Degradation rate affords a dynamic cue to regulate stem cells beyond varied matrix stiffness

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Abstract

While various static cues such as matrix stiffness have been known to regulate stem cell differentiation, it is unclear whether or not dynamic cues such as degradation rate along with the change of material chemistry can influence cell behaviors beyond simple integration of static cues such as decreased matrix stiffness. The present research is aimed at examining effects of degradation rates on adhesion and differentiation of mesenchymal stem cells (MSCs) in vitro on well-defined synthetic hydrogel surfaces. Therefore, we synthesized macromers by extending both ends of poly(ethylene glycol) (PEG) with oligo(lactic acid) and then acryloyl, and the corresponding hydrogels that were obtained after photo-polymerization of the macromers were biodegradable. Combining the unique techniques of block copolymer micelle nanolithography with transfer lithography, we prepared a nanoarray of cell-adhesive arginine-glycine-aspartate peptides on this nonfouling biodegradable hydrogel. The biodegradation is caused by hydrolysis of the ester bonds, and different degradation rates in the cell culture medium were achieved by different stages of accelerated pre-hydrolysis in an acidic medium. For the following cell culture and induction, both the matrix stiffness and degradation rate varied among the examined groups. While adipogenic differentiation of MSCs can be understood by the lowered stiffness, the osteogenic differentiation was contradictory with common sense because we found enhanced osteogenesis on soft hydrogels. Higher degradation rates were suggested to account for this interesting phenomenon in the sole osteogenic/adipogenic induction and even more complicated trends in the co-induction. Hence, the degradation rate is a dynamic cue influencing cell behaviors, which should be paid attention to for degradable biomaterials.

1. Introductions

Cell-material interactions are fundamental issues for the development of next-generation biomaterials [1–4]. Various chemical, physical and biological cues can influence cells [5–9]. For instance, functional groups [10–12] and surface topography [9] are known to regulate cell adhesion and differentiation; selective decoration with cell-adhesive ligands to restrict cell spreading area [13,14] and using material techniques to control cell shape [15–19] can well adjust cell behaviors. Besides, cell cues like cell–cell contact [20,21] and cell density [22] have also been found to tune lineage commitment of stem cells. A particularly important biomechanical cue revealed in the last decade is matrix stiffness [23–25]. To date, it has become common knowledge that a soft material favors adipogenic differentiation of mesenchymal stem cells (MSCs) and a stiff matrix favors osteogenic differentiation, especially in two-dimensional (2D) systems [26,27].

Most of the known cues are static parameters. In a biomaterial-based system, these parameters are inevitably varied when material chemistry changes during cell culture or tissue regeneration [28,29]. It is critical to understand whether or not a change of one parameter can be understood simply from summation of a series of static cues, that is to say, whether or not the change rate of that quantity affords an additional parameter as a dynamic cue. Biodegradability becomes a key factor when designing new biomedical materials for potential applications in tissue engineering and tissue regeneration. Degradation of biomaterials has inspired much research [30–35]. It is particularly meaningful to study cell-material interactions in a biodegradable microenvironment [36–41].

Degradation rate might be a candidate for a dynamic cue that influences cell behaviors. Recently, some valuable efforts have been...
made to examine the effects of biodegradation on cells encapsulated in three-dimensional (3D) hydrogels. Anseth et al. [42] introduced a photodegradable hydrogel based on a poly(ethylene glycol) (PEG) derivative containing nitrobenzyl ether photolabile moieties, and found that human MSCs spread much better in hydrogels upon light exposure (and thus upon degradation) than in the control group that was not exposed to light. Burdick et al. [43] prepared permissive/restricted hydrogel networks through distinct crosslinking protocols, and introduced matrix metalloproteinase (MMP)-sensitive peptides as degradable crosslinkers of hyaluronic acid (HA); they found that human MSCs preferred spreading and favored osteogenesis in permissive systems, which were degraded by cells; when a later crosslinking changed the permissive system to a restricted one, they observed enhanced adipogenesis. Bian et al. [44] extended this study and further found that cell-mediated degradation regulated chondrogenesis of human MSCs and hypotherpy in MMP-sensitive HA hydrogels, independently of cell spreading. Some other dynamic material-related cues were also studied recently. For instance, Mooney et al. [45,46] revealed that stress relaxation of alginate hydrogels enabled the encapsulated cells to remodel the matrix and cluster the cell-adhesive ligands, resulting in more spreading.

While these elegant studies have revealed the roles of biodegradation on cells by comparing biodegradable versus non-biodegradable or permissive versus restricted material systems, a study of the effects inflicted by the degradation rate on stem cells would be more attractive. This kind of study should be based on well-controlled hydrogels with a series of degradation profiles in a cell culture medium. Herein, we pay attention to the effects of rates of biodegradation on adhesion and differentiation of stem cells. The present report is dealing with 2D cell behaviors based on well-designed nanopatterned surfaces of synthetic hydrogels of varied degradation rates. The nanopatterned surfaces are powerful 2D model material systems for fundamental research. Our test system comprises a cell-adhesive arginine-glycine-aspartate (RGD) nanoarray on an adhesion-resistant biodegradable hydrogel with nonfouling PEG as the major component and a hydrolysis-labile oligoester motif as the minor component. The idea is schematically shown in Fig. 1.

The hydrogels that we utilized in this study were PEG-based derivatives. PEG hydrogels have been widely used in biomedical fields for their high fidelity towards extracellular matrices (ECMs) and their excellent biocompatibility [47–49]. Introducing hydrolysis-labile or enzyme-sensitive segments makes PEG-based materials degradable [50–52]. In this study, we used the block copolymer PLA-b-PEG-b-PLA as a PEG derivative that combines the advantages of PEG and poly(lactic acid) (PLA) [48,53]. Its admirable properties have rendered it a good candidate as a tissue engineering scaffold [54,55] and for drug delivery [56–58]. PLA, a polyester with hydrolytically labile chemical bonds, has been generally considered to be a biodegradable material that mainly undergoes bulk erosion [59]. Incorporating oligomers of lactic acid (LA) as extension of PEG allows for tunable degradability by changing the number of the LA units and the crosslinking density of the hydrogels [50–52]. Furthermore, introducing alkenes at both ends of the oligoLA-PEG-oligoLA chain through acryloyl functionalization can make the diacrylate (DA) macromer chemically cross-linkable. Thus, a hydrogel with tunable degradability can be achieved, as schematically indicated in Fig. 1C.

Since PEG is chemically relatively inert, it is very difficult to generate a stable array of other chemicals on the surface of a PEG hydrogel. Nevertheless, we have combined block copolymer micelle nanolithography and transfer lithography to develop a unique patterning technique to generate a gold array on a PEG hydrogel [63,64]. In the present study, we first constructed a quasi-hexagonal nanoarray of gold on glass via self-assembly of micelles of block copolymers loaded with chloroauric acid upon dip-coating followed by plasma treatment. Then, a bifunctional linker with one thiol (–SH) end and one acryloyl end was grafted on the gold nanodots and joined in the photopolymerization of the macromers with acryloyl ends. Prior to cell experiments, RGD peptides were grafted on the gold nanodots, providing cell-adhesive sites.

It seems worthy to note that while the synthesis of the hydrolysis-labile macromers to prepare biodegradable hydrogels and the transfer strategy to prepare RGD nanoarrays on PEG hydrogels have been known [60,63], the model material of RGD-nanoarray-decorated biodegradable hydrogels examined in the present study has never been reported. Most importantly, this article examines the cell behaviors on surfaces of biodegradable synthetic materials with a series of well-controlled degradation rates. In this context we used an acidic medium to speed up hydrolysis, and by controlling the accelerated hydrolysis stages we found that the obtained samples exhibited different degradation rates in the cell culture medium, which enabled us to examine the potential effect of degradation rates on cell behaviors in a parallel way. The change of other parameters such as matrix stiffness should, of course, also be taken into consideration to fully understand the results of the cell experiments.

MSCs from the bone marrow of Sprague Dawley (SD) rats were employed as the cell model. Osteogenic and adipogenic induction were carried out in vitro. Both cell adhesion and lineage commitments were recorded on the surfaces of the hydrogels with well-controlled degradation rates leading to some interesting results.

2. Materials and methods

2.1. Synthesis and characterization of the biodegradable macromer

The synthesis route of the macromers is showed in Fig. 1A. Before introducing the chemically crosslinkable alkenes into the PEG chains, oligoLA-PEG-oligoLA was synthesized via ring-opening polymerization of $\text{D}_{14}$-lactide (Zhejiang Medzone), PEG with a number average molecular weight ($M_n$) of 2000 (Sigma) with hydroxyl (–OH) at both ends initiated the reaction, and stannous octoate (Sigma) was used as the catalyst. PEG was added into a three-neck flask and dried under vacuum at 120 °C for 3 h. Then the reaction system was cooled down to 80 °C, $\text{D}_{14}$-lactide and SnOct2 were added to molten PEG. The reaction was allowed to proceed at 140 °C under stirring. Twelve hours later, the acquired product was cooled to room temperature, diluted by chloroform (CHCl3) and purified by precipitation from diethyl ether. After filtration and lyophilization, the dry solid polymer was stored at −20 °C for the next step.

The cross-linkable macromer was obtained via the nucleophilic substitution reaction of oligoLA-PEG-oligoLA and acryloyl chloride (Tokyo Chemical Industry). In order to avoid moisture, the reaction was carried out under argon using standard Schlenk techniques; dichloromethane (DCM) and TEA were obtained from commercial sources and were purified according to standard procedures before use. OligoLA-PEG-oligoLA was dissolved in DCM, and TEA was added at 0 °C under argon. After stirring in an ice bath for 30 min, the mixture was treated with acryloyl chloride through a quantitative injection pump with a constant addition rate. During the injection process, the reaction system was kept in an ice bath until the acryloyl chloride was completely added. The reaction was firstly run in an ice bath for 5 h, and stirring was continued at room temperature for 18 h. The product mixture was filtered through a diatomite column to remove the precipitated salts, and then eluted with ethyl acetate (EtOAc). The filtrate was concentrated and gave a yellow mixture. The residue was then dissolved in a small amount
of DCM and diluted by EtOAc to separate the residual salts. After filtering through a diatomite column, the filtrate was added to ice-cold diethyl ether for precipitation. Finally, acrylated oligoLA-PEG-oligoLA was gained through filtration and lyophilization, and the white powder was stored at −20°C.

The feed ratio of each step are listed in Table S1. The structures of the macromers obtained from the two-step protocol were determined by 1H nuclear magnetic resonance (1H NMR) in CDCl3 at 400 MHz (Bruker, AVANCE III). The scan number of each sample was 16. The NMR spectra are shown in Fig. S1.

2.2. Photopolymerization of diacrylated oligoLA-PEG-oligoLA to form chemical hydrogels

Diacylated oligoLA-PEG-oligoLA powder was dissolved in Milli-Q water at room temperature to prepare a macromer solution with a concentration of 45% (w/w). After stirring for 12 h, 0.1 wt% photoinitiator 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropionic acid (D2959, Sigma-Aldrich) was added into the macromer solution. The mixture was stirred for 0.5 h. The whole process described above was run in the absence of light.

![Fig. 1. Schematic presentation of the fabrication process of an RGD nanoarray on a PEG-based yet hydrolysis-labile hydrogel and the underlying scientific question about cell-material interactions to be examined. (A) Synthesis routes of oligoLA-PEG-oligoLA macromers. In the first step, PEG with two free hydroxyl end groups triggered the ring-opening polymerization of d,l-lactide induced by catalytic amounts of stannous octoate Sn(Oct)2; then, oligoLA-PEG-oligoLA was terminated with acryloyl chloride, using triethylamine (TEA) as the base. (B) Fabrication process of an RGD-grafted gold nanoarray on a hydrogel synthesized by polymerization of the above described macromers. The nanoarray was first formed on glass through block copolymer micelle nanolithography and then transferred to the synthesized hydrogel; finally, cyclic RGD with a thiol end group was grafted on each gold nanodot to form an RGD-grafted nanopattern on the biodegradable hydrogel. (C) Biodegradation of the hydrogel via hydrolysis of the oligoester segments. (D) Schematic illustration of the cell experiments and the corresponding key scientific question. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)](image-url)
Photopolymerization was conducted under ultraviolet (UV) radiation (365 nm). The prepared solution was pipetted to tunnel in a mould which was covered by glass slides. The depth of each tunnel was 0.7 mm, and each mould was filled with around 500 μL of solution. Then the samples were submitted to 7 mW/cm² UV irradiation under nitrogen atmosphere for 1 h. Finally, hydrogels with a thickness of approximately 0.7 mm were gained by peeling off from the glass.

2.3. Measurements of compressive moduli and swelling ratios of the biodegradable hydrogels

Hydrogel slices (26 mm × 22 mm with thickness of 0.7 mm) obtained from photopolymerization were soaked in phosphate buffered saline (PBS, pH 7.4, Hyclone) and then moved to a 37 °C water bath shaker. When the slices reached a swelling balance, the initial linear swelling ratio (SR_initial) was measured and calculated from the specific length of the hydrogels. SR_initial was defined as the specific length of the hydrogel measured after swelling in a large amount of an aqueous medium such as PBS divided by the length after hydrogel synthesis yet before swelling (Lg, 26 mm).

Degradation experiments were conducted immediately after swelling. Besides the normal yet slow hydrogel degradation in PBS, we also carried out the accelerated degradation by immersing the hydrogels in 2 mol/L hydrochloric acid (HCl) at 37 °C for varied time periods (0 h, 2 h, 4 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h). Samples taken out at different points in time were washed with PBS for 5 times until the final pH of the solution was equal to 7.4. After washing, the hydrogel samples were immersed in PBS for immediate use. Considering the envisioned use in cell induction experiments, hydrogels were then immersed into a cell culture medium in a 37 °C incubator to explore their degradability in cell culture conditions.

The linear swelling ratio (SR) was obtained by measuring the length of the hydrogels at different degradation stages and calculated by the equation

\[ \text{SR} = \text{SR}_\text{initial} \times \text{SR}_\text{degradation} \]

Here, SR_degradation was defined as the final length after degradation for a certain time divided by the swelling length before degradation.

The volume swelling ratio was measured to evaluate the bulk swelling of the degraded hydrogels. Samples being washed with PBS were weighed and put into a vacuum drying oven for at least 24 h to fully evaporate the water in the hydrogels. Then the dry hydrogels were weighed, and the volume swelling ratio (QV) was calculated by the equation

\[ Q_V = \frac{m_1}{m_0} \]

\[ Q_V = 1 + \frac{\rho_0}{\rho_s} (Q_m - 1) \]

Here, Qm denotes the mass swelling ratio of a hydrogel, m1 refers to the mass of a hydrogel swollen in PBS, m0 refers to the mass of the corresponding dry hydrogel, ρ0 represents the density of the dry polymer, and ρs represents the density of PBS.

A stress-controlled rheometer (Kinexus Pro, Malvern, UK) was used to measure the compressive moduli of the hydrogels. The upper parallel plate attached to the rheometer slowly moved down with velocity v = 5 × 10⁻³ mm/s. After the upper plate contacted the top surface of the hydrogel slice, the normal force increased with the gap narrowing down. The original force-gap curve was collected in real time and converted into a stress-strain curve through calculation, as presented in Fig. S2. Compressive moduli of the hydrogels were obtained from the stress-strain curve at a low range of strain (ε ≤ 5%).

2.4. Fabrication of an RGD nanoarray on the biodegradable hydrogel

The fabrication process of the nanopattern is depicted in Fig. 1B. Before obtaining the nanoarray on the hydrogels, we needed to prepare a nanoarray on glass. Glass slides (26 mm × 22 mm with thickness of 0.5 mm) were washed thoroughly with piranha solution (H₂SO₄:H₂O₂ = 3:1) and immersed in Milli-Q water before use. Block copolymer poly(styrene-b-2-vinyl pyridine) (PS-b-P2VP, Polymer Source) was dissolved in toluene to form a micelle solution. The hydrophilic or lipophobic component P2VP constituted the micelle cores while the hydrophobic or lipophilic part PS constituted the coronas. Hydrophilic hydrogen tetrachloroaurate(III) trihydrate (HAuCl₄·3H₂O, Alfa Aesar) was added to the polymer solution as an Au precursor, and migrated into the cores of PS-b-P2VP micelles. Subsequently, glass slides were dipped in the micelle solution and pulled up. Thus, a self-assembled monolayer of micelles was coated on the glass surface. After treatment with oxygen plasma, the polymer segments of the micelles were thoroughly removed from glass, and the loaded gold acid was reduced to gold. Finally, a quasi-hexagonal array of Au nanodots was obtained. The preparation parameters are demonstrated in Table S2.

To fabricate nanoarrays on the hydrogels, a transfer lithography strategy was applied. Glass slides with nanopatterns were bathed in an ethanol solution of the linker N,N'-bis(acryloyl) cystamine (2 mM, Sigma) at 35 °C for 1 h and for further 12 h at room temperature to ensure complete grafting on Au nanodots through Au–S bonding. Then, we added a macromer aqueous solution and triggered photopolymerization. After peeling off the hydrogel, we obtained an array of gold nanodots on the biodegradable hydrogel.

Before the cell experiments, the cell-adhesive RGD sequence was introduced to improve cell adhesion on the hydrogel. A cyclic peptide c(-RGDFK)-thiol (f: d-phenylalanine, K: L-lysine; Synpeptide) was utilized to form an Au–S bond between the peptides and the gold nanodots. The gold-nanopatterned oligoLA-PEG-oligoLA-DA hydrogels were soaked in 25 μM peptide solution at 4 °C for 4 h. Excess peptides were removed by multiple rounds of PBS rinsing. Finally, the biodegradable hydrogel with an RGD nanoarray on the surface was obtained.

2.5. Observation of nanopatterns

A transmission electron microscope (TEM, Tecnai G2 20 TWIN, FEI) was employed to observe the dispersion and morphology of the micelles. The block copolymer solution was dropped on a copper grid and evaporated naturally. The accelerating voltage was set to 200 kV.

The gold nanoarray on glass was observed with a field-emission scanning electron microscope (SEM, Ultra 55, Zeiss, Germany). The accelerating voltage was set at 1.5 kV, working distance at 3.5 mm, magnification at 8 × 10⁴ and an aperture size of 30 μm. The secondary electron imaging mode was utilized.

2.6. Isolation and culturing of MSCs

MSCs were obtained from 7-day-old SD rats. The femurs and tibias were isolated from muscle tissue of the rat’s hind legs. A syringe filled with low-glucose Dulbecco’s modified Eagle medium (DMEM, Gibco) was inserted into the bone marrow cavity to thoroughly flush out the marrow. Through centrifugation, primary cells...
were acquired and seeded in cell culture flasks. The cell growth medium consisted of 90% low-glucose DMEM with additional 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco) and 2 mM l-glutamine (Gibco). Cells grew and proliferated at 37 °C in a humid condition with 5% CO₂, and were passaged when the confluence of cells reached up to 70–80%. The second-passage cells were used in subsequent cell experiments.

2.7. Seeding of stem cells on nanopatterned hydrogels

The nanopatterned hydrogels were fully swollen under wet environment and then punched into discs in a diameter of 20 mm to match the 12-well tissue culture plates (TCPs). Before being seeded with cells, all hydrogel specimens were disinfected with a 75% ethanol solution for 30 min. Hydrogels were then washed with PBS for 6 times, each time for 30 min to completely replace the ethanol.

MSCs were digested from cell culture flasks and seeded on hydrogels at a density of 3000 cells per square centimeter. All the following cell experiments employed the same cell density.

2.8. Measurement of cell viability on nanopatterned hydrogels

The hydrogels with nanopatterns were treated with 2 mol/L HCl and washed as mentioned in section 2.3. Viabilities of MSCs on the hydrogels obtained at different degradation stages were quantitatively determined by Cell Counting Kit-8 (CCK-8, DOJINDO) assays. The cell viability referred to the activities of succinate dehydrogenase (SDH) on mitochondrial inner membranes of all cells in each sample. Cell viability on glass was chosen as a positive control. Wells with only DMEM and CCK-8 solution were selected as blanks to avoid background interference.

After seeding the cells on surfaces for 1 day, the cell culture medium was replaced by a fresh medium with 10% CCK-8 solution for another incubation time of 2.5–3 h. Then the upper reacted medium was pipetted to 96-well TCPs. A microplate reader (Multiskan Mk 3) was used for characterizing solution absorbance of each well at 450 nm. The relative optical density (OD) was calculated to quantify the cell viability. The control group with the cells seeded on glass was as defined as of 100% viability. The results of the experimental groups were calculated from OD values deducting the background absorbance values.

2.9. Immunofluorescence staining, observation and analysis of stained cells on nanopatterned biodegradable hydrogels

Filamentous actin (F-actin), vinculin and nuclei of cells were stained to observe cell adhesion on the hydrogels. After seeding of MSCs on the hydrogels in the cell growth medium for 1 day, all samples in 12-well TCPs were gently rinsed with PBS for once. Then samples were fixed by 4% paraformaldehyde for 12 min and soaked in 0.1% Triton X-100 solution for 10 min to improve permeability of the cell membrane. Once a new reagent was used, samples needed to be rinsed with PBS in order to allow the next reagent to function well. To label vinculin, specimens were firstly blocked with 5% bovine serum albumin (BSA) solution for 20 min to diminish nonspecific protein staining. Then a 1:100 dilution of vinculin primary antibody (2 μg/mL, Santa Cruz) was used to incubate the sample at 4 °C overnight. After rinsing by PBS twice, the Alexa fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen) was added at a concentration of 10 μg/mL to conjugate with the primary antibody. The application conditions of the second antibody were at room temperature for 1 h. To visualize cytoskeleton and observe F-actin, samples were processed in 1 μg/mL phalloidin-tetramethyl rhodamine B isothiocyanate (phalloidin-TRITC, Sigma) for 30 min at room temperature. Subsequently, 2 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) was added to the samples to stain the nuclei in a reaction time of 5 min. At last, all samples were rinsed with PBS for 6 times to fully remove the fluorescent reagents.

The fluorescently stained cells were observed under a fluorescence microscope (Axiovert 200, Zeiss) and photographed by a charge couple device (CCD, AxioCam HRC, Zeiss). In order to ensure quantitative analysis, the voltage of the excitation light source and exposure times of photographing were kept constant for all groups.

To explore the cell adhesion on the surfaces at different biodegradation stages, single cells were chosen for comparison. For each fluorescence picture, cells were outlined using the software ImageJ to obtain cell spreading areas, circularity and aspect ratios. Photographs with stained nuclei were chosen to count the cells on the samples to obtain the cell density.

In order to semi-quantify the cytoskeleton tension, the integral intensity of F-actin per cell was obtained by calculation from the following equation:

\[ \text{Intensity of F-actin} = S_{cell} \times (I_f - I_0) \]

Here, \( I_f \) refers to the grey value of one cell in a pixel, and \( I_0 \) to the mean grey value of the whole cell; \( I_0 \) indicates the mean grey value of the background near the cell; \( S_{cell} \) is the pixel number in a cell outlined with ImageJ.

2.10. Osteogenic, adipogenic and co-induction of MSCs on nanopatterned biodegradable hydrogels

The second-passage MSCs were seeded on hydrogels that had undergone different degrees of acid-promoted degradation. On the first day, cells were cultivated in the growth medium (low-glucose DMEM with 10% FBS) to guarantee their adhesion to the hydrogel surfaces. The growth medium was replaced by the induction medium in the following induction period.

In terms of the osteogenic induction, cells were exposed to the osteogenic induction medium following a 1 d growth phase. The osteogenic medium contained high-glucose DMEM, 10% FBS, 50 μM ascorbic acid-2-phosphate (Sigma), 10 mM β-glycerophosphate (Sigma) and 100 nM dexamethasone (Sigma). The medium was changed at the second, fourth and sixth days after cell seeding.

For the adipogenic induction, cells were initially incubated in the adipogenic induction medium for 3 d, then in the maintenance medium for 2 d, and furthermore in the adipogenic induction medium for 2 d. The adipogenic induction medium consisted of high-glucose DMEM, 10% FBS, 1 μM dexamethasone, 200 μM indomethacin (Sigma), 10 μg/mL insulin (Sigma) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). The adipogenic maintenance medium was composed of high-glucose DMEM in addition to 10% FBS and 10 μg/mL insulin.

As for the osteogenic and adipogenic co-induction, we used a 1:1 mixture of the osteogenic and adipogenic medium. The change of the induction medium was consistent with the sole induction process.

The total induction period was 7 days. In order to avoid interference of cell proliferation in the cell differentiation experiments, 0.5 μg/mL aphidicolin (Sigma) in dimethylsulfoxide (DMSO, Sigma) was added to the medium at the fourth to fifth day of induction.

2.11. Determination of the extents of osteogenesis and adipogenesis

After 7 d of induction, the osteoblasts and adipocytes were
to the mean grey value of the cells; protons existed in oligoLA-PEG-oligoLA: protons from the methine chemical structure of the resultant polymer with LA units was sticky solid at room temperature and easily dissolved in DCM. The background near the outlined cells with the same area; (2.12. Statistical analysis

One-way ANOVA tests. A significant difference among different groups were analyzed by for each sample, approximately 200 cells were measured. Data here, the area of a single cell or cell groups outlined by the software ImageJ; \(I_0\) refers to the mean grey value of the background near the outlined cells with the same area; \(I\) refers to the mean grey value of the cells; \(N\) is the number of cells in the outlined region.

2.12. Statistical analysis

Four independent experiments in each group were performed and averaged in the induction experiments (\(n = 4\)). In cell adhesion analysis, six independent experiments were carried out (\(n = 6\)), and for each sample, approximately 200 cells were measured. Data from above experiments among different groups were analyzed by One-way ANOVA tests. A significant difference was denoted as **\(**(p < 0.05), ****(p < 0.01) and *****(p < 0.001, very significant difference).

3. Results

3.1. Synthesis of oligoLA-PEG-oligoLA and diacylated macromers

Block copolymer oligoLA-PEG-oligoLA was obtained via ring-opening polymerization of lactide triggered by the hydroxyl end groups of PEG and catalyzed by Sn(Oc)2. The product was a white sticky solid at room temperature and easily dissolved in DCM. The chemical structure of the resultant polymer with LA units was confirmed by \(^1H\) NMR, as demonstrated in Fig. S1. Three different protons existed in oligoLA-PEG-oligoLA: protons from the methine (–CH) and methyl (–CH\(_3\)) of the LA units had chemical shifts of 5.2 ppm and 1.4–1.6 ppm, respectively; the characteristic peaks in the ranges of 3.45–3.81 ppm and 4.25–4.35 ppm belong to the methylene (–CH\(_2\)-CH\(_2\)) protons of the PEG segment. The two shifts indicate that the two kinds of protons in the PEG segment are not chemically equivalent. The average number of LA units at each side of PEG chains is about 2.5, as shown in Table S1.

Diacrylated macromer (oligoLA-PEG-oligoLA)-DA was gained by treating the oligo-block copolymer oligoLA-PEG-oligoLA with acryloyl chloride, using TEA as an acid acceptor during the reaction process. The resulting product was also a white solid, but with powder-like appearance possibly caused by the alkene part of the acryloyl groups. The presence of alkenes was confirmed by \(^1H\) NMR, as shown in Fig. S1. The plane structure of the alkene’s double bond lay in different protons (peak e and two f peaks). The –CH = proton peak can be found at 6.1–6.2 ppm and the proton peaks from = CH\(_2\) are at 5.89–5.92 ppm and 6.45–6.5 ppm. The modified ratio of double bonds is approximately 70%.

3.2. Photopolymerization of oligoLA-PEG-oligoLA diacrylate macromers and degradability of the resultant hydrogels

The as-synthesized macromers were photopolymerized to form hydrogels. The resulting chemically crosslinked hydrogels were transparent and resilient. After swelling, the equilibrium linear swelling ratio of the hydrogel in 37 °C PBS was determined to be 1.13, owing to retained water after photopolymerization.

Hydrogels were first treated with 2 mol/L HCl solution to quickly observe degradation. After soaked in HCl, hydrogels were taken out at given time points and rinsed with PBS. We then detected moduli and swelling ratios of hydrogels in cell culture medium.

The approach to measure the compressive modulus of the hydrogel is demonstrated in Fig. 2A and Fig. S2. For the compressive modulus, an obvious decrease (approximately 35 times) appeared after 12 h treatment.

Untreated samples (0 h) and samples after 2 h and 6 h of acid treatments were selected as three groups (samples A, B and C) in the later cell experiments. In order to monitor degradation behaviors under in vitro biomimetic conditions, all of the samples were washed with PBS for five times, and then put into a cell culture medium (DMEM with 10% PBS, 37 °C) for 7 days. The three groups exhibited the same trend of declining moduli during biodegradation but different degradation rates, as illustrated in Fig. 2B. The moduli and degradation rates of different samples are summarized at the bottom of Fig. 2B. The stiff hydrogel (sample A) showed a small change of modulus which was defined as slow degradation, while the soft sample C showed a fast degradation; hydrogels from sample B held moderate properties.

Swelling ratios varied also at different acid-treatment stages. Nevertheless, the linear swelling ratios (SR) didn’t change so much as moduli. Among the three groups used in the later cell experiments, the softest sample C changed the most albeit still less than 1.6 (Fig. S3).

3.3. Preparation of nanopatterns on the biodegradable hydrogels

Biodegradable hydrogels with an RGD array on their surfaces were acquired via block copolymer micelle nanolithography to prepare a gold nanooarray on glass, and transfer lithography to transfer the gold nanooarray from the glass to the hydrogel, which was formed by photopolymerization of the as-synthesized biodegradable macromer. Amphipathic block copolymer chains were self-assembled into micelles in a selective solvent, as shown in a TEM observation (Fig. 3A). The polymeric micelles loaded HAuCl\(_4\) in their cores. After plasma treatment, the polymer was removed, the precursor of gold was reduced to gold nanodots, and the micelle array formed after dip-coating served as an ordered template of the resultant gold nanooarray. The array of gold nanodots on glass was observed by SEM, as shown in Fig. 3A. The spacing between nearest-neighbor nanodots was around 30 nm. The mean and
standard deviation of nanospacing are presented in Table S2.

A naturally formed dipline divided the glass surface into two parts: one side with nanopattern and the other without nanopattern. In addition to the successful transfer of the gold nanopattern to the oligoLA-PEG-oligoLA hydrogel, the dipline would also be transferred to the hydrogel. Cell seeding on the hydrogel with the properties mentioned above achieved an adhesion contrast on both sides of the dipline (Fig. 3B). The labeled vinculin (stained in green) of the cells was distributed at one side of the dipline. Even after 7 days of cell culturing, the dipline could be well observed, which illustrated both the persistently nonfouling property of the hydrogel background and the cell-adhesive potency of the RGD nanoarray.

3.4. Cell adhesion on nanopatterned hydrogels at different degradation stages

MSCs were cultured on the hydrogels of different degradation stages with varied stiffness and degradation rates (samples A, B and C). Hydrogels were seeded with cells of similar densities. Non-adherent cells were washed away before cell staining. Representative fluorescence micrographs of the stained cells and corresponding statistics after 1 day of cell adhesion are shown in Fig. 4. While cell density was lower in soft hydrogels, slightly complicated trends were observed for spreading area and shape (Fig. 4A). It is very interesting that although the softest group (sample C) led to relatively more confined cell spreading, the cytoskeleton was not weaker, as judged from the F-actin staining images shown in the upper row of Fig. 4B.

3.5. Cell viabilities on nanopatterned surfaces at different degradation stages

We also did some statistics on the cell cytoskeleton. The F-actin intensity of single cells was measured. After being seeded on hydrogels for 1 day, the cytoskeleton of the cells was fluorescently stained and photographed with consistent exposure time. For semi-quantitatively analyzing cell tension on different samples, the integral intensity of F-actin per cells was calculated. According to the graph demonstrated in Fig. 5A, cells on softer sample B showed a decrease of intensity compared to on sample A; but cells on the softest sample C presented a different trend. The intensity of cells on sample C surpassed that on sample B, which implied that cell tension increased on matrices of a higher degradation rate.

As for the overall activities of cells adhering on those hydrogels, a CCK-8 kit was utilized to quantify the whole cell viability. After seeding of the cells for 1 day, cells adhered and spread well on glass which constituted the maximum cell viability (Fig. 5B). Among the three samples, cells on degrading hydrogels in samples B and C showed increased viabilities; and the moderate and high degradation rates of the hydrogels activated cells much more than the low degradation rate in sample A.
3.6. Sole osteogenic or adipogenic induction of MSCs on samples at different degradation stages

To evaluate the effects of biodegradation on stem cell differentiation, MSCs were seeded on samples A, B and C, and underwent osteogenic or adipogenic induction. Osteogenically induced cells were stained by Fast Blue RR Salt. The osteogenesis extents among all samples were compared by ALP activities, which were quantitatively calculated by the relative grey values of stained cells. Meanwhile, red-stained oil droplets that appeared in the cells were considered as a marker of adipocytes. The results are presented in Fig. 6A and B.

Both osteogenesis and adipogenesis exhibited enhancement in the sequence of samples A, B and C in Fig. 6A and B. Such trends on degrading hydrogels are very interesting and abnormal, because the previous common knowledge is that a stiff hydrogel favors osteogenesis but a soft hydrogel favors adipogenesis on static matrices. It seems also worthy of noting that the same trends do not mean no selectivity. The difference between adipogenesis and osteogenesis was raised in our well designed co-induction experiments, with the results presented in the next subsection.

3.7. Complicated lineage commitments in co-induction of MSCs on samples at different degradation stages

We further examined stem cell differentiation in a mixed osteogenic/adipogenic medium. The co-induction results are very complicated owing to competition between the two lineage commitments. As shown in Fig. 6C, the optimal adipogenesis occurred for sample B with a moderate matrix stiffness and degradation rate while the osteogenic extent was at a minimum. The softest hydrogel showing the fastest degradation (sample C) led to more significant osteogenic differentiation than the hardest hydrogel that show the slowest degradation (sample A).

4. Discussion

It is very important to reveal various cues to regulate stem cells [7,9,11,13,65]. Certain cues might be predominant over others in some cases of lineage commitment. In the present study, we prepared RGD nanoarrays on PEG-based yet biodegradable hydrogels by combining the techniques of extension of PEG by oligoester and then acryloyl to synthesize a biodegradable macromer, block copolymer micelle nanolithography to obtain a gold nanoarray on glass, transfer lithography assisted by a bifunctional linker to transfer the gold nanoarray to the biodegradable hydrogel, and eventually, linking RGD peptides using a self-assembled monolayer. The preparation principles and the basic steps are schematically presented in Fig. 1. In this way, we generated a well-controlled nanopatterned surface with PEG as a nonfouling background and RGD peptides affording cell-adhesive sites. The excellent control of cell adhesion is demonstrated in Fig 3. Another key new issue of material chemistry comes from the introduction of oligoester into the macromer prior to the nanopatterning, which enables the
The biodegradation made the hydrogels softer, as demonstrated in Fig. 2. We also found that the three samples which were prepared at different accelerated hydrolysis stages led to different biodegradation rates in the cell culture medium. So, we eventually obtained three well-controlled samples with both varied stiffness.

Fig. 4. Cell adhesion on hydrogels with different degradation rates. (A) Statistic results of cell adhesion parameters after 1-day culture on indicated samples. (B) Corresponding fluorescence micrographs of stained MSCs with F-actin in red, vinculin in green, and nuclei in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. Statistics concerning the cytoskeleton of single cells and cell viabilities of the whole cells on indicated hydrogels 1 day after seeding of the MSCs. (A) Statistic results of integral intensity of F-actin per cell adhering on hydrogels. Intensity of F-actin was normalized to the mean value of sample B. (B) CCK-8 results of cell viabilities on different hydrogel samples, using culture on glass as a control. Cell viability was obtained from the measurements of activities of the enzyme SDH in all cells on each sample and normalized to the mean of the control group.
and degradation rates, as highlighted in the bottom of Fig. 2.

As the stiffness effects on cell adhesion and differentiation are concerned, it seems necessary to indicate that real differences in stem cell differentiation are typically observed at moduli in the low kPa (1–2 kPa) to 20–30 kPa range as reported in the literature [28,46]. But they are for cells encapsulated in some 3D hydrogels. Different material systems have different appropriate modulus ranges with significant stiffness effects, and we found that the stiffness range for cells on 2D surfaces decorated with a nanogold array might shift to higher moduli. According to our previous publications about the significant stiffness effects on adhesion of MSCs and the following adipogenic/osteogenic induction in nano-patterned 2D systems [24,25], the relatively soft and hard static PEG hydrogels were of moduli about 130 kPa and 3200 kPa, respectively. The present studies of dynamic hydrogels of PEG derivatives during biodegradation in cell culture medium exhibited moduli

Fig. 6. Bright-field images and statistics of MSC differentiation on samples at different degradation stages. (A) Osteogenesis of MSCs after osteogenic induction for 7 d. Cells experienced ALP staining and nucleus staining prior to the optical microscopic observations. (B) Adipogenesis of MSCs after adipogenic induction for 7 d. Cells were submitted to oil-droplet staining and nucleus staining prior to observations. (C) Co-induction of MSCs in a mixed induction medium for 7 days. Cells experienced ALP staining, oil-droplet staining and nucleus staining prior to the microscopic observations.
among 70–1800 kPa, which is sufficient to reflect the underlying stiffness effect if any.

It is very interesting that cell behaviors on our degrading hydrogel cannot be fully understood by those on static hydrogels with also varied stiffness. Our adipogenic induction experiments agree very well with the normal trend that soft matrices benefit adipogenesis, as shown in Fig. 6A. However, the osteogenic induction results are contradictory with the normal assumption, which implies the impact of some unknown material cues. Meanwhile, we found that cell adhesion behaviors on our degradable hydrogels did not completely follow those on static hydrogels with different matrix stiffness either. MSCs exhibited smaller adhesion density and spreading area (Fig. 4A), which are consistent with the previous publications on nonbiodegradable PEG hydrogels of two stiffness values [24]. However, the cytoskeleton on the softest sample (sample C) seemed more stringent (Fig. 4B).

Besides matrix stiffness, another parameter that changed during hydrogel degradation in our experiments is the enlarged RGD nanospacing along with hydrogel swelling. RGD is a ligand in ECM to promote specific cell adhesion after conjugating with its receptor, integrin, on the cell membrane [66,67]. The critical nanospacing is about 70 nm: cells can only adhere well on surfaces with an RGD nanospacing < 70 nm [68–72]. We have reported that the enlarged RGD nanospacing can enhance both osteogenesis and adipogenesis when the spacing changes from <70 nm to >70 nm [65,73]. The initial RGD nanospacing on glass in the present experiment was 30.9 nm as listed in Table S2. Considering the linear swelling ratio of the initial hydrogel obtained from photopolymerization, the RGD nanospacing on our biodegradable hydrogel was about 34.9 nm. Even with the highest linear swelling ratio of 1.6 for sample C after degradation for 7 days as shown in Fig. 53, the resulting RGD nanospacing might still be less than 70 nm. A more important evidence to rule out the interference of the changed RGD nanospacings comes from the facts that larger RGD nanospacings always lead to significantly decreased cell viability and weaker cytoskeletons [24,65] and in contrast higher cell viability and stronger cytoskeleton could be observed with degradation of our hydrogels. So, our present experiments implied a new cue beyond both decreased matrix stiffness and different RGD nanospacing.

Material degradation itself could contribute to the cell results. The degradation byproducts such as lactic acid afforded new components in culture medium. Our previous research on the byproducts of hydrolysis of polyesters illustrates that only a large amount of lactic acid could affect cells and it is the decreased pH instead of the lactate ion that influences the differentiation of MSCs [74]. In the present hydrogel system, the oligoLA modification to PEG chains resulted in only a little amount of degradation byproducts of lactic acid, and the buffered cell culture medium would stable pH around neutral according to our experimental record. Hence, the byproducts cannot be the main factor of the hydrogel degradation to influence the cell behaviors in this study.

So, the rate of biodegradation, another factor closely related to the dynamic process of degradation should be taken into consideration. The highest degradation rate in sample C exerted a dynamic stimulus to cells. Such a stimulus was so strong that shaded the otherwise weakened adhesion of cells on soft matrices, and sometimes a more stringent cytoskeleton was raised and even the cell viability was increased, as reflected in Figs. 4 and 5.

Fig. 7. Schematic presentation of the fate decision for stem cells on hydrogels during biodegradation. (A) In the cases of sole osteogenic or adipogenic induction of MSCs on the material surfaces; (B) In the case of co-induction in a bipotential mixed medium.
extent of osteogenesis is related to cell tension while that of adipogenesis is not [17]. So, the sensitivities of these two lineages to the cytoskeleton and cell viability are quite different.

We thus interpret our sole induction results as follows: in the adipogenic induction on samples A, B and C, the matrix stiffness is predominant over the degradation rate; and in the osteogenic induction, the degradation rate is predominant over the matrix stiffness. These two trends are schematically presented in Fig. 7A.

More complicated results were found in co-induction, as seen in Fig. 6C. Here competition should be taken into consideration. The competition between osteogenesis and adipogenesis in the mixed induction medium has already been studied [13,22]. The two kinds of sole induction led to the same increased differentiation trends (Fig. 6A and B), and thus it is not unreasonable that a peak might, under co-induction, appear at sample B in Fig. 6C. Since osteogenesis is more sensitive to the dynamic stimuli caused by material degradation, sample C with the highest degradation rate exhibited a higher extent of osteogenesis than sample A with the slowest biodegradation. The combined effects in the co-induction experiments are also schematically shown in Fig. 7.

The present report is in agreement with the previous pertinent studies. Burdick’s group found that cells extended more, showed a stronger cell tension, generated more traction force to the HA matrix around cells, and exhibited osteogenesis lineage tendency in cell-mediated degradation of enzyme-degradable and HA-based hydrogel [43]; they pointed out that the key factor to influence differentiation was the degradation rather than the initial cell spreading. Bian et al. confirmed and extended the viewpoint, and reported a more significant differentiation under the chondrogenic induction medium in enzyme-degradable HA hydrogels than in non-degradable hydrogels [44]. Anseth’s group found that osteogenesis and chondrogenesis were more sensitive to the degradation extent than the adipogenesis of MSCs in MMP-degradable and PEG-based hydrogel [52]. So, our experiments of cells on 2D patterned surfaces are consistent with those 3D studies of MSCs encapsulated in hydrogels.

The degradation rate affords a new cue to regulate cell adhesion and differentiation. Such a dynamic cue should be considered during both 2D culturing and 3D encapsulation, although dimensionality might influence signaling of MSCs in hydrogel environments [76]. The main difference of a cell in 3D or 2D systems comes from the presence or absence of steric hindrance. Our in vitro experiments on 2D surfaces are helpful for understanding various experiments in cell culture when the matrix has a dynamic characteristic. We can also reasonably anticipate that degradation rates can influence cell fate significantly during tissue engineering and tissue regeneration after implanting biodegradable materials. Even in the case of nonbiodegradable implants, ECM remodeling might also alter matrix stiffness [77], and thus the change rate of the matrix stiffness should be considered as a dynamic cue. Recently, matrix remodeling during degradation was found to support the cell-cell contact and spreading, thus promoting stemness of neural progenitor cells (NPCs) [78]. Different from NPCs, more contractile lineages of MSCs showed the dependence upon cell-adhesive ligand clustering and the traction force generated by cells in the degradation process [79]. The present study illustrated that under fast degradation cells might sense the impact of changes of the hydrogel and turn out to be activated along with the reconstruction of the cytoskeleton and a higher traction force.

5. Conclusions

In order to examine stem cells on well-controlled biomaterials during biodegradation, we built nanoarrays of cell-adhesive RGD peptides on hydrolysable hydrogels synthesized from biodegradable diacrylated oligoLA-PEG-oligoLA macromers. The accelerated hydrolysis in an acidic medium led to a series of samples with varied matrix stiffness and degradation rates during the subsequent treatment in the cell culture medium for several days. The cell density and spreading area declined on samples at the late stage of acidic hydrolysis, but cytoskeleton and cell viability were found to be enhanced on softer samples with a higher degradation rate. While more adipogenesis happened on softer samples, osteogenesis was also more significant, which cannot be attributed to the well-known stiffness effect. We thus put forward that it was the degradation rate that stimulated cells and then enhanced osteogenesis of MSCs. The cell adhesion and differentiation on the biodegradable materials were found to be determined by the combinatory effects of degradation rate, matrix stiffness, competition of commitment between different lineages, etc. The present study reveals that degradation rates cannot be interpreted simply by the changed static cues. Such a dynamic cue should be paid much attention to in the understanding of cell-matrix interactions and development of next-generation biomaterials.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biomaterials.2018.04.021.

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