Micropatterned Substrates for the Growth of Functional Neuronal Networks of Defined Geometry

Angela K. Vogt,[†] Lars Lauer,[†] Wolfgang Knoll,[†] and Andreas Offenhäusser*[,‡]

Max-Planck Institute for Polymer Research, Ackermannweg 10, D-55128 Mainz, Germany, and Institute of Thin Films and Interfaces, Bio and Chemosensors (ISG2), Forschungszentrum Jülich, D-52425 Jülich, Germany

The in vitro assembly of neuronal networks with control over cell position and connectivity is a fascinating approach not only for topics in basic neuroscience research but also in diverse applications such as biosensors and tissue engineering. We grew rat embryonic cortical neurons on patterned substrates created by microcontact printing. Polystyrene was used as a cell repellent background, onto which a grid pattern of physiological proteins was applied. We printed laminin and a mixture of extracellular matrix proteins and additionally both systems mixed with polylysine. Attachment of cells to the pattern with high fidelity as well as the formation of chemical synapses between neighboring cells on the pattern could be observed in all four cases, but cell attachment was strongly increased on samples containing polylysine. Neurons grown on patterned substrates had a membrane capacity smaller than that of neurons on homogeneously coated controls, which we attributed to the geometrical restrictions, but did not differ either in resting membrane potential or in the quality of synapses they formed. We therefore believe that the cells attach and differentiate normally on the pattern and form functional, mature synapses following the predefined geometry.

Introduction

One of the major challenges in studying development, activity, and dynamics of naturally forming neuronal networks lies in their enormous complexity. A promising approach to this problem is the creation of simplified networks of controlled and reproducible geometry as a model system. Such a model has multiple potential applications in cell-based biosensors, neuroelectronic circuits, neurological implants, and pharmaceutical testing as well as fundamental biological questions. These range from investigations on neurite extension *(1)* to topics concerned with neuronal signaling and network behavior. To present a suitable model, the following criteria must be fulfilled. First, a high fidelity of the cells to the applied geometry is needed such that connections can only form along defined pathways, ensuring control over the connectivity in the resulting network. Additionally for many applications, e.g., when extracellular recording devices are to be used, an exact positioning of the cell bodies is important, such that the cells can be placed onto the sensitive areas of the recording device *(2)*. Third, the health and viability of the patterned neurons should remain unimpaired, and fourth, the neurons should form functional, mature chemical synapses not different from those found in more complex networks. Chemical synapses—in contrast to electrical ones—exhibit plasticity and thus are thought to be responsible for processes such as learning and memory. Therefore, their presence in the system is absolutely crucial for the examination of network behavior, network plasticity, and activity-dependent changes.

Imposition of geometry onto an in vitro neuronal network can occur either by topographical guidance *(3, 4)* or by chemical guidance, offering the cell a pattern of cell-attracting components contrasting a cell-repellent background. Such a pattern can be created, e.g., by photolithography *(4−7)* or by microcontact printing *(8−12)*. Several groups have studied electrophysiological properties of patterned neuronal networks: Liu et al. used line patterned silane surfaces to orient adhesion and outgrowth of hippocampal neurons *(7)*. Synaptic currents were recorded from interconnected neurons, but the cells adhered randomly along the line. Chang et al. describe a system in which cells were grown on stripes of polylysine and recorded using a multielectrode array *(13)*. Synaptic activity was detected, although the cell density was too high to track down single cell connections. Merz et al. report the formation of neuronal networks from the snail *Lymnaea stagnalis* on a topographical pattern, containing electrical synapses *(14)*. Wyart et al. *(15)* describe a network of synaptically connected neurons on a patterned surface. However, the cells could not be confined to areas smaller than 80 μm in diameter such that an exact positioning of the cell bodies was not achieved and extensive branching of the neurites within the adhesion area may result in uncontrolled autaptic signaling.

In this article, we describe a system that enabled us to grow networks from rat embryonic cortical neurons in a controlled geometry, confining neuronal cell bodies precisely to the nodes of a grid pattern. Our substrates consisted of a pattern of extracellular matrix (ECM) proteins applied onto a background of polystyrene by microcontact printing. We tried out different types of

* To whom correspondence should be addressed. Tel: +49-2461-61-2330. FAX: +49-2461-61-8733. e-mail: a.offenhaeusser@fz-juelich.de.

[†] Max-Planck Institute for Polymer Research.

[‡] Institute of Thin Films and Interfaces.

10.1021/bp034016f CCC: $25.00
Published on Web 08/01/2003

proteins for stamping; the use of a physiological mixture of ECM proteins with polylysine proved to give the best results in terms of pattern transfer as well as recruitment of cells to the surface. The cells grew along the guidance of the pattern with a high compliance while the density was low enough for individual cell contacts to be observed. Double patch clamp measurements showed that the cells grown on patterned substrates had resting membrane potentials as low as cells grown on control dishes. In addition, the synapses that formed between neighboring cells on the pattern did not show any obvious differences from those formed on controls, indicating no impairment of cell function or development by the geometrical constraints imposed by the patterned substrate.

Methods and Materials

Microcontact Printing. Microstamps were produced by photolithography and molding. An electron beam writer transposed the grid structure to a chrome mask. Applying UV-photolithography, master stamps were produced out of spin coated 12.5 μm thick photoresist layers (AZ 4562, Clariant) on 0.6 mm thick silicon wafers (MEMC Electronic Materials). Poly(dimethylsiloxane) (PDMS) microstamps were then fabricated curing Sylgard 184 (Dow Corning) in 2 mL eppendorf tubes for 48 h at 55 °C upside down on the master stamps. After master stamp release, final curing was performed during 1 h at 110 °C.

PDMS stamps were stored for 24 h in deionized water before using and then decontaminated in a 70% ethanol bath for 1 min. Inking took place for 30 s in the respective protein solution (25 μg/mL laminin (1243217, Boehringer Mannheim GmbH) dissolved in PBS or each 12.5 μg/mL laminin and D-polylysine (Sigma, P6407) in PBS or ECM-gel (Sigma E1270) diluted 1:100 in Dulbecco's Modified Eagle's Medium (GIBCO 11880-028); for PECM substrates 10 μg/mL D-poylylysine was added to the solution). The inked stamp was dried in a nitrogen airstream and pressed to the substrate for 10 s. Non-tissue-culture polystyrene Petri dishes of 35 mm diameter (Greiner) were used.

Grid patterns established in earlier experiments *(16)* were applied. These patterns have nodes with a diameter of 12−14 μm in equidistant steps of 100 μm in the *x*-direction and 50 μm in the *y*-direction; grid lines were 4−6 μm in width. Unstructured additional adhesion areas were created by applying drops of the inking solution to the edge of the dish. After 1 h, the drop was removed by aspiration; the substrates dried overnight at room temperature.

Evaluation of Pattern Transfer. To evaluate how well different solutions transferred during printing, the inking solutions were mixed each with 10 μg/mL sulforhodamine (Molecular Probes, S-359). Substrates were then printed as usual and viewed under fluorescent light with a standard microscope (IX 50, Olympus). Pictures of the substrates were taken (AxioCam color 3.0; 8 bit AD-convertion, exposure time 8 s) and evaluated using the gray scale analysis of an imaging software (ImageProPlus). For this evaluation, areas represented by pixels of an intensity 50% or more of the maximum were considered to be covered with protein. The program then calculated the total covered area per picture. For data analysis 64 pictures per substrate type were evaluated and averaged.

Isolation of Embryonic Neurons. Rat embryonic cortical neurons were obtained as described by Brewer et al. *(16)*. Briefly, embryos were recovered from pregnant CD rats at 18 days gestation. Cortices were dissected from the embryonic brains; cells were mechanically dissociated by trituration in Hank's Balanced Salt Solution (without Ca^{2+} and Mg^{2+}, GIBCO 14170-088), 0.035% sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 20 mM glucose, pH 7.4 with a firepolished siliconized pasteur pipet. Two volumes HBSS (GIBCO 24020-091) 0.035% sodium bicarbonate, 1 mM pyruvate, 10 mM HEPES, 20 mM glucose, pH 7.4 were added. For 3 min, nondispersed tissue was allowed to settle; the supernatant was centrifuged at 200*g* for 5 min. The pellet was resuspended in 1 mL of Neurobasal Medium (GIBCO 21103-049), 1X B27 (GIBCO 17504-044), 0.5 mM L-glutamine (GIBCO 35050-038) per hemisphere isolated.

An aliquot was diluted 1:1 with trypan blue and dye-excluding cells were counted in a Neubauer counting chamber. The remaining cells were diluted in NB medium with the above supplements and plated onto the substrates at a density of 16 000 cells per cm^2. Half of the medium was changed every 3−4 days.

Electrophysiology. Patch-clamp recordings were performed using a triple patch-clamp setup (EPC9/3, Heka Elektronik) in current clamp mode; typically at DIV 11−15. Borosilicate micropipets (1810016, Hilgenberg) with a resistance of ∼6 MΩ were pulled using a micropipet puller (P-97, Sutter Instrument Company). Stepper motor based micromanipulator units (Mini 25, Luigs & Neumann) under microscopic control (IX 50, Olympus) were used for manipulation. For current-clamp recordings, membrane current was set to zero; action potentials were stimulated with 20 or 100 ms pulses of 130 pA. The extracellular solution contained (mM): KCl, 5; NaCl, 150; $MgCl_2$, 1; HEPES, 10; $CaCl_2$, 2.5; glucose, 10; pH 7.4, adjusted with 1 M NaOH. The intracellular solution contained (mM): potassium gluconate, 125; KCl, 20; $CaCl_2$, 0.5; $MgCl_2$, 2; HEPES, 10; EGTA, 5; ATP, 4; pH 7.4, adjusted with 1 M KOH.

Antibody Staining. Cells were fixed in 2% paraformaldehyde for 20 min on ice and then permeabilized with 5% acetate in 95% ethanol for 7 min at −20 °C. Incubation with primary antibody (rabbit anti-neurofilament-M, Chemicon AB1987), diluted 1:200 in PBS containing 10% FCS and 0.02% NaN_3 (Sigma, F2442 and S2002) followed after three washes with PBS (GIBCO 14190094). Cells were washed again before incubation with secondary antibody (Cy3 conjugated donkey anti rabbit, Dianova 711-166-152). After washing, cells were mounted in mounting medium from DAKO (S 3023). The substrates were viewed using an Olympus IX50 microscope, pictures were taken with AxioCam color (Zeiss).

Evaluation of Cell Adhesion to Different Protein Patterns. We counted cells adhering to the nodes of the grid pattern on randomly chosen photographs, taken under an IX50 microscope with a 10× objective. We only counted single adherent cells; lumps of cells were ignored. Counted as "empty nodes" were line intersections where protein transfer has apparently taken place since neurites followed the pattern, but where no cell bodies adhered. The substrates were between 11 and 15 days old when the evaluation was performed.

Results

Sample Preparation. Patterned substrates were created by microcontact printing as described previously *(10)*. We used polystyrene culture dishes as a highly hydrophobic and therefore cell-repellent background and applied grid patterns consisting of 4−6 μm wide lines and nodes that were 12−14 μm in diameter; this geometry

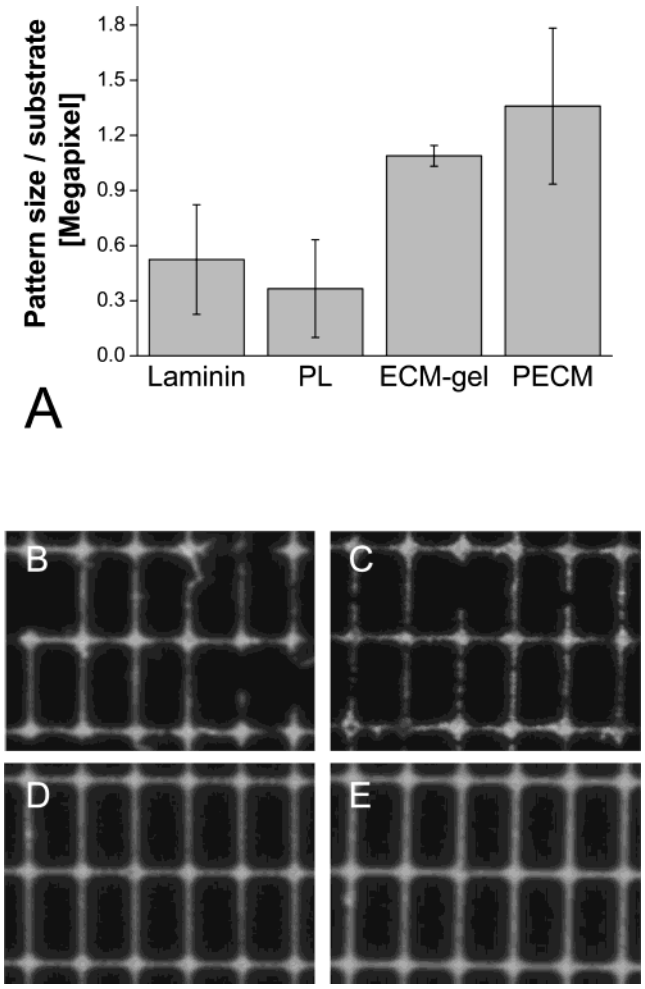

Figure 1. Comparison of different inking solutions for microcontact printing. Pattern transfer was evaluated by adding sulforhodamine to the solution before staining and visualizing the patterned substrates under fluorescent light. Pictures were taken and the size of the patterned area was determined using standard imaging software. ECM-gel and PECM show a higher rate of pattern transfer than laminin and PL. (A) Transfer quantification. (B−E) Typical pictures of patterns stamped with different inks: (B) laminin, (C) PL, (D) ECM-gel, (E) PECM.

has been shown before to be most suitable for the placement and confinement of neurons to a pattern *(12)*. Initially, two different types of ink were used. In the first instance we printed laminin, which is a well-established system for patterning neuronal cells *(8, 11, 12, 18)*. Next, we used a commercially available mixture of ECM proteins extracted from native tissue (ECM-gel), since we believed such a mixture to be closer to in vivo conditions (where the cells encounter a highly complex substrate that can influence cellular behavior in multiple ways *(2, 19)*) than a purified protein. As positively charged polymers are known to enhance cell adhesion *(20)*, we also used both systems mixed with polylysine. (The mixture of polylysine/laminin will be referred to as PL and the mixture of polylysine and ECM-gel as PECM).

We then wanted to compare whether protein transfer during microcontact printing was different for the different inking solutions. Quantification of pattern transfer was performed by adding sulforhodamine to the respective solution before stamping. Pictures of the labeled substrates were taken under fluorescent light, and the size of the patterned area was determined using standard image evaluation software.

As shown in Figure 1, ECM-gel transferred significantly better than laminin, and this effect was not notably changed by the addition of polylysine to either solution. Differences in transfer efficiency could arise at two steps, either as a result of a different adsorption rate of the inking solution to the stamp. The more material adsorbs, the more material is available for patterned transfer. Stamp wetting is a difficult issue as PDMS is a hydrophobic material and thus not the ideal interaction partner for water-soluble proteins. A more hydrophilic stamp on the other hand would impede transfer to the equally hydrophobic substrate, which is a similarly bad interaction partner. A compromise was taken by soaking the stamps in water before printing in order to increase hydrophilicity. Alternatively, the efficiency with which the molecules transferred in the printing step could be divergent between different inks. Transfer to the substrate is a similarly critical step as wetting since the hydrophobic substrate is as unattractive for interaction with water-soluble compounds as the stamp itself. Determining which of the two steps limited the different transfer efficiency was beyond the scope of this work.

In addition to the stamped pattern in the middle of each culture dish, small areas at the edge were coated with the respective protein in an unstructured manner. These areas served as additional adhesion sites for neurons and glia in order to increase the overall cell density and thus cell longevity on each substrate.

Network Formation. Dissociated cortical neurons from E18 rat embryos were seeded onto the patterned substrates. The contrasting surface areas of cell-repellent background and attracting pattern forced the neurons to place themselves onto the nodes and to extend their processes along the lines only. To avoid the deposition of serum proteins onto the hydrophobic background, which would reduce the contrast between the two areas, a serum-free medium was used *(17)*. As shown in Figure 2a, the aligning of the cell bodies onto the nodes was achieved with a high compliance, as well as the guidance of the processes along the lines. In the resulting network, any given cell could physically contact and thus form synapses with its direct neighbors on the pattern. Apart from morphological and electrophysiological evidence, the neuronal identity of the cells was confirmed by antibody staining, using an antibody against the neuron-specific cytosceletal protein Neurofilament-M (Figure 2b). The networks looked similar whether laminin or ECM-gel was used for stamping and were stable for 3 weeks. After this time, the cells tended to overgrow the pattern and form connections over the nonpermissive areas.

Trying to increase the yield of adhering cells, we mixed polylysine with both inking solutions in order to add positive charge to the pattern, since positively charged polymers have been described to promote cell adhesion. After 2 weeks in culture, photographs were taken of randomly chosen areas from each of the four substrate types and the amount of adhering cells was evaluated.

As shown in Figure 3a and b, the number of cells adhering to the pattern increased dramatically when polylysine was added, although this effect was much more pronounced in the ECM system. Figure 3a compares the number of grid nodes per photograph that were occupied by a neuron, while Figure 3b shows the ratio of occupied versus empty nodes (where protein had transferred as verified by the growth of neurites, but no cell bodies adhered). Thus, Figure 3a displays the total success of a given substrate type in recruiting cells to the surface, also considering the amount of pattern that had transferred and thus was available as an adhesive area for the cells. By comparison in Figure 3b the

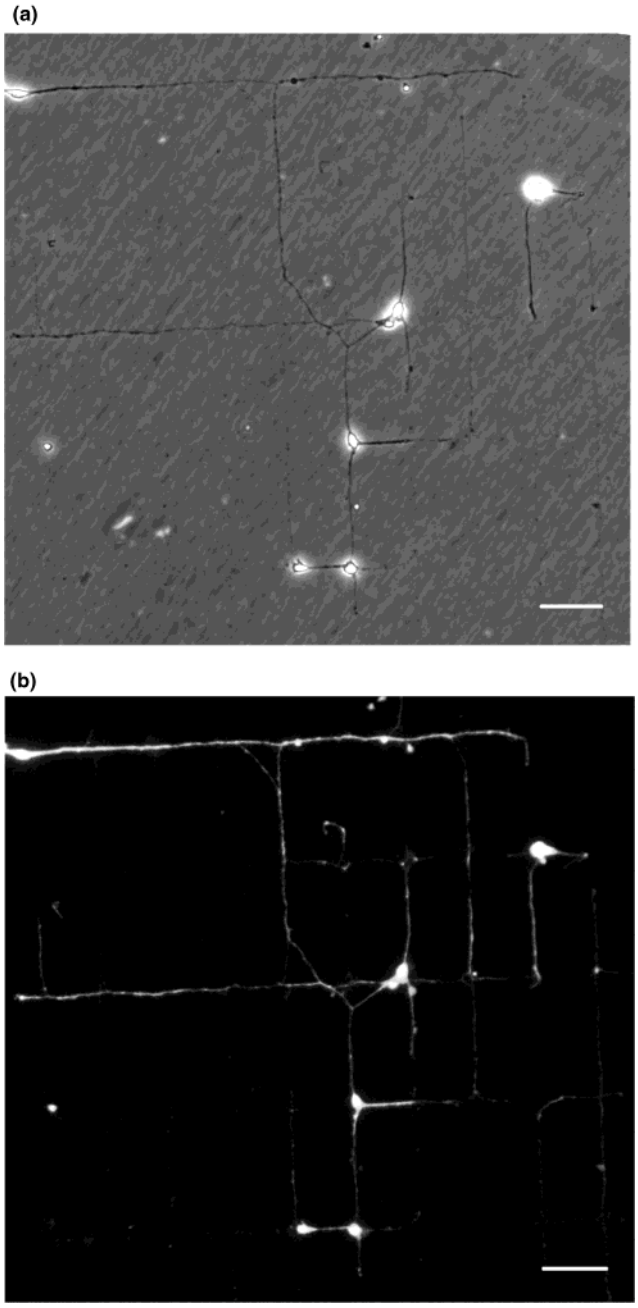

Figure 2. Cells growing on an ECM-gel grid pattern with 6 μm wide lines and 14 μm nodes on a background of polystyrene. Pictures were taken at DIV 7; scale bars are 50 μm. (a) Phase contrast micrograph and (b) immunostaining of the aligned cells with antibodies against Neurofilament-M.

adhesion rate per available surface area is displayed for the different inking solutions. Clearly, not only the total rate of cell adhesion but also the rate of adhesion per surface area increased with the addition of polylysine to both inking solutions. Also, the ECM-gel system was more successful in recruiting cells than the laminin system, again both in total adhesion as well as in adhesion per area.

Electrophysiological Characterization. To test the electrical properties of the patterned cells, patch-clamp recordings were performed. As shown before *(12*), the average membrane capacity of neurons on patterned substrates is 22% smaller than on unpatterned controls (Table 1), probably because of the geometrical confinement restricting the cells. However, the average resting membrane potential is very similar between patterned

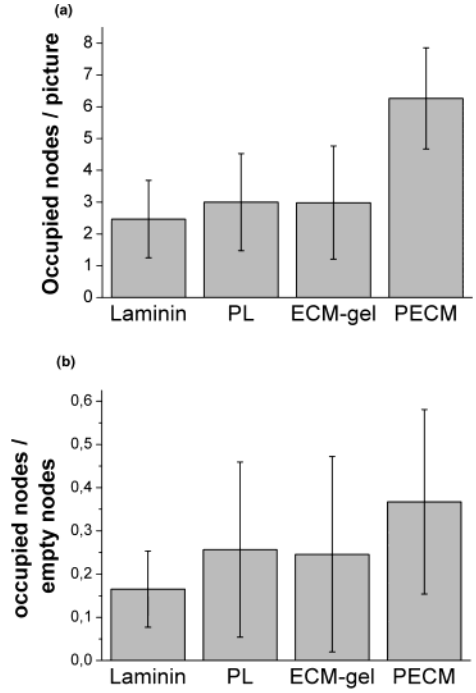

Figure 3. Adhesion of neurons to different types of micropatterned proteins. Recruitment of cells to the surface is by far best on PECM samples. (a) Number of cells occupying a node in the grid pattern per evaluated photograph and (b) ratio of occupied versus empty nodes per photograph.

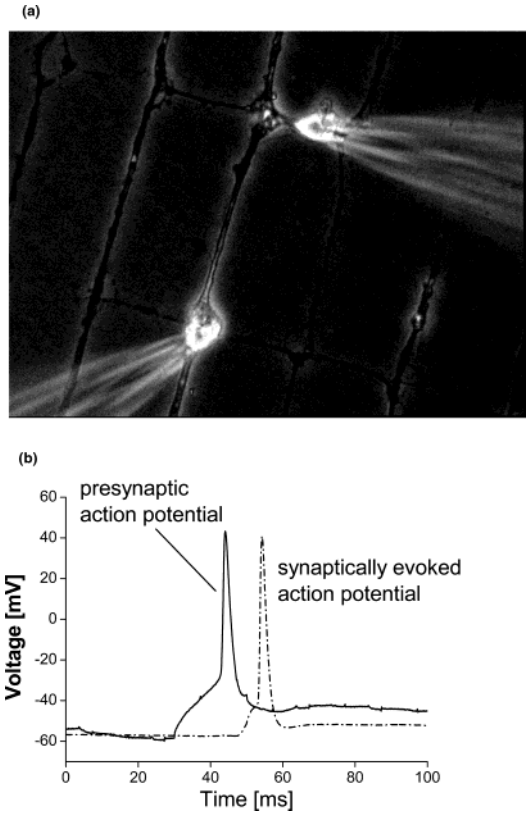

Figure 4. (a) Neurons grown on micropatterned substrates are able to form chemical synapses with their neighbors on the pattern. (b) Exemplary current clamp recording of two synaptically coupled cells growing on an ECM pattern, DIV 12.

and unpatterned substrates, as well as the average sodium and potassium currents per surface area (Table 1), indicating no impairment of cell function by the growth on patterned surfaces.

Table 1.

	patterned	unpatterned
membrane capacity	24.6 ± 11.5 pF	31.4 ± 15.6 pF
resting membrane potential	−61.7 ± 11.0 mV	61.1 ± 10.8 mV
Na^+ current/ surface area	56 ± 44 pA/pF	65 ± 41 pA/pF
K^+ current/ surface area	58 ± 35 pA/pF	57 ± 39 pA/pF

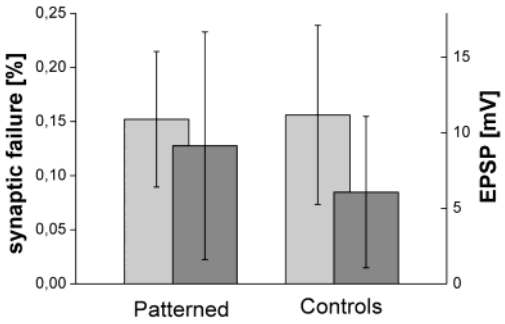

Figure 5. The rate of synaptic failure (shown in light gray) is not markedly changed on patterned substrates in comparison to homogeneously coated controls. Average EPSP height (dark gray) is also similar in the two groups.

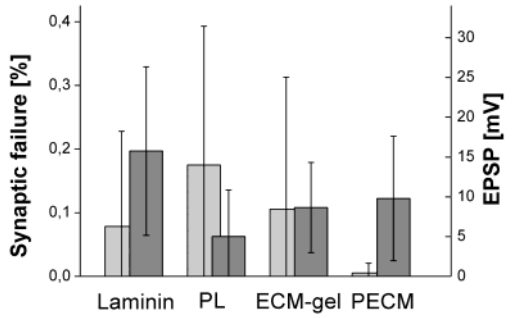

Figure 6. Synaptic efficacy and synaptic strength on different micropatterned proteins. PECM shows by far the lowest rate of synaptic failure of all samples tested. (Rate of synaptic failure is shown in light gray, average EPSP height in dark gray).

For the examination of synaptic connectivity, double patch clamp measurements were performed. We patched two cells on neighboring nodes on the pattern simultaneously in the current clamp mode. For each recording, one cell was stimulated to fire an action potential by applying a current pulse, while the response of the other cell was recorded without injecting current. As shown in Figure 4, we found chemical synapses some of which were strong enough to elicit an action potential in the postsynaptic cell.

We then wanted to address the question whether the synapses formed on patterned substrates differed in any respect from synapses we found on unpatterned controls. Two aspects were evaluated: average rate of synaptic failure as a criterion for synaptic efficacy *(21)* and average EPSP height as an indicator for the strength of the synapse.

As shown in Figure 5, the average rate of synaptic failure was similar in patterned and unpatterned cultures, while the average EPSP height was somewhat higher on patterned substrates.

We therefore conclude that the geometrical restriction of the pattern does not prevent the cells from forming functional, mature synapses that do not differ significantly from synapses formed on unpatterned substrates.

Impact of Protein Solution Used for Stamping. To find out whether the type of protein used for microcontact printing had an impact not only on the adhesion of the cells but also on their development and the formation of synapses, we compared the synapses formed on the four different surface modifications used. As shown in Figure 6, synaptic failure on PECM substrates was by far lower than on the other three surfaces, which showed similar values, while the average EPSP height was in the same range for all groups.

Discussion

Microcontact printing has been used successfully before to pattern neuronal cells in vitro. Using this technique with a low density cell culture protocol and a serum-free medium, we were able to reproducibly grow small neuronal networks of geometrically well-defined cell position and connectivity. As shown by double patch-clamp measurements, the cells in these networks seem to be physiologically normal and communicate through chemical synapses while cell density could be kept low enough to observe connectivity on the single cell level. These synapses do not appear to differ significantly from those found under control conditions. This finding is not in complete agreement with the results Ravenscroft et al. *(22)* reported on the synaptic development of hippocampal neurons on patterned substrates. However, the experimental conditions used in their work are different from ours in regard of substrate, age of the cells, and cell type. The only difference we observed between cells grown on patterned substrates and controls lies in their average size. On the micropattern, the cells were smaller on average, probably restricted by the limited adhesion area. However, we do not see this fact as a drawback to our system, since in vivo neurons may be restricted similarly. In an intact organism, the extracellular matrix is anything but a homogeneous surface and apart from the size of the adhesion area, other factors such as steric limitations or the interaction with other cell types (e.g., in competition for adhesive molecules) may restrict the expansion of a neuron. We therefore believe our system to be suitable for the examination of fundamental questions about neuronal signal transduction and processing.

We also tested the suitability of different protein solutions in their use as inks for microcontact printing. One clear advantage of ECM-gel over laminin was the notably better transfer to the surface, which may be due to the higher viscosity of the solution that improves wetting of the stamp. Although the transfer of protein was not markedly changed by adding polylysine to either solution, at least in terms of the size of the patterned area, the adherence of cells to the pattern increased dramatically when polylysine was added, particularly in PECM substrates. The ability of positively charged polymers to improve cell attachment has been known for a while, although it remains unclear whether the positive charge itself or an associated molecular structure are responsible for the effect *(20)*. In our application, one possible explanation for the increase in adhesion to polylysine-containing patterns may be a difference in protein transfer during microcontact printing; electrostatic interactions, for example, could improve the adhesion of protein to the substrate dish, resulting in thicker or denser protein layers that offer more potential interaction sites. Alternatively, the presentation of the proteins on the surface could be influenced by the presence of

polylysine, which may have an impact on the orientation or conformation of the molecules. However, these ideas remain highly speculative and will be subject of further studies.

The combination of excellent protein transfer during stamping and a high ability per surface area to recruit cells makes the PECM mixture particularly suitable for the patterning of small neuronal networks. Especially for applications in which the network is grown on an extracellular recording device like a FET, where a high occupancy of the gates is crucial, PECM patterns present the most suitable substrate.

Comparison of synaptic efficacy on the four substrate types shows somewhat unexpectedly that synapses formed on laminin, PL, and ECM-gel had a fairly similar rate of synaptic failure. We had expected that the ECM-gel, as a complex substrate rather than one single protein, might improve the formation and maturation of synapses, e.g., through signaling pathways triggered by the engagement of specific cell-matrix receptors, particularly since different types of adhesion molecules have been implicated in the formation and maturation of synapses *(23*−*25*). However, no such effect was observed. Amazingly though, the rate of synaptic failure was greatly reduced on PECM substrates. As polylysine did not cause an increase in synaptic efficacy when mixed with laminin, it seems unlikely that it is directly responsible for the increase seen in PECM over ECM. Again, we cannot exclude that the surface chemistry is influenced by PL in a way that may be beneficial to the interaction of the cells with the surface bound proteins. In addition the greater number of adhering cells, which causes a greater local density of neurons and maybe also glia cells, may be responsible for the effect, since the presence of glia cells is known to reduce synaptic failure as shown by Pfrieger et al. *(21*).

Conclusion

Control over the assembly and geometry of simplified neuronal networks is a topic of growing interest in many areas of biotechnology. We were able to grow rat embryonic cortical neurons on a pattern, highly constraining the geometry of the resulting network and precisely controlling the location of cell bodies. We were able to show that the cells are physiologically intact and form mature chemical synapses that do not appear to differ from those formed on unpatterned substrates. We found that a mixture of polylysine and ECM-gel has multiple advantages over the other substrates we used in that it transfers best and recruits most cells to the pattern. In addition, the synapses formed on this substrate showed by far the lowest rate of synaptic failure, which may be an effect caused by the greater local cell density on these substrates.

Acknowledgment

This work was partly supported by SONY International (Europe) GmbH, Advanced Technology Center, Stuttgart. The authors wish to thank Prof. Alfred Maelicke for the possibility to use his animal facility. We are particularly grateful to Prof. Greg Brewer and John Torricelli for their generous help and advice with the cell culture. We are indebted to Andrew Pattinson for a critical reading of our manuscript.

References and Notes

(1) Tai, H.; Buettner, H. M. Neurite outgrowth and growth cone morphology on micropatterned surfaces. *Biotechnol. Prog.* **1998**, *14*, 364−370.

(2) Chen, C.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Inber, D. E. Micropatterned surfaces for control of cell shape, position, and function *Biotechnol. Prog.* **1998**, *14*, 356−363.

(3) Maher, M.; Dvorak Carbone, H.; Pine, J.; Wright, J. A. Tai, YC Microstructures for studies of cultured neural networks *Med. Biol. Eng. Comput.* **1999**, *1*, 110−118.

(4) Kleinfeld, D.; Kahler, K. H.; Hockberger, P. E. Controlled outgrowth of dissociated neurons on patterned substrates. *J. Neurosci.* **1988**, *8*, 4098−4120.

(5) Ma, W.; Liu, Q. Y.; Jung, D.; Manos, P.; Pancrazio, J. J.; Schaffner, A. E.; Barker, J. L.; Stenger, D. A. Central neuronal synapse formation on micropatterned surfaces. *Dev. Brain Res.* **1998**, *111*, 231−243.

(6) Stenger, D. A.; Hickman, J. J.; Bateman, K. E.; Ravenscroft, M. S.; Ma, W.; Pancrazio, J. J.; Shaffer, K.; Schaffner, A. E.; Cribbs, D. H.; Cotman, C. W. Microlithographic determination of axonal/dendritic polarity in cultured hippocampal neurons *J. Neurosci. Methods* **1998**, *82*, 167−173.

(7) Liu, Q.; Coulombe, M.; Dumm, J.; Shaffer, K.; Schaffner, A.; Barker, J.; Pancrazio, J.; Stenger, D.; Ma, W. Synaptic connectivity in hippocampal neuronal networks cultured on micropatterned surfaces. *Dev. Brain Res.* **2000**, 223−231.

(8) Wheeler, B. C.; Corey, J. M.; Brewer, G. J.; Branch, D. W. Microcontact printing for precise control of nerve cell growth in culture. *J. Biomech. Eng.* **1999**, *121*, 73−78.

(9) Branch, D. W.; Wheeler, B. C.; Brewer, G. J.; Leckband, D. E. Long-term maintenance of patterns of hippocampal pyramidal cells on substrates of polyethylene glycol and microstamped polylysine. *IEEE Trans. Biomed. Eng.* **2000**, *47*, 290−300.

(10) Lauer, L.; Ingebrandt, S.; Scholl, M.; Offenhausser, A. Aligned microcontact printing of biomolecules on microelectronic device surfaces. *IEEE Trans. Biomed. Eng.* **2001**, *48*, 838−842.

(11) Scholl, M.; Sprossler, C.; Denyer, M.; Krause, M.; Nakajima, K.; Maelicke, A.; Knoll, W.; Offenhausser, A. Ordered networks of rat hippocampal neurons attached to silicon oxide surfaces. *J. Neurosci. Methods* **2000**, *104*, 65−75.

(12) Lauer, L.; Vogt, A.; Yeung, C.; Knoll, W.; Offenhausser, A. Electrophysiological recordings of patterned rat brain stem slice neurons. *Biomaterials* **2002**, *23*, 3123−3130.

(13) Chang, J. C.; Brewer, G. J.; Wheeler, B. C. Modulation of neural network activity by patterning. *Biosens. Bioelectron.* **2001**, *7*−*8*; 527−533.

(14) Merz, M.; Fromherz, P. Polyester microstructures for topographical control of outgrowth and synapse formation of snail neurons. *Adv. Mater.* **2002**, *2*, 141−144.

(15) Wyart, C.; Ybert, C.; Bourdieu, L.; Herr, C.; Prinz, C.; Chatenay, D. Constrained synaptic connectivity in functional mammalian neuronal networks grown on patterned surfaces *J. Neurosci. Methods* **2002**, *117*, 123−131.

(16) Lauer, L.; Klein, C.; Offenhausser, A. Spot compliant neuronal networks by structure optimized micro-contact printing. *Biomaterials* **2001**, *22*, 1925−1932.

(17) Brewer, G. J.; Torricelli, J. R.; Evege, E. K.; Price, P. J. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **1993**, *35*, 567−576.

(18) Branch, D. W.; Wheeler, B. C.; Brewer, G. J.; Leckband, D. E. Long-term stability of grafted polyethylene glycol surfaces for use with microstamped substrates in neuronal cell culture. *Biomaterials* **2001**, *22*, 1035−1047.

(19) Burden Gulley, S.; Payne, H. R.; Lemmon, V. Growth cones are actively influenced by substrate-bound adhesion molecules. *J. Neurosci.* **1995**, *15*, 4370−4381.

(20) Makohliso, S.; Valentini, R. F.; Aebischer, P. Magnitude and polarity of a fluoroethylene-propylene electret substrate charge influences neurite outgrowth in vitro. *J. Biomed. Mat. Res.* **1993**, *27*, 1075−1085.

(21) Pfrieger, F.; Barres, B. A. Synaptic efficacy enhanced by glia cells in vitro. *Science* **1997**, *277*, 1684−1687.

(22) Ravenscroft, M. S.; Bateman, K. E.; Shaffer, K. M.; Schessler, H. M.; Jung, D. R.; Schneider, T. W.; Montgomery, C. B.; Custer, T. L.; Schaffner, A. E.; Liu, Q. Y.; Li, Y. X.; Barker, J. L.; Hickman, J. J.1998. Developmental neurobiology implications from fabrication and analysis of hippoc-

ampal neuronal networks on patterned silane-modified surfaces. *J. Am. Chem. Soc.* **1998**, *120*, 12169−12177.
(23) Brusés, J. Cadherin-mediated adhesion at the interneuronal synapse. *Curr. Opin. Cell Biol.* **2000**, *12*, 593−597.
(24) Chavis, P.; Westbrook, G. Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse. *Nature* **2001**, *411*, 317−321.
(25) Ranscht, B. Cadherins: molecular codes for axon guidance and synapse formation. *Int. J. Dev. Neurosci.* **2000**, *18*, 643−651.

Accepted for publication June 9, 2003.

BP034016F