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UV-Imprint of micro-textured polymer films for biomedical disposables

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Abstract
In the life science sector, increasing demands on the reproducibility and accuracy of lab-on-chip systems require new manufacturing technologies for the production of polymer films with multifunctional surfaces. A mass-production capable and cost-effective way to pattern microfluidic films is the manufacturing process of UV injection molding. Therefore, the aim of the project is to develop microstructured polymer films with multifunctional surface properties by means of an injection molding UV plate-to-plate (P2P) replication process. For this purpose, a special film injection molding system was developed that allows filling with different formulations, curing by UV light and molding of the required microstructures. In respect to the application of the microstructured polyacrylate films in cell-based assays as well as biosensor disposables, microstructures of various geometries were designed and produced in the P2P process. In addition, cell tests were performed in this study in order to evaluate the biocompatibility and optical quality of the substrates, and to verify the functionality of the microstructures. Here, we show that the structured polyacrylate foil is a suitable substrate that can be implemented in plastic microfluidic chips for cell-based assays and biosensor disposables.

Keywords: replication, biomedical disposables, UV-Imprint

1. Introduction

The development of smart diagnostic medical devices, accompanied by a growing share of integrated functionalities and decentralization in their application (Point of Care), poses major challenges to the transformability of corresponding value chains in production. On the one hand, additive manufacturing of complex diagnostic medical devices requires new production technologies to address adequately the increasingly complex product functions in the future. On the other hand, small and medium-sized companies face enormous challenges in integrating completely new production technologies aimed at miniaturizing and extending the functions of existing product lines with regard to high-throughput systems and single-cell analytics.

For example, hot embossing and injection molding are currently used for the production of microfluidic films in particular. However, a major limitation of hot embossing is the long cycle time of at least 10 min, which is required regardless of the number of individual structures on the film for their replication. Injection molding has limitations in terms of miniaturization (mold filling capacity) and surface modification or functional integration. Compared to hot embossing and injection molding, UV-Imprint offers not only working under ambient conditions, but also a reduction of cycle time up to 50 % as well as flexibility regarding adjustment of the mechanical and/or chemical properties of the resulting polyacrylate film. However, shrinkage as a function of the formulation must also be taken into account when designing the molds for UV injection molding.

Therefore, we aim to develop UV replication as a P2P production process for microfluidic film fabrication. For this purpose, a new tooling concept for P2P UV injection molding has been developed, which enables the production of polymer films with multifunctional surface properties. The polyacrylate film was qualified for use for cell-based assays and biosensor disposables based on investigations of, for example, molding quality, transparency, shrinking, cytotoxicity test, cell staining, autofluorescence, live cell imaging.

2. Design and development of tool system

2.1 Layout microstructure and cell cultivation

In respect to the application of the microstructured polyacrylate films in cell-based assays as well as biomedical disposables, microstructures of two different geometries were designed and produced in the P2P process. The microstructures were 1) pyramidal wells (400x400x800 µm) for culturing 3D cell aggregates (e. g., tumor spheroids), and 2) arrays of 30x30x30 µm microwells for immobilising and culturing single suspension cells.

The structured films were combined with plastic microfluidic chips of specific geometries corresponding to the planned applications (Figure 1). The pyramidal microwells were integrated into a perfundable chip for long term cultivation of 3D tissue samples (µ-Slide III 3D Perfusion, ibidi). Supplementing the cultivation chambers of the chip with an array of the pyramidal microstructures makes it possible to generate spheroids directly in the chip by self-organisation of the cells. Besides, positioning of the spheroids in the microstructures facilitates optical analysis of the cells. Since the spheroids can not escape the field-of-view, the image acquisition and analysis can be automated. Similarly, single suspension cells can be immobilized in the arrays of the smaller, square microstructures. The immobilisation on an array facilitates an automated, high-throughput evaluation of cell dynamics by fluorescent or phase contrast microscopy, for example upon addition of drugs [1, 2].

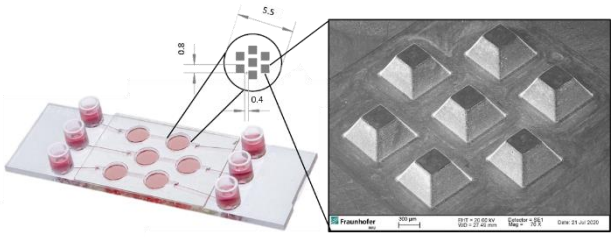

Figure 1. Structure 1: Arrays of pyramidal wells for spheroid culture under flow were integrated into the cultivation chambers of the µ-Slide III 3D Perfusion (ibidi)

2.2 Development of tool system

In order to meet the existing technological challenges, such as the realization of reliable filling and venting of the mold system as well as the implementation of the demolding device, the existing mold system [3] was fundamentally redesigned and further developed. Polyacrylate films were prepared using a customized mold made of tool-steel with a float glass slide on top, as shown in Figure 2.

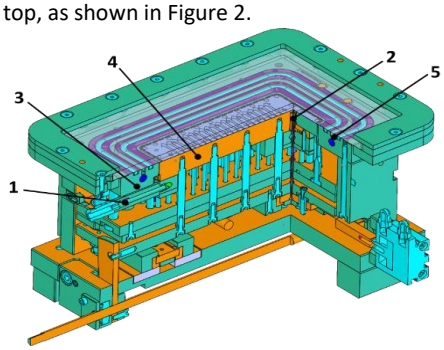

Figure 2. Developed tool system for plate to plate UV imprint

The mold consists of a mold base with integrated vacuum channels, ejectors, a lifting device for the center part and has a needle valve gated system for filling the mold. The mold can be filled via several acrylic filling channels (1), which are opened and closed by means of a needle valve gated system (2). The main mold insert (3), in which the microstructure to be molded is placed, was equipped with a movable center part (4) for multi-component filling. Due to the significant differences in microstructure layouts, various technologies were used to machine the microstructure. In particular, micromilling and µ-laser ablation in combination with laser polishing processes in special areas were used, as explained in [3].The inserts were coated with a non-adhesive coating (Dursan®, SilcoTek) and the glass slide was silanized with Dynasylan®F 8263 (abcr GmbH, Karlsruhe, Germany) in order to facilitate the demolding of the polyacrylate film.

The glass plate together with the frame is lowered onto the main mold insert. By applying a vacuum between the two sealing cords (5), the glass plate is sucked onto the main mold insert. This creates a mold cavity of 350 µm and closes the system. By opening the feed needles, the acrylate flows into the resulting mold cavity and is molded by closing without the feed point shown. Filling of the mold is ensured by injecting the acrylate through syringe pumps. In order to cure the first component, it is necessary to irradiate the acrylate with UV light at 395 nm (100 % intensity) moving the UV lamps with a velocity of 10 mm/s. Therefore, two UV LED lamps (12 W/cm^2, 395 nm, Firejet FJ 200, Phoseon Technology, Hillsboro, Oregon, USA) were mounted on a linear stage with a stepper motor in order to move the lamps over the mold. Vacuum, stepper motor, lamps and syringe pumps as well as pneumatic cylinder were controlled by a programmable logic controller.

After completion of this process step, the movable center part is lowered by 150 µm via a slide mechanism and pneumatic cylinder to release the second mold cavity for the second acrylate. This mold cavity is also filled by a needle valve gated system. Once the second UV irradiation is complete, the mold is ventilated and the glass plate is lifted off the bottom part by means of an ejector movement. Finally, the one-sided structured, multifunctional polyacrylate film can be removed. A more detailed description of the process steps is given in [4].

Since the final mold concept was developed iteratively, the polymer films were initially molded with corresponding test molds in the study. Therefore, the results presented below refer to polymer films molded with the test molds. For example, the acrylate mixture was injected into the mold using a plastic syringe connected to the acrylate filling channel.

3. Material and methods

3.1 Preparation of acrylate mixture

A typical acrylate mixture was composed of a urethane acrylate main compound at 40 wt% to 80 wt%, a diluting acrylate at 5 wt% to 50 wt% and a polythiol at 0 wt% to 15 wt%. Acrylate samples were received from Allnex (Allnex Germany GmbH, Wiesbaden, Germany), Sartomer (Sartomer, Cedex, France) and Miwon (Miwon Europe GmbH, Straelen, Germany). Polythiols were sample orders from Bruno Bock Thiochemicals (Bruno Bock Chemische Fabrik GmbH & Co. KG, Marschacht, Germany). 1 wt% of photo-initiator Omnirad TPO-L (IGM Resins, Waalwijk, Netherlands) was added to the mixture, followed by stirring for 30 minutes at 5000 rpm using a lab disperser. In a next step, air bubbles were removed by centrifugation (4700 rpm, 2 min) and degassing under reduced pressure (30 mbar).

3.2. Characterization of the polymer films for application in biosensor disposables

For application in biosensor disposables, the structured polyacrylate films were compared to reference standard hot-embossed polycarbonate (PC) samples. They had to fulfill the following requirements: high optical transparency, low shrinkage, high molding quality and mechanical properties comparable to those of polycarbonate, i.e. a high stiffness at moderate flexibility that makes the material well suitable for the preparation of mechanical stable sensors on the one hand, but without causing problems during the final cutting process on the other hand.

Molding quality, transparency and shrinking

Transparency, shrinking and filling behavior of the prepared films were investigated using a stereo microscope (Stemi 2000-C, Fa. Zeiss) combined with an additional video camera system (AVT-Horn, MC009/S). Shrinking of the polyacrylate film was measured by a glass scale of the type 1972-200 (Fa. M-Service & Geräte Peter Müller e.K., Meckenheim, Germany) enabling a resolution of 0.1 mm.

Wetting properties

Wetting properties of the double measuring chamber was monitored using a dye test solution containing a volume fraction of 0.01 % of the surfactant Triton X-100 (Sigma Aldrich GmbH, Steinheim, Germany) and Coomassie Brilliant Blue R-250 (Merck KGaA, Darmstadt, Germany). In order to ensure equal measurement conditions, the flow rate was determined in a defined section of the channel (here length of the working electrode). All measurements were analyzed by counting the frames of the video sequences (Software Debut Video Capture and Virtual Dub 1.9.11).

Mechanical properties

Brittleness of the polymer films was evaluated visually at defined cutting edges prepared by an Ideal 2035 paper cutter (Fa. Krug & Priester GmbH & Co. KG, Balingen, Germany). In

order to get information about the bending stiffness of the material, the force offered by an unstructured polymer stripe (50 mm x 7 mm x 0.3 mm) to deformation via bending of 180° was measured. Therefore, a custom-built device (SensLab GmbH) equipped with a force sensor (Type 9203, Fa. Kistler Instrumente GmbH, Sindelfingen, Germany) connected to a charge amplifier (Type 5995A, Fa. Kistler Instrumente GmbH, Sindelfingen, Germany) was used. The resulting bending force was normalized by the film thickness. The influence of selected monomers was evaluated systematically with Design of Experiments (DoE software Design Expert®, version 13, STATCON GmbH, Witzenhausen, Germany), and using an I-optimal mixture design, optimized parameters (maximum stiffness, no brittleness) were found for a composition of 50 wt% urethane acrylate, 40 wt% acrylic monomer and 10 wt% thiol.

Stability tests

Stability of the polyacrylate foils and the acrylate mixture was studied under ambient conditions (air, temperature) over a period of 60 days. Furthermore, bending force and brittleness of the foils were evaluated after thermal incubation at -20 °C and 60 °C.

3.3. Characterization of the polymer films for application in cell cultivation chips

Cell culture

L929 murine fibroblasts and Jurkat T-cell leukemia cells (both DSMZ, Braunschweig, Germany) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamin and supplemented with 10 % fetal bovine serum (FBS). The cultures were maintained at 37 °C in 5 % CO_2 humidified atmosphere. The L929 cells were cultivated until 80-90 % confluence and then harvested using 0.5 ml accutase. The Jurkat culture was maintained at the cell concentration between 0.1–1.5x10^6 cells/ml. For experiments, the cells were resuspended in RPMI medium supplemented with 10 % FBS and Penicillin/Streptomycin antibiotics, adjusting the cell concentration to 1.5–2.5 x 10^5 cells/ml.

All cell culture reagents were purchased from Gibco (Waltham, MA, USA).

Cytotoxicity test and cell staining

For the cell tests, the polyacrylate substrates were bonded with bottomless microfluidic chips from ibidi (Gräfelfing, Germany), using a double-sided adhesive tape. The cells were seeded in the chips in the RPMI medium with antibiotics, and cultured in the microstructures for 2 – 14 days in standard cell culture conditions (37 °C, 5 % CO_2, >95 % humidity). Afterwards, the viability of cells was determined by differential live and dead staining with fluorescein diacetate (FDA) and propidium iodide (PI). The cell culture medium in the microfluidic chips was carefully exchanged for a staining solution containing 8 µg/ml FDA and 20 µg/ml PI. The cells were incubated with the staining solution for 10 min at 37 °C. Before fluorescent imaging, the staining solution was exchanged for serum-free RPMI medium. For fluorescent imaging of the cells in the microstructures, the cells were stained with the live and dead staining solution as described above and the cell nuclei were labelled with NucBlue (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer.

Autofluorescence and Live Cell Imaging

All fluorescent and phase contrast microphotographs were taken with the inverted microscope Nikon Eclipse Ti (Nikon GmbH, Düsseldorf, Germany) equipped with a 4x or a 10x objective (CFI Plan Fluor DL Phase; Nikon), V-2A, TexRed, Cy3, Cy5, and FITC filter sets (Nikon), and a CCD camera ORCA-Flash 4.0-LT (Hamamatsu Photonics, Hamamatsu City, Japan). Autofluorescence was evaluated by taking fluorescent images of the polyacrylate substrates with the different filter sets. Standard microscopic glass and plastic coverslips were included as controls. The mean grey value of the microphotographs was evaluated with the National Institute of Health ImageJ software [5]. The spheroid formation in the pyramidal microwells was documented by videomicroscopy, using the same microscope with a Stage Top Incubation System (ibidi) to ensure stable cell culture conditions for the duration of the experiment (37 °C, 5 % CO2, 80 % humidity). Phase contrast images were taken with the time-lapse interval of 30 min for 48 hours.

4. Results and discussion

4.1. Polyacrylate foils for application in biosensor disposables

UV-imprinted polyacrylate films with double measuring chambers prepared in the costumized mold had a reproducible thickness of approximately 315 µm to 340 µm (mold cavity test mold 320 µm), which was tolerable for the following processing steps. Variation of thickness could be further reduced by a fully- instead of semi-automatized molding step, which is planned for the future. Transparency of the tested polyacrylate foils was comparable to that of hot-embossed PC foils according to the optical evaluation as well as the detection of the registration marks. However, it depends on the quality and surface roughness of the tool, as well as from the acrylate compostition itself. The same applies to shrinkage.

The tested polyacrylate foils were characterized by a high molding quality of the microstructures, which was reproducible for small series of replication. Demolding defects in the resulting polyacrylate foils were reduced to a minimum due to the Dursan® coating of the tool and occurred independently from the replication order. Polymer residues, as particles or filaments at the walls, were discovered sporadically and could be removed by an ultrasonic cleaning step. In few cases, defects of the structure were found, such as missing segments of the walls or measuring chambers, which could be attributed to a damage of the non-adhesive coating over time.

The tested polyacrylate foils showed an even filling of the double measuring chambers and wetting properties comparable to reference hot-embossed PC. Flow rates were in the range of 80 % to 122 % with respect to reference PC and depended from the wettability of the polyacrylate material itself, deformation, proper sealing and defects of the polymer foil. Reproducibility of the filling performance was affected by leakages due to sealing failures, which were mainly caused by the shrinking of the polyacrylate foils (up to 2 %) during the UV-replication process.

Crucial challenge in the development of the final acrylate mixture was the optimization of the mechanical properties – high stiffness and moderate flexibility without brittleness during cutting. Here, a huge improvement was achieved by applying Design of Experiments. The final polymer foils had a bending force of 140 N/mm (PC: 215 N/mm) and were stable in the following cutting/punching process.

No significant aging of the foils was detected, neither after long-term incubation (several months) under ambient conditions, nor short-term thermal incubation (< one week) at -20 °C or 65 °C. The acrylate mixture was stable over a period of 60 days, i.e. molded foils of this mixture maintained their properties.

The potential applicability for biosensor production was evaluated by amperometric measurements with sensors prepared from the optimized polyacrylate foil. It was demonstrated, that the optimized polyacrylate foil was biocompatible for the reaction mixture and that a substitution of the standard hot-embossed PC foil by a UV-imprinted polyacrylate foil is principally possible.

4.2 Polyacrylate foils for application in cell cultivation chips

Optical properties of micro-structured polyacrylates

To verify the suitability of the structured polyacrylate substrates for application in cell assays, which are generally based on optical documentation of cell behaviour by phase contrast or fluorescent microscopy, the optical properties of the structured substrates were evaluated (Figure 3).

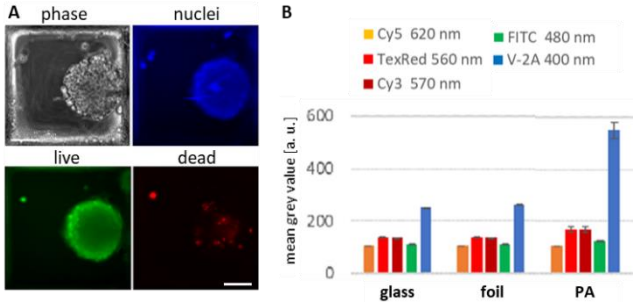

Figure 3 Fluorescent microscopy on microstructured polyacrylates. A: The microphotographs show a fibroblast spheroid in the 400x400x800 µm microstructure in phase contrast (left), and the fluorescent signal of cell nuclei (blue), live cells (green), and nuclei of dead cells (red). Scale bar = 100 µm. **B:** The graph shows the background fluorescent signal of standard microscopic substrates (glass, plastic foil), and the structured polyacrylate foil in fluorescent channels that respond to commonly used fluorophores. The bars signify the measured mean grey value ± SEM (n=3).

The autofluorescence was measured in the middle of the pyramidal microstructures without cells and should not be affected by the production process, but is dependent on the final thickness of the substrate. The evaluation of the mean grey value showed an increased fluorescence signal of the polyacrylates mainly in the blue channel, compared to the standard glass and plastic foil microscopic coverslips (Figure 3B). However, fluorescent microscopy of labelled cells in the microstructures showed that the background signal of the substrate did not significantly interfere with the imaging, and the cells could be easily recognized by both phase contrast, and fluorescent microscopy (Figure 3A). The two microphotographs at the right of Figure 3A (live and dead staining) also show that the polyacrylate of the chosen formulation is biocompatible, as there was only a low number of dead cells in the centre of the spheroid. Parallel experiments with cells growing in a confluent 2D layer on unstructured polyacrylate substrates showed an overall viability (i.e., the ratio of living cells) of > 98 %, which is comparable with the viability of cells growing on the standard glass or plastic microscopic substrates (data not shown).

Functionality of the microstructured substrates

L929 fibroblasts in a single cell suspension were seeded in the assembled µ-Slide III 3D Perfusion with the pyramidal microstructures, and the formation of the 3D cell aggregates was documented by time-lapse microscopy. As shown in Figure 4, the cells in the microwells clustered together fast, and formed compact, round spheroids within several hours after seeding. The spheroids remained stable for the whole duration of the experiment (48 hours).

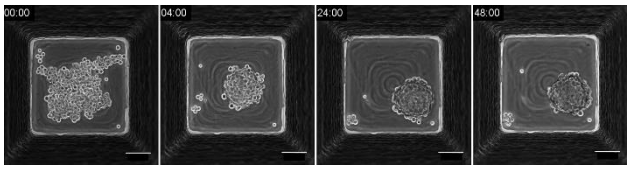

Figure 4 Spheroid formation in pyramidal microstructures. The microphotographs show the 3D aggregate of L929 fibroblast at the beginning of the time-lapse experiment (ca. 30 min after cell seeding), and after 4, 24, and 48 hours. Within several hours, the cells formed a compact spheroid which remained stable for the entire duration of the experiment. Scale bar = 100 µm.

The suspension cells Jurkat were seeded in the channel chips assembled with the single cell microwell array test substrates and cultivated for 14 days in standard cell culture conditions. After two weeks, the cells were stained with a live and dead staining solution to asses their viability. Figure 5 shows that the majority of the cells in the microstructures were alive (green). At the end of the experiment, some of the microstructures were occupied with more than one cell, although the initial occupancy was one cell per well in most of the microstructures. This is the result of cell division that took place in the microstructures during the time of the experiment.

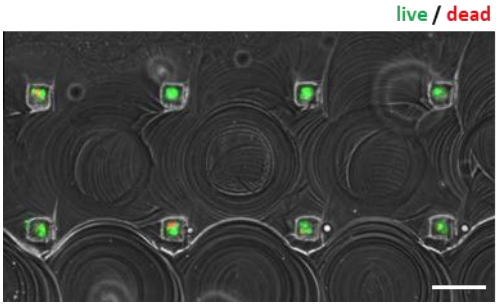

Figure 5 Immobilization of suspension cells in the single cell microstructures. The merged microphotograph shows the Jurkat cells after 14 days cultivation in the 30x30x30 µm square microstructures. The living cells were labelled green, and the dead cells red. The microphotograph shows the phase contrast image of the substrate with the microwells. Scale bar = 100 µm.

5. Summary

Structured polyacrylate films have been successfully fabricated by a UV imprinting process using a customized mold. We were able to optimize the properties of the formed polyacrylate films, such as transparency, mechanical stiffness, low autofluorescence, and biocompatibility, with respect to their future application. Thus, the potential application of the optimized polymer films for use in biosensors could be demonstrated.

The results of the cell tests showed that the structured polycrylates are suitable for application in cell-based assays. Formation of 3D cell spheroids could be observed in pyramidal microwells, and suspension cells could be immobilized and cultivated in arrays of small, single-cell microwells.

Acknowledgements

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