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On-line observation of cell growth in a three-dimensional matrix on surface modified microelectrode arrays

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Abstract

Despite many successful applications of microelectrode arrays (MEAs), typical two-dimensional *in-vitro* cultures do not project the full scale of the cell growth environment in the three-dimensional (3D) *in-vivo* setting. This study aims to on-line monitor *in-vitro* cell growth in a 3D matrix on the surface-modified MEAs with a dynamic perfusion culture system. A 3D matrix consisting of poly(ethylene glycol) hydrogel supplemented with poly-D-lysine was subsequently synthesized *in-situ* on the self-assembled monolayers modified MEAs. FTIR spectrum analysis revealed a peak at 2100 cm⁻¹ due to the degradation of the structure of the 3D matrix. After 2 wks, microscopic examination revealed that the non-degraded area was around 1500 μm² and provided enough space for cell growth. Fluorescence microscopy revealed that the degraded 3D matrix was non-cytotoxic allowing the growth of NIH3T3 fibroblasts and cortical neurons *in vitro*. Time-course changes of total impedance including resistance and reactance were recorded for 8 days to evaluate the cell growth in the 3D matrix on the MEA. A consistent trend reflecting changes of reactance and total impedance was observed. These *in-vitro* assays demonstrate that our 3D matrix can construct a biomimetic system for cell growth and analysis of cell surface interactions.

Keywords

MEA; Perfusion culture system; Degradation; 3D matrix; Impedance measurement

1. Introduction

With the advancement of Micro-Electro-Mechanical Systems (MEMS) techniques, various lab-chips, such as microelectrode arrays (MEAs), have been designed as

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electrophysiological recording devices in *in-vitro* studies [1-3]. On-line and long-term *in-vitro* monitoring the electrophysiological activity of excitable cell cultures on MEAs is a compromise between patch-clamp and animal experiments [4, 5]. In addition, several groups have pursued the investigations of the properties and mechanisms of two-dimensional (2D) cell culture systems on MEAs [2, 6-9]. However, 2D culture systems involve exposure of cells to rigid substrates, such as glass, silicon, or plastic surfaces and do not mirror the *in-vivo* environment in comparison to 3D culture system with microfluidic perfusion system [10-13]. Previous studies have demonstrated that the proliferation and morphology of cells cultured in 3D is more physiologically relevant than in 2D culture models [14, 15]. A reformed 3D microcavity silicon chip with multicellular spheroids cultures was established for mimicking *in-vivo* conditions [16], but it is complicated due to the requirement for manually positioning the spheroids in the cavity with a micropipette. Taken together, these drawbacks highlight the importance of incorporating proper 3D culture systems with MEAs that more adequately reflect the *in-vivo* environment.

Biocompatible materials, including collagen [14], alginate [10, 17], gelatin [18], hydrogel chemistry [17-20], have been developed into diverse 3D cell culture systems in order to more closely resemble *in-vivo* physical environments. These materials possess porous structures that can support cell growth, interconnection, and differentiation on or within their structure. One special hydrogel chemistry that has been developed into a scaffold for 3D culture is photopolymerization of poly(ethylene glycol) (PEG) [19, 20]. Because of the non-cytotoxicity and biodegradation of the PEG hydrogel, a solid cross-linked PEG hydrogel has been easily synthesized *in-situ* and widely applied. In addition, PEG matrix has been shown to remain at the original application site as demonstrated in a model of injured nerve repair [21] and a therapeutic cardiomyocyte matrix transplant [22].

Although the biological responses can be characterized after fixing and staining cells [23, 24], currently there is lack of approaches for on-line evaluation of 3D cell growth other than the measurement of electrophysiological activity. Among the various electrical properties of cells, impedance measurement is an electrochemical method usually expressed as the capacitive and resistive components that is used to monitor changes in biological environments [25-27]. Among various biological applications, impedance measurement has been utilized to estimate the effects of electrode materials on neural prosthetic devices *in vitro* [10, 28], to study changes in impedance of stratified cell layers on MEAs [1, 16, 25, 26], and to assess variations in tissue resistances *in vivo* [27, 29]. In addition, impedance measurements have been used in cell-based sensing applications such as alteration of cell membranes or morphological changes of adherent cell layers [1, 11, 16, 25]. Furthermore, changes in impedance have been measured *in vivo* and have been correlated with immunohistochemical observations [27]. Therefore, impedance measurement is a useful tool for assessing the electrical properties of tissues or cell-electrode interfaces and for interpreting changes of equivalent components based on the recorded impedance.

The aim of this study is to develop a perfusion type 3D culture system on surface-modified gold microelectrodes of MEA for long-term, on-line investigation of cell growth from impedance measurements. The properties of synthesized 3D matrix were first systematically evaluated by assessments of chemical bonding, swelling rate of 3D matrix, and subsequent

degradation tests before cell culture. After culturing fibroblasts and cortical neurons within the 3D matrix on modified MEA, cell growth rates were analyzed with respect to cell numbers. The morphology and cellular distribution was observed by immunofluorescence microscopy. In addition, the variations of impedance were recorded once every day for 8 consecutive days before the deliberate termination of cell culture.

2. Materials and methods

2.1 3D matrix synthesis and MEA fabrication

3D matrix was synthesized by photochemical cross-linking polymerization of hydrogel-precursor solution containing PEG precursor supplemented with 100 µg/ml poly-D-lysine (PDL) (30,000-70,000 MW, Sigma). PEG precursor solution consisted of 20% phosphate-buffer saline (PBS), 20% PEG (7,000-9,000 MW, Fluka), 60% PEG acrylate (Aldrich), 1 mM eosin Y (Sigma), 100 mM triethanolamine (Sigma), and 4% 2,2-dimethyl-2-phenyl-acetophenone (DMPA, Aldrich) in 1-vinyl-2-pyrrolidone (VP, 600mg/ml, Aldrich). The PEG precursor solution is considered biocompatible since eosin Y is a visible photo-initiator and a common drug colorant. Triethanolamine is a co-catalyst commonly used in pharmaceutical formulations, DMPA is a photocuring agent, and VP is a bioactive polymer.

The gold-based planar MEAs with a substrate of biocompatible polyimide (PI, Durimide 7320SE, Arch Chemicals) were fabricated on a cleaning glass substrate (4.5×4.5 cm²) by using standard MEMS lithography technology as described previously [25]. PI was chosen as a substrate based on its insulation properties for the layout of stimulation/sensing microelectrodes and its biocompatibility for *in-vitro* cell culture. In brief, the first layer consisted of photosensitive PI, which was spun and coated on the glass. A gold film (200 nm) was then deposited by e-beam evaporation onto the PI-spun glass and used for constructing the circuits of planar MEAs. Subsequently, photosensitive PI was respun to create the protective layer. After fabrication and hard bake (baked at 100°C for 1 hr), planar MEAs were then surface modified by 11-mercaptopundecanoic acid (MUA, COOH-(CH₂)₁₀-SH, Aldrich) SAMs and PDL in accordance with previous experimental protocols [25]. Afterwards, 3D matrix was synthesized by pouring 500 µl hydrogel-precursor solution into the planar surface-modified MEA and exposing to visible light for 3 min.

2.2 Characterization of 3D matrix degradation

2.2.1 Quantification of degradation—In order to quantify the degradation of 3D matrix, MEAs coated with 3D matrix were incubated in PBS for 0 and 3 hrs as well as 1, 3, 5, 7, 14, 21, and 28 days. Eluates were collected at each time point and used to determine the release of eosin Y, which was used to infer the degradation of the 3D matrix. An ultraviolet-visible (UV-VIS) spectrophotometer (Jasco-530, Japan) was employed to record the absorption spectra of collecting solution. The absorbance at 518 nm, a maximum light absorbance of eosin Y, was utilized for calibration and quantification.

After 3D matrix was incubated for 0 to 28 days in PBS, samples were dehydrated in a graded series of ethanol and finally treated by three 15 min washes in absolute ethanol. Samples were then stored in a desiccator until further analysis. The weight of dehydrated

sample was measured by using a Sartorius moisture balance MA30 as described previously [30]. The weights of degraded samples were obtained and subtracted from the weight of the original 3D matrix. Degradation rates were calculated by dividing the weight of degraded samples by the weight of original.

2.2.2 FTIR analysis for degraded 3D matrix—The degradation characteristics of the 3D matrix were examined by using Fourier transform infrared (FTIR) microscope. FTIR spectra of 3D matrix were obtained with a Bio-Rad Meline 3.1 IR-Microscope UMA-500 (Digilab C); 64 scans were averaged with a resolution of 2 cm^{-1} at RT. Spectra were recorded with a computer connected to the IR device ($n=6$) using the Win-IR Pro 3.1 spectrometer software.

2.2.3 Swelling test and degraded area evaluation of 3D matrix—The pre-weighed samples of 3D matrix were placed in pH 7.4 PBS at 37°C . The swollen 3D matrix was taken out at regular time intervals, wiped superficially with filter paper to remove surface-water, weighed and then placed in the same solution. The modulus of weight difference was obtained from which the weight of swelling 3D matrix subtracted the one of initial 3D matrix. The percentage of swelling rate was calculated by dividing the modulus of weight difference by the weight of initial 3D matrix as described previously [20]. After culturing in PBS, the pore area indicative of degraded structure in the 3D matrix, was determined from digital images using image analysis software *Image J* (Java image processing program, National Institute of Health, USA). In addition, the time-course of swelling rate and degraded area were recorded and expressed as mean $\pm$ SEM for 28 consecutive days. The appearance of the degraded 3D matrix was further observed by scanning electron microscopy (SEM) (Philips XL-30 FEG, FEI company). 3D matrix was dried and coated with gold in a sputter coater (Auto 108, Cressington, Watford, UK) before examining by SEM at an accelerating voltage of 10 keV.

2.3 Cell culture on 3D matrix MEA

The experimental setup of impedance recording and perfusion cell culture system is shown in Fig. 1. After cell culture on the 3D matrix MEA, a peristaltic pump was used to transport culture medium in a closed-loop perfusion system over the chip with a flow rate of $1\text{ }\mu\text{l/min}$. NIH3T3 fibroblasts (ATCC Cat. No.: CCL-92) and primary cortical neurons were used to investigate the culture environment of the 3D matrix. All animal protocols were reviewed and approved by the Yale Institutional Animal Care and Use Committee. Cell culture media and supplement were purchased from GIBCO-BRL (Gaithersburg, MD); all other chemicals and reagents were purchased from Sigma.

NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum (FBS) and $100\text{ }\mu\text{g/ml}$ penicillin-streptomycin (P/S) in a humidified atmosphere 5% CO_2 , 95% air at 37°C . Fibroblast (3×10^5 cells/ml) were then mixed with $500\text{ }\mu\text{l}$ hydrogel-precursor solution and poured onto surface-modified planar MEA. The samples were exposed to visible light for 3 min. To generate control cultures, fibroblasts were placed in tissue culture dishes at the same density and culture conditions. Cell growth

assay, fluorescent observation, and time-course impedance measurement were performed after 1 wk *in vitro*.

For primary cortical neurons, cerebral cortices were dissected and removed from embryonic mice at gestational day 18 under sterile conditions. Cortex pieces were dissociated by enzymatic digestion in Trypsin 0.25% for 30 min at 37°C and then triturated with a smoothly-fire-polished Pasteur pipette. Neurons suspension at a density of 3×10^5 cells/ml were then mixed with 500 μ l hydrogel-precursor solution and poured onto surface-modified planar MEA. The samples were exposed under visible light for 3 min. Neurons were cultured overnight in MEM containing 10% FBS, 20% glucose, and 100 μ g/ml P/S in a humidified atmosphere 5% CO₂, 95% air at 37°C. Afterwards, neurons were incubated with 0.25% glutamine, 100 μ g/ml P/S, 2% B-27 supplemented neurobasal medium, in a humidified atmosphere 5% CO₂, 95% air at 37°C. Neurons cultured at the same density in regular dishes served as control. Cultures were maintained by replacing half of the neurobasal medium every 3 days. Cell growth assay, immunofluorescent observation, and real-time impedance measurement were performed on neuron culture after 1wk *in vitro*.

2.4 Cell growth assay

NIH3T3 fibroblasts and cortical neurons cultured on culture dish and on 3D matrix MEA were examined under a phase-contrast upright microscope (Olympus 51) equipped with an intensified charged coupled device camera. Cell numbers were determined using image analysis software *Image J* (Java image processing program, *National Institute of Mental Health*, USA) by enumerating the number of cells per high power field from captured digital images taken from six random fields. Cell growth ratio was calculated by dividing the cell numbers on culture dish or on 3D matrix MEA by the number of seeded cells. Statistical significance was performed using one-way ANOVA followed by Tukey's pairwise comparisons.

2.5 Fluorescent observation

Cells on 3D matrix MEAs were stained with the fluorescence and observed and photographed using a phase-contrast upright microscope (Zeiss Axioplan 2 Imaging MOT) equipped with fluorescent optics and an intensified charged coupled device camera. For preparation of cells for staining, the 3D matrices containing cells were fixed on d 7 of culture with 4% paraformaldehyde for 20 min at RT. Subsequently, the 3D matrix with NIH3T3 fibroblasts was washed with PBS and treated with 0.1% Triton X-100 (American Bioanalytical). Samples were then stained with DAPI (1 μ g/ml, Sigma) and Rhodamine-Phalloidin (1:100, Molecular Probes) in PBS for 45 min. Samples were washed twice with PBS and examined by fluorescence microscopy.

The 3D matrix containing cortical neurons was incubated with anti-NeuN antibody (1:10, Chemicon International, Germany) in 2% BSA and 0.1% saponin in PBS for 2 hrs at RT. Samples were washed with PBS and then incubated with secondary antibody anti-mouse IgG-FITC (1:50, Sigma) and DAPI (1 μ g/ml) in 2% BSA and 0.1% saponin in PBS for 1 hr. Following three washes with PBS, samples were examined by fluorescence microscopy.

2.6 Impedance measurement

Impedance measurements were recorded from MEAs, which were connected to a perfusion culture system. First, we measured the AC impedance of surface-modified gold MEAs and 3D matrix MEA in contact with the culture medium at RT. After NIH3T3 fibroblasts and cortical neurons were cultured on 3D matrix MEA, the changes in impedance were recorded daily under the same conditions for 8 consecutive days. The variations of total impedance, including resistance and reactance between the cell-matrix-microelectrode-medium interfaces, were measured by using an HP 4284A Precision LCR meter under a constant 50 mV ac voltage at 1 kHz. Analytical results in terms of total impedance and its constituents, resistance and reactance, were expressed as mean $\pm$ SEM. Statistical significance was determined with the unpaired *t*-test.

3. Results

3.1 Characterization of 3D matrix on planar MEA

3.1.1 Evaluation of 3D matrix degradation—Time-course measurements of 3D matrix degradation as well as release profiles of eosin Y were recorded during 4 wks of incubation in PBS (Fig. 2(a)). The degradation rate increased rapidly in the first few days in PBS due to the destruction of the structure of the 3D matrix and the majority of eosin Y was also released over this period. The degradation rate exhibited a gradually rising pattern up to 40 ± 5.49 % in the first week of incubation and reached 45% by 4 wks. In addition, the release of eosin Y increased to 0.49 ± 0.09 mg/ml within 1 wk, reached a value of 0.6 ± 0.06 mg/ml at 2 wks, and remained constant up to 4 wk.

3.1.2 Characterization of chemical bonding of degraded 3D matrix—The 3D matrix, containing PEG precursor solution supplemented with PDL, was photochemically polymerized on the modified MEA and was then characterized by FTIR. FTIR spectra in the range of $1000 - 2500 \text{ cm}^{-1}$ are shown in Fig. 2(b). Specific N-H bending of amide bond can be observed near 1500 cm^{-1} in 3D matrix. In addition, the peak at 1700 cm^{-1} shifted to 1650 cm^{-1} because of the amide bond existing in PDL. After the 3D matrix on the modified MEA was incubated in PBS at 37°C for 1-4 wks, the particular frequency around 2100 cm^{-1} appeared in Fig. 2(b) ($\text{C}=\text{C}$ and $\text{C}\equiv\text{C}$) due to the degradation of the structure of 3D matrix. Except for this particular bond, degraded 3D matrix possessed all the main chemical bonds present in original 3D matrix.

3.1.3 Swelling test and degraded-area evaluation of 3D matrix—After incubation in PBS for 3 hrs, the 3D matrix started to degrade indicated by the appearance of small pores. Representative SEM image (inset in Fig. 2(c)) shows the appearance of pores in the 3D matrix at 1 wk. To quantify the degraded area of the 3D matrix, the pore areas were determined during 4 wks of incubation in PBS. The pore area increased to $980 \pm 97.5 \mu\text{m}^2$ in the first wk of incubation and reached $1445 \pm 191.9 \mu\text{m}^2$ at 2 wks after which it remained at approximately $1500 \mu\text{m}^2$. Fig. 2(c) also shows the dynamic uptake of water by the 3D matrix in PBS at 37°C . The ratio of swelling was calculated by dividing the difference in weight between the swelling 3D matrix and the initial 3D matrix. The swelling ratio for 3D matrix abruptly increased to 119.1 ± 2.2 %, with an initial large burst during the first 2 days

of culture. Furthermore, the swelling ratio declined to $97.9 \pm 1.5\%$ and stayed at approximately 96% by 2 wk. The swelling test indicated that 3D matrix sustained hydrophilic and moist properties during the entire incubation period.

3.2 Cell growth on 3D matrix MEA

Cell culture was performed to gain insight into the ability of the 3D matrix MEA to promote cell survival and growth. NIH3T3 fibroblasts and cortical neurons were cultured on 3D matrix MEA and their growth was compared to that achieved in conventional culture dishes as controls. Fig. 3 shows cell growth in the first wk of culture to assess the short-term effect of 3D matrix on NIH3T3 fibroblasts and cortical neurons. Fibroblasts on culture dish and 3D matrix MEA indicated an exponential growth rising from initial $92 \pm 10\%$ and $93 \pm 9\%$ to $8258 \pm 890\%$ ($p < 0.001$) and $8483 \pm 918\%$ ($p < 0.001$), respectively. Furthermore, fibroblasts in 3D matrix displayed substantial growth after 4 days of culture. On the other hand, primary cortical neurons grew on culture dish as well as 3D matrix MEA, but did not reveal an exponential growth trend during the entire period. The cell growth of cortical neurons showed that there was no significant difference between initial value (98%) and those either on culture dish (83%) or on 3D matrix MEA (84%).

3.3 Fluorescent analysis of cells in 3D matrix MEA

Figures 4(a) and 4(b) show representative phase-contrast images of NIH3T3 fibroblasts and cortical neurons cultured on 3D matrix MEA, respectively. Denser distribution of fibroblasts (Fig. 4(a)) was observed on 3D matrix MEA in contrast to neurons (Fig. 4(b)) and cells in contact with electrodes could be observed in both cultures. To further observe cell morphology on 3D matrix, NIH3T3 fibroblasts were labeled with fluorescent rhodamine-phalloidin (red) and DAPI (blue) on day 7 of culture (Fig. 4(c)). The growing configuration of NIH3T3 fibroblasts revealed a denser pattern in the 3D porous structure as shown in Fig. 4(c). On the other hand, cortical neurons cultured under the same conditions and visualized with primary anti-NeuN antibody (green) and DAPI (blue) displayed neurite outgrowth and neuron-like morphology in the 3D matrix (Fig. 4(d)).

3.4 Impedance evaluation for 3D matrix on MEA

The analytical results of the resistance (real part, R), reactance (imaginary part, X), and total impedances (Z) for SAMs surface-modified MEA and those with 3D matrix on SAMs-modified MEA are shown in Table 1. Compared to surface-modified MEA ($293.5 \pm 45.6\text{ k}\Omega$), the resistance of 3D matrix on modified MEA was slightly higher ($321.9 \pm 51.3\text{ k}\Omega$) but the difference was not significant. However, the reactance of 3D matrix on modified MEA increased to $551.3 \pm 51.2\text{ k}\Omega$, which was significantly higher than that of surface-modified MEA ($419.3 \pm 79.8\text{ k}\Omega$). Total impedance, the complex sum of resistance and reactance, for 3D matrix on modified MEA was $638.4 \pm 57.1\text{ k}\Omega$, which was greater than that of surface-modified MEA ($514.7 \pm 65.3\text{ k}\Omega$) ($p < 0.05$, $N = 20$).

Fig. 5(a) shows the time-course changes in resistance during the 8 d culture of NIH3T3 fibroblasts and cortical neurons on 3D matrix MEAs. The resistance of fibroblasts exhibited a similar trend to that of neurons during the entire recording period. The first resistance values, measured at 2 hr post-seeding on the 3D matrix MEA, were $341.6 \pm 50.8\text{ k}\Omega$ for

fibroblasts and $338.4 \pm 48.0 \text{ k}\Omega$ for cortical neurons. After 8 d of culture the cell culture environment was disrupted by changing the osmolarity of media leading to sharp drop in resistance of fibroblasts and neurons to $860.9 \pm 75.4 \text{ k}\Omega$ and $660.9 \pm 75.4 \text{ k}\Omega$, respectively.

Figures 5(b) and 5(c) show the time-course changes in reactance and total impedance for the entire 8 d culture period of NIH3T3 fibroblasts and cortical neurons on 3D matrix MEAs, respectively. The first reactance values of fibroblasts and neurons were obtained at 2 hrs of cells seeding on the 3D matrix MEA and found to be $569.1 \pm 80.8 \text{ k}\Omega$ and $561.0 \pm 80.5 \text{ k}\Omega$, respectively. The reactance of fibroblasts on the 3D matrix MEA reached a peak value of $2716.9 \pm 84.2 \text{ k}\Omega$ at 7 d. During the same period, the total impedance of fibroblasts on the 3D matrix MEA exhibited an abrupt rising pattern to a peak value of $3047.6 \pm 305.3 \text{ k}\Omega$. In contrast, the reactance and total impedance of neurons on 3D matrix MEA increased to a maximum of $1405.4 \pm 81.0 \text{ k}\Omega$ and $1755.2 \pm 206.9 \text{ k}\Omega$ by d 3, respectively. Before the destruction of culture environment, the total impedance of neurons on 3D matrix MEA was $1755.2 \pm 206.9 \text{ k}\Omega$ in comparison with the initial $656.4 \pm 109.6 \text{ k}\Omega$. Furthermore, the values of reactance of fibroblasts and cortical neurons suddenly dropped after the destruction of the cell culture environment on d 8 to $1036.7 \pm 89.8 \text{ k}\Omega$ and $679.0 \pm 88.9 \text{ k}\Omega$, respectively. Accordingly, the values of total impedance of fibroblasts and cortical neurons abruptly dropped to $1347.6 \pm 199.5 \text{ k}\Omega$ and $947.6 \pm 199.6 \text{ k}\Omega$, respectively. The reactance and total impedance values measured on d 8 decreased to the levels that were slightly higher than those obtained at 2 hrs.

4. Discussion

A biocompatible material suitable for 3D culture matrix should have an appropriate degradation rate, non-cytotoxicity, and suitable pore size. In general, the 3D matrix should degrade at a rate that would cells to embed and grow within the structure [19, 22, 31]. The degradation of our engineered 3D matrix was evidenced by a series of experiments, including the release of eosin Y, chemical bonding of FTIR analysis, and appearance of pores. In Fig. 2(a), the degradation rate of the 3D matrix calculated by weight difference was similar to that estimated by measuring the release of eosin Y with UV-VIS. In addition, both methods revealed a pattern of rapid degradation during the first days followed by a plateau after 14 d. The specific chemical bondings of the degraded 3D matrix were observed in the FTIR spectrum (Fig. 2(b)). Although the ingredients of our PEG precursor solution are very complex, it is important to note that the visible-light photoinitiator, eosin Y and DMPA, and co-catalyst triethanolamine, eventually would turn into passive products in our experiment owing to their participation in the chemical reaction only once [21]. Considering this fact and the results of FTIR spectra, the main constituents of our 3D matrix are PEG (C-H asymmetric stretching), PEG acrylate (C-H asymmetric stretching, C=C asymmetric stretching, C=O stretching), and VP (C-H asymmetric stretching, C-N stretching, C=C asymmetric stretching, C=O stretching, and C=C bond in ring). Moreover, the presence of specific amine and amide bonds, as shown in Fig. 2(b), indicates that PDL molecules have been incorporated into the 3D matrix. This is not surprising since it is common to supplement PEG hydrogel with biological molecules in order to expand its utility and to improve its non-cytotoxicity [22, 31, 32]. In accordance with FTIR spectra of the degraded

3D matrix, we conclude that the degradation rate eventually slowed due to the occurrence of a solid bulky carbon backbone.

The expanding range of techniques to manipulate natural and synthetic materials has allowed the design of 3D matrices at multiple scales, which have been categorized into 3 types: macro-scale ($10^{-1} - 10^{-3}$ m), micro-scale ($10^{-3} - 10^{-6}$ m), and nano-scale ($10^{-6} - 10^{-9}$ m) [33]. Since the average size of animal cells is generally around $10 - 100 \mu\text{m}$ in diameter [33, 34], micro-scale architecture is considered proper for 3D culture. In Fig. 2(c), we demonstrate that the pore area of 3D matrix reached a steady state around $1500 \mu\text{m}^2$ after 2 wks and possessed enough moisture, suggesting that our engineered 3D matrix could provide enough space for cell growth and possesses essential properties for 3D-matrix according to previous studies [31-33].

Previously, it was shown that determination of cell damage, cell growth, and cell morphology, could serve as preliminary indices for evaluating the non-cytotoxicity of medical devices [25, 35, 36]. In our study, characterization of cells cultured in the degraded 3D matrix with a perfusion system on MEA indicated cell survival and growth in the *in-vitro* 3D matrix. Observations of cell growth ratio (Fig. 3) and morphology (Fig. 4) demonstrated substantial differences in cell growth between NIH3T3 fibroblasts and cortical neurons. NIH3T3 fibroblasts showed robust growth (from initial 92 % to above 8400 %) and formed fibrous-like structures either on culture dish or 3D matrix MEA. Since the cell growth of NIH3T3 fibroblasts can continuously proliferate in a serum-supplemented medium. By contrast, cortical neurons maintained almost the same initial cell density and grew into typical neuronal network structures. The fluorescent images showed that NIH3T3 fibroblasts (Fig. 4(c)) and cortical neurons (Fig. 4(d)) could grow in a 3D configuration in the degraded porous matrix. There have been attempts to culture cells in various 3D matrices by confining them in microfluidic perfusion systems, which can provide fresh medium to support cell growth all the time [11-13]. The flow rate of our recording and perfusion culture system is $1 \mu\text{l}/\text{min}$, which did not affect cell morphology or growth in comparison to other studies [12, 37, 38]. In the past, researchers have applied commercially available material blending with extracellular matrix, biological molecule such as growth factors, which can be costly, to construct a 3D culture matrix *in vitro* [15, 17, 33]. Although our 3D material needs to be supplemented with PDL, it possesses the main features of the *in-vivo* micro-environment for cell growth and has the added advantage of being low cost and easy to prepare in the laboratory.

The impedance measurement is essential for validating successful fabrication and chemical modification of self-designed MEAs when AFM or SEM methods are generally unsuitable for all electrode samples [25, 26, 36, 39]. In accordance with our previous experience, it is commonly suggested that the total impedance exceeding $800 \text{ k}\Omega$ after surface modification should be considered a modification failure [25]. Our results, tabulated in Table 1, indicate changes in resistance, reactance, and total impedance between SAMs surface-modified MEA and 3D matrix on modified MEA. Although the reactance and total impedance of 3D matrix MEA have a significant difference as compared with SAMs surface-modified MEA, these changes are below $800 \text{ k}\Omega$. Thus, the 3D matrix on MEA still possesses enough sensitivity and qualification for electrophysiologic stimulation and recording.

Another advantageous application of impedance spectroscopy that has not been possible through optic approaches is to infer the function of cells or tissues on the electrode contact [1, 11, 16, 25-28, 36, 40, 41]. In our study, the resistances of NIH3T3 fibroblasts and cortical neurons abruptly increased during the first 3 days because of cell adhesion and outgrowth on the electrode contact [1, 25]. While the cells completely covered the electrode contact or outgrew in a steady state [1, 25], resistance changed slowly for the rest of the culture period (Fig. 5(a)). After deliberate destruction of the cells by changing the osmolality of media, the resistances suddenly dropped to a level slightly higher than their initial values. The slight difference could be due to the degraded 3D matrix or cellular debris. In addition, the time-course recording of reactance, the other constituent of total impedance, was different between the two cell types in Fig. 5(b). Due to the proliferation of NIH3T3 fibroblasts, the value of reactance kept rising during the 7 d of culture. In contrast, the reactance of cortical neurons reached a plateau after 3 d of culture. Moreover, the debris from large numbers of NIH3T3 fibroblasts caused higher reactance in contrast with cortical neurons after the culture environment was destroyed on d 8. The value of total impedance, combined resistance with reactance, can also be used to determine whether cells grow on the electrodes. For both cell types, the total impedance suddenly decreased after deliberately destruction of the cells on d 8 as shown in Fig. 5(c). These sudden decreases of total impedance provide evidence that these changes were due to the fact that cells were in contact with microelectrodes. Furthermore, the trend of total impedance was similar to reactance suggesting that the latter had a great impact on total impedance.

Surface-modified MEA has been simplified to be a parallel model in which the electrode impedance is presumably composed of electrode resistance (R_e) and shunt capacitance (C_e) from SAMs modification [25, 42, 43]. The SAMs-covered electrode is considered as an ideal capacitor with a plate distance that depends on the number of carbon atoms. In addition, extra resistance (R) and capacitance (C) of the sealing gap, which interpreted the interface between cells and electrodes, also had to be introduced after cells were cultured on the electrodes [1, 25]. According to our previous study [25], the equivalent circuit representing the parallel circuit of R_e and C_e for surface modified microelectrodes and the series circuit of R and C for cells covering the MEA is depicted in Fig. 6(a). In terms of the changes of impedance before and after the destruction of culture environment, the new equivalent circuit is therefore proposed in Fig. 6(b) for interpreting the system of cells cultured with 3D matrix on the surface-modified MEA. Except parallel circuit of R_e and C_e for surface modified microelectrodes, a series circuit of R_c and a parallel capacitance, C_m and C_c , are respectively depicted. The role of R_c in Fig. 6(b) represents the changes of resistance recording attributed to cells growing in the 3D matrix. Higher R_c is ascribed to numbers of cells on the electrode contact. Since our synthesized 3D matrix would finally retard its own degradation, the remainder of the matrix contributes C_m in our proposed new circuit. C_c is considered as layers of live or dead cells in our 3D-matrix culture system. Under the new assumption of the equivalent circuit in this study, we infer that R_c was affected by cell numbers according to time-course change of resistance recording (Fig. 5(a)). In addition, C_m and C_c influenced the values of reactance recording (Fig. 5(b)). The trends of total impedance in Fig. 5(c) are similar to those in reactance recording, and this phenomena imply that C_m and C_c have an effect upon total impedance.

5. Conclusions

Our engineered 3D matrix on the SAMs-modified MEA with a dynamic perfusion system was undertaken in order to evaluate the material properties and non-cytotoxicity as well as electrophysiological impedance measurements after *in-vitro* cell culture. The appearance and degradation properties indicate that the 3D matrix would degrade but keep enough skeleton and moisture for cell growth. Our results show that the 3D matrix on the SAMs-modified MEAs provided an environment compatible with cell growth. In addition, we show that impedance measurements can be applied to interpret the rate of cell growth. Our non-destructive and immediate monitoring of 3D cell outgrowth using impedance measurement challenges to some extent the traditional use of 2D culture on MEA to assess the relatively dynamic response of living cells. It is worthwhile to note that our 3D matrix need to be supplemented with PDL for providing cells an adequate non-cytotoxic environment. It is therefore possible to culture other kinds of cells by blending with other biomolecules into our engineered 3D matrix. More importantly, a design of on-line and lab-on-a-chip recording system can be generated by synthesizing the degradable 3D matrix on surface-modified MEA.

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Fig. 1. Schematic diagram of the experimental setup of impedance recording and perfusion culture system. Cells and 3D matrix were in custom-made vertically-oriented fluid reservoir with a seal cap on the top of the MEA. A peristaltic pump was used to circulate culture medium in perfusion culture system with flow rate of 1 $\mu\text{l}/\text{min}$.

Fig.2.

Characterization of 3D matrix degradation. 3D matrices on the SAMs-surface-modified MEAs were characterized for degradation in PBS for a 4 wk period. (a) The degradation rate of 3D matrix and release of eosin Y from degraded 3D matrix were measured. (b) The specific chemical bonds of 3D matrix before and after culture were analyzed and marked in FTIR scanning. (c) The degraded area and swelling test of 3D matrix were evaluated from 3D matrix incubated in PBS. Error bars represent mean ± SEM (n=20). The inset in (c) is a SEM image of a representative profile of degraded area which was taken at 1 wk of culture.

Fig. 3.

Assessment of cell growth of fibroblasts and cortical neurons in conventional dish and 3D matrix MEA. Fibroblasts and neurons were placed on culture dish as control. Fibroblasts and neurons were respectively mixed with hydrogel-precursor solution to form a 3D matrix on surface-modified MEAs for observing the cell growth, which was monitored for 7 d. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Error bars represent mean $\pm$ SEM (n=20).

Fig. 4. Optical microscopic images and fluorescent images of cells after 5 d on 3D matrix MEA. Representative images of NIH3T3 fibroblasts (a) and cortical neurons (b) are shown. Arrows point individual cells and arrowheads with a dash circle point microelectrodes in 3D matrix MEA. Fluorescent image of fibroblasts stained with DAPI (blue) and rhodamine-phalloidin (red) is shown in (c). Neurons were visualized with primary anti-NeuN antibody (green) and DAPI (blue) as shown in (d).

Fig. 5.
Time-course of impedance recordings. (a) resistance, (b) reactance, and (c) total impedance for NIH3T3 fibroblasts and cortical neurons on 3D matrix MEA were measured for 8 d.

Fig. 6. Schematic diagrams of impedance models for cells growing on surface-modified MEA (a) and cells growing on 3D matrix surface-modified MEA (b).

Table 1

The total impedance (Z) as a combination of resistance (R) and reactance (X) for surface-modified MEA and 3D matrix on MEAs

	Surface-modified MEA	3D matrix on modified MEA
R (kΩ)	293.5±45.6	321.9±51.3
X (kΩ)	419.3±79.8	551.3±51.2 ([*])
Z (kΩ)	514.7±65.3	638.4±57.1 ([*])

N = 20; Value = mean±S.E.M.

^{*}
p<0.05 (t-test)