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Evaluation of cell culture in microfluidic chips for application in monoclonal antibody production

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ABSTRACT

Microfluidic chips are useful devices for cell culture that allow cell growth under highly controlled conditions, as is required for production of therapeutic recombinant proteins. To understand the optimal conditions for growth of cells amenable of recombinant protein expression in these devices, we cultured HEK-293T cells under different microfluidic experimental conditions. The cells were cultured in polymethyl methacrylate (PMMA) and polydimethylsiloxane (PDMS) microdevices, in the absence or presence of the cell adhesion agent poly-D-lysine. Different microchannel geometries and thicknesses, as well as the influence of the flow rate have also been tested, showing their great influence in cell adhesion and growth. Results show that the presence of poly-D-lysine improves the adhesion and viability of the cells in continuous or discontinuous flow. Moreover, the optimal adhesion of cells was observed in the corners of the microchannels, as well as in wide channels possibly due to the decrease in the flow rate in these areas. These studies provide insight into the optimal architecture of microchannels for long-term culture of adherent cells in order to use microfluidics devices as bioreactors for monoclonal antibodies production.

1. Introduction

Microfluidics allows miniaturization of basic conventional biological or chemical laboratory operations. Lab-on-a-chip technology has been well accepted by biological and medical research communities as a promising tool for engineering microenvironments at molecular, cellular and tissue levels [1]. In the early 1990s the first microfluidic devices for biochemical applications were developed, and since then the field has been rapidly expanding [2,3]. These microfluidic chips have been used on a broad range of cell-oriented applications including monitoring cellular activity [4], cell-based assays to test drug sensitivity [1], cell-free protein synthesis [5] or monoclonal antibodies production [6,7] among others.

In contrast to conventional static approaches, microfluidic-based cell cultures are not only able to maintain well defined cell culture conditions, but more importantly, allow to continuously provide cells with fresh media containing oxygen, carbon dioxide and nutrients while removing metabolic products at a controlled flow rate [1,4,8].

Alternatively to early glass microfluidic chips, today polymers have become the popular choice offering a wide range of chemical and mechanical properties as well as better biocompatibility [2,9–11]. Commonly employed polymers are polydimethylsiloxane (PDMS) and polymethyl-methacrylate (PMMA). Garza-Garcia and collaborators engineered a chip with PMMA body and PDMS cover plate to produce the monoclonal antibody Infliximab [6]. Recombinant monoclonal antibodies are used for treatment grave diseases including autoimmune disorders and cancer [12], becoming one of the fastest growing areas in biopharmaceutical industry. Currently, monoclonal antibodies commercial production and other biotherapeutics are based on the synthesis in bioreactors with suspended mammalian cells with agitation operated in fed-batch or perfusion mode [13,14]. The monoclonal antibodies production in stirred tanks faced challenges related to product quality and process such as demand for higher productivity, glycosylation control and reproducibility. Most of these challenges are related to large spatial and temporal variability of intrinsic fermenters conditions. One way to improve control is to reduce the scale of the system by miniaturization in the form of micro devices [15]. A micro device provides several advantages, including shorter time response, a higher surface/volume ratio and a more homogeneous and controllable microenvironment.

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Moreover, PDMS devices offer surfaces that can be modified through oligopeptides, polysaccharides, proteins adsorption or via plasma processing to obtain specific surface features. Mimicking extracellular matrix is a challenge that has been addressed by texturing microchannels with fibronectin [6,7] and collagen [4,16] to achieve a better surface attachment of cells.

In this study, antibody production cells HEK-293T [17] were cultured in PMMA and PDMS microchannels in presence or absence of cell adhesion agent poly-D-lysine to assess their compatibility for mammalian cell culture and the effect of microchip geometry on cell growth. Coating with poly-D-lysine was chosen because it increases the number of positively-charged sites on chip surface, enhancing electrostatic interactions with the negatively charged groups on cell surface, therefore improving adsorption while preserving biological activity.

2. Materials and methods

2.1. Microfluidic devices design and fabrication

Two different microfluidic devices have been designed using Layout editor software (http://www.layouteditor.net). The first chip consists in three different microchannel shapes 40 μm height × 0.4 mm width (linear, zigzag and square waves), with an internal volume of 3.68 μL (Fig. 1A).

In the second microfluidic chip, channels of 40 μm height, with different serpentine shapes 100 μm wide and lengths between 12 and 80 mm, feed by a central channel (40 × 1.9 mm), and spacer channels (12 × 1.2 mm) between serpentines, were designed. The internal volume is 17.8 μL (Fig. 1B). These different microchannel shapes and widths have been designed and fabricated to test if cell adhesion and growth depend on geometry.

The microdevices were built in PDMS. To do this, a mold of the design in high relief was made by photolithography in a silicon wafer 700 μm thick (Virginia Semiconductor, Inc.), by using the negative resin SU-8 (MicroChem). The silicon substrate was cleaned by sonication in acetone and isopropylic alcohol, and substrate surface was dehydrated for 10 min at 200 °C. Then, SU-8 resist was dispensed on the substrate and spun in two cycles. The spinner was accelerated for 5 s at 100 rpm·s^{-1} until 500 rpm, and held at 500 rpm for 5 s. In the spin cycle, a ramp of 300 rpm·s^{-1} was applied until 2000 rpm, and held for 30 s. The resist was soft baked firstly at 65 °C for 20 min, and secondly at 95 °C for 50 min. The substrate was aligned and the resist was exposed to near UV at 650 mJ. The first step of a post-exposure bake consisted on 65 °C for 12 min, and the second step at 95 °C for 15 min. Finally, the resist was developed for 15 min under agitation. PDMS chip fabrication steps are described in supplementary material. The first chip was also constructed from PMMA, which was manufactured using a Class 2 CO_2 laser etching system (Megalaser ML-609), operating at 60 W and 200 mm·s^{-1} scan speed. Designs of the plano-convex lenses were created using Layout editor software, interfaced directly with the CO_2 laser. Finally, the inlet and outlet of the microdevice linked the microchannels with a syringe needle.

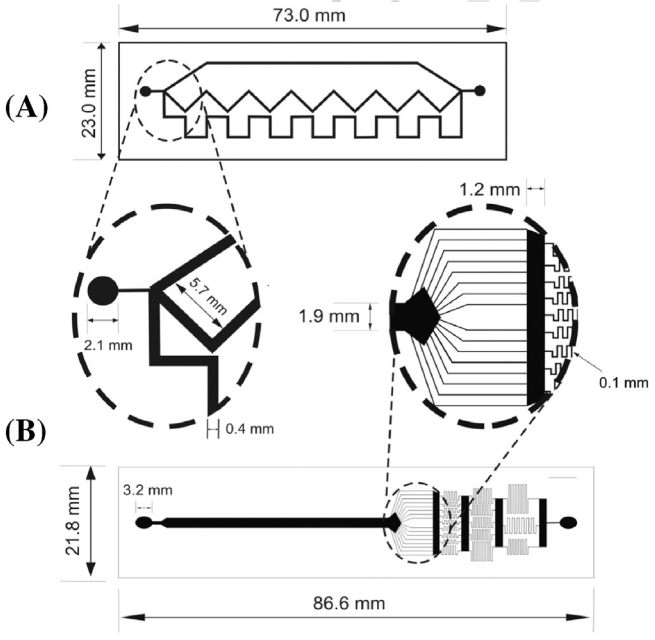

Fig. 1. Microfluidic chips designs (A) lineal, zigzag and square wave microchannels, internal volume of 3.68 μL, (B) serpentine microchannels, internal volume of 17.8 μL.

Two flow types have been used: continuous and discontinuous. Continuous flow experiments consisted in connecting microfluidic chips to a peristaltic pump (APEMA) and a bubble trap using PVC tubes in a recirculation mode (See a scheme in supplementary material), this set-up was inserted into an incubator at a constant temperature of 37 °C, whereas discontinuous flow studies were carried out by renewing culture medium each 24 h. Cells were cultured in an incubator (Ciberbay) that is commonly used to incubate eggs as it allows to control temperature and humidity (See supplementary material).

2.2. Cell culture and distribution

The microfluidic chips were disinfected using NaOH 0.1 mol·L^{-1} for 24 h, and then rinsed with sterile water. Before cell seeding, chip was treated with poly-D-lysine hydrobromide 0.1 mg·mL^{-1} (Sigma) sterile solution to improve cells attachment. The microdevice was incubated with poly-D-lysine solution for one hour at 37 °C. The solution was then removed and let dry 24 h at 4 °C. HEK-293T cells (ATCC CRL-3216) were cultured in complete DMEM medium (Gibco), supplemented with fetal calf serum heat-inactivated (FBS) 10%(w/v) (Internegocios SA), L-glutamine 2 mmol·L^{-1} (Gibco), penicillin 100 units·mL^{-1}, streptomycin 100 μg·mL^{-1} and fungizone 0.250 μg·mL^{-1} (Gibco) at 37 °C in an incubator with 5% CO_2. Cells were resuspended with trypsin 0.50 mg·mL^{-1} and EDTA-4Na 0.2 mg·mL^{-1} (Gibco), and incubated at 37 °C for 3 min. Trypsin was inactivated with FBS and cells were washed with phosphate buffer solution (PBS) (NaH_2PO_4 50 mmol·L^{-1}, NaCl 300 mmol·L^{-1}, pH = 7.6) and centrifuged at 1000 rpm for 5 min. Finally, cells were resuspended in the same complete DMEM medium, supplemented this time with 20% FBS at 10^7 cells·mL^{-1}.

The microchannels and PVC tubes were filled with 15 mL complete DMEM medium, and the system was purged for 2 h. Next, HEK-293T cells in suspension were seeded into the syringe needle in the inlet of the microfluidic device. Cells were allowed to settle and microfluidic device was incubated at 37 °C in incubator overnight. A flow rate of 5 μL·min^{-1} was applied to the peristaltic pump to constantly refresh DMEM medium, so each channel flow rate was 1.67 μL·min^{-1}. The microchannels were visualized using an inverted Olympus microscope CKX41. Brightfield images were taken with Olympus objectives LUCPlan FLN 40×/0.60; LCAch N 20×/0.40; PlanC N 10×/0.25; and PlanC N 4×/0.10 with an Olympus QColor 5; and processed with QCapture Pro 6.0 software. Cell density inside microchannels with linear, zigzag, or square wave (Fig. 1A) treated with poly-D-lysine was quantified. After two days incubation with peristaltic pump, cell density in each configuration was determined through quantification using Open CFU software [18] of 10× images acquired in triplicates. Bar graph analysis was performed with Excel and the significant differences analysis between the microchannels was made by *t*-test with the Statistica Package software.

The analysis of cell distribution over time in multiple configuration microdevices was determined through area quantification using Image J software of 4× images acquired in quadruplicates for wide channels and serpentine channel overtime, respectively. Bar graph and

significant differences analysis between microchannels was made by *t*-test performed with Excel (See supplementary material).

3. Results and discussion

The study of cell adhesion in the PMMA microdevice, in presence of cell adhesion molecules such as poly-D-lysine, has been carried out. In absence of poly-D-lysine, cells did not attach on any surface of the PMMA microdevice channel and were washed away. In contrast, when poly-D-lysine was present, cells were attached to the bottom of the microchannel, as it can be seen in Fig. 2A (see videos in supplementary material depicting cell flow in absence or presence of poly-D-lysine). In the case of adhered cells, 5 $\mu L \cdot min^{-1}$ continuous-flow culture medium was provided by peristaltic pump.

Since presence of flow washed away cells in the chip without poly-D-lysine we tested whether discontinuous flow allowed cell adhesion. HEK-293T cells were seeded in a PDMS microdevice without poly-D-lysine addition and in discontinuous flow with fresh media pulses every 24 h (Fig. 2B). Although most of the cells were washed away by the first discontinuous flow, after 24 h some cells attached to channel walls instead of glass floor. Cells remained attached to the channel walls after the second day even though PDMS does not have intrinsic charge. It is possible that the mild texture of walls provides enough support for cells to attach, but they did not grow further.

In contrast, when poly-D-lysine was present in PDMS chips, cells showed good adhesion to the bottom. In one of the chips, three different microchannel geometries (lineal, zigzag and square wave, Fig. 1A) were assessed. Before starting flow rate, cells settled and the distribution was homogeneous trough all microchannels. When flow rate started to run, it was observed that zigzag and square waves configurations presented cell accumulation at corners due to the lower local flow velocity and vorticity field in these areas, facilitating cell deposition and attachment, as previously described by Garza-Garcia et al. [6,7]. As expected, linear channel showed less cell adhesion. Fig. 2C shows differences in cell density after two days incubation in different microchannel configurations. As thought, the lineal channel showed significantly lower cell density compared to zigzag and square wave channels. This could be associated to the fact that linear microchannel exhibit the fastest lineal speed ($1.1\ mm \cdot s^{-1}$) in comparison with zigzag ($0.57\ mm \cdot s^{-1}$) and square waves ($0.47\ mm \cdot s^{-1}$), which have the lowest lineal speed. We also observed that there was a significant difference in density ($p < 0.0001$) between zigzag versus square waves microchannels.

Different serpentine designs separated by wide channels were tested in a PDMS microdevice in a discontinuous flow rate. In this case, after 16 h of cells deposition, the DMEM culture medium was renewed each 24 h under 5% CO_2 and 37 °C (Fig. 3).

Under this condition, at seeding day (day 0) cells were not distributed uniformly through all microchannels (Fig. 3). As expected, it was observed that cell localization is more frequent in the wide channels than in the serpentine channels. In fact, at day 0 cells cover a significantly larger area (1.5 higher) in wide channels than serpentine channels. Two days after cells seeding, the most of cells in serpentine channels were washed and the individual cells were adhered to the wide channels bottom. High fluid velocity of the narrow microchannels seems to result in constant washing of cells, favoring their carrying and deposition to quieter areas (See supplementary material). Over time, cells began to form clusters and to extend processes around the clusters. At day five cells consolidate their growth in clusters on the floor of the wide microchannels, with almost no cells in the serpentine channels (Fig. 4), resulting an area covered by cells that is 29.5 fold higher in wide channels compared to serpentine channels (See supplementary material). At day eight, cells began to show signs of stress, with granulations in the cytoplasm and the experiment was concluded.

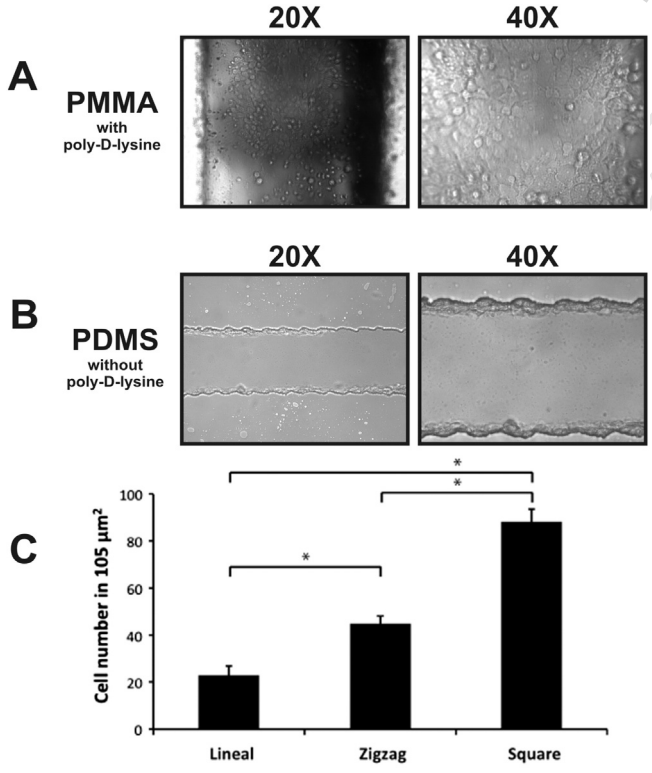

Fig. 2. Cells incubation on microdevices with and without poly-D-lysine coating. Panel A shows inverted microscope optical images of HEK-293T cells, attached to PMMA microchannel bottom. HEK-293T cells were attached to microchannels bottom, coated with poly-D-lysine ($0.1\ mg \cdot mL^{-1}$), at 37 °C. Panel B shows HEK-293T cells incubated for two days on a PDMS chip without poly-D-lysine coating. HEK-293T cells were attached to walls but not to glass floor. Cells were cultured under the following conditions: discontinuous flow, atmosphere of 5% CO_2, 37 °C. Panel C shows a bar graph of the quantification of HEK-293T cell densities in linear, zigzag and square waves microchannels geometries in PDMS chips coated with poly-D-lysine. Error bars indicate standard deviation of triplicate determinations and significance in difference was determined by *t*-test for non-paired data, with two tails and unequal variances. Significant difference is represented by * $p < 0.001$.

4. Conclusions

HEK-293T cells have been cultured in microdevices with different materials, microchannel geometries and experimental conditions. Usefulness of poly-D-lysine as cell adhesion molecule in microfluidic chips has been confirmed in both PMMA and PDMS materials. Besides, channel geometry significantly influences cells adhesion and growth,

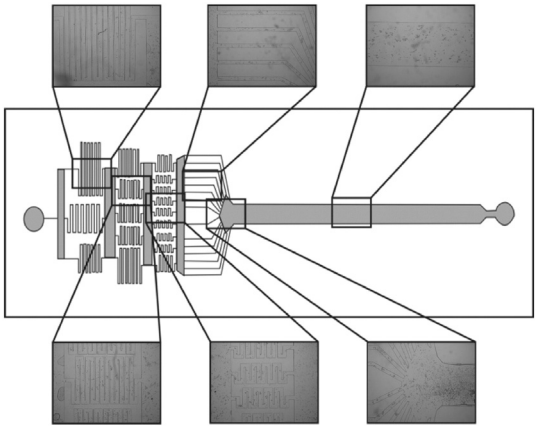

Fig. 3. Distribution of cells along microchannels with multiple configurations. HEK-293T cells were not distributed uniformly through microchannels on day 0. Microchannels were coated with poly-D-lysine and cells were cultured in discontinuous flow at 5% CO_2 and 37 °C.

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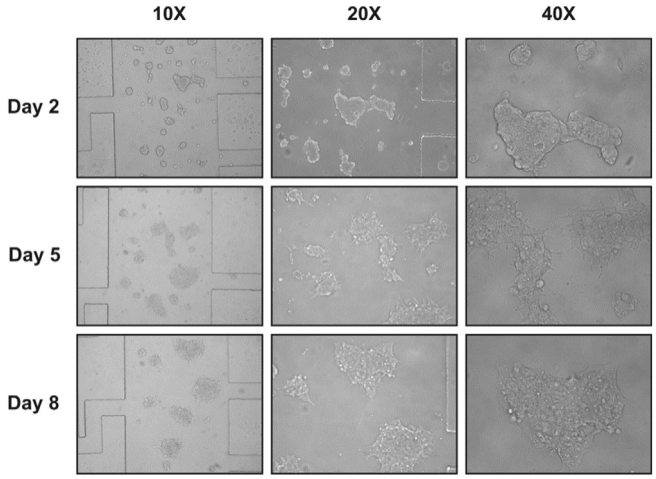

Fig. 4. Incubation of cells on PDMS microdevices with poly-D-lysine coating. Images of HEK-293T cells cultured for eight days attached to the bottom of the wide microchannels coated with poly-D-lysine.

showing that cells tend to grow in the microchannel corners, and consequently in the square waves channel. Moreover, in another chip with multiple configurations it has been observed that wide channels are more suitable for cell stabilization, providing adequate environment for cells to attach and grow. This seems to be associated to reduction in fluid speed in wide microchannels favoring cells retention, accumulation, and attachment.

This paper provides new insights in cells growth for monoclonal antibodies production, using microfluidics chips as potential bioreactors. These results show that HEK-293T cells can attach and grow in microfluidic devices preferably in areas with reduced flow for at least eight days, demonstrating the usefulness of microfluidics chips as bioreactors, which defines a direction for future work. Although we did not show antibody expression by the cultured cells, an extensive characterization of cell growth as well as antibody production study will be the subject of future publications.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mee.2016.03.059.

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