MICROSTRUCTURED THERMORESPONSIVE POLYMER COATINGS AS A PROMISING TOOL FOR CONTROLLING NEURITE OUTGROWTH IN ARTIFICIAL NEURONAL NETWORKS

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ABSTRACT
Despite extensive research worldwide, the spatial and temporal control of neurite outgrowth in artificial neuronal networks is still unsolved. Here, we evaluated the potential of thermoresponsive polymer (TRP) coatings as a neuronal cell culture substrate that could allow for elegantly switching on and off neurite pathways by application of simple temperature cues. Immunochemical and morphological analysis of human SH-SY5Y neuroblastoma cells that had been differentiated on TRP coatings demonstrated a high biocompatibility of our substrates. By precisely controlling the temperature of microstructured TRP coatings, we managed to pattern the cells and were able to precisely trigger neurite outgrowth.

KEYWORDS: Cell Patterning, Thermoresponsive Polymers, Artificial Neuronal Networks, Controlled Neurite outgrowth

INTRODUCTION
The elucidation and medical treatment of human brain (dys)function is one of the big challenges of our time and subject of extensive research. The analysis of artificial neuronal networks is raising great expectations with regard to the understanding of neuronal information processing and for the establishment of in vitro pharmacological test systems. In order to reproduce the highly organized architecture of neuronal tissue from dissociated neuronal cells in vitro, it is essential to precisely control cell position and neurite outgrowth in time and space.

Many strategies that have been developed to achieve this goal (i.e., micropatterning of cell adhesive proteins[1], presentation of topographical cues[2], laser-assisted axonal outgrowth[3] or dielectrophoretic forces[4]) either provide only static cues, offer low spatial resolution or require complex manipulation protocols which cannot be adapted to the formation of complex networks with large amounts of cells.

Here we investigated the applicability of micropatterned surface coatings of thermoresponsive polymers (TRP)[5,6] for controlling neurite outgrowth in time and space. Since TRP undergo a phase transition from a cell-repellent into a cell-friendly state in a temperature-dependent manner, they could allow for the elegant activation or deactivation of defined pathways for neurite outgrowth.

We evaluated the biological compatibility of our TRP coatings by analyzing both the expression of a neuronal marker protein as well as the morphological marker neurite length in SH-SY5Y neuroblastoma cell cultures that were grown on TRP. We then produced micropatterned TRP surfaces and identified culture conditions for the use of these surfaces for cell patterning as well as for triggering neurite outgrowth by precisely setting ambient temperature cues.

EXPERIMENTAL

Cell culture: Cells of the SH-SY5Y human neuroblastoma cell line (DSMZ, Germany) were grown at 37 °C and 5% CO₂ in basal culture medium (i.e., DMEM supplemented with 15% FCS, 2 mM L-α-nalyl-L-glutamine and 100 U / 0.1 mg ml⁻¹ Penicillin / Streptomycin) and were passaged every 6 to 7 days. For differentiation into a neuronal phenotype, cells were grown for 5 days in basal culture medium supplemented with 10 µM retinoic acid (RA). After this, the medium was replaced with serum-free DMEM containing 50 ng ml⁻¹ BDNF.

Surface modification: An Auto 500 Electron Beam Evaporation System (Edwards, UK) was used for coating glass slides (20x20 mm², Menzel, Germany) with 3 nm chromium and 47 nm gold. TRP were coupled to the gold layer by incubating the slides at 25 °C for 3 h in an ethanolic solution containing 50 µM of the TRP disulfide-poly(MEO2MA-co-OEGMA) followed by an extensive washing step to remove unbound polymers. Prior to TRP coupling, the gold layer was eventually microstructured by UV laser ablation employing a pulsed 248 nm KrF excimer laser with a fix pulse duration of 10 ns (Lambda-Physik, Germany).

Analysis of differentiation markers: Cells were seeded onto either TRP-coated glass slides or untreated standard cell culture petri dishes (StPD, TPP, Switzerland) at cell densities of 1.1 x 10⁴ cells cm⁻². Over time periods of 5 to 11 days, the cells were either differentiated into a neuronal phenotype or left undifferentiated. Neurite lengths were evaluated every 1 - 2 days using the NeuronJ plug-in (E. Meijering, University Medical Center Rotterdam, Netherlands) of the image processing software ImageJ (http://rsbweb.nih.gov/ij/). Alternatively, cells were fixed after 5 days of differentiation and stained with anti-β-Tubulin3 primary antibodies (detection via Cy3-labeled secondary antibodies) and DAPI before they were analyzed with an Olympus IX51 fluorescence microscope equipped with a 10x phase contrast objective.
**Triggered neurite outgrowth:** Differentiated SH-SY5Y were seeded onto microstructured TRP surfaces at 34 °C (i.e., below the lower critical solution temperature (LCST) of TRP: TRP in cell-repellent state) such that cells only adhered to TRP-free glass patches. After gently washing away excess and non-attached cells from the surface, the slides were cultivated at 34 °C for 4 days. After this, the cultivation temperature was shifted to 37 °C (i.e., above the LCST of TRP: TRP in cell-friendly state) followed by another 24 h cultivation period. Finally, the temperature was lowered to 34 °C again. The cells were analyzed by brightfield microscopy 96 h, 24 h and 2 h after setting either of the three temperatures, respectively.

**RESULTS AND DISCUSSION**

Analysis of biochemical and morphological markers for neuronal cells revealed virtually no difference between SH-SY5Y cells that were differentiated into a neuronal phenotype on either TRP or StPD, which shows the exceptional biocompatibility of the TRP substrates: Immunostaining against the neuronal marker protein β-Tubulin3 exhibited strong immunofluorescence in both cases after day 5 of differentiation while undifferentiated cells (on StPD) showed only little β-Tubulin3 immunoreactivity (Figure 1A). Similarly, the evolution of mean neurite lengths throughout 11 days of differentiation was comparable in cell populations that were differentiated on either TRP or StPD surfaces. Mean neurite lengths for cells on TRP were 24 ± 1.8 µm and 116 ± 12.1 µm at the beginning and after 11 days of differentiation, respectively, while corresponding values for cells on StPD calculated to 24 ± 3.7 µm and 144 ± 7.8 µm (Figure 1B).

**Figure 1:** Biocompatibility of the TRP surface. SH-SY5Y human neuroblastoma cells were differentiated on either thermoresponsive polymer coatings (TRP) or standard cell culture petri dishes. (A) After differentiation for five days with retinoic acid into a neuronal phenotype, cells were stained against the neuronal marker protein β-Tubulin3. (B) Cells were differentiated over 5 days with retinoic acid followed by 6 days of cultivation in serum-free medium supplemented with BDNF. Throughout the whole cultivation period, the cells were analyzed for mean neurite length. Scale bar, 50 µm

We then produced micropatterned TRP surfaces by laser ablation of gold-coated glass slides such that the surface comprised an array of (temperature-independent) cell-adhesive glass spots of 25 µm in diameter and 200 µm distance to each other (see Figure 2A). Thiolated TRP were coupled to the remaining gold coating via thiol/gold chemistry before the substrates were used for neuronal cell culture.

By precisely controlling the temperature of the chip surface we were able to pattern cells onto the glass spot array and to successfully trigger neurite outgrowth: When setting the temperature of the chip surface below the TRP phase transition temperature (i.e., 34 °C: TRP in cell-repellent state, see Figure 3A) during cell seeding, cell adherence was restricted to the cell-adhesive glass spots. Throughout four days of cultivation at 34 °C, the cells remained inside the spots without any neurites growing out. After setting the surface temperature above the TRP phase transition temperature (i.e., 37 °C, TRP in cell-friendly state) neurites grew out from the spots and spread over the whole surface (Figure 3B). Switching the surface temperature back to 34 °C forced the cells to retract their neurites which shows the reversibility of the process (Figure 3C). In a control experiment with cells cultivated at 34 °C and over 4 days on uncoated glass substrates, cells and neurites spread normally which shows inhibition of neurite growth in Figure 1A was due to cell-repellence of TRP and not due to temperature-dependent cell physiological aspects.
Figure 2: Microstructured surface for triggering neurite outgrowth. The chip comprises cell-adhesive glass spots (light) and a backfill of thermoresponsive polymer (TRP, dark). (A) At 34 °C (i.e., TRP in cell-repellent state) cell adhesion is restricted to the glass spots (A, red circles). (B) After 96 h cultivation time, the surface temperature was shifted to 37 °C (i.e., TRP in cell-friendly state) which let the cells growing out their neurites. (C) 24 h later the temperature was lowered back to 34 °C again which made the cells retracting their neurites to the glass spots. (D, control) Cells that were cultivated at 34 °C on uncoated glass substrate showed normal cell spreading and neurite growth.

CONCLUSION
We evaluated the applicability of microstructured thermoresponsive polymers coatings as a cell culture substrate for artificial neuronal network formation. As shown by biochemical differentiation markers and cell morphology, our substrates showed a high biocompatibility and could be successfully used for both patterning cells as well as precisely triggering neurite outgrowth in time. To conclude, micropatterned TRP coatings serve as a promising tool for the formation of artificial neuronal networks since they allow controlling the accessibility of cells and outgrowing neurites to the substrate in time and space.

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REFERENCES

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