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2018

Wang, L., Cai, P., Luo, J., Zhang, F., Liu, J., Chen, Y., . . . , Wang, S. (2018). Engineering subcellular-patterned biointerfaces to regulate the surface wetting of multicellular spheroids. *Nano Research*, 11(10), 5704–5715. doi:10.1007/s12274-018-2117-6

<https://hdl.handle.net/10356/137813>

<https://doi.org/10.1007/s12274-018-2117-6>

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Engineering Subcellular-Patterned Biointerfaces to Regulate Surface Wetting of Multicellular Spheroids

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Subcellular-patterned biointerfaces were applied to inhibit the surface wetting of multicellular spheroids.

Engineering subcellular-patterned biointerfaces to regulate the surface wetting of multicellular spheroids

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KEYWORDS

topography,
wetting,
multicellular spheroids,
cell adhesion,
collective migration,
biointerfaces

ABSTRACT

Studying the wetting behaviors of multicellular spheroids is crucial in the fields of embryo implantation, cancer propagation and tissue repair. Existing strategies for controlling the wetting of multicellular spheroids mainly focus on surface chemistry and substrate rigidity. Although topography is another important feature in biological micro-environment, its effect on multicellular spheroids wetting has seldom been explored. In this study, the influence of topography on the surface wetting of multicellular spheroids has been investigated by using subcellular-patterned opal films with controllable colloidal particle diameters (from 200 nm to 1500 nm). The wetting of hepatoma carcinoma cellular (Hep G2) spheroids is impaired on opal films compared with that on flat substrates, and the wetting rate decreases as colloidal particle diameter increases. The decrement reaches 48.5% when the colloidal particle diameter is 1500 nm. The subcellular-patterned topography in opal films dramatically reduced the cellular mobility in precursor films, especially the frontier cells in the leading edge. The frontier cells failed to form mature focal adhesions and stress fibers on micro-patterned opal films. It was resulted from the gaps between colloidal particles leave adhesion vacancies, causing weak cell-substrate adhesion and consequent retardant migration of Hep G2 spheroids. Our study manifests the inhibiting effects of subcellular-patterned topography on the wetting behaviors of multicellular spheroids, providing new insights for tissue wetting-associated treatments and biomaterial design.

1 Introduction

Cell-substrate interactions contribute to many physiological processes, ranging from embryogenesis [1], cancer metastasis [2, 3], wound repair and immune surveillance [4, 5]. Cells sense the physiochemical signals of substrates [6-9], and implement biochemical responses such as protein synthesis [10, 11], gene expression and cytoskeleton rearrangement to adapt to the extracellular micro-environment [12-15]. Modulating substrate properties, such as surface chemistry, substrate rigidity and topography, is proved to be a potent strategy to alter the extracellular micro-environment, which could regulate cell-substrate interactions to guide cell fates. For example, soft hydrogels favor neural stem cells to become neurons while hard hydrogels promote glial differentiation [16, 17], heparin and heparin-like molecules modified surfaces promote endothelial cells proliferation [18], and slanted nanocilia arrays induce unidirectional polarization and migration of fibroblasts [19]. Traditional researches on cell-substrate interactions mainly concentrate on the individual cell, which misses the information from neighbor cells and be exposed to medium thoroughly. Recently, multicellular spheroids, which re-establish cell-cell contact and tissue architecture, are emerging as vivid in vitro models for cell-substrate interactions between tissues and biointerfaces [20, 21]. The cell-substrate

multicellular spheroids can wet the cytophilic molecules coated surfaces but not the cytophobic molecules modified surfaces [22]; decreasing substrate rigidity would inhibit and finally stop the wetting transition [23, 24]. However, as one of the most important features of biointerfaces, how the surface topography influences the wetting behaviors of multicellular spheroids still remains unclear.

Herein, we reported a subcellular-patterned biointerface from self-assembled opal films to manipulate the surface wetting of multicellular spheroids by simply altering the sizes of colloidal particles. The opal films supply long-range ordered subcellular-scale patterns by colloidal particle diameters ranging from 200 nm to 1500 nm. As a proof of concept, Hep G2, a human hepatoma carcinoma cell line, was chosen as a model system. Interestingly, the wetting of multicellular spheroids is dampened on opal films, comparing with that on flat substrate (Scheme 1). The wetting velocities decrease as the colloidal particle sizes increase, and reduced 48.5% in contrast with the velocity on flat substrate when the particle diameter reaches 1500 nm. Particle image velocimetry (PIV) analysis displays the frontier cells in precursor films are less motile on opal films. Microscope observations reveal that lamellipodia in the frontier cells attach to the top of colloidal particles but span across the particle gaps, resulting in discontinuous adhesion

Scheme 1 The surface wetting of multicellular spheroid on opal film are decelerated compared with that on flat substrate.

adhesion competes with cell-cell cohesion and drives the multicellular spheroids to wet the unoccupied surface [22]. Those substrate properties direct individual cell behaviors, such as surface chemistry and rigidity, also affect the surface wetting of multicellular spheroids. For example,

and retardant migration. This work unveils the surface topography is another factor that affects the wetting of multicellular spheroids, and provides new approaches for the control of tissue wetting velocity.

2 Results and Discussion

We prepared a series of opal films with particle diameters of 200 nm, 500 nm, 1000 nm and 1500 nm (Fig. 1(a) and Fig. S1 in the Electronic Supplementary Material (ESM)) by the self-assembly of polystyrene (PS) colloidal particles on glass slides with subsequent silica fixation [25-27]. The silica was produced by the hydrolysis of tetraethyl orthosilicate (TEOS), which built Si-O bonds with glass and carboxylated particle surfaces to glue the colloidal particles together [28]. Energy dispersive X-ray spectroscopy analysis verifies the micro-particle assemblies were covered by a silica layer (Fig. S2 in the ESM). The flat glass slides were

chosen as the control. We characterized the surface topography and roughness of the sample substrates by atomic force microscope (AFM). The surface of flat substrate is quite smooth, while the opal films are close-packed by hemisphere structures and the structure heights increase with particle sizes (Fig. 1(b) and Fig. S3 in the ESM). As a result, the roughness of the opal films also increase with particle sizes (Figure 1c). In sum, we fabricated ordered subcellular-patterned surfaces with controllable topography, to modulate the collective cell-substrate interactions.

Figure 1 Surface topography and chemistry of opal films and flat substrate. a) SEM images of flat substrate, opal films with particle diameters of 500 nm and 1500 nm, respectively. b) AFM 3D height images of flat substrate, opal films with particle diameters of 500 nm and 1500 nm, respectively. c) Surface roughness of sample substrates. d) The average contact areas of individual cells cultured on Fn modified flat substrates after 1 h seeding (n=100). The error bars represent the standard error of the mean.

Since material stiffness plays an important role in cell adhesion and migration [29, 30], we measured the apparent stiffness of opal films. The modulus of opal films with particle diameters of 200 nm, 500 nm, 1000 nm and 1500 nm are 96.5 ± 9.6 MPa, 74.8 ± 13.3 MPa, 54.0 ± 12.0 MPa, and 48.9 ± 9.8 MPa, respectively (Fig. S4 in the ESM). The cell areas and migration speed increase with substrate rigidity and plateau at a certain threshold, which ranges from 10 kPa to 100 kPa [31-38]. Moreover, multicellular spheroid keeps a highly cohesive precursor film when the substrate stiffness is above 500 kPa [23]. Obviously, the opal films are too rigid for cells to perform their mechano-sensitivity. Hence, the stiffness difference of opal films was not

taken into account in this study.

To facilitate the cell surface adhesion, cytophilic fibronectin (Fn) was coated on the opal films and glass slides. Laser scanning confocal microscopy demonstrates Alexa Fluor 488-Fn had successfully attached to the opal films and flat substrate after 4 h deposition (Fig. S5 in the ESM). Furthermore, all sample substrates show hydrophobicity before Fn coating (Fig. S6 in the ESM), since the incompletely hydrolysis of TEOS leaving ethyl groups on the coating surfaces [39]. After Fn treatment, all substrates become hydrophilic. To optimize the coating condition, we compared the contact areas of individual cells on Fn coated flat substrates, with Fn modification concentration varied among 1

Figure 2 Wetting behaviors of Hep G2 spheroids on opal films and flat substrate. a) Representative snapshots during the spreading process of Hep G2 spheroids. The contours of spreading spheroids are highlighted by yellow. b) Time evolution of the wetting area A normalized by the initial aggregate radius R_0 of multicellular spheroids on sample substrates ($n=13, 10, 11, 8$ and 11 experiments for flat substrate and opal films with 200 nm, 500 nm, 1000 nm and 1500 nm-size particles, with spheroid diameters ranging from 100 μm to 200 μm). c) Spreading velocity (V^*) of Hep G2 spheroids on opal films and flat substrate. The error bars represent the standard error of the mean.

$\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ (Fig. 1(d)). The cellular contact areas increase with Fn concentration and culminate in 10 $\mu\text{g/ml}$ condition. Therefore, we chose 10 $\mu\text{g/ml}$ as the modification concentration in the following experiments.

To better understand the topography effect, the dynamic wetting behaviors of Hep G2 spheroids were observed by a living cell imaging system (Fig. 2(a)). The Hep G2 spheroids were cultured on sample substrates for 1 h to ensure they stuck to the substrates before observation. Snapshots show the spheroids flattened and enlarged like viscoelastic drops initially, then gradually generated precursor films surrounding the central aggregates. The presence of precursor films illustrates all the sample substrates would allow the complete wetting of multicellular spheroids. As time went on, the precursor films grew and expanded persistently. Nevertheless, the expansions of precursor films on flat substrate and nano-patterned substrates (opal films with particle diameters of 200 nm and 500 nm) were much faster than that on micro-patterned surfaces (opal films

with particle diameters of 1000 nm and 1500 nm). After 12 h spreading, the spreading areas on flat substrate and nano-patterned substrates showed more than 4-fold increase compared with the initial areas, while on opal film with particle diameter of 1000 nm the area increase was about 3.5-fold and on opal film with particle diameter of 1500 nm the increase was only 2.5-fold.

Multicellular spheroids wetting can be analogized to viscoelastic droplets spreading [40]. The spreading coefficient S can be written as $S = W_{cs} - W_{cc}$, where W_{cs} and W_{cc} denote the cell-substrate and cell-cell adhesion energies per unit area, respectively. When the cell-substrate adhesion overwhelms the cell-cell cohesion, namely $S > 0$ ($W_{cs} > W_{cc}$), complete wetting occurs; otherwise, partial wetting is observed, with multicellular spheroids remaining spherical caps. The wetting process is directed by a balance between the adhesive surface energy gain when the leading edge advances and the viscous dissipation when cells flow. There are two cellular flows

Figure 3 Cell motion analysis of Hep G2 spheroids by PIV. Velocity fields of Hep G2 spheroids on a) flat substrate and b) opal film with particle diameter of 1500 nm. The arrow scale in b) is 3.3-fold as that in a). The velocity maps (upper) show migration directions and heat maps (lower) demonstrate the spatial distribution of velocity fields. c) Mean velocity of frontier cells on flat substrate and opal film with particle diameter of 1500 nm. The blue circles corresponds to the limit within which the substrate is entirely occupied by cells.

during the wetting process, (i) the entry of the cells from the central “reservoir” into periphery monolayer (permeation) and (ii) the slippage as the precursor film expands (friction) [24, 41, 42]. When the dissipation is dominated by the permeation, spreading area A increases linearly with time t

$$A = \frac{kSR_0}{\eta}t \quad (1)$$

where k is a constant coefficient, η is the viscosity of the spheroid, R_0 is the initial radius of the spheroid. We measured the spreading areas of Hep G2 spheroids and fitted time evolution of the A/R_0 after precursor films began to appear on all substrates (Fig. 2(b)), which shows a linear relationship in good agreement with Eq. (1). $V^* = \frac{kS}{\eta}$ (i.e. the slope

of the straight line in Fig. 2(b)), an intrinsic parameter independent upon the sizes of multicellular spheroids, represents the typical spreading velocity. V^* undergoes monotonic decrease as particle sizes increase (Fig. 2(c)). On opal film with particle diameter of 1500 nm, $V^* = 37.6 \mu\text{m/h}$, which is only a half of V^* on flat substrate. It indicates that the subcellular-patterned topography would decelerate the wetting of Hep G2 spheroids. Considering the cell viscosity η is constant, aforementioned results proves the spreading coefficient S is dependent on the surface topography, apart from chemical modification and substrate rigidity.

To investigate the cell migration mode in multicellular spheroids, we used PIV to analyze the cellular flow fields on flat substrate and opal film with particle diameter of 1500 nm (Figs. 3(a) and 3(b)). Multicellular spheroids on both substrates show radical velocity, and cells in precursor films present much higher mobility than cells in central aggregates. On flat substrate, periphery cells began to extend into free space after hours spreading, and all cells in precursor films became highly motile after 10 h. However, cells on opal film with particle diameter of 1500 nm were quite stagnant, even those in precursor film. By analyzing the velocity distribution in Hep G2 spheroids, we discovered that on flat substrate the velocity magnitude rises as the position goes farther from the center and reaches maximum at the front of precursor film

(Fig. S7 in the ESM). This is in accordance with traditional studies about collective cell migration, which observed cells at the front are extremely active [43-45]. However, on opal film with particle diameter of 1500 nm, the most motile cells appear at the rear rather than the front. Then we calculated the mean velocity magnitude of frontier cells and found frontier cells on flat substrate always moved at a high speed, while frontier cells on opal film with particle diameter of 1500 nm migrated quite slowly (Fig. 3(c)). These results suggest that the progress of frontier cells in precursor films are impeded by the subcellular-patterned opal films.

The frontier cells act as pioneers during the advancing of collective cells [45, 46]. They generate protrusions and form large lamellipodia to build firm interaction with the substrate [47, 48]. In addition, they transfer force signals to adjacent cells through cytoskeleton and cell-cell adheren junctions [49-52], and drag the rear cells to follow [53-55]. To in-depth unravel how surface topography affects the adhesion and migration of the frontier cells, immunofluorescence was adopted to investigate adhesion- and motion-related proteins in the frontier cells after spreading for 20 h (Figs. 4(a) and 4(b)). On flat substrate and nano-patterned opal films, dense focal adhesions emerged at the edge of frontier cells, with size between 1-5 μm , suggesting the maturation of focal adhesion [56]. Whereas, only scarce focal complexes with sizes smaller than 1 μm appear on micro-patterned opal films. The adhesion sizes decline as the colloidal particle diameters increase (Fig. 4(c)). Fibrillar adhesions, whose lengths exceed 5 μm , appear only on the flat substrate (Fig. S8 in the ESM). Actin filaments aggregated into stress fibers along the migration direction on flat substrate and nano-patterned opal films, while these large actin bundles were extremely rare on micro-patterned opal films. Besides, we measured the contact areas of the frontier cells and they also decrease as the colloidal particle diameters increase (Fig. 4(d)). These results indicate that microscale particles perturb the focal adhesion assembly and actin network rearrangement, inducing frontier cells to expand less, and finally inhibiting the wetting of multicellular spheroids.

The morphology of pseudopodial protrusions in the frontier cells were characterized by environmental scanning electron microscope (ESEM) (Figs. 4(e) and 4(f)). Broad lamellipodia formed on flat substrate with short filopodia, and the lamellipodia on opal film with particle

diameter of 1500 nm were less extended with long filopodia stretching out. It suggests the adhesion is weaker on micro-patterned opal films. The cell-substrate interfaces were observed by taking advantage of focused ion beam and scanning electron microscope (FIB-SEM) (Figs. 4(g) and 4(h)).

Figure 4 Cell-substrate adhesion between the substrates and frontier cells of Hep G2 spheroids. Immunofluorescence images of frontier cells on a) flat substrate and b) opal film with particle diameter of 1500 nm. c) Length measurement of adhesions in frontier cells on sample substrates (n=30). d) Contact area measurement of frontier cells on sample substrates (n=20). Protrusion morphology of frontier cells on e) flat substrate and f) opal film with particle diameter of 1500 nm. Cell is pseudocolored by pink and substrate is pseudocolored by green. FIB-SEM images show the cross sections of lamellipodia on g) flat substrate and h) opal film with particle diameter of 1500 nm. Pt deposition was assumed for protection in the viewing zone by EBID and FIBID. The cell is pseudocolored by pink. The error bars represent the standard error of the mean.

Before milling, Pt deposition was assumed to prevent the ion beam from damaging the lamellipodia. Viewing from the cross section, we discovered that on opal films lamellipodia only covered the top of colloidal particles but spanned across the gaps between particles. These adhesion vacancies in the gaps were ubiquitous on all opal films and even on the smallest 200 nm-size particles (Fig. S9 in the ESM), which were quite different from the adhesion on flat substrate, where lamellipodia adhere to the substrate thoroughly. Therefore, the gaps between colloid particles construct subcellular-scale obstacles for protrusions and lead to discontinuous cell-substrate adhesion. According to aforementioned results, we speculate

adhesion at edge of protrusions [58]. The nascent adhesions gradually assemble into focal complexes and further into focal adhesions, accompanied by the bundling and cross-bridging of actin filaments [59]. The adhesions stabilize the protrusions, which drive the cell migration [60, 61]. Accordingly, on opal films the discontinuous cellular contact can prevent focal complexes from aggregating together into mature focal adhesion and perturb actin filament aggregation, which dampen the cell-substrate adhesion (Scheme 2). The inhibition enhances as the gap sizes increase. The weakened adhesion finally prevent the migration of frontier cells. Therefore, the wetting behaviors of multicellular spheroids could be controlled by

Scheme 2 Model explaining how subcellular-patterned topography decelerates the wetting of multicellular spheroids. The multicellular spheroids spread on the Fn coated substrates and the precursor films advance progressively. The frontier cells in precursor films orchestrate adhesion formation and cytoskeleton reorganization in the wetting process. On flat substrate (left), nascent adhesions cluster to form large focal adhesions, and actin filaments assembled into bundles, facilitating the migration of frontier cells. On subcellular-patterned substrate (right), cellular protrusion adheres the substrate discontinuously, contributing to the failure of focal adhesion maturation and actin assembly. Consequently, the weakened cell-substrate interactions impair the mobility of frontier cells and thereby hinder the wetting of multicellular spheroids.

that discontinuous cell-substrate interaction originate the deceleration of surface wetting of multicellular spheroids. When a multicellular spheroid wets a planar surface, a monolayer precursor film expands around the spheroid, under the leadership of frontier cells. The frontier cells attach to the free substrate by the activation of integrin-matrix linkages [57], and formed nascent

designing subcellular-patterned biointerfaces.

3 Conclusion

In summary, this study experimentally and theoretically demonstrates that subcellular-patterned opal films is able to manipulate the wetting behaviors of multicellular spheroids. The gaps between the colloidal particles in opal films impair the continuous cellular

adhesion, leading to the failure of focal adhesion assembly and actin rearrangement in the frontier cells. Weak cell-substrate anchorage and force transduction in the frontier cells impede the advancing of the leading edge of precursor films, and eventually induce the deceleration of surface wetting of multicellular spheroids. Ultimately, our finding provides a more comprehensive understanding of the collective cell-substrate interactions, and open new doors to the mechanical design of biointerfaces for facilitating embryo implantation, preventing cancer metastasis and accelerating wound healing [62].

4 Experimental

4.1 Opal films fabrication

Opal films could be fabricated according to previous methods, either by evaporative deposition of colloidal particles suspended within hydrolyzed silicate sol-gel precursor solution [25], or fixation of the monolayer colloidal particle self-assembly by TEOS hydrolysis [26, 28, 63]. Before the surface modification, opal films were treated by O₂ plasma. The activated opal films were immersed in Fn (Sigma-Aldrich) solution for 4 h at 37 °C. After Fn coating, the substrates were rinsed by PBS for 3 times.

4.2 Stiffness tests of opal films

The apparent stiffness of opal films were tested by TI-900 triboindenter (In-Situ Nanomechanical Test System) from Hysitron. A 400 μm radius conospherical tip was employed. Displacement-controlled mode was assumed with maximum excursion of 100 nm.

4.3 Cell culture

Hep G2 cells (Beijing Xiehe Hospital) was cultured in complete Dulbecco's Modified Eagle Medium (DMEM) (HyClone®) supplemented with 1% penicillin-streptomycin (HyClone®) and 10% fetal bovin serum (Biological Industries) and maintained at 37 °C in a humidified atmosphere with 5% CO₂. When the culture reaches 90% confluency, 0.25% trypsin-EDTA was used to digest the cells.

4.4 Multicellular spheroids culture

2% (w/v) agarose powder was added in PBS and

then autoclaved for sterilization and dissolution. The molten agarose was pipette into the micro-molds for MicroTissues® 3D Petri Dishes® (Sigma-Aldrich). The 3D Petri Dishes® were formed after agarose gelling. The 3D Petri Dishes® were taken out from the micro-molds and equilibrated by incubating in cell culture medium for 15 min or longer. Before cell seeding, medium surrounding the 3D Petri Dishes® was removed. Hep G2 cell suspension (7.5×10^5 cells/ml) was seeded into the chambers of 3D Petri Dishes®. 10 min later, additional medium was added to immerse the 3D Petri Dishes®. The 3D Petri Dishes® were incubated for 3 d to allow cells to aggregate into multicellular spheroids, replacing fresh medium every 1-2 days.

4.5 Living cell observation

Mature multicellular spheroids were harvested from the 3D Petri Dishes® and pipetted on the Fn coated substrates. Prior to observation, the samples were incubated for 1 h until the multicellular spheroids stick to the substrates. After washing gently by culture medium for 3 times, the sample substrates were placed in a living cell observation system, with 37 °C environment temperature and 5% CO₂ supply. Long-time wetting behaviors were observed by using an inverted microscope (Nikon Ti-E) under time-lapse mode.

4.6 Immunostaining

Multicellular spheroids were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton X-100 for 5 min and blocked by 2% bovine serum albumin (BSA) (Sigma-Aldrich) for 1 h. For vinculin visualization, the multicellular spheroids was coated in anti-vinculin mouse monoclonal (Sigma-Aldrich, 1:100 diluted in 1% BSA solution) for 1 h. Subsequently, FITC-conjugated goat anti-mouse (Sigma-Aldrich, 1:8 diluted in PBS) was employed to interact with primary antibody for 30 min. To stain actin, 100 nM working stock of rhodamine phalloidin (Cytoskeleton) was applied to incubate with the multicellular spheroids for 30 min. 4'-6-Diamidino-2-phenylindole (DAPI) (Coolaber) was used to stain cell nuclei for 5 min. Finally, the fluorescence images were visualized by a laser scanning confocal microscope (Nikon Ti-E).

4.7 Cellular morphology characterization

The multicellular spheroids were fixed firstly with 4% paraformaldehyde for 15 min and subsequently with 2.5% glutaraldehyde for 4 h at room temperature. Then the samples were dehydrated through a series of ethanol/water solution with ethanol concentration (30%, 50%, 70%, 85%, 95% and 100%), each for 15 min. The samples were treated by supercritical CO₂ drying to remove the ethanol inside cells. 60° tilted stages were employed for ESEM analysis.

Two beam systems, Nova 200's FIB and NanoLab's SEM were used to analyze the interaction between substrate and multicellular spheroid. Pt deposition was assumed for protection in the viewing zone by electron beam-induced deposition (EBID) and subsequent focused ion beam induced deposition (FIBID). The entire Pt layer was about 1 µm in thickness. The specimen stage was tilted at 52° during milling and cross-section observation.

4.8 Measurement and PIV analysis

The contact areas of cells, spreading areas of multicellular spheroids and lengths of adhesion are measured by using Image J. The velocity fields of multicellular spheroids are analyzed by PIVlab.

Acknowledgement

This research is supported by National Natural Science Foundation of China (21425314, 21434009, 21421061), National Program for Special Support of Eminent Professionals, Beijing Municipal Science & Technology Commission (Z161100000116037), and MOST (2013YQ190467).

Electronic Supplementary Material: Supplementary material (SEM images of the opal films with particle diameters of 500 nm and 1500 nm (Fig. S1), EDS analysis of opal films (Fig. S2), AFM images of the opal films with particle diameters of 500 nm and 1500 nm (Fig. S3), stiffness measurement of opal films (Fig. S4), microscopic images of substrates absorbing Fluor-conjugated Fn (Fig. S5), contact angle measurement of sample substrates before and after Fn modification (Fig. S6), velocity fields distribution of multicellular

spheroids (Fig. S7), fibrillar adhesions on flat substrate (Fig. S8), cross section of the lamellipodium on opal film with particle diameter of 200 nm (Fig. S9)) is available in the online version of this article at http://dx.doi.org/10.1007/s12274-***-****- (automatically inserted by the publisher)

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