

Effects of Cell Culture Flexible Substrate on Cardiomyocytes

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Abstract

Major considerations in the design of engineered cardiac patches are the regenerative and electrophysiological properties of cardiac cells. The objective of this study is to compare key cardiomyocyte functional properties for cardiac repair such as proliferation, migration and displacement, and conduction velocity of atrial murine cells (HL-1-myocytes) in two different substrates: (1) rigid Petri dishes and (2) flexible PDMS (polydimethylsiloxane) wells. Results from this study confirm that the use of flexible substrates induces a more similar cardiac phenotype to that shown in vivo, especially characterized by higher conduction velocities. This result was supported by a higher expression of genes involved in action potential generation and propagation. Flexible membranes will allow further studying the effect of simultaneous mechanical and electrical stimulation on cardiac properties in vitro.

1. Introduction

Cardiovascular diseases are the first leading cause of death worldwide, being ischemic heart disease and myocardial infarction the most common [1]. If cardiomyocyte depletion occurs, it can cause further heart failure, as remodelling occurs with heterogeneous tissue. This tissue presents lower conduction velocities that provoke asynchronous electric conduction, thus increasing the risk of future re-entries that can lead to cardiac arrhythmias [2].

Engineered cardiac patches seem a promising tool to solve this problem. Furthermore, they allow adequate simulation of cardiac tissue dynamics *in vitro* to obtain models and realistic responses of pharmacological agents and therapies. To serve those purposes, cardiac patches must have optimal structural, mechanical and electrophysiological properties, and to populate them, cell proliferation, migration and displacement are also important. Myocardial tissue has complex architecture and cell interconnections, and to mimic the Extra Cellular Matrix (ECM) environment is one of the main challenges of tissue engineering, as it is crucial to obtain *in vivo* like phenotypes, such as higher conduction velocities and more efficient and synchronous contractions [3].

The purpose of this work is to study and quantify the effect of using flexible membranes as substrate on different properties that play a key role in cardiac patches development and functioning. The flexible membranes employed are PDMS (polydimethylsiloxane) wells. Its

flexibility is closer to that of the ECM in contrast to conventional Petri dishes, providing a more *in vivo*-like environment. The use of these membranes will also offer the possibility of studying the impact of mechanical and electrical stimulation during cell culture, which may result in further enhancement of these desired properties.

2. Methods

2.1. Cell culture

HL-1 cells were maintained, grown and proliferated according to the standard protocol established by Claycomb et al. [4].

2.2. Proliferation assay

To study proliferation of HL-1 cells, alamarBlue® (oxidation-reduction colorimetric indicator) was used. Cells were seeded in three p35s (Petri dishes) and three PDMS wells at an initial concentration of 35000 cells/cm² on day 1 and cultured with 208μL of medium/cm². From day 2 to day 5, equal amount of media was changed at 5 pm and incubated overnight. In the following morning, alamarBlue® was added at 9 am in a concentration of 1 μL/10 μL of medium and left in the incubator for 4 hours. After this time, 300 μL of each sample (medium + alamarBlue®) were loaded into 96-well plates (100 μL/well, 3 replicates). Absorbance for each well was measured using EMax® Plus Microplate Reader from bioNova científica. In order to obtain the percentage of reduction (marker of proliferation) from absorbance at two different wavelengths, the formula in the product specifications was applied.

2.3. Migration and displacement

Migration and displacement properties of HL-1 cells were measured by quantifying the time needed by the cells to close an injury. Specifically, a linear wound was generated after reaching full confluence by scratching with the tip of a pipette in three p35s and three PDMS wells. After wound induction, cell migration and displacement were recorded using time-lapse equipment (Lumascope 400 iVue, etaluma) by taking an image every 5 minutes during 60 hours. To process those images, custom software in MATLAB (The MathWorks) was developed to obtain graphs of confluence over time in the wound area and displacement velocity values.

2.4. Conduction velocity

Four confluent samples of HL-1 on each substrate were employed to measure electrophysiological conduction velocity. Specifically, calcium transients were recorded using optical mapping technique, and results were further analysed by custom software developed in MATLAB to obtain impulse propagation velocity measurements.

For calcium transient imaging, each HL-1 sample was incubated for 30 minutes in 1.5 mL of Tyrode solution, 8 μ L of Probenecid and 5 μ L of rhod-2 dye. After several washes, in order to excite rhod-2, cell cultures were illuminated with two filtered green LED light sources (CBT-90-G, peak output 58W, peak wavelength 524nm; Luminus Devices, Billerica, USA), with a plano-convex lens (LA1951; focal length=25.4mm, Thorlabs, New Jersey, USA). The green excitation filter used was D540/25X (Chroma Technology, Bellows Falls, USA). Fluorescence emitted was recorded using an EMCCD camera (Evolve-128: 128x128, 24x24 μ m-square pixels, 16 bit; Photometrics, Tucson, AZ, USA), with a custom emission filter (ET585/50-800/200M; Chroma Technology) in front of a high-speed camera lens (DO-2595; Navitar Inc., Rochester, USA).

Once electrical activity was confirmed, lines were made in the cell culture by scratching in order to allow electric conduction to happen only in a certain direction. This simplifies the electric activity and eases future measurements. Moreover, cells were stimulated by bipolar point stimulation with two electrodes in order to produce a linear propagation over the cell culture.

The movies were further processed in custom software developed in MATLAB. In order to obtain conduction velocity values, two pixels are selected and their two fluorescence signals along time are correlated. With a calibration distance (spatial information) previously indicated and the electric impulse correlation distance (temporal information), conduction velocity is calculated. This process can be observed in Figure 1.

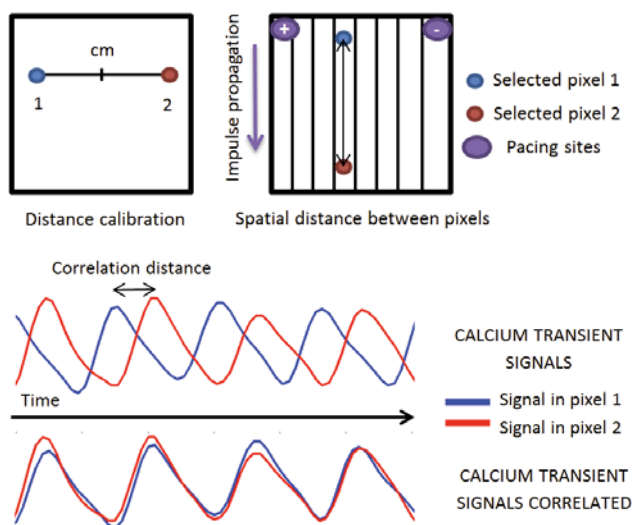


Figure 1. Process for calculating conduction velocity. (1) Calibration. (2) Pixel selection. (3) Correlation of fluorescence signal in time of pixel 1 and pixel 2 (4) Conduction velocity obtaining as Spatial distance (cm)/Correlation distance (s).

2.5. Gene expression analysis

In order to evaluate the effects of the cell culture substrate on main ion channels gene expression, RNA from three samples in each substrate was isolated using Tri-reagent (Sigma). Transcripts were quantified in a two-step RT-PCR. First strand cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The samples were later run using SYBR Green oligonucleotides and the CFX Real Time PCR detection Systems (Bio Rad). Analysis was performed with two technical replicates for each sample. Gene expression values were normalized to two standard housekeeping genes (36b4 and Cyclophilin) as internal controls, and expressed as relative mRNA levels (relative expression). Primer sequences are shown in Table 1.

Gene	Protein	Forward Primer (5'→3')	Reverse Primer (5'→3')
SCN5A	Nav 1.5	CACCTTCACGCCATCTACA	AAGGTGCGTAAGGCTGAGAC
CACNA1C	Cav 1.2	CCTCGAAGCTGGGA GAACAG	TGTGTGGGAGTCAATGGAGC
KCNJ2	Kir 2.1	GACGCCTTCATCATTGGTGC	CCGGACATGAGCTTCACAA
GJA5	Con 40	ATACCATTACGCTGTGTTGC	GGTGGGCCTCTTTAGCTTC
GJA1	Con 43	GGACTGCTTCTCTCACGTC	CAGCTTGACCCAGGAGGAG
GJA7	Con 45	TTTGTGTGCAACACAGAGCA	GGTCTCTTCCGTTTCTTCC
36B4		GCGACCTGGAAGTCCAACATA	ATCTGCTGCATCTGCTTGG
CYCLOPHYLIN		ACAGGTCTCTGGCATCTTGTC	CATGGCTTCCACAATGTTCA

Table 1. Primers used for reverse transcription polymerase chain reaction (RT-PCR).

3. Results

3.1. Proliferation

HL-1 cells were able to grow, attach and keep spontaneous contraction over both substrates. In fact, proliferation was high because of the cell tumorous origin [4], as 100% of reduction was achieved within 6 days. Although not significant differences were observed, in Petri highest proliferation rates occurred at early stages of the cell culture, whereas in PDMS at late stages. Moreover, in Petri dishes a more reproducible response was observed, as in PDMS wells standard deviation between the three samples was considerably greater.

3.2. Migration and displacement

The ability of HL-1 cells to close induced wounds was demonstrated to be limited over both substrates, as it is characteristic of adult cardiomyocytes. After 60 hours, none of the injuries were completely closed in any of the materials (Figure 2). The analysis of maximum displacement velocities (i.e. 0.40 μ m/min in PDMS and 0.25 μ m/min in Petri) demonstrated the reduced ability of these adult cardiomyocytes to migrate, suggesting that these cells colonize only by proliferation.

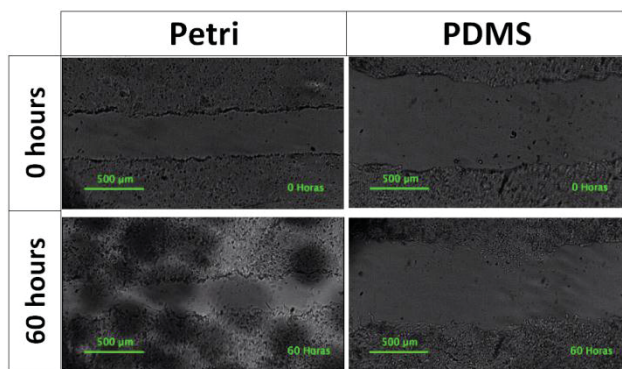


Figure 2. Wound test images in both substrates at two different times.

3.3. Conduction velocity

A representative example of isochrones maps for Petri and PDMS generated from the optical mapping movies data is shown in Figure 3. Uniform impulse propagation can be observed in both bands, confirming that the cells were confluent and that conduction followed a straight path. Notice that the time impulse takes to reach the end of the band in the PDMS well is shorter than in the Petri dish, suggesting faster conduction velocities.



Figure 3. Isochrones maps of a significant band in each substrate.

Average conduction velocity values of HL-1 cells grown in Petri and PDMS can be observed in Figure 4. Impulse propagated significantly faster when cells were grown in flexible membranes as the conduction velocity was 156% greater in PDMS wells (2.4 cm/s) than in Petri dishes (1.5 cm/s).

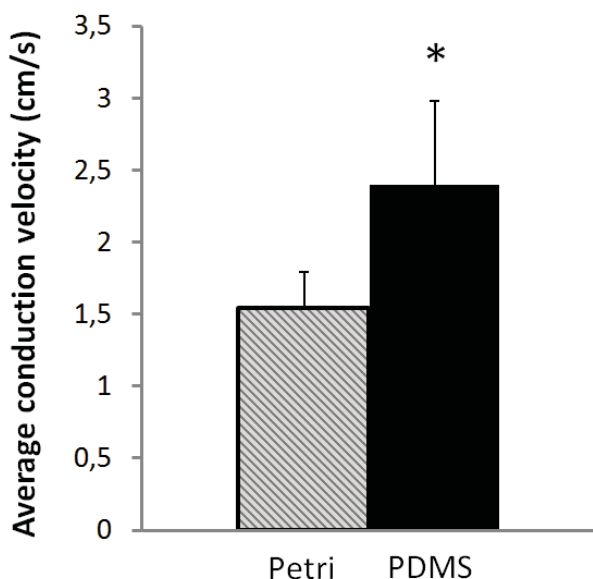


Figure 4. Average conduction velocity for each substrate. Differences are statistically significant when $p < 0.05$ (*) (TTest).

3.4. Gene expression analysis

The gene expression analysis supported what was observed at a functional level, as genes analysed codifying for proteins involved in action potential generation and propagation were higher expressed in cells grown over PDMS wells (Figure 5).

A higher expression of genes codifying for molecular components of sodium, calcium and potassium channels (SCN5A, CACNA1C and KCNJ2) and of genes codifying for connexins forming part of gap junctions (GJA5, GJA1, GJA7), may indicate a greater presence of these proteins in HL-1 cells. These results confirm the influence of the flexible substrate on the gene expression corresponding to proteins related to electrophysiological cardiomyocytic properties.

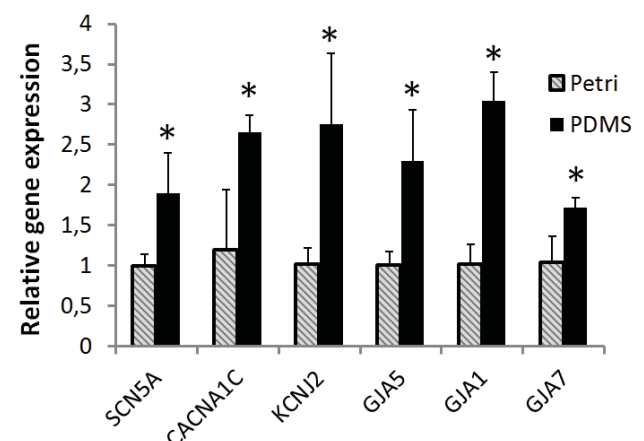


Figure 5. Expression levels of ion channels in HL-1 cells grown in PDMS versus Petri substrates. SCN5A, CACNA1C and KCNJ2 genes codify for proteins which are subunits of ion channels associated to I_{Na} , I_{CaL} and I_{K1} respectively. GJA5, GJA1, GJA7 genes codify for components of the gap junctions: connexin 40, connexin 43 and connexin 45 respectively. Differences are statistically significant when $p < 0.05$ (*) (TTest).

4. Discussion

4.1. Main contributions

The results of this study demonstrate that the culture of cardiac cells on flexible membranes has an impact on their phenotype. This impact has shown to be especially significant in electrophysiological properties, as conduction velocity has increased considerably. The similar flexibility of PDMS with that of the ECM may be the reason behind the gene expression profile more characteristic of adult cardiomyocytes. These changes in gene expression may have led to a higher presence of gap junctions and ion channels that ease impulse generation and propagation.

Proliferation, migration and displacement have not shown relevant differences between both substrates. Proliferation was in general elevated because of the nature of HL-1 cells, while migratory capacity and displacement were nearly absent in both substrates as it is characteristic of adult cardiomyocytes. From the data obtained, it could be concluded that HL-1 cells colonize merely by

proliferation. This low ability to repair wounds may pose further problems for the population of cardiac patches.

The use of flexible membranes opens new insights into cardiac patches for cardiac regeneration, as obtaining higher conduction velocities while maintaining other general cell properties is possible. As impulses propagate faster, the possibility of future re-entries will diminish and the patch will integrate better within healthy tissue. Flexible membranes also allow future studies of electrophysiological properties during cell culture under simultaneous mechanical and electrical stimulation, which is otherwise impossible with rigid Petri dishes. These conditions may improve characteristic cardiomyocyte properties even further, leading to better quality patches.

4.2. Comparison with previous studies

Conduction velocities in rat ventricular myocyte monolayers are close to 25 cm/s, but *in vitro* growing of cardiac cell lines and iPSCs (induced Pluripotent Stem Cells) has shown very inferior speeds (1-2.5 cm/s). The use of flexible membranes had already been suggested to have an impact on impulse propagation, as maximum conduction velocity obtained *in vitro* has been 21 cm/s using genetically purified human cardiac myocytes and flexible membranes together [5]. However, the impact of flexible membranes alone on electrophysiological properties was carried out through this systematic study.

4.3. Applications

The generation of cardiac patches that mimic *in vivo* properties have two main applications: (1) as therapy for cardiac regeneration of scar tissue after an infarct and (2) as models of cardiac tissue for *in vitro* drug and therapy testing.

In order to serve as therapy, one of the main limitations of current cardiac patches is their low conduction velocity values. In addition to that, cells which present high proliferation and migration capacity are required in order to populate the patches effectively. To obtain considerable migration capacity, immature cardiomyocytes such as cardiopoietic cells or iPSCs not terminally differentiated into cardiomyocytes should be contemplated. To reach higher conduction velocity values these immature cells should be further differentiated.

The use of these patches as realistic models of *in vivo* behaviour offers many advantages. First, they may reduce the number of animals and the time required. Secondly, they ease procedures and present better simulation of cardiac tissue dynamics, as conduction velocities of HL-1 cells grown over flexible membranes are closer to *in vivo* values. Low migration capacity observed also mimicks accurately adult cardiomyocyte behaviour.

4.4. Limitations

The results obtained for conduction velocity measurements, although significantly higher for flexible membranes, are still far from *in vivo* impulse propagation

velocities. This may be due to the limited number of cells interconnected in monolayers. The use three dimensional *in vitro* structures such as decellularized matrices could contribute to the improvement of cardiomyocytic properties, as it provides an ideal environment for cells to grow [6].

With the objective of clarifying if a higher expression of the studied genes has led to a higher presence of proteins related to unique cardiomyocyte properties it will be convenient to use direct protein detection techniques. This is necessary to confirm that this higher protein presence is the responsible of the higher conduction velocity values.

5. Conclusions

The use of flexible membranes has shown to play a role in cardiac cell phenotype, especially inducing faster impulse propagation. At the molecular level, expression of genes codifying for proteins involved in ion channels and gap junctions were also more expressed in cells grown over flexible membranes, suggesting that this may be the cause behind the higher conduction velocity measurements.

These results open new insights into cardiac patches, as its use allows further research on the impact of simultaneous electrical and mechanical stimulation during cell culture, mimicking even better the *in vivo* environment of cardiac cells.

Acknowledgements

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