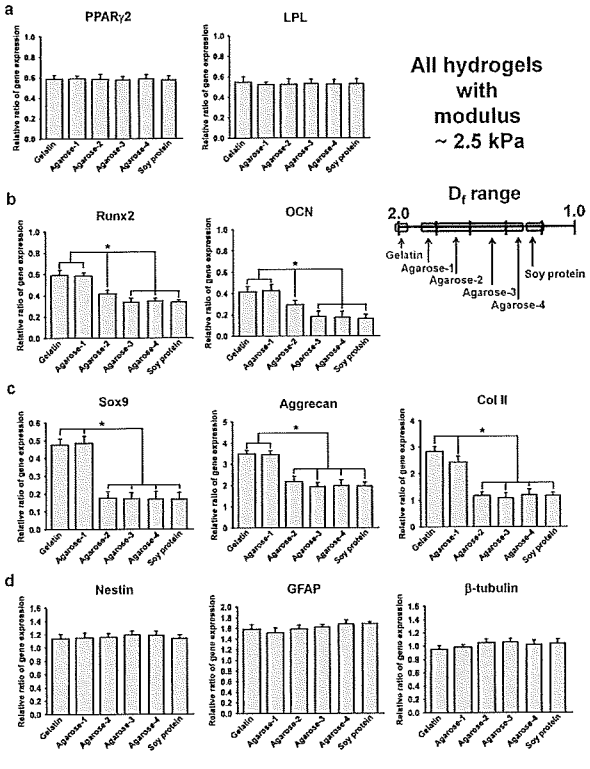

Figure 3. Stem cell differentiation in hydrogels with same stiffness (~2.5 kPa) but different D_f (~1.2–2.1). (a) Adipogenic (LPL and PPARγ2), (b) osteogenic (Runx2 and OCN), (c) chondrogenic (Sox9, aggrecan, and Coll II), and (d) neural (nestin, GFAP, and β-tubulin) representative gene expression of MSCs grown in the softer (~2.5 kPa) gels after 3 weeks of induction. *$p < 0.05$ among the indicated groups.

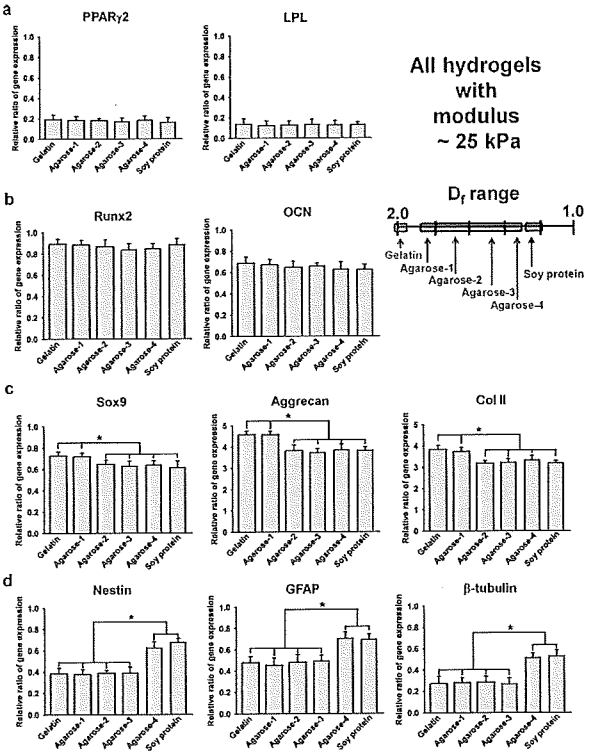

Figure 4. Stem cell differentiation in hydrogels with the same stiffness (~25 kPa) but different D_f (~1.2–2.1). (a) Adipogenic (LPL and PPARγ2), (b) osteogenic (Runx2 and OCN), (c) chondrogenic (Sox9, aggrecan, and Coll II), and (d) neural (nestin, GFAP, and β-tubulin) representative gene expression of MSCs grown in the stiffer (~25 kPa) gels after 3 weeks of induction. *$p < 0.05$ among the indicated groups.

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Information). Adipogenic differentiation remained similar among all groups, but the expression level was lower than that in softer hydrogels (Figures 4a and S8a, Supporting Information). Osteogenic differentiation (Runx2 and OCN expression) in stiffer hydrogels was generally greater than that in softer hydrogels and appeared to be favored by higher D_f, but not significantly (Figure 4b). The results of calcium quantification and Alizarin red staining followed a similar tendency to those of gene expression (Figure S8b, Supporting Information). Chondrogenic differentiation (Sox9, aggrecan, and Col II expression) was greater in gelatin and agarose-1 than that in the other hydrogels of the same stiffness (~25 kPa). This tendency was the same as that observed in softer hydrogels (~2.5 kPa); however, the expression levels in stiffer hydrogels were greater and the D_f dependency was not so remarkable as compared to softer hydrogels (Figure 4c). Judging from the gene expression as well as the Safranin O staining and GAG quantification (Figure S8c, Supporting Information), a greater D_f still favored the chondrogenic differentiation. In contrast, neurogenic differentiation (nestin, GFAP, and β-tubulin expression) was significantly greater (1.4–1.9×) in agarose-4 and soy protein hydrogels. The expression levels, however, were lower than those in the respective softer hydrogels (Figure 4d). This tendency was confirmed by the immunostaining of β-tubulin in Figure S8d (Supporting Information).

Cell behavior and fate are controlled and regulated by the extracellular matrix. The literature focuses mostly on the critical role of substrate stiffness.[15] Stiffness of 1 kPa is favorable for neural differentiation of MSCs. Higher stiffness of 25–34 kPa promotes chondrogenic differentiation. The stiffness suitable for osteogenic differentiation is 100 kPa.[25] The above applies for cells grown as monolayer on substrates. Regarding hydrogels, fibroblasts grow faster in softer ones.[26] The appropriate stiffness range of a hydrogel was 0.6–1, 2.5–5, 3–30, and 11–60 kPa for neural, adipogenic, chondrogenic, and osteogenic differentiation, respectively.[23,27−29] The extracellular matrix is a three-dimensional network composed of polymer chains and high contents of water. The fractal structure of the hydrogel, thus, may have a profound impact on cells but has never been explored so far.

According to our study, cells grew the fastest in gelatin (higher D_f). Gelatin is the denatured form of collagen, a major component of extracellular matrix. Gelatin and soy protein may provide bioactive signals that contribute to cell proliferation,[30,31] and the observations with gelatin and soy protein may not be completely ascribed to D_f. Yet, they had very distinct data in cell proliferation and differentiation, suggesting that D_f affect cell fate. Meanwhile, agarose is polysaccharide polymer composed of D-galactose and 3,6-anhydro-L-galactopyranose. The bioactivity is less evident, if any.[32,33] The difference in D_f of agarose is attributed to the amount of free chain ends, the molecular weight, and the extent of chain entanglement.[34] The four types of agarose allow the effect of D_f to be studied more systematically. In agarose with the same stiffness, cells proliferated faster in that with a greater D_f. When the stiffness of agarose increased from 2.5 to 25 kPa, cell proliferation was reduced. Earlier studies demonstrated that MSCs on the surface of the soft hydrogel had lower proliferation rate than those on the surface of the stiff hydrogel because of less cell spreading.[35−37] This rule applied to cell culture on the surface of hydrogel (2D substrates). In this study, MSCs were cultured within the 3D hydrogel. Other literature also reported that MSCs grown in soft 3D hydrogels had faster proliferation rate than those grown in stiff 3D hydrogels.[38] The observations suggested that MSCs may respond differently to the 2D versus 3D environment.[38,39] Our finding also demonstrated that higher D_f promotes cell proliferation in both softer and stiffer hydrogels. The D_f value of hydrogels, which depends on the ratio of folding and curling of polymer chain. The D_f value is close to 1 for straight chain and close to 2 for formation of plane. When the ratio of chain folding and chain curling increases, the D_f value increases. The higher ratio of folding and curling of molecule chain may promote the cell–material interaction and further affect the cell proliferation. In addition to cell proliferation, one of the most important measures of stem cell function is the differentiation, which was examined in this study. Meanwhile, cell viability and metabolism are also important. Because cells were embedded in the gel, the protocol for evaluation of cellular metabolism, such as the assay of mitochondrial function using optical methods, must be modified to avoid influence from the gel. This will be a future subject of study.

Our study also established that MSCs were more osteogenic in hydrogels with $D_f \sim 1.6–1.8$ or greater and were more chondrogenic in hydrogels with $D_f \sim 1.8$ or greater. Investigation on the fractal structure of trabecular bone revealed D_f values in the range of 1.68–1.86.[40] This range was matched to that observed for better osteogenesis in our study. The D_f of linear polymers described by self-avoiding walk is 1.67.[41] The D_f of branched polymers described by lattice animal is 2.0. Although the complex aggrecan molecules have $D_f \sim 2.7$, the structure formed by aggrecan clustering has $D_f \sim 1.9$.[42] The latter D_f value was close to that favorable for chondrogenic differentiation of MSCs in our study. Hydrogels formed by the mechanism of DLCA had experimental D_f values in the general range of 1.75–1.95.[43] The close match of D_f between DLCA gels and skeletal tissues may implicate the potential use of DLCA gels in MSC differentiation and skeletal tissue formation. Meanwhile, our study established that MSCs were more neurogenic in hydrogels with $D_f \sim 1.4$ or smaller. Literature showed the neural network in human brain had $D_f \sim 1.1–1.2$.[44] Neural cells have D_f of 1.1–1.5.[45] These D_f values were closely matched to those favorable for neural differentiation in our study.

It was also revealed that the effect of D_f on MSC fate may be shielded by gel stiffness. The influence of D_f on osteogenesis was less obvious in stiffer gels. The influence of D_f on neurogenesis was less obvious in softer gels. Although the effect of D_f on chondrogenesis was also less remarkable in stiffer gels, its effect remained significant ($p < 0.05$). The sensitivity of chondrogenesis to D_f may be attributed to the mesenchymal tissue condensation process that is essential for chondrogenesis.[46] In contrast, the effect of D_f on adipogenesis was not observed in the present study. The fat crystal network has a D_f 2.7–2.9.[47] Natural hydrogels offer limited choices of D_f and relatively low stiffness in general. The available range of D_f (~1.2–2.1) may be too low compared to that of adipose tissue (Figure S9, Supporting Information) for adipogenesis in this study. An investigation can be extended in the future to include synthetic hydrogels[48,49] for a more comprehensive understanding regarding the effect of D_f on cell behavior.

In summary, the D_f of a polymeric hydrogel can affect the cell growth rate. Cells grow faster in hydrogels with a higher D_f. Moreover, D_f influences stem cell differentiation in a tissue "matching" manner, that is, hydrogels with D_f matched to that

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of a specific tissue favor the tissue-specific differentiation, as summarized in Table 2. Chondrogenesis, osteogenesis, and

Table 2. Regulation of Stem Cell Differentiation by the Gel Modulus and D_f Based on the Results of the Current Study

	gel modulus (kPa)	D_f
adipogenesis	~2.5	≫2.1
osteogenesis	≥25[a]	≥1.6
chondrogenesis	~25	≥1.8
neurogenesis	≤2.5[a]	≤1.4

[a]Predicted from the tendency.

neurogenesis are each preferred in hydrogels with $D_f \sim 1.8$ or greater, ~1.6 or greater, and ~1.4 or smaller. Adipogenesis is favored by softer hydrogels (~2.5 kPa), but D_f dependency is not clearly observed unless at a higher D_f (~2.4). The pioneer study offers a new design rationale for polymeric hydrogels intended for controlling stem cell growth and differentiation. It also helps in selecting suitable hydrogels for cell culture and tissue regeneration.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.5b00597.

Detail of materials and methods, supporting figures, and supporting table (PDF)

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Notes

The authors declare no competing financial interest.

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