An Evaluation of Different Coatings for TiN Microelectrode Chambers used for Neonatal Cardiomyocytes

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Abstract

Neonatal myocytes are a widely used model for modern in-vitro pharmacological screening and tissue engineering of cardiac tissue. The recording of neonatal myocyte electrophysiology in real time has still not been ideally optimised, however. The aim of this study was to evaluate the impact of using different substrates in culture (MEA) chambers on the surviving and functional electrophysiological activity of the myocytes after the first five days after isolation.

Neonatal cardiomyocytes were isolated from two day-old neonatal rats and plated on 12×12 ITO microelectrode chambers (Multi-Channel Systems) and cultured for five days.

Four coating methods were used. The PDL + fibronectin treatment displayed the highest cardiomyocyte adhesion to the electrodes (20% of total cells in chamber after seeding) and developed contractible cells during the five days of pre-cultivation with a measurable electrical response. Nevertheless, the cells adhered to all surface-treated MEA chambers, creating typical cell morphology, but with different success rates.

1. Introduction

Neonatal cardiomyocytes permit the study of many of the morphological, biochemical, toxicological, and electrophysiological characteristics of heart cells and their interactions [1,2]. The recording of neonatal myocyte actions and analysis of the signals from single cells or small aggregates in real time is one of the modern methods. Microelectrode arrays (MEAs) are one of the most modern tools; these systems are based on extracellular recordings and they have many advantages over traditional methods (e.g. patch clamp, indirect recording of ion flash by fluorescence methods). On the other hand, the preparation of the recording system and interpretation of the data have still not been ideally optimised. Ineffective preparation of MEA samples can negatively affect the results from mathematical modelling of electrophysiology and future biomedical applications. The coating of the electrode array has a significant role, not only in the attachment of the cells, but also in cell signalling and the development of cardiomyocyte electrophysiology during the first few days after seeding.

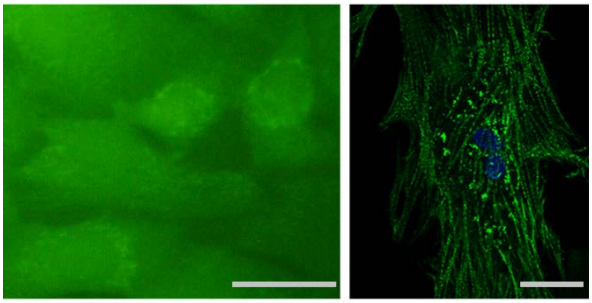

Figure 1: Typical neonatal myocytes in vitro. Left: typical geometry of cell aggregate (colour visualisation of cells using Calcein AM dye); Right: visualisation of alfa-actinin and nucleus of neonatal cardiomyocyte, day 5 after seeding. Recorded by epifluorescence microscope Olympus IX73, bar 20 µm.

Nowadays, there are many different MEA setups. The MEA head-stage can be wireless, which is easier to manipulate, e.g. in the cell incubator. Current MEA systems are very compact and usually have integrated temperature control, a pulse generator for sample electrical stimulation, an amplifier, and real-time signal analysis and

feedback. Moreover, higher throughput from parallel recording is also an advantage. MEAs can also monitor action potential as well as sub-threshold responses [3,4].

Multi-electrode array chambers have many different designs, which are highly application-specific. The number of substrate-integrated recording electrodes (channels) is in the range from units to thousands in recently presented applications [5-7]. The size, shape and electrode spacing might also vary from units to hundreds of micrometres. The electrodes' active surface is usually made from Au, Pt or TiN, which are chemically inert and easy to clean. Tracks and pads are usually made from transparent polymer, in which indium tin oxide (ITO) is used [8]. The cells are seeded on the surface of the pads, which is covered by a 50 µm-high (approx.) layer of coating substrate (collagen, PLL etc). Electrodes do not have direct contact with cells and they are usually several µm above the electrode surface.

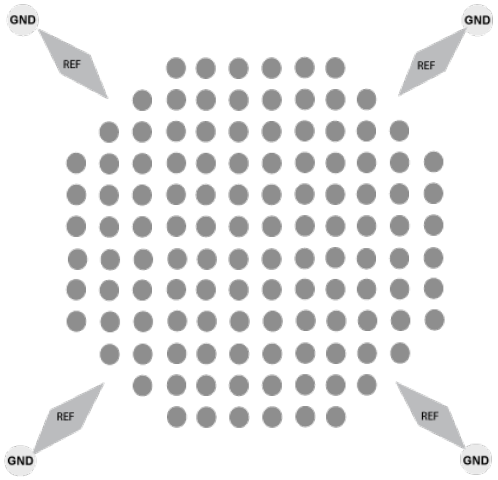

Figure 2: 120 MEA chamber layout (Multi-Channel Systems). Electrode spacing 100 µm.

2. Material and methods

2.1. Preparation of cells

Cardiomyocytes were isolated from two day-old neonatal rats by trypsin digestion (0.2% w/v), after which the cells were re-suspended in a medium containing Iscove's modified Dulbecco's medium (IMDM) (Sigma-Aldrich, I6529) and Medium 199 (Sigma-Aldrich, M4530) in a 4:1 ratio and supplemented with horse serum (10%), fetal calf serum (5%), penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were plated for 1.5-2 hours to allow attachment and/or separation of non-myocardial cells (heart fibroblasts). The non-adhesive cells (cardiomyocytes) were transferred into a centrifugation tube, washed, then centrifuged at 150 × *g* for 10 min. Suspension enriched in non-adhesive CMs was transferred to coated MEA chambers with different substrates (density 4×10^6 cells/cm^2). The culture medium consisted of DMEM and medium 199 (4:1) with penicillin (100 U×ml^{-1}). Cultures were incubated in conditions of 21% O_2 and 5% CO_2 at 37°C. The cultivation medium was removed after 72 hours. The cell electrophysiology was studied after 120 hours from seeding.

2.2. Multielectrode chamber coating

Two different 120-electrode chamber designs (Multi Channel Systems) were tested (12×12 electrode layout, 4 reference and 4 ground electrodes): i) 30 µm electrode diameter with 100 µm electrode spacing and ii) 30/10 µm variation. The electrodes were made from TiN (max 2% w/v of N) and the tracks and contact pads from ITO, covered with SiN isolator. Chambers were cleaned with Terg-A-Zyme (Sigma-Aldrich, Z273287) and sterilised with 70% ethanol and UV light before use.

MEA chambers were treated in four ways: i) collagen I (originated from rat tails); ii) 500 µl of 0,01% poly-L-lysine (PLL) (Sigma-Aldrich, P4707); iii) 500 µl of 50 µg/ml poly-D-lysine (PDL) (Sigma-Aldrich, P7886) were added to the chamber, keeping the O/N at 4°. After the treatment, the chamber surface was rinsed twice with PBS and dried on a sterile lab bench with UV light on; iv) PDL coating was used as a substrate and covered with 400 µl of 12.5 µg/ml porcine fibronectin (Sigma-Aldrich, F4759) for 1 hour at 37°C and 5% CO_2. After this, the surface was rinsed once with PBS and cells were plated immediately.

Cell adhesion and morphology were monitored with an Olympus IX73 microscope and Nikon D3100 camera. Calcein AM dye (Thermo Fischer Scientific, C3099) was used for cell viability staining.

2.3. Electrical recording and analysis

120 channels USB 2.0 MEA2100-System (Multi-Channel Systems, Fig. 3) were used for electrical recording, with 25 kHz sampling frequency and ±10 mV input range.

The MEA chamber was placed in the head-stage and signal baselines were recorded in 0.09% glucose buffer for 300 seconds, thereafter the buffer was replaced with 0.9% glucose and recorded for 300 seconds for spontaneous cell activity. After that, a biphasic electrical pulse from an integrated pulse (stimulus) generator was applied for a duration of 200 µs with different amplitudes (250, 500, 1000 and 2000 mV) and frequencies (16.67, 100, 200 and 333.33 mHz). Records were then analysed for point of shape, frequency, and amplitude (MC_Rack, Multi-Channel Systems).

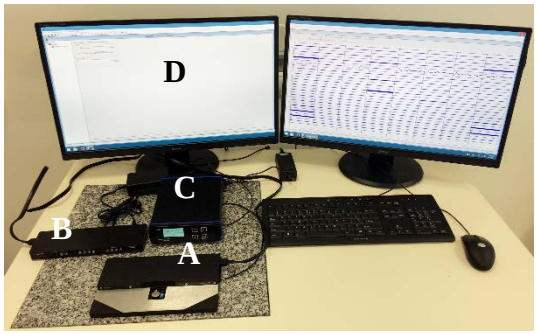

Figure 3: USB MEA2100 System measurement setup. A) Chamber holder with integrated amplifier and pulse generator. B) Interface board. C) Temperature control. D) MC Rack software with real-time signal visualisation and analysis.

3. Results and discussion

The neonatal cardiomyocyte display adhered to all of the coated surfaces in the MEA chamber, creating a typical cell shape (Fig. 4), but the ratio of adhered to non-adhered cells was different depending on the coating.

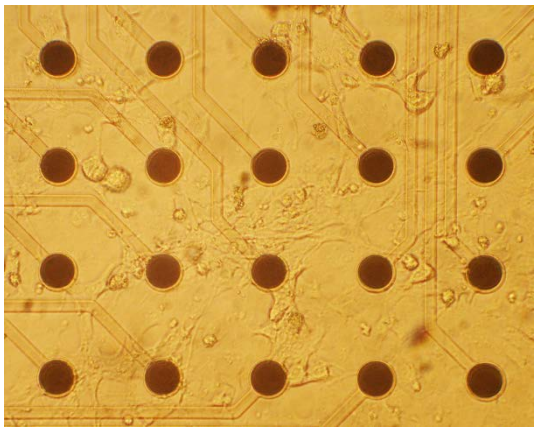

Figure 4: Neonatal cardiac cells cultured for five days on PDL+fibronectin 30/100 µm MEA chamber

The PDL+fibronectin surface treatment displayed the highest levels of cardiomyocyte adhesion to the electrode surface (20% of cells on 30/100 µm chamber, as shown in Fig. 4; 18% on 10/30 µm chamber respectively) after five days of pre-cultivation. Pure PDL also provided good cell adhesion (about 15%). Collagen I and PLL displayed the weakest interactions with the surface, showing very low cell adhesion (0.5~5%).

The viability of the adhered cells after five days was >95% (Calcein AM method, illustrated in Fig. 1). The pulse stimuli were always delivered through the neighbouring electrode to the electrode with the adhered cells. The developing of functional contractive structures in the first few days after seeding was proved by alfa-actinin staining (Fig. 1 – right window) and also by visual detection of rhythmical spontaneous pulsing of some cells. No spontaneous electrical activity was detected during records analysis, however; only evoked potentials were identified.

Table 1: 30/100 µm chamber neonatal cardiomyocytes adhesion

Surface treatment	Mean adhesion	Evoked response	Desensiti-sation
Collagen I	5%	No	No
PLL	1%	No	No
PDL	16%	Yes	No
PDL+fibronectin	20%	Yes	Yes

PDL+fibronectin surface treatment on 30/100 µm chamber enabled the development of contractible cells which were electrically deactivated (desensitized) with ≥ 500 mV pulses at 16.67 mHz frequency and recovered after 10 minutes in 0.9% glucose buffer.

Table 2: 10/30 µm chamber neonatal cardiomyocyte adhesion

Surface treatment	Mean adhesion	Evoked response	Desensiti-sation
Collagen I	3%	No	No
PLL	0.5%	No	No
PDL	15%	Yes	No
PDL+fibronectin	18%	Yes	No

Only the PDL+fibronectin and PDL samples allowed the creation of a measurable cell response. The PDL-only samples were lower in amplitude than those combined with fibronectin. Collagen I and PLL displayed very low cell adhesion (0.5~5%) with no visible response. We therefore hypothesise that the main reason could be the low stability of both the collagen and PLL layers on our chamber's bottom surface.

The amplitude of cell response to single external stimuli from 30/100 µm MEA chamber was 198±18 mV in the case of the PDL+fibronectin sample and 179±12 mV in case of pure PDL. Because the MEA chamber was not fully covered with cells, the evoked cell response could not be detected on all channels.

In this study, the 30/100 µm MEA chamber with PDL+fibronectin coating was the most convenient.

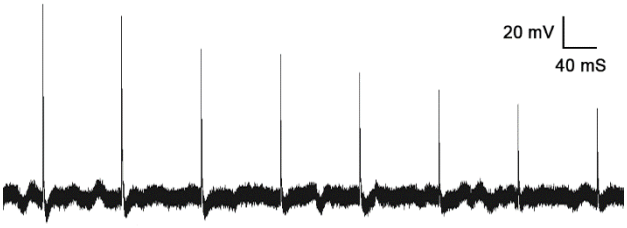

Figure 5: Cell desensitization (500 mV, 16 mHz external stimuli)

The response amplitude from the 10/30 µm chamber was always lower (15~20%) than in 30/100 µm one. This was due to the smaller active electrode area and higher input resistance of the 10/30 µm chamber. In these samples, we did identify that one cell adhered to more than one electrode (2-4) several times. In these cases, the same response could be detected on parallel channels with different amplitudes (due to the different quality of cell adhesion). Unfortunately, time variances caused by different longitudinal and transverse cell membrane depolarisation conductivity could not be identified due to the low sampling frequency.

Our results reveal the importance of carefully selecting the culture substrate for cardiomyocyte adhesion, survival, and physiology. In future, simple coatings of collagen I, PDL, PDL +fibronectin and PLL should be tested over a longer time period after cardiomyocyte seeding. We also wish to investigate another modern coating based on naturally occurring complete extracellular matrix (a sophisticated mix of collagen and other components, which mimics the actual extracellular matrix in the heart).

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