Video Article

Dendrimer-based Uneven Nanopatterns to Locally Control Surface Adhesiveness: A Method to Direct Chondrogenic Differentiation

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Abstract

Cellular adhesion and differentiation is conditioned by the nanoscale disposition of the extracellular matrix (ECM) components, with local concentrations having a major effect. Here we present a method to obtain large-scale uneven nanopatterns of arginine-glycine-aspartic acid (RGD)-functionalized dendrimers that permit the nanoscale control of local RGD surface density. Nanopatterns are formed by surface adsorption of dendrimers from solutions at different initial concentrations and are characterized by water contact angle (CA), X-ray photoelectron spectroscopy (XPS), and scanning probe microscopy techniques such as scanning tunneling microscopy (STM) and atomic force microscopy (AFM). The local surface density of RGD is measured using AFM images by means of probability contour maps of minimum interparticle distances and then correlated with cell adhesion response and differentiation. The nanopatterning method presented here is a simple procedure that can be scaled up in a straightforward manner to large surface areas. It is thus fully compatible with cell culture protocols and can be applied to other ligands that exert concentration-dependent effects on cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/56347/

Introduction

Here we describe a simple and versatile dendrimer-based nanopatterning procedure to obtain cell culture surfaces that allow the control of local adhesiveness at the nanoscale. Nanoscale details of ECM organization have been reported,[1,2,3] and the nanopatterning of cell adhesion surfaces has provided deep insights into the cellular requirements related to adhesion[4,5]. Experiments using micellar lithography-based nanopatterns revealed a threshold value of around 70 nm for RGD peptide nanospacing, cell adhesion being significantly delayed above this value[6,7,8,9]. These studies also highlighted the greater influence of local than global ligand density on cell adhesion[9,10,11].

During morphogenesis, cell interactions with the surrounding environment trigger the first differentiation events, which continue until final complex tissue structures have been formed. Within this framework, nanopatterned surfaces have been used to tackle the influence of the initial cell-surface interactions on morphogenesis. Lithography-based RGD nanopatterns with a lateral spacing of 68 nm in β-type Ti-40Nb alloys help to maintain the undifferentiated phenotype of non-committed stem cells[12], while RGD nanospacings of between 95 and 150 nm enhance the differentiation of mesenchymal stem cells (MSCs) towards adipogenic/osteogenic[13,14,15] and chondrogenic fates[16]. Also, self-assembling macromolecules modified with signaling components have been shown to direct cell adhesion and differentiation by providing nanoscale architectural regulation of the signaling cues[17]. In this regard, the deposition of dendrimers with cell-interacting moieties in their outer sphere[18,19,20] onto surfaces has been used to study cell adhesion[21,22], morphology[23,24], and migration events[25,26]. Nevertheless, the lack of surface characterization in these studies makes it difficult to establish any correlation between dendrimer surface configuration and cell response.

Dendrimer nanopatterns with liquid-like order and defined spacing can be obtained when dendrimers adsorb to low-charged surfaces from solutions with low ionic strengths.[27] On the basis of this property, here we present a method to obtain large-scale uneven nanopatterns of RGD-functionalized dendrimers on low-charged surfaces that permit the nanoscale control of local RGD surface density. Water contact angle (CA), X-ray photoelectron spectroscopy (XPS) and scanning probe microscopy techniques (STM and AFM nanopatterns) show that local ligand densities can be adjusted modifying the initial dendrimer concentration in solution. The local RGD surface density is quantified from AFM images by probability contour maps of minimum interparticle distances and then correlated with cell experiments. Compared with other nanopatterning techniques[4], dendrimer-based nanopatterning is straightforward and can be easily scaled up to large surface areas, thus being fully compatible with cell culture applications. Nanopatterns are used as bioactive substrates to evaluate the effect of the local RGD surface density on cell adhesion[28] and on the chondrogenic induction of adult human MSCs[29]. Our results show that RGD dendrimer-based nanopatterns sustain cell growth and that cell adhesion is reinforced by high local RGD surface densities. In the differentiation experiments, intermediate adhesiveness of cells to the substrates favored MSC condensation and early chondrogenic differentiation. Due to the ease with which dendrimer peripheral groups can be modified, the method described here can be further extended to other ECM ligands that exert concentration-dependent effects on cells.

Protocol

1. Substrate Preparation

1. **Annealing 1.4 x 1.1 cm Au(111) on Mica Substrates.**
    1. Place the Au(111) substrate on a glass-ceramic hob and anneal it with a butane flame for 3 min. Allow the substrate to cool under an argon atmosphere. Repeat this step for each Au(111) substrate.
    NOTE: Au(111) substrates should be used immediately after annealing.

2. **Preparation of Poly(L-Lactic Acid) (PLLA)-Coated Glass Substrates.**
    1. Cut and wash the glass slides.
        1. Cut microscopy slides into 18 slides of 1.25 cm x 1.25 cm with a diamond-tip cutter. Make a small indentation on the lower side of each slide, so that the upper and lower sides can be later distinguished.
        2. Wash the slides thoroughly with deionized water followed by 96% ethanol. Allow them to air-dry.

    2. Prepare the 2% PLLA solution.
        1. Add 200 mg of PLLA to 10 mL of 1,4-dioxane in a pressure tube. Add a stir bar and close the tube tightly.
        2. Place the pressure tube into a glycerin bath on a hot plate at 60 °C under gentle stirring for 24 h, and then transfer the solution to a 15-mL glass vial.

    3. Coating the glass slides with the PLLA solution.
        1. Place the glass slides and the vial with the PLLA solution on a clean hot plate at 60 °C. Make sure to place the slides facing upward and allow them to remain for at least 10 min to reach the necessary temperature.
        2. Prepare the spin coater and set the program specified in **Table 1**.
        3. Place one of the slides face up on the spin coater, using a vacuum system. With a Pasteur pipette, apply 0.25 mL of the PLLA solution to the slide, making sure that the whole surface is covered. Run the coating program. Repeat this step for each slide.

2. Dendrimer Nanopatterning

1. **Preparation of RGD-Functionalized Dendrimer (RGD-Cys-D1) [28] Solutions.**
    1. Dissolve 5 mg of the dendrimer in 6.494 mL of deionized water. This is solution A.
    NOTE: Use dendrimer stock solution within 6 months of preparation.
    2. Sonicate solution A for 10 min and prepare solutions B and C following **Table 2**.
    3. Sonicate solution C for 10 min and prepare solutions D, E and F following **Table 2**.
    4. Store solutions B, C, D, E and F at 4 °C until use. Solution A can be stored at -20 °C for later use.

2. **Nanopatterning of RGD-Cys-D1 Dendrimers on the Substrates**
    1. In a tissue culture hood, sterilize the substrates by irradiating them with UV light for 13 min.
    NOTE: This step is only necessary when nanopatterned substrates are going to be used as cell culture substrates. In this case, maintain the sterile conditions (tissue culture hood, sterile materials, solutions and techniques) for the following steps.
    2. Place each substrate face up in the wells of a plate, handling them carefully with tweezers.
    3. Sonicate solutions B, C, D, E and F for 10 min.
    4. Pass the RGD-Cys-D1 dendrimer solutions through a 0.22-µm diameter filter using a syringe directly in the wells containing the substrates (2 mL/well). At least three replicas per dendrimer concentration are recommended. Close and seal the plate and leave it at room temperature (RT) for 16 h.
    5. Remove and discard the solutions. Wash the substrates with sterile deionized water and dry. Store substrates at 4 °C.
    NOTE: The protocol can be paused here.

3. Preparation of Control Substrates

Note: All steps were performed in a sterile tissue culture hood, and only sterile materials, solutions and techniques were used. At least, six control substrates were used (three replicas of the positive control and three replicas of the negative control).

1. In a tissue culture hood, sterilize the substrates by irradiating them with UV light for 13 min.
2. **Control Substrates for Fibroblast Adhesion Experiment.**
    1. Use flame-annealed Au(111) substrates as the negative control. Gold has a well-known protein-denaturation effect that confers anti-cell adhesive properties[28]. Sonicate all solutions and filter prior to substrate incubation.
    2. For the positive controls, immerse flame-annealed Au(111) substrates in a solution of RGD-modified PEG thiol, Ac-Gly-Arg-Gly-Asp-Ser-Asp-NH-ethylene glycol mono-11-mercaptoundecanamide (RGD-PEG-SH)[30] and triethylene glycol mono-11-mercaptoundecyl ether (PEG-SH) at a 1:100 molar ratio in 96% ethanol for 16 h at RT.
    3. Wash substrates thoroughly in ethanol and dry them with argon. Store substrates at 4 °C.

3. **Control Substrates for Chondrogenesis.**
    1. Use pristine PLLA substrates as the negative control. Without any surface treatment, PLLA shows poor interfacing with living cells.[31]
    2. For the positive controls, prepare 5 mL of 0.1 mg/mL fibronectin in phosphate-buffered saline (PBS) and incubate each PLLA substrate with 1.6 mL of the fibronectin solution for 1 h at RT.
    3. Remove and discard the fibronectin solution, and wash the substrates with PBS. Store substrates at 4 °C.

4. Surface Characterization

1. **AFM Imaging**
    1. Perform AFM imaging of PLLA substrates for a roughness analysis. Since dendrimers have a diameter of 4-5 nm, roughness values of 1 nm should be obtained for the proper visualization of the nanopatterns. Also, perform AFM imaging of the nanopatterns. In both cases, perform AFM in tapping mode in air.
    2. Select a silicon cantilever with a spring constant k = 40 N/m and a resonant frequency v = 300 kHz and mount it onto the AFM equipment.
    3. Mount the sample on the stage of the atomic force microscope. Depending on the set-up of the apparatus, tuning and imaging specifications may vary. Consult the manufacturer's handbook.
    4. Approach the sample with the tip of the microscope until contact.
    5. To image PLLA for roughness analysis, select at least four representative areas of 20x20 µm per substrate from three independent substrates. To image dendrimer nanopatterns, choose at least three representative images of 5×5 µm per substrate from three independent substrates per condition (initial dendrimer concentration in solution). To avoid damaging the dendrimer layer while imaging, adjust the set point to keep the force at a minimum.
    NOTE: The choice of the imaging area also depends on the working range of the piezoelectric scanner. Please consult the instrument manual in this regard.
    6. Process the AFM height images by fitting each scan line to polynomial leveling functions using the proprietary software of the manufacturer of the AFM apparatus, and analyze those from PLLA to calculate surface roughness. Root-mean square (RMS) analysis may provide a suitable option.

2. **Local RGD Surface Density Measurement.**
    1. Obtain the image thresholds of the processed AFM height images to select the dendrimers on the surface.
    2. Determine the particle positions using an image processing software and use them to obtain the minimum interparticle distances (d_{min}).
    3. Plot d_{min} values in z to the corresponding particle positions to obtain the probability contour maps for d_{min}. Adjust the color scale of the plot to visualize the regions of highest local RGD surface density (d_{min} < 70 nm).
    4. Quantify the area of the regions with the highest local RGD surface density using an image processing software. Include the areas of dendrimer aggregates in the calculation.

3. **STM Imaging.**
Note: STM imaging can be performed only for nanopatterns on conductive Au(111) substrates. Measurements are done in air.
    1. Place the nanopatterned substrate in the sample holder of the STM equipment. Check the electrical connection between sample and holder with a tester.
    2. Etch a tip of the selected probe material (*i.e.* Pt 0.8: Ir 0.2) with a diameter that ensures proper fitting on the head of the STM equipment. Etching can be performed either by manual cutting or by electrochemical etching.[32]
    NOTE: Electrochemical etching renders more symmetric tips.
    3. Mount the tip in the head of the STM equipment and connect the sample.
    4. Set "current" in the feedback channel (in this type of measurement, current will be constant by feedback tuning). Adjust the bias voltage and current set point and scan size to obtain a well resolved image once the tip is engaged.
    NOTE: The choice of the imaging area also depends on the working range of the piezoelectric scanner. Please consult the instrument manual in this regard.
    5. Process the STM topographical images by fitting each scan line to polynomial leveling functions using the proprietary software of the manufacturer of the STM apparatus.

4. **CA Measurements.**
    1. Measure CA on the nanopatterned substrates by the sessile-drop method at three different positions on three independent substrates per condition (initial dendrimer concentration in solution) with an optical CA system.
    2. Fill the microsyringe with deionized water and set the parameters in the CA software to produce drops of 1 µL.

3. Place the sample on the stage so that the surface of the sample is clearly visible on the computer for imaging.
4. Move the syringe with the micromanipulator until it gets close to the surface and dispense the drop onto the surface. Record the image immediately after droplet stabilization.
   NOTE: In CA measurements, it is very important to control humidity in order to achieve reproducible results.
5. Analyze the CAs measured with the proprietary software of the manufacturer of the CA apparatus. The fitting method can be adjusted to user requirements. The elliptic fitting method can be an option.

5. Cell Culture

NOTE: All steps were performed in a tissue culture hood, and only sterile materials, solutions and techniques were used.

1. **Fibroblast Adhesion Experiment.**
   1. Culture NIH 3T3 mouse embryonic fibroblasts from early passages (< 10) at 37 °C and 4.6% CO_2 atmosphere in basal medium with high glucose supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin and 1% sodium pyruvate (growth medium). T75 flasks for a total seeding of 500,000 cells per flask in 10 mL of growth medium are recommended.
   2. Remove old medium with a 10 mL pipette every 2 days and replace with 10 mL of freshly prepared growth medium.
   3. Culture cells in the growth medium until they reach around 80% confluence, then remove the medium and add 5 mL of trypsin per flask. To ensure proper cell detachment from the bottom of the flask, maintain the trypsin solution in contact with the cells for 5 min at 37 °C.
   4. Add 5 mL of growth medium per flask and collect the cells in a centrifuge tube. Centrifuge the cells at 470 x *g* for 5 min. Remove the supernatant and resuspend the cell pellet in 10 mL of growth medium. Determine the concentration of cells using a hemocytometer.
   5. Transfer the substrates to well plates not treated for tissue culture (non-adherent).
   6. Seed the cells on the substrates at a density of 4,000 cells/cm^2 in growth medium and incubate them for 4.5 h at 37 °C and 10% CO_2 atmosphere.

2. **Chondrogenic Induction of MSCs.**
   1. Culture human MSCs from early passages (<5) at 37 °C and 4.6% CO_2 atmosphere in MSC growth medium. T75 flasks for a total seeding of 500,000 cells per flask in 10 mL of growth medium are recommended.
   2. Change medium every 3 days (as described in 5.1.2).
   3. Trypsinize cells before 80% confluence is reached, and centrifuge and resuspend them in MSC growth medium (as described in 5.1.3). Determine the concentration of cells using a hemocytometer.
   4. Centrifuge the cells again and resuspend them in 10 mL of chondrogenesis-inducing medium.
   5. Transfer the substrates from the plates to new well plates not treated for tissue culture (non-adherent). Seed the cells on the substrates at a density of 3,000 cells/cm^2.
   6. Change the chondrogenic medium every 3 days (as described in 5.1.2).

6. Cell Fixation and Immunostaining

NOTE: The following steps can be performed in non-sterile conditions.

1. Remove the media and wash the cells gently with PBS. Fix them by adding a 10% formalin solution for 20 min at RT.
2. Remove the formalin and wash the cells with PBS.
3. Block the free aldehyde groups by adding a 50 mM solution of ammonium chloride (NH_4Cl) in PBS. Leave the cells for 20 min at RT. Remove NH4Cl solution and wash the cells with PBS.
   NOTE: The protocol can be paused here. Store samples in PBS at 4 °C.
4. Remove PBS and permeabilize the cells by adding a 0.1% solution of saponin in the blocking solution (1% albumin in PBS) for 10 min at RT. Wash the cells with PBS and transfer samples to a new well plate.
5. Incubate the cells with a solution of primary antibodies in the blocking solution (**Table 3**) for 1 h at RT. Then, remove the primary antibody solution and wash the cells with PBS.
6. Incubate the cells with secondary antibodies in the blocking solution (**Table 3**) for 1 h at RT.
   NOTE: Avoid light exposure.
7. Remove the secondary antibody solution and wash the cells with PBS and dry.
   NOTE: The protocol can be paused here. Store the samples in PBS at 4 °C in the dark.
8. Sample mounting for microscope observation: Using a diamond-tip cutter, cut coverslips into 1.25 cm x 1.25 cm. Apply 50 µL of microscopy mounting medium onto the samples and cover them gently with the cut coverslips.
9. Transfer the samples to a convenient recipient, cover it with aluminum foil and store in the dark at 4 °C until observation.

7. Cell Imaging and Data Analysis

NOTE: For opaque Au(111) and thick microslide-based substrates, an upright microscope must be used.

1. **Fibroblast Adhesion Experiment.**
   1. Use an epifluorescence microscope equipped with a digital camera and low (i.e. 10X) and high (i.e. 40X) magnification objectives. Perform measurements in air.
   2. Place the sample on the stage and image cell nuclei with the 10X objective using an ultraviolet excitation, longpass emission filter to visualize the Hoechst/DAPI stain.

3. Image cell cytoskeleton and focal adhesions (FAs) with the 40X objective, selecting the microscope filter that matches the corresponding antibody fluorochrome specifications.
4. For FA quantification, use an image processing software to convert images to 8-bit files. Remove the background and convert images to binary by setting a threshold. Compute at least 30 images per sample and consider FAs from 1 μm^2 for calculation.

2. **Chondrogenic Induction of MSCs.**
   1. Image cell condensates at the initial stages of chondrogenic induction (< 5 days) with an upright epifluorescence microscope equipped with a digital camera and a low (*i.e.* 10X) magnification objective. Use ultraviolet excitation and longpass emission filter to visualize the Hoechst/DAPI stain.
   2. For measuring the condensate area, use an image processing software, convert images to 8-bit files, remove the background and select a threshold that highlights the aggregate contour. Calculate the area of the particles.
   3. Image immunostained samples for FAs, cytoskeleton actin fibers, and cartilage-specific collagen type II alpha 1 (COL2A1) with an upright confocal microscope at high magnification (i.e. 40-60X). Collect sections at a representative interval (*i.e.* 0.5–1 µm).
   4. To process the stack, use an image processing software. Convert images to 8-bit files, remove the background, and make them binary by setting a threshold. Treat a minimum of three cell condensates per condition of three independent samples.
   5. Quantify the FA protein stained areas from the basal zone of cell condensates and express them as the corresponding percentage of area divided by the number of cell nuclei in the image.
   6. To measure COL2A1 staining, use confocal z-projections and quantify the sum of the projected area (maximum COL2A1 area per sample). Normalize the area value obtained against the area of the corresponding condensate.

8. Quantitative Reverse Transcription-polymerase Chain Reaction (qRT-PCR) Analysis

NOTE: To prevent RNase contamination, use disposable, sterile plastic ware and wear disposable gloves while handling reagents and RNA. Always use proper microbiological aseptic techniques and use an appropriate decontamination solution to remove RNase contamination from work surfaces and non-disposable items such as centrifuges and pipettes.

1. Isolate total RNA and pulverize it in a RNA disrupter. Treat RNA samples with DNase and convert them into cDNA using a cDNA synthesis kit.
2. Conduct qRT-PCR using primers for the transcription factor *SOX9*. Beta-2-microglobulin (B2M) and ribosomal protein L13a (RPL13a) can be used as housekeeping genes.
3. Calculate the expression levels and normalize them against the negative control (PLLA).

Representative Results

We present a nanopatterning method that allows surface adhesiveness to be addressed at the nanoscale (**Figure 1**). The chemical structure of RGD-Cys-D1 is shown in **Figure 1A**. Dendrimers were patterned on electrical conductive Au(111) surfaces for high resolution STM characterization. Low dendrimer concentrations in solution (up to 10^{-5}% *w/w*) rendered isolated dendrimers of 4-5 nm in diameter (**Figure 1B**), while highly packed dendrimer aggregates formed at higher concentrations (**Figure 1C**). AFM surface characterization (**Figures 1D-G**, upper row) revealed that the surface distribution of RGD-Cys-D1 dendrimers can be adjusted as a function of the initial dendrimer concentration in solution. On the probability contour maps for d_{min}, this results in different local RGD nanoscale densities on the surface (**Figures 1D-G**, lower row).

In cell culture experiments, the cell-membrane receptor integrins of around 10 nm in diameter recognized the RGD sequence on the dendrimers periphery. Although eight copies of this sequence were provided per dendrimer, only one dendrimer interacted per integrin due to its size (4-5 nm measured by STM; **Figure 1B**). That is, each dendrimer provided a single site for integrin binding, thereby allowing a direct correlation from dendrimer distribution (as seen in the AFM images) and the RGD distribution available for cell adhesion. Moreover, no significant variations were found in the CA values obtained for the different nanopattern configurations that may influence cell adhesion[29]. These characteristics make dendrimer nanopatterns on biocompatible surfaces suitable substrates through which modulate and study cell behavior.

Cell adhesion to nanopatterned Au(111) was tested with fibroblasts within the first 24 h of culture (**Figure 2A**) and with MSCs on nanopatterned PLLA (**Figure 2B**). In both cases, the percentage of area stained for the FA protein paxillin (pax) increased progressively with dendrimer concentration, as did local RGD surface density (the percentage of nanopatterned area with d_{min} below the 70-nm threshold for efficient cell adhesion; **Table 4**). For an initial dendrimer concentration of 10^{-2}% *w/w* and positive controls, the percentage of area stained for pax per cell decreased and the correlation with the percentage of nanopatterned area with d_{min} < 70 nm was lost.

The percentage of surface area occupied by d_{min} <70 nm (**Figure 1D-G** lower row and **Table 4**) is a good indication of local RGD surface density for nanopatterns up to 10^{-5}% *w/w* initial dendrimer concentration but not of those up to 10^{-2}% *w/w*, due to dendrimer aggregation. Aggregation produced highly heterogeneous samples in terms of ligand distribution, with only a small percentage of the surface with d_{min} values below the 70-nm threshold (**Figure 1D** lower row and **Table 4**). XPS results showed that these surfaces present a global RGD density comparable to 10^{-5}% *w/w*-derived nanopatterns[28]. This observation indicates that maximum RGD density was achieved in the regions containing dendrimer aggregates and that the percentage of surface area occupied by d_{min} <70 nm is not representative of the local RGD surface density in this case.

The observation that positive controls (homogeneous coatings) with maximum local RGD surface density did not show the expected increase in cell adhesion can be attributed to a steric hindrance effect. These results indicate that, compared to the corresponding homogeneous surfaces commonly used in cell culture, RGD nanopatterns sustain cell adhesion more efficiently. This finding thus highlights the relevance of local ligand density.

Interactions with the surrounding environment in morphogenesis trigger the first cell differentiation events and propagate them until the establishment of the final complex tissue structures. To evaluate the effects of dendrimer nanopatterns on cell adhesion and differentiation, we

used the chondrogenic induction of MSCs as a model. During chondrogenesis, there is active matrix remodeling, which plays an instructive role in directing cells through the different stages of cartilage formation.

MSCs underwent chondrogenesis on the nanopatterns (**Figure 3**). Chondrogenic differentiation begins with a cell condensation step, which involves cell recruitment to form dense condensates, the establishment of cell-to-cell communication, and concomitant changes in cell morphology[33]. **Figure 3A** shows that cell condensation occurred in all the substrates and that condensates increased in area with increasing dendrimer concentrations up to 2.5 x 10^{-8}% *w/w* The condensate area then decreased again for a dendrimer concentration of 10^{-2}% *w/w* and for the positive control. The cell condensation step in chondrogenesis occurs through active cell movement rather than through an increase in cell proliferation[34]. This cell movement is favored by flexible adhesion with the substrate: stable adhesions should be formed to permit traction forces to move the cell body, and at the same time, these adhesions should be weak enough to allow cell release from the substrate during movement. Cell condensation is favored by an increase in local RGD surface density up to 2.5x10^{-8}% *w/w*-derived nanopatterns, with a good balance between cell adhesion and cell movement. For 10^{-2} and the positive control, the local RDG surface density was too high, thereby impairing the condensation event.

Chondrogenic differentiation proceeds from prechondrogenic condensates: cells in condensates become more rounded and start the synthesis of cartilage specific markers such as the ECM protein COL2A1 and the transcription factor SOX9[33,34,35]. Accordingly, substrates with an intermediate adhesiveness, favoring the formation of cell condensates, showed higher COL2A1 staining (**Figure 3B**) and higher levels of *SOX9* mRNA expression (**Figure 3C**).

Our results highlight the influence of cell-cell matrix interactions during the early stages of chondrogenic differentiation. Such interactions can be easily addressed through dendrimer-based nanopatterns.

Step	Time (s)	Speed (rpm)	Acceleration (rpm/s)
1	5	500	300
2	30	3,000	1,500

Table 1: Spinner program steps used to coat the glass slides with the prepared PLLA solution. A first homogenization step was used at 500 rpm with an acceleration of 300 rpm/s for 5 s. followed by a last step at 3,000 rpm with an acceleration of 1,500 rpm/s for 30 s.

Solution	RGD-Cys-D1 (mg/mL)	RGD-Cys-D1 (mg)	MQ water (µL)
A	0.77	5	6,494
	RGD-Cys-D1 (% w/w)	**Solution A (µL)**	
B	10^{-2}	779	5,220
C	10^{-5}	0.78	6,000
		Solution C (µL)	
D	2,5 x 10^{-8}	15.0	5,985
E	10^{-8}	6.0	5,994
F	4 x 10^{-9}	2.4	5,998

Table 2: Details of the preparation of the RGD-Cys-D1 solutions used. A stock solution of RGD-Cys-D1 dendrimers was prepared at a final concentration of 0.77 mg/mL (solution A), from which solutions B and C were prepared at 10^{-2} and 10^{-5}% *w/w* concentrations. Solution C with a 10^{-5}% *w/w* concentration of RGD-Cys-D1 dendrimer was used to prepare solutions D, E and F at final concentrations of 2.5 x 10^{-8}, 10^{-8} and 4 x 10^{-9}% w/w, respectively.

	Name	Volume (µL)	Blocking buffer (mL)
Primary mix	Rabbit mAb to pax	65	12.870
	Mouse mAb to COL2A1	32.5	
Secondary mix	Alexa Fluor 488 goat anti-mouse	13	12.961
	Alexa Fluor 568 goat anti-rabbit	13	
	Hoechst solution	13	

Table 3: Antibody solution preparation. The primary antibody mixture contained monoclonal antibody against pax produced in rabbit diluted 1:200 in the blocking solution with monoclonal antibody against COL2A1 produced in mouse diluted 1:400 in the blocking solution. The secondary antibody mixture contained the cell nuclei stain Hoechst and the secondary antibodies produced in goat against mouse and rabbit respectively (labeled with Alexa Fluor 488 and 568 fluorophores, respectively).

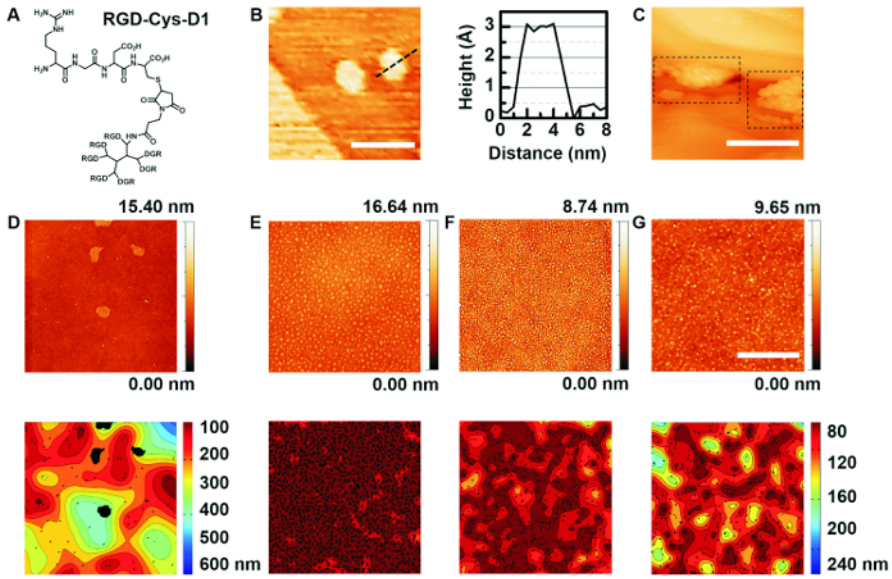

Figure 1: RGD-Cys-D1 dendrimer nanopatterning for the nanoscale control of local RGD surface density. (A) RGD-Cys-D1 dendrimer structure containing up to eight copies of the cell adhesive RGD peptide. Representative STM images (bias = 200 mV, set point = 0.5 nA) of RGD-Cys-D1 nanopatterns on Au(111) from an initial aqueous solution of 10^{-8}% *w/w* (Scale bar = 3 nm, **B**) and the corresponding height-distance profile obtained on the dashed region indicated in **(B)**, and **(C)** of 10^{-2}% *w/w* showing dendrimer aggregation (Scale bar = 20 nm). **(D-G)** Representative AFM images of RGD-Cys-D1 nanopatterns on PLLA obtained from the corresponding dendrimer aqueous solutions of 10^{-2}%, 2.5 x 10^{-8}%, 10^{-8}% and 4 x 10^{-9}% *w/w*, respectively (Scale bar = 1 µm). The corresponding minimum interparticle distance (d_{min}) probability contour map is shown below each AFM image, with high density RGD regions shown in dark red (d_{min} <70 nm). Dendrimers and dendrimer aggregates are depicted in black for clarity. Please click here to view a larger version of this figure.

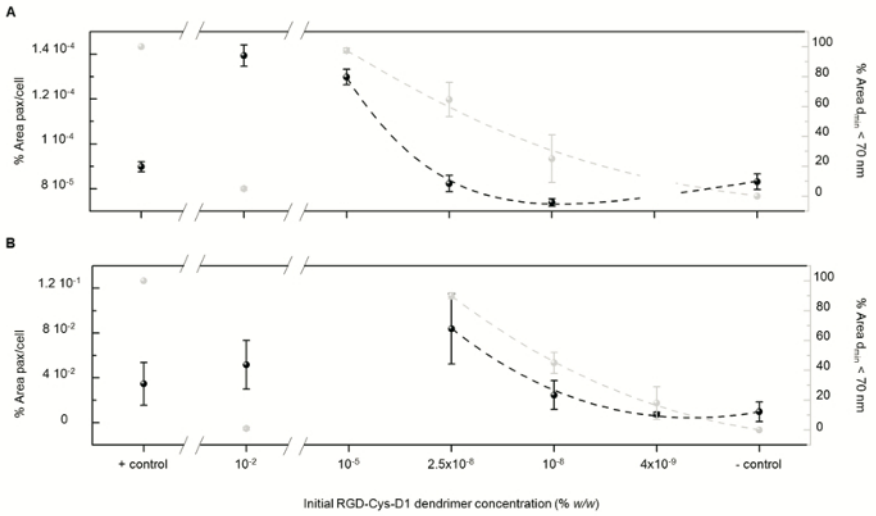

Figure 2: Cell adhesion on the RGD-Cys-D1 nanopatterns. Plot of the percentage of area stained for the FA protein pax per cell for the cells in contact with the substrate (left axis) with initial dendrimer concentration. Values are compared with the local RGD surface density (percentage of surface area in the nanopatterns containing d_{min} values below the 70 nm threshold) (right axis) with 100 and 0 percentages assigned to the positive (+ control) and negative (- control) controls, respectively. **(A)** Fibroblast adhesion on dendrimer nanopatterns on Au(111) after 4.5 h of culture. **(B)** MSC adhesion on dendrimer nanopatterns on PLLA evaluated at day 1 of chondrogenic induction. Values are given as the mean with the standard deviation. Please click here to view a larger version of this figure.

RGD-Cys-D1 (% w/w)	Area (< d_{min} = 70 nm) (%) on Au(111)	Area (< d_{min} = 70 nm) (%) on PLLA
10^{-2}	5 ± 1	2 ± 2
10^{-5}	97 ± 2	
$2{,}5x10^{-8}$	65 ± 11	90 ± 2
10^{-8}	25 ± 16	45 ± 7
$4x10^{-9}$		18 ± 11

Table 4: Percentage of area with d_{min} values below 70 nm obtained for RGD-Cys-D1 dendrimer deposition on Au(111) and on PLLA surfaces as a function of the initial dendrimer concentrations used.

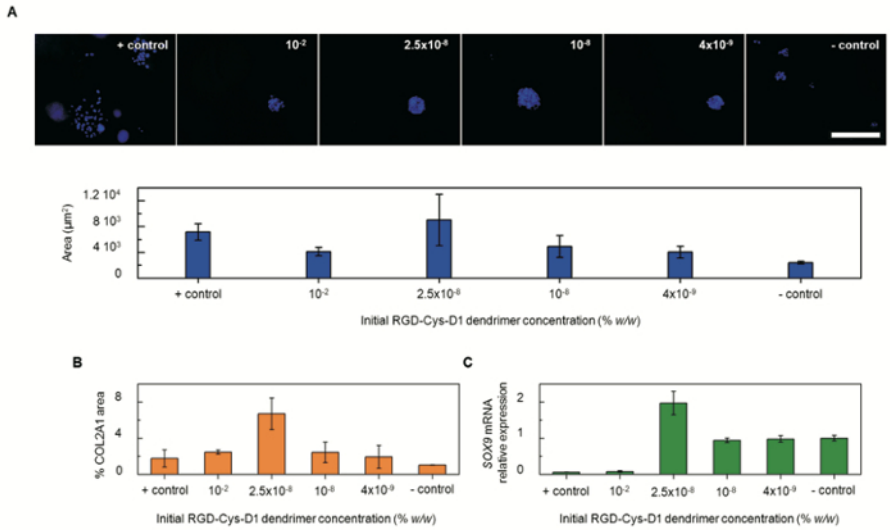

Figure 3: Chondrogenic differentiation of MSCs on the RGD-Cys-D1 nanopatterns. (A) Condensation of MSCs cultured on RGD-Cys-D1 nanopatterns on PLLA after 5 days of chondrogenic induction. Representative epifluorescence images of stained cell nuclei (Hoechst; Scale bar = 300 µm; upper row) and plot of the area of cell condensates (lower row) obtained on RGD-Cys-D1 nanopatterns from different initial dendrimer concentrations. Differentiation proceeds from cell condensation step with the expression of specific chondrogenic markers: **(B)** Percentage of the area of COL2A1 staining normalized with the area of the cell condensate from the corresponding confocal z-projections obtained after 5 days of chondrogenic induction. **(C)** Relative *SOX9* mRNA expression (against negative control) after 3 days of chondrogenic induction. Values are given as the mean with the standard deviation in **(B)** and **(C)**. Please click here to view a larger version of this figure.

Discussion

During the development of the described protocol, a number of critical steps should be considered. The first refers to nanopattern characterization with scanning probe microscopy techniques. To visualize the nanopatterns, the surface where patterning is produced must have a roughness value below the mean diameter of the dendrimers, which is around 4–5 nm as measured by STM (**Figure 1B**). Also, it should be taken into account that high resolution STM imaging is restricted to conductive substrates, in this case Au(111). Any lifting of the polymer from the corners of the slide after the spin-coating can be rectified using biocompatible glue.

Dendrimer nanopatterning is a process through which to achieve controlled local cell adhesiveness at the nanoscale. Based on the self-assembly of dendrimers on the surface by adsorption, this technique does not require any complex nanopatterning equipment, thus contrasting with previously described lithography-based methods[36,37,38]. Dendrimer nanopatterning can be easily scaled up to large surface areas and is fully compatible with cell culture protocols.

The dendrimer nanopatterning method described here can find future applications in regenerative medicine. The control exerted by dendrimer nanopatterning on cell adhesiveness makes this technique suitable for the conditioning of biomaterials prior to implantation, thereby facilitating their integration into host tissues. Moreover, the ease with which dendrimer peripheral moieties can be modified makes dendrimer nanopatterning appropriate for other ligands that exert concentration-dependent activity on cells.

Disclosures

The authors have nothing to disclose.

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