USING A MICROFABRICATED HYDROGEL TO STUDY THE EFFECT OF EXTRINSIC FACTORS ON DRUG RESPONSE

M. Håkanson1*, S. Kobel2, M. Charnley1, M. Lutolf2 and M. Textor1

1 Surface Science and Technology, Department of Materials, ETHZ, Zurich, SWITZERLAND and
2 Laboratory of Stem Cell Bioengineering, Inst. of Bioengineering, EPFL, Lausanne, SWITZERLAND

ABSTRACT

The emergence of drug resistance remains a major hurdle in cancer treatment. Therefore it is important to identify signaling pathways that lead to the occurrence of resistance, including signaling from the extracellular environment. Here we apply a microfabricated hydrogel as a tool to study the effect of extrinsic factors on drug response. This platform is compatible with high-resolution microscopy, which allows for analysis of cell health on the single cell level. An initial experiment using this hydrogel shows that the 3D organization of breast carcinoma cells is one factor that affects their response to treatment.

KEYWORDS: Cancer cells, 3D culture, hydrogel, drug resistance, confocal microscopy

INTRODUCTION

Recent research has strengthened the hypothesis that the extracellular context of cancer cells affects the progression of cancer [1]. In line with these findings, extrinsic factors have also shown to have an effect on the cellular response to treatment. Several in vitro studies have found that signaling from different constituents of the extracellular environment induces de novo drug resistance, which could be a pre-state to MDR (multi drug resistance) [2]. In order to better understand the relationship between anti-apoptosis signaling and the extracellular environment, predictive in vitro methods need to be developed.

Until today, in vitro studies of the effect of environmental parameters on drug response have typically focused on one parameter at a time, such as the interaction with matrix proteins [3] or the effect of enhanced cell-cell contacts in 3D organized cells [4]. However, it is likely that there is not one dominant factor but rather that there exist an inter-relationship between the different factors. Therefore we are working on an in vitro platform, with which it is possible to independently study the effect of several extrinsic factors. This platform consists of a microwell array molded into a polyethylene glycol (PEG) hydrogel [5]. The material properties of this hydrogel make it possible to mimic tissue-like stiffness. Hence, this platform allows the exploration of many parameters of the environment, such as the 3D organization of the cells, composition of the interfacing protein matrix and rigidity.

Typically, high content analysis of drug response has been performed on cells cultured in 2D. Therefore new assays or adapted assays need to be developed for the study of drug response in 3D cell cultures. While population based assays such as measuring the width of multi-cellular spheroids or solution-based measurement of metabolic activity are commonly used, high resolution imaging for analysis on the single cell level has been less applied. Here we present two methods to analyze the 3D cultured cells on a single cell level. The analysis of 2D confocal images is one method that has been used for all drug response data presented in this paper. The second method is to analyze 3D images, for which a matlab program was developed and tested.

EXPERIMENTAL

Hydrogel microwell arrays were prepared using a micro molding procedure as described previously [5]. In short, the PEG gel precursors were mixed at a stoichiometrically balanced ratio. The hydrogels in this work were prepared to have a polymer concentration of 5 %. Subsequently the PEG precursor solution was pipetted onto a microstructured PDMS stamp and a thin polystyrene slide (Ibidi, Germany) was placed on top. A 100-µm-thick plastic spacer assured a defined thickness of the gel. After 1 hour of polymerization the slide with the attached hydrogel was carefully peeled of the PDMS stamp, rinsed thoroughly and left to equilibrate overnight in PBS. Before cell seeding the arrays were sterilized 20 min under UV-light and incubated for 1 hour in PLL(20kDa)-g-[3.4]-PEG(2kDa) solution (0.25 mg/ml in PBS) to render the polystyrene non-adhesive to cells. Flat substrates were produced according to the same procedure, but using a flat PDMS stamp.

Matrix proteins may be attached to the bottom of the microwells in a microcontact printing procedure [5]. In this work we used collagen-I (non-functionalized) and laminin (functionalized with a PEG-maleimide linker). Solutions of proteins (0.3 mg/ml) were first incubated on a flat polyacrylamide gel, which was dried until no liquid drops could be seen on the gel surface. Then this gel was placed in contact with the PDMS stamp for 30 min. By this procedure the proteins will be incorporated into the gel during the molding step only at the bottom of the microwell. The quality of the coating was visualized with immunohistochemistry

MCF-7 breast cancer cells were seeded into the wells at a density of 750 000 cells / ml. After 1 hr of adhesion the samples were rinsed to remove the cells that had not assembled inside microwells. The cells were pre-cultured for 24 hrs to form dense cluster in the microwells. Thereafter 10 nM Taxol or vector only was incubated with the samples for another 24 hrs. After completed culture time the samples were rinsed with PBS, fixed in 2% paraformaldehyde and permeabilized with 0.1 % Triton-X100. Finally the nuclei were stained with the DNA binding dye Hoechst 33342. Images of the cell clusters were obtained using a Leica SP2-AOBS confocal microscope with a water immersion 0.7 NA 20x objective. Each 3D cell cluster was imaged at 3 different z-positions separated by 15 µm using a solid state 405 nm laser.
amount of apoptosis at the different positions was detected as the percentage of fragmented nuclei in the 2D images. The experiments were performed in duplicates and repeated three times.

Within the frame of this work we also developed a Matlab program for 3D object segmentation, based on the gradient vector flow algorithm proposed by Li et al. [6]. To test this program, images of nuclei in non-drug treated cells were obtained using the confocal microscope as described above. To render 3D reconstructions of sufficient quality, z-slices were obtained with a 1 μm spacing.

RESULTS AND DISCUSSION

Both collagen I and laminin were successfully coated onto the bottom of the PEG microwells as shown in figure 1. The laminin coating was more homogenous than the collagen I coating. This could possibly be assigned to the self polymerizing capability of collagen I. After 24 hrs of culture within the 100 μm wide microwells, MCF-7 breast cancer cells had formed dense clusters similar in size, figure 2. This result was obtained for both collagen I and laminin. It was found that cells organized in 3D clusters were significantly more resistant to Taxol treatment compared to cells cultured on flat substrates, figure 3. The cell death was 23 ± 3 % and 35 ± 4 % in microwells and on flat substrates respectively, \( p < 0.05 \). On the other hand, the presence of different matrix proteins, in this case collagen I and laminin, did not significantly affect the level of drug response, figure 3. Hence, the 3D organization seems to be the more important factor in governing resistance. Finally we determined the distribution of apoptosis throughout the cell cluster. In this experiment there were no significant differences in cell death ratios over the clusters (data not shown). With similar amount of cell death occurring inside and at the outside of the clusters we can assume that all cells were exposed to similar levels of the drug, indicating that the effect observed was not due to differences in drug uptake.

![Figure 1](image1.png)  ![Figure 2](image2.png)  ![Figure 3](image3.png)

**Figure 1.** Collagen I (A) and laminin (B) coating at the bottom of the microwells visualized by immunohistochemistry.

**Figure 2.** Hydrogel array containing 100 μm wide microwells coated with collagen I. MCF-7 cells form similar size clusters in the wells. The image is from 48 hours post seeding.

**Figure 3.** A. Cells cultured on collagen coated hydrogel show a higher response to Taxol on the flat gel (2D) compared to when grown in 100 μm microwells. This indicates that the 3D organization of the cells provides an anti-apoptotic signal. B. The matrix coating (collagen I and laminin) does not influence the response to Taxol in 3D grown cells.
All drug response data presented in this paper was obtained by 2D image analysis. In addition, a matlab program for automatic 3D object segmentation, based on gradient flow tracking, was developed and tested, figure 4. It was found that this method was superior to conventional methods in detecting touching objects. However, there are several disadvantages of the program such as the large amount of calculations needed for a single 3D image and lost objects due to different intensities of objects in an image. We propose that adding object discriminating functions in addition to improving the quality of the images would positively affect the results of this program.

Figure 4. A. z-reconstruction of a confocal image of the nuclei in a cell cluster. The cells were fixed and stained with Hoechst prior to imaging on a Leica confocal microscope. B. The output image of a matlab program for 3D object segmentation based on gradient flow tracking. The input image is shown in (A) and the different colors show the separation of the nuclei.

CONCLUSION

This work aims at the development of a tool to study the dependence of drug response on extrinsic factors. We show that the use of a hydrogel based microwell array is a promising approach to obtain detailed information of drug response in 3D cultured cells. The preliminary results demonstrate that the 3D organization of cancer cells induce an anti-apoptotic signal. We propose that cell culture platforms with better control of the extrinsic factors, such as this microwell array, can be useful in the development of new cancer therapies, as understanding and targeting signaling from the extracellular environment may be a way to overcome drug resistance.

ACKNOWLEDGEMENTS

The authors would like to thank the Competence Center for Materials Science and Research, Switzerland, CCMX, for their kind support of this work.

REFERENCES


CONTACT
*M.Håkanson. ETH Zürich, tel: +41-44-633 62 91; hakanson.maria@mat.ethz.ch

294