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Three Dimensional Conjugation of Recombinant N-Cadherin to a Hydrogel for *In Vitro* Anisotropic Neural Growth

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Abstract

Living cells are extensively being studied to build functional tissues that are useful for both fundamental and applied bioscience studies. Increasing evidence suggests that cell-cell adhesion controlled by intercellular cadherin junction plays important roles in the quality of the resulting engineered tissue. These findings prompted efforts to interrogate biological effects of cadherin at a molecular scale; however, few efforts were made to harness the effects of cadherin on cells cultured in an *in vivo*-like three dimensional matrix. To this end, this study reports a hydrogel matrix three dimensionally functionalized with a controlled number of Fc-tagged recombinant N-cadherins (N-Cad-Fc). To retain the desired conformation of N-Cad, these cadherins were immobilized and oriented to the gel by anti-Fc-antibodies chemically coupled to gels. The gels were processed to present N-Cad-Fc in uniaxially aligned microchannels or randomly oriented micropores. Culturing cortical cells in the functionalized gels generated a large fraction of neurons that are functional as indicated by increased intracellular calcium ion concentrations with the microchanneled gel. In contrast, direct N-Cad-Fc immobilization to microchannel or micropore walls of the gel limited the growth of neurons and increased the glial to neuron ratio. The results of this study will be highly useful to organize a wide array of cadherin molecules in a series of biomaterials used for three-dimensional cell culture and to regulate phenotypic activities of tissue-forming cells in an elaborate manner.

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INTRODUCTION

The engineering of functional tissues is a promising strategy to assemble advanced biological tools to better understand and modulate biological processes in development, regeneration, and pathogenesis^{1,2}. Engineered tissue can also be used to treat various acute and chronic wounds and tissue defects³. Typically living cells of interests are cultured in a microporous or hydrogel matrix that was designed to recapitulate chemical and mechanical properties of a natural extracellular matrix^{4,5}. However, increasing evidence suggests that the quality of the engineered tissue greatly depends on cell density in the matrix, likely due to changes of intercellular adhesion junctions⁵. Cadherins mediate intercellular adhesions and regulate differentiation and morphogenesis⁶. Blocking cellular cadherin expression leads to limited tissue development⁷, suggesting that cadherins influence differentiation in engineered tissues. These findings suggest that a cell culture matrix assembled to present recombinant cadherin molecules may allow us to regulate cellular activities independent of cell density⁸.

Recently, efforts were increasingly made to regulate cadherin-mediated cellular activities using recombinant cadherin molecules^{8,9}. However, most of these studies focused on coupling the cadherin molecules to 2D substrates^{8,9,10}. There have been few efforts to fabricate matrices in which cadherin molecules are three dimensionally organized to culture cells in *in vivo*-like microenvironment^{11,12,13}. Accordingly, there are no documented improvements in the quality of engineered tissue using cadherin molecules.

To this end, this study systematically examines the biological impact of a 3D hydrogel matrix functionalized with a controlled number of recombinant cadherin in regulating activities of neurons towards 3D neural network formation. We hypothesized that coupling Fc-tagged cadherin to chemically conjugated Fc antibodies in a hydrogel would improve cadherin bioactivity in a 3D matrix, compared with direct chemical conjugation of cadherin (Scheme 1). Moreover, cell response to N-Cad conjugated to uniaxially aligned microchannels of a hydrogel would be more apparent than in gels that present randomly oriented micropores with poor interconnectivity.

We tested this hypothesis by coupling N-Cad-Fc to Fc-antibodies chemically conjugated to walls of uniaxially aligned microchannels or to randomly oriented micropores within a hydrogel. We denote this process as biochemical conjugation. Alternatively, we denote the direct conjugation of N-Cad-Fc to the gel using carbodiimide chemistry as chemical conjugation¹⁴. Hydrogels with microchannels or with micropores were prepared via uniaxial freeze-drying process and isotropic freeze-drying process, respectively¹⁵. Primary hippocampal neurons isolated from the rat cerebral cortex were cultured in these N-Cad-Fc functionalized hydrogels. In this study, we used alginate hydrogel formed from covalent cross-linking with adipic acid dihydrazide (AAD) as a model hydrogel system. We evaluated the quality of the resulting neural networks by examining the interconnection between neurons, the glial to neuron cell ratio, and intracellular calcium levels as indicative of neuronal activity. Based on these findings, we expect that these biomaterial systems will greatly advance both fundamental and applied neurobiology studies and further improve treatment quality of neurologic diseases.

EXPERIMENTAL SECTION

Synthesis of Fc-tagged N-Cadherin (N-Cad-Fc)

N-Cad-Fc was purified from the supernatant of HEK293 cells engineered to secrete the recombinant N-Cad-Fc⁷. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 0.4 mg/mL G418 (Sigma) as a selection marker. The cell culture supernatant was collected and filtered. Then, N-Cad-Fc was isolated by using the protein A affinity column (Bio-Rad) followed by gel filtration chromatography, as described previously¹⁶. Protein functional activity was confirmed by bead aggregation tests¹⁷.

Hydrogel Preparation

2 wt% alginate (Mw ~ 250,000 g/mol, FMC Biopolymer) solution was prepared by dissolving the polymer in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer with pH 6.5, followed by filtration. Then, 1-hydroxybenzotriazole (HOBT, Fluka), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Thermo Scientific), and adipic acid dihydrazide (AAD, Sigma-Aldrich) were sequentially added to the alginate solution. Molar ratio of EDC, HOBT, and AAD was kept constant at 1.0:0.5:0.2. Then, the solution was cured between two glass slides separated by a 0.5 mm spacer. The resulting hydrogels were punched out in the form of disk with a diameter of 5 mm. The gel disks were incubated in deionized (DI) water at room temperature overnight.

To fabricate the microchanneled gel, the alginate hydrogel that was incubated in DI water was placed on a copper plate cooled by liquid nitrogen and surrounded by polystyrene foam. The resulting frozen gel was lyophilized to fabricate cryogels with uniaxially aligned microchannels. Separately, to fabricate the microporous gels with randomly oriented micropores, the hydrogel was placed in a copper container cooled by liquid nitrogen and then lyophilized. This process was previously reported elsewhere^{15,18}. The resulting cryogels were kept dehydrated until they were used for characterization and cell culture. The microchannel and micropore architecture of the cryogel were characterized by cryo-fracturing the gels and imaging the cross-sections with a field emission scanning electron microscopy (FE-SEM, S-4800, Hitachi)¹⁵.

Biochemical/Chemical conjugation of N-Cad-Fc to alginate hydrogels

The resulting microchanneled and microporous cryogels were modified by N-Cad-Fc as follows. Hydrogels were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Thermo Scientific) and 1-hydroxybenzotriazole (HOBT, Fluka). Biochemical conjugation was conducted by dissolving EDC and HOBT in DI water at a concentration of 0.4 mg and 1.1 mg per 1 mL of DI water, respectively. Cryogels were rehydrated with this solution and left overnight at 4 °C, while maintaining the hydrated state. Subsequently, the gel was incubated overnight in PBS containing anti-Fc-antibody (Sigma), denoted as Fc-antibody, at 250 µg/mL. Then, the gel was washed with PBS three times to remove the unbound anti-Fc-antibody. Finally, the gel was incubated overnight at 4 °C with N-Cad-Fc in calcium containing HEPES buffer (20 mM HEPES, 50 mM NaCl, 5 mM CaCl₂) at 250 µg/mL. At this dilute concentration, cadherin molecules minimally self-associate to each

other in the buffer. The gel was washed with PBS three times to remove unbound N-Cad-Fc. Chemical conjugation of N-Cad-Fc to the alginate hydrogel was processed by sequentially incubating the cryogel with EDC, HOBT, and then N-Cad-Fc in calcium containing HEPES buffer.

Analysis of the surface density of N-Cad-Fc

Hydrogels functionalized with fluorescein (FITC)-labeled N-Cad-Fc were used to measure the density of immobilized cadherin (molecular number/area). The FITC-labeled N-Cad-Fc was prepared by reacting 1 mg/mL of N-Cad-Fc dissolved in HEPES buffer with fluorescein isothiocyanate (Sigma-Aldrich) for 12 h at 4 °C. Following the reaction, FITC-labeled N-Cad-Fc was purified using centrifugal filtration (3 kDa MWCO, Millipore), followed by dialysis in HEPES buffer at 4 °C to remove unreacted FITC. While FITC is reactive towards amine groups on the protein, it most stably binds to the N-terminus¹⁹, which ensures that the protein is mostly unaltered, and thus minimally alters the immobilization of the protein onto the hydrogel. Then, the FITC-labeled N-Cad-Fc was conjugated to a hydrogel in calcium containing HEPES buffer. The concentration of FITC-N-Cad-Fc was varied from 0 to 250 µg/mL. Then, the gel was washed three times with fresh PBS to remove excess FITC-N-Cad-Fc. The fluorescence from the gels was measured using the microplate reader (Tecan). Three gels per condition were analyzed. Separately, fluorescence yield of the HEPES buffer dissolved with known amounts of FITC-labeled N-Cad-Fc were measured to prepare a standard curve. This standard curve was used to back-calculate the density of N-Cad-Fc immobilized within the gel.

Cortical Neuron Culture

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (protocol #15254) at the University of Illinois Urbana-Champaign in accordance with the guidelines of the U.S. National Institutes of Health. Cortices were obtained and dissected from rat embryos at 18–19 days (E18-E-19) and the cortical neurons were isolated as described previously²⁰. Cortices were incubated with 3 mg/mL protease 23 (Sigma) in 1× slice dissection solution (82 mM Na₂SO₄, 30 mM K₂SO₄, 10 mM HEPES, 10 mM Glucose, 5 mM MgCl₂ and 0.001 % Phenol Red pH 7.4) for 10 minutes at 37 °C followed by washing with plating medium (10 % Fetal Bovine Serum (FBS), 20 % (w/v) glucose, 1% sodium pyruvate (100 mM), and 2 mM L-Glutamine in MEM mixed with Earle's BSS without L-glutamine). Dissociation into single cells took place in 3 mL of plating medium. Cells were seeded onto microporous and microchanneled alginate gels, so that the cells would migrate into the micropores or microchannels, respectively. The cell seeding density was constant at 1×10^5 cells/cm². The medium was replaced after 4 hrs with the maintenance medium (i.e., neural basal serum free media (Invitrogen) containing B27 (Invitrogen) extract, 2mM L-Glutamine and 100 units/ml Penicillin Streptomycin).

Immunofluorescence imaging of neural networks

At three weeks after seeding the cells, cells were fixed with 4% paraformaldehyde for 1 hr, and rinsed with PBS three times. Following permeabilization with 0.3 % Triton-X in PBS for 30 min at room temperature, neurons were blocked with 5% goat serum diluted in PBS overnight, and sequentially incubated with primary and secondary antibodies. The primary

antibodies used were rabbit polyclonal antibodies against neuronal marker microtubule-associated protein-2 (anti-MAP2, 1:1000, Sigma Aldrich) and mouse monoclonal antibodies against the glial marker glial fibrillary acidic protein (anti-GFAP, 1:5000, BD Bioscience). Secondary antibodies used were Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit antibodies²¹. Cells were rinsed 3 times with PBS and incubated with 100 ng/ml DAPI solution for 20 minutes. The DIC and fluorescence images were acquired with laser-scanning confocal microscope (LSM700, Zeiss).

Calcium imaging of neural networks

The Fluo-4 NW calcium assay kit (Molecular Probes) was used to evaluate and quantify the calcium activity in the cortical neuronal cultures. After three weeks of cell culture in the gel, the cell-gel construct was washed with PBS to remove any baseline fluorescence. Then, the construct was incubated with the mixture of Hank's balanced salt solution (HBSS) and HEPES buffer dissolved with Fluo-4 NW, a calcium indicator, for 60 min. All samples were then washed with the HBSS twice at room temperature. Finally, all samples were placed on a sterile coverslip, and mounted on the stage of a confocal microscope (LSM700, Zeiss). A 20× objective lens was used to visualize cells in the gel matrix. Laser excitation light was provided at a wavelength of 488 nm, and fluorescent emissions were collected at wavelengths above 515 nm. For image acquisition, an exposure time of 0.8 s was adopted. Approximately 300 cells were imaged in 10 randomly chosen separate images.

Statistical Analysis

All average data are presented as means $\pm$ SE. To determine significance, comparisons between groups were performed by one-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$).

RESULTS AND DISCUSSION

In this study, we present a method to systematically examine the role of cell-cell adhesive cues by assembling a three-dimensional hydrogel matrix that presents biologically active N-Cad. The recombinant N-Cad-Fc was either conjugated to the gel by using Fc-antibody as a linker or chemically linked to the gel in uniaxially aligned microchannels or randomly oriented micropores.

Fabrication of hydrogels

Hydrogels formed from cross-linking between uronic acids of alginate and AAD were modified to present uniaxially aligned microchannels or randomly oriented micropores¹⁵. Placing the gel on a copper plate cooled by liquid nitrogen and surrounded by insulating the polystyrene foam resulted in uniaxial growth of multiple ice columns perpendicular to the plate surface (Fig. 1A–I). Subsequent lyophilization of the frozen gel resulted in cryogels with uniaxially aligned microchannels with an average diameter of 100 micrometer, as confirmed with cross-sectional image of the gel captured with SEM (Fig. 1A–II).

In contrast, the alginate hydrogel placed on a copper container in which both bottom and wall were cooled by liquid nitrogen displayed randomly oriented growth of ice crystals (Fig.

1B-I). Therefore, subsequent lyophilization created a cryogel with randomly oriented micropores with irregular cross-sectional shape (Fig. 1B-II). A large number of micropores in the gel fell into groups of non-passing or isolated pores. For convenience, throughout the manuscript, the hydrogel with uniaxial microchanneled gel and the gel with randomly oriented micropores are denoted as the microchanneled gel and the microporous gel, respectively.

Functionalization of hydrogels with recombinant N-Cad-Fc

Intercellular cohesion is mediated by binding between cadherin extracellular domains on adjacent cells²². These interactions are crucial, as cadherin knockouts are embryonically lethal, and conditional knockouts significantly impair developmental processes and wound healing²³. The extracellular domains mediate both cell-cell cohesion and trigger signaling cascades that regulate cell proliferation and differentiation⁶. Biochemically conjugating N-Cad-Fc to hydrogels using the Fc-antibody ensured optimal presentation of the N-Cad binding site for binding to N-Cadherins on neuronal cells⁸. Here we demonstrated the ability to conjugate functional, biochemically oriented N-Cad-Fc within 3D hydrogels.

First, the microchanneled gel was modified to present N-Cad-Fc via biochemical conjugation. An Fc-antibody that could biochemically associate with Fc tags of the N-Cad-Fc was used as a linker. Through incubation of the gel in the aqueous buffer dissolved with HOBt, EDC, and Fc-antibodies, the gel presented chemically immobilized Fc-antibodies in the microchannel (Fig. 2A). Subsequent incubation of the gel with media dissolved with FITC-labeled N-Cad-Fc resulted in microchannels functionalized with N-Cad, as confirmed with positive fluorescence captured in confocal microscopic images (Fig. 3A-I). In contrast, the microchanneled cryogel incubated with FITC-labeled N-Cad-Fc without chemically coupled Fc-antibodies displayed little fluorescence (results not shown here).

In addition, the N-Cad-Fc could be immobilized onto microchannel walls via chemical conjugation (Fig. 2B). Cryogels incubated with an aqueous mixture of HOBt, EDC, and FITC-labeled N-Cad-Fc exhibited positive fluorescence from the unidirectional microchannel walls (Fig. 3A-II). According to the image analysis used to quantify total N-Cad-Fc immobilized on the microchannel walls, there was little difference in the amount of immobilized N-Cad obtained with either conjugation methods (Fig. 4).

In parallel, microporous gels were also modified to biochemically or chemically modified with N-Cad-Fc. The FITC-N-Cad-Fc clearly localized in randomly oriented micropore walls in the gel when using either immobilization method (Fig. 3B-I-II). According to the image analysis used to quantify N-Cad loading, the amount of N-Cad-Fc coupled to hydrogel micropores via the Fc-antibody was slightly higher than that chemically coupled to the microchanneled gel (Fig. 4).

3D neuronal cell culture within the gels

To demonstrate the capabilities of the micro-channel and micro-porous gels with either chemical or biochemical conjugation methods to support 3D cell culture, the cortical neurons were seeded into the various gel constructs. Hydrogels without N-Cad-Fc were used as negative controls. Within 24 hours, most cells migrated into the N-Cad-Fc functionalized

microchannels or micropores. In contrast, few cells migrated into microchannel or micropores without N-Cad-Fc. Here we demonstrated the ability to conjugate functional, biochemically oriented N-Cad-Fc within 3D hydrogels to promote neuronal cell attachment and differentiation.

According to SEM images of cells captured after three weeks of cell culture, there were few cells in gels without N-Cad-Fc (Fig. 5A-I). Cells cultured in the microchannels with chemically conjugated N-Cad-Fc aggregated (Fig. 5A-II). In contrast, cells cultured in biochemically conjugated channels displayed neuronal extensions along microchannels (Fig. 5A-III).

Immunostaining cells for MAP2 (neuron marker) and GFAP (glial cell marker) confirmed the limited adhesion of neuronal cells in the gels free of N-Cad-Fc (Fig. 5B-I). More interestingly, cells cultured in microchannels chemically conjugated with N-Cad-Fc exhibited a larger number of GFAP-positive glial cells than MAP2-positive neuron cells (Fig. 5B-II). Conversely, biochemical conjugation of N-Cad-Fc to the microchannel walls resulted in greater numbers of MAP2-positive neurons than GFAP-positive glia (Fig. 5B-III).

Neurons cultured in the microporous gel aggregated to each other, as seen in both SEM and immunostaining images (Fig. 6). In contrast to the microchannels, there was a higher ratio of glial cells to neurons in the microporous gel, regardless of the N-Cad-Fc conjugation method.

The quantitative analysis of immunostained images displayed in both Figs 5 and 6 confirmed that the dependence of the ratio of GFAP-positive glial cells to MAP2-positive neuronal cells on both conjugation method of N-Cad-Fc and gel architecture is significant (Fig. 7). With the microchanneled gel, the biochemical conjugation resulted in a smaller glial to neuron ratio than chemical conjugation. In contrast, with the microporous gel, the difference in the glial to neuron ratio obtained by biochemical versus chemical conjugations was statistically insignificant. This result implicates that the microporous alginate gel provides a porous architecture permissive for glia cells to adhere independently of the N-Cad-Fc immobilized on the pore walls^{24,25}.

The formation of more glia on gels with chemical conjugated N-Cad-Fc is likely due to degraded N-Cad-Fc function due to the direct chemical conjugation. Both neurons and glia express endogenous N-cadherin⁶; however, glial cells express only a limited number of N-cad. The greater glial population may be due to nonspecific adhesion. These 3D results are very similar to our previous findings with 2D cultures of cells on hydrogel surfaces modified with N-Cad-Fc⁸.

Apart from the previous study conducted with 2D cell culture, this 3D cell culture study demonstrates that biochemical conjugation of N-Cad-Fc to the microchanneled gel results in synergy to improve adhesion and growth of neurons and, finally, formation of interconnected neuronal networks. In contrast, the microporous architecture of the gel diminishes or neutralizes the role of N-Cad in regulating neural network formation. According to our previous study, the microchanneled hydrogel is more permeable than the microporous gel,

thus increasing diffusivity of Fc-antibody and N-Cad-Fc sequentially delivered through the gel matrix⁸. Therefore, the microchanneled gel should increase the number of Fc-antibody linked to the gel and also the probability of binding between Fc-antibody and N-Cad-Fc, as confirmed with fluorescently labeled N-Cad-Fc. Accordingly, there should be increased number of cells bound to the microchanneled gel via cadherin-cadherin bonds.

Functional analysis of the neural networks

To analyze the functionality of neurons in the gels, we examined their basal electrical activity by optical imaging of intracellular calcium concentration. Electrical activity of neurons leads to increases in intracellular calcium levels via calcium influx through voltage-dependent calcium channels²⁶. The rise in intracellular calcium levels was measured with fluorescence arising from calcium indicator Fluo-4 AM bound with calcium ions. The microchanneled gel free of N-Cad-Fc presented few cells positively stained for calcium ion (Fig. 8A-I).

The microchannels biochemically conjugated with N-Cad-Fc led to the interconnected neurons charged with high concentration of calcium ions (Fig. 8A-III). Active neuronal extensions were also observed. In contrast, neural cells cultured in the microchanneled gel chemically bound with N-Cad-Fc developed fewer numbers of interconnected neurons with lower calcium signals (Fig. 8A-II). According to quantitative image analyses, the microchannels biochemically conjugated with N-Cad-Fc led to 1.7-fold increase of intracellular calcium level compared to the gel chemically conjugated with N-Cad-Fc (Fig. 9).

In addition, neurons cultured in the microporous gel displayed limited development of interconnected neurons (Fig. 8B). Little difference was made from the gel free of N-Cad. There were few neurites in the gel. Intracellular calcium concentration was also lower than cells cultured in the microchanneled gel. In particular, with the biochemical conjugation of N-Cad-Fc, cells in the microchanneled gel exhibited 1-fold higher level of calcium than those in the microporous gel (Fig. 9).

These results indicate that microchannel alginate gels biologically modified with N-Cad-Fc had a greater effect in stimulating cell adhesion, neural activity and overall neural network formation. According to previous studies, abilities of neurons to extend axons and to form interconnected neural networks are supported by N-cadherin on the surface of other axons or non-neuronal cells^{27,28}. We propose that the recombinant N-cadherin immobilized on the gel recapitulates this cell-cell adhesion. In particular, the results of this study implicate that the microchanneled hydrogel synergistically facilitates binding between cellular N-cadherin and the recombinant N-Cad-Fc immobilized on the microchannel wall. It is likely that the smooth surface and uniaxial alignment of microchannels lead to better disclosure of cell adhesion binding domains of N-Cad-Fc than microporous gels.

We propose that the results of this study provide an invaluable guideline of materials used to recapitulate N-Cad-induced cell signaling in engineered environments, and in addition, be readily used with a wide array of biomaterials used for 3D cell culture. These findings should be broadly useful for immobilizing a series of cadherin molecules and for better

understanding and regulating the biological role of cell-cell adhesion in differentiation. The fusion of Fc-domain to N-cadherin, to attach the N-cadherin to the gel, has several advantages²⁹. The Fc-domain is able to fold independently of the protein, improve physiological stability of the recombinant protein^{29,30}, and allows for easy and cost-effective purification via a protein G/A affinity chromatography²⁹. We envision that the 3D gel functionalized with cadherin would be readily modified to present integrin-binding proteins (e.g., fibronectin, collagen, vitronectin, etc), in order to orchestrate effects of cell-cell adhesion and cell-matrix adhesion for enhanced wound healing and tissue regeneration. Mechanical properties of the gel including softness can be also readily modulated to examine the biomechanical functionality of cadherin molecules³¹. Overall, the platforms assembled herein would be also highly useful to study and regulate cellular emergent behavior involved in tissue/organ development, wound healing, and pathology.

CONCLUSION

In conclusion, we have shown that the biochemical conjugation of recombinant N-cadherin extracellular domains to a microchanneled hydrogel appears to recapitulate the role of cell-cell adhesion in guiding the formation of neural networks. The biochemical conjugation of N-Cad-Fc with the Fc-antibody appeared to better preserve the biochemical activity of the immobilized N-Cadherin. We attribute the increased neuronal extensions into the microchannels to greater N-Cad densities, due to enhanced mass transfer of Fc-antibody and N-Cad-Fc. This orchestrated control of N-Cad-Fc binding and microstructure of the gel created a gel matrix that promoted adhesion and growth of neuronal cells and, ultimately, formation of interconnected neuronal networks in the 3D matrix.

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Figure 1.

Fabrication of hydrogels with uniaxially aligned microchannels and those with randomly oriented micropores. (A) (A-I) Schematic of the assembly and (A-II) SEM image of the cross-section of the cryo-fractured microchanneled gel. (B) (B-I) Schematic of the assembly and (B-II) SEM image of the cross-section of the cryo-fractured microporous gel.

Figure 2. Schematics of (A) the biochemical conjugation chemistry of N-Cad-Fc and (B) the chemical conjugation chemistry of N-Cad-Fc to microchannel walls of the gel.

Figure 3. Confocal images of FITC-labeled N-Cad-Fc conjugated to (A) microchanneled gels and (B) microporous gels using (I) biochemical and (II) chemical conjugations.

Figure 4. Image analysis of the mass of N-Cad-Fc conjugated to microporous and microchanneled gels with different conjugation methods. The values and error bars represent average values and standard deviation of three different samples per condition.

Figure 5.

Neural networks formed by cortical neurons after three weeks of culture in microchanneled gels. (A) SEM images and corresponding (B) confocal images of cells with different conjugation methods: (I) no N-cad, (II) chemical conjugation, and (III) biochemical conjugation. In (B), cell nucleus, neuron, and glia were stained by DAPI (blue), MAP2 (green), and GFAP (red), respectively.

Figure 6.

Neural networks formed by cortical neurons after three weeks of culture in microporous gels. (A) SEM images and corresponding (B) confocal images of cells with different conjugation methods: (I) no N-cad, (II) chemical conjugation, and (III) biochemical conjugation. In (B), cell nucleus, neuron, and glia were stained by DAPI (blue), MAP2 (green), and GFAP (red), respectively.

Figure 7.

Comparison of glia/neuron ratio between microporous and microchannel gels with different conjugation methods. The values and error bars represent average values and standard deviation of three different samples per condition. * represents the statistical significance of the values between conditions (* $p < 0.05$)

Figure 8. Intracellular calcium imaging of neurons cultured in the (A) microchannel gels and (B) microporous gels with (I) no N-cad, (II) chemical conjugation, and (III) biochemical conjugation of N-Cad-Fc.

Figure 9.

Quantitative analysis of the intracellular calcium concentration. The values and error bars represent average values and standard deviation of three different samples per condition. * represents the statistical significance of the values between conditions (* $p < 0.05$)

Scheme 1. Strategy to culture cells in a three dimensional hydrogel conjugated with the Fc-N-Cad

Cells are cultured in microchannels functionalized with a controlled number of recombinant N-Cad-Fc. N-Cad-Fc biochemically binds with Fc-antibodies that are chemically linked to the microchannel walls.