Chemical micropatterning of polymeric cell culture substrates using low-pressure hydrogen gas discharge plasmas

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Micropatterned cell cultures will allow a new quality of bioartificial systems. Here, an approach to chemical micropatterning of polymer substrates is presented, which is completely based on low pressure gas discharge processes. Well expressed micropatterned cell cultures on polystyrene and poly (ether ether ketone) were obtained with many different cell types. No impairment of typical cell behavior was observed.

1. Introduction

A new class of cell culture substrates became available by the development of chemical micropatterning techniques. They allow control of cell adhesion on a microscopic scale. Thus, the development of novel biosensors and bioartificial systems is possible. These systems require compact two- and three-dimensional microstructured substrates with a chemically suited surface in order to mimic the basic functions of natural tissue. Examples of first experiments on chemically micropatterned cell culture substrates are bioartificial neuronal networks [1], culture systems with improved oxygen supply and a bioartificial liver in sandwich structure [2].

The first experiments to generate chemical contrasts on cell culture substrates were performed in 1965 [3]. Deposition of palladium vapor on cellulose acetate led to structured mouse fibroblast cultures. Using this technique cell motility was studied [4]. The generation of chemical contrasts using technologies from microelectronics opened up a new era of research. Since 1988 [5] chemical patterns in tissue-like dimensions of 1–50 μm can be produced on silicon, quartz and glass using masking techniques from microelectronics. The contrast is generated by silanes with amino functional groups which enhance cell adhesion and by silanes with hydrophobic alkyl groups to suppress adhesion. Meanwhile, structures of comparable sizes can also be generated by either photochemical or micro contact printing techniques. The latter use self-organization effects of amphiphilic molecules [6] (self-assembled monolayer, SAM). These techniques have been compared to gas discharge plasma assisted ones [7, 8] recently [9].

Here, a new approach to chemical micropatterning is presented which is completely based on gas discharge plasma processing. In contrast to the techniques mentioned before, this technique is well suited for polymer substrates with proven biocompatible properties. Physical plasmas consist of chemically reactive, ionized gases. Their suitability for the generation of topological microstructures is well known. This could be of value for the creation of microarchitectures mimicking tissue *in vivo* [10, 11]. Moreover, they allow a surface to be directly chemically modified by introducing functional groups while preserving the bulk properties of polymers. Plasma processing does not require additional intermediate layers for chemical patterning as for example, SAM-like techniques. Most importantly, it enables covalent bonding for the fixation and immobilization of biomolecules on various substrates.

2. Materials and methods

2.1. Substrates and substrate treatment

The results presented focus on chemical patterning of polystyrene (PS). For this, commercially available cell culture dishes with 60 mm diameter were used as delivered. Their surface was either untreated (PS) or plasma-functionalized (Primaria^{TM} [12]) by the supplier (Becton Dickinson, NJ, USA). In addition to PS, untreated poly(ether ether ketone) (PEEK) foils (Reichelt Chemie, Heidelberg, Germany) were used as an alternative substrate material.

The plasma process developed splits up into two subsequent steps. First, the whole surface is modified by a process which generates functional groups capable of bonding biomolecules. Second, patterning is performed by covering the surface with a laser-cut metallic mask (see Fig. 1) exhibiting characteristic dimensions down to 30 μm and partially removing the functional groups by a soft plasma etching process.

The chemistry of the surface modifications was analyzed by contact angle (H_2O, sessile drop) and X-ray photoelectron spectroscopy (XPS) measurements.

2.2. Cell culture

Different cell types were randomly seeded on the patterned substrates with densities well below those of confluent monolayers. Five cell lines and two types of primary cells were cultured. Two of the cell lines, L929 cells (DSM ACC2, DMSZ Braunschweig, Germany; RPMI 1640, 10% fetal calf serum (FCS)) and 3T3 cells (DSM ACC 173, DMSZ Braunschweig, Germany; (DMEM), 5% FCS), are mouse fibroblasts, whereas the other cell lines, KB cells (nasopharyngeal carcinoma, ICN, Costa Mesa, CA, USA; minimum essential medium (MEM), 10% FCS), RT112 cells (bladder cancer, provided by J. Masters, St Paul's Hospital, London, UK; RPMI 1640, 10% FCS) and HaCaT cells (keratinocytes, provided by N. Fusenig, German Cancer Research Centre, Heidelberg, Germany; Dulbecco's modified MEM (DMEM), 10% FCS) are derived from human epithelial cells. The primary cells were human fibroblasts (HBF, conjunctiva, Eagle's MEM(EMEM), 20% FCS) and human umbilical vein endothelial cells (50% DMEM, 50% Ham's F12). Note that standard serum-containing culture conditions (humidified atmosphere of 95% air and 5% CO_2 at 37 °C) were always applied. Phase contrast microscopy and fluorescence staining techniques were used to monitor cell morphology and cell function.

3. Results and discussion

Different processing sequences were identified which lead to efficient chemical micropatterning of the substrates.

For PS, the first processing step was performed downstream of a low pressure Ar/NH_3 microwave excited plasma (50 Pa pressure, $[Ar]/[NH_3] = 4/1$ partial pressure ratio, $2\,W\,cm^{-3}$ plasma power density). It typically creates surfaces containing around 4% nitrogen-containing functional groups, 50% of them being amino groups (Fig. 2). This approach of plasma-induced chemical functionalizing can be extended to other materials. Also a suitable process was found for PEEK, a chemically inert high performance polymer with possible medical uses (see Fig. 3). Comparison of cell densities on PS and PEEK functionalized by this process with Primaria^{TM} shows similar, well-expressed cell adherence on all three substrates.

The second micropatterning processing step was applied to all three materials equipped with functional groups, Primaria^{TM} and plasma-treated PS and PEEK, in a similar manner. Etching of functional groups through a metallic mask was done downstream of a low-pressure

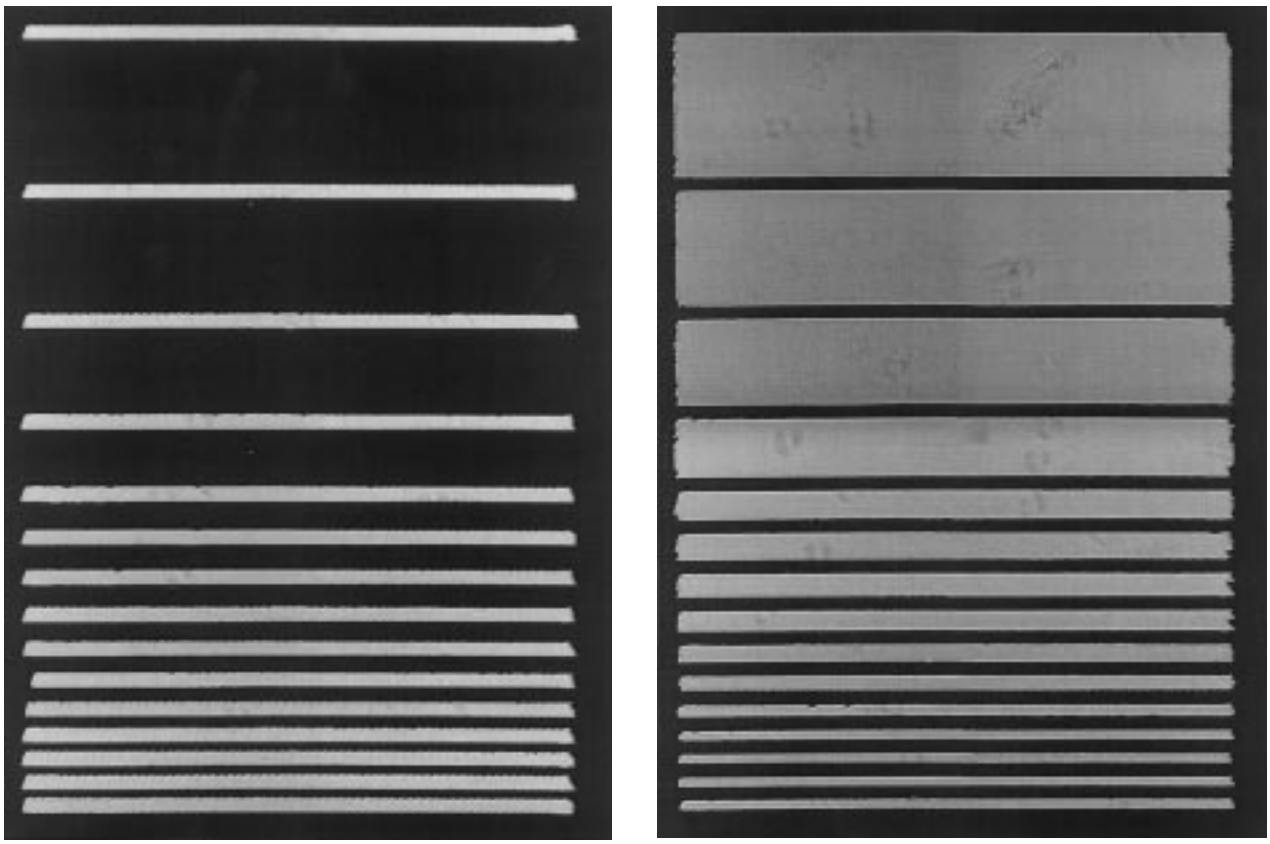
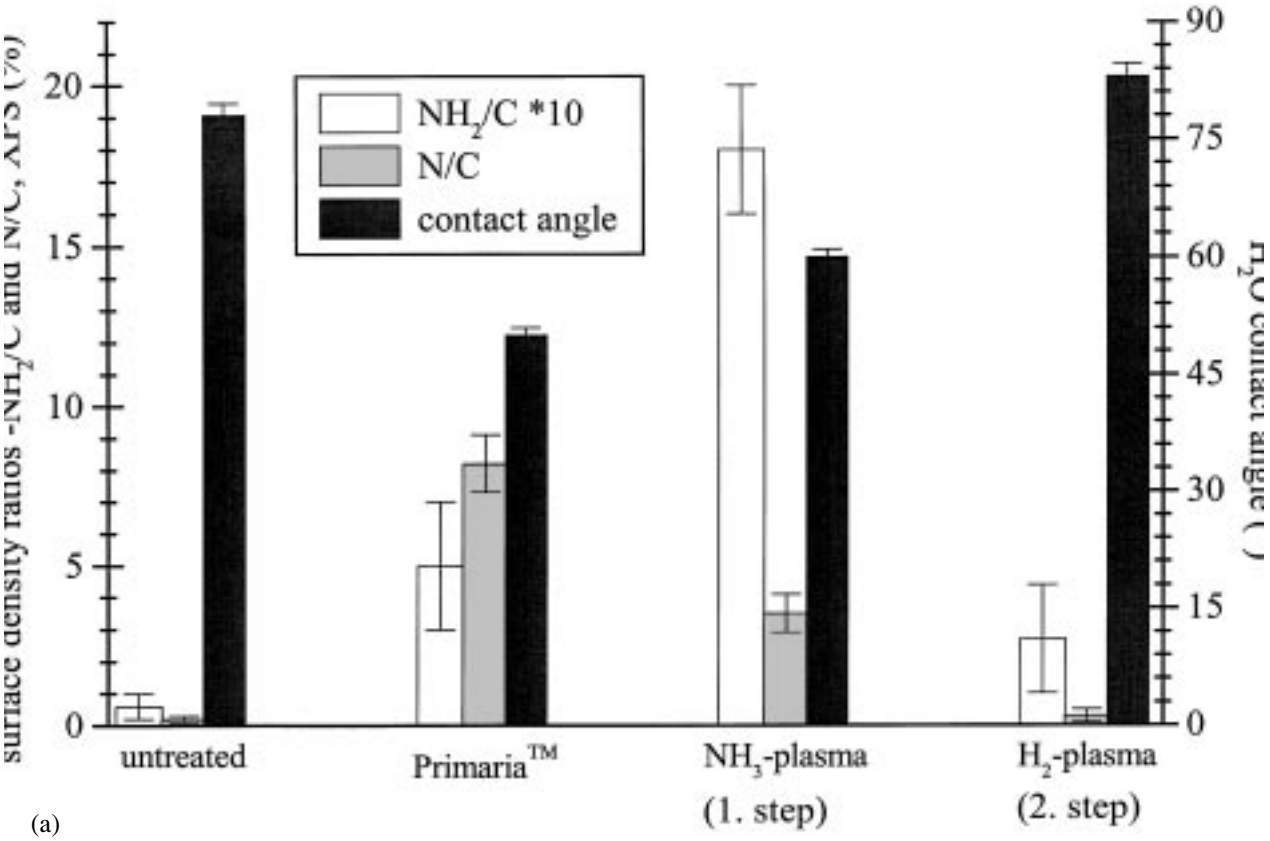


Figure 1 Microphotography of laser cut metal mask. The logarithmic variation of slotted holes or bridges (30 to 500 μm) lies in dimensions typical for tissues. This variation was chosen to investigate the influence of pattern dimensions on pattern reproduction by growing cell cultures.



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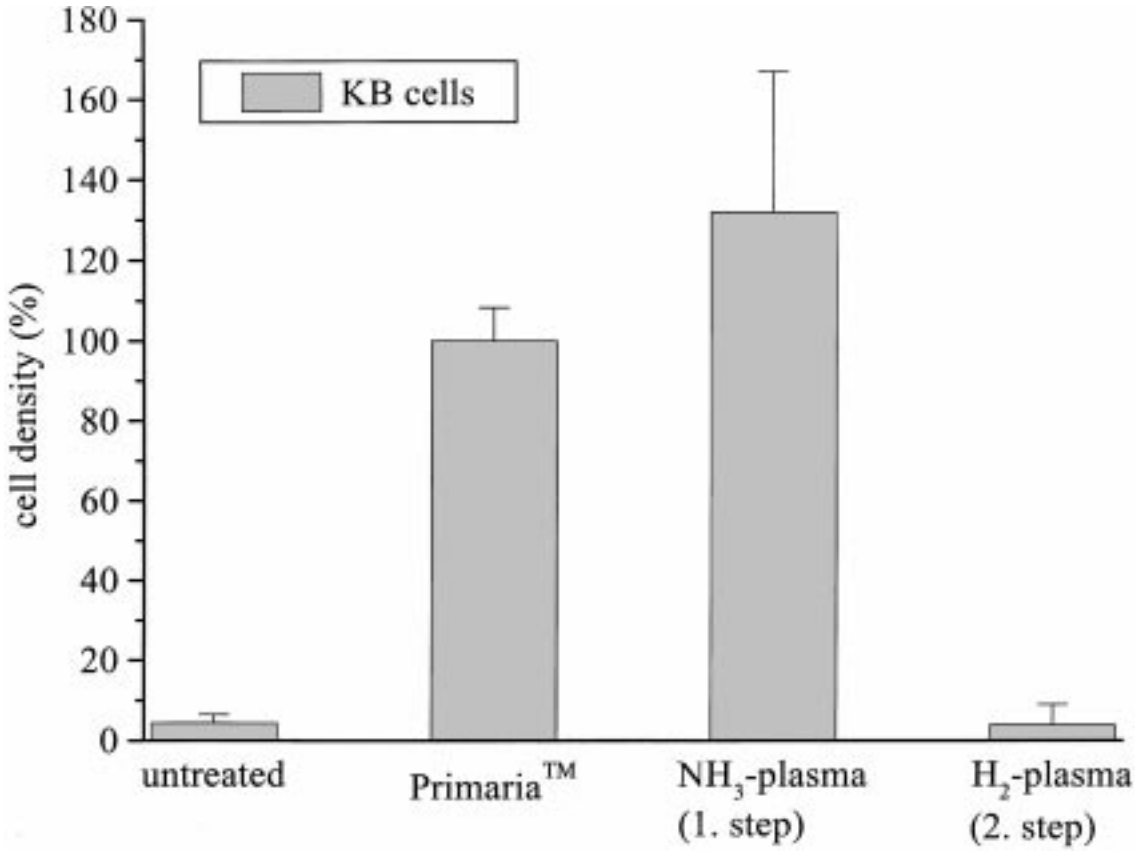
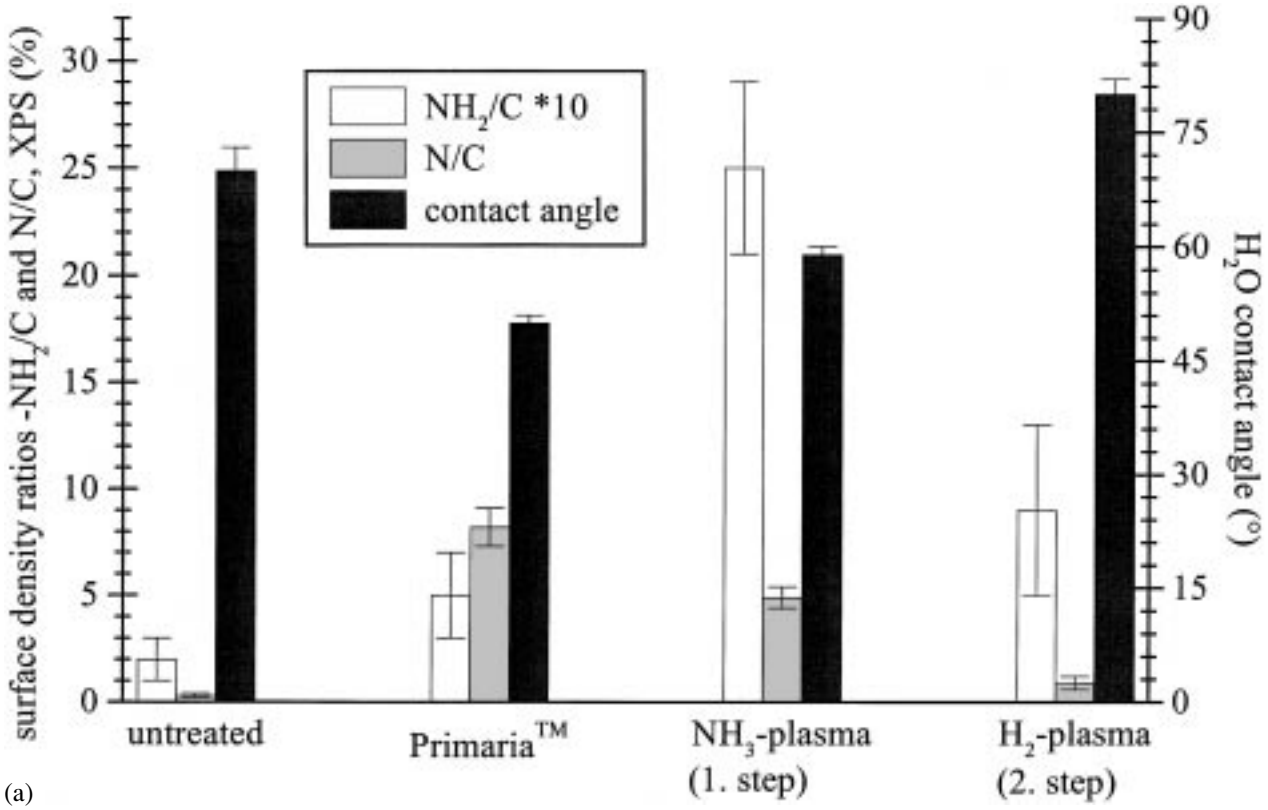


(b)

Figure 2 (a) Characteristic surface properties (chemical composition, measured by XPS, and H_2O contact angle) and (b) cell densities of untreated PS, functionalized PS after the first processing step (with NH_3-plasma or with N_2/O_2-plasma: Primaria™) and etched PS after the second processing step (with H_2-plasma). The increased wettability after introduction of nitrogen containing groups (functionalization), especially amino groups, lead to an enhanced cell density 24 h after seeding. H_2-plasma modification yields hydrophobic surfaces. The cell density on these surfaces is drastically reduced.



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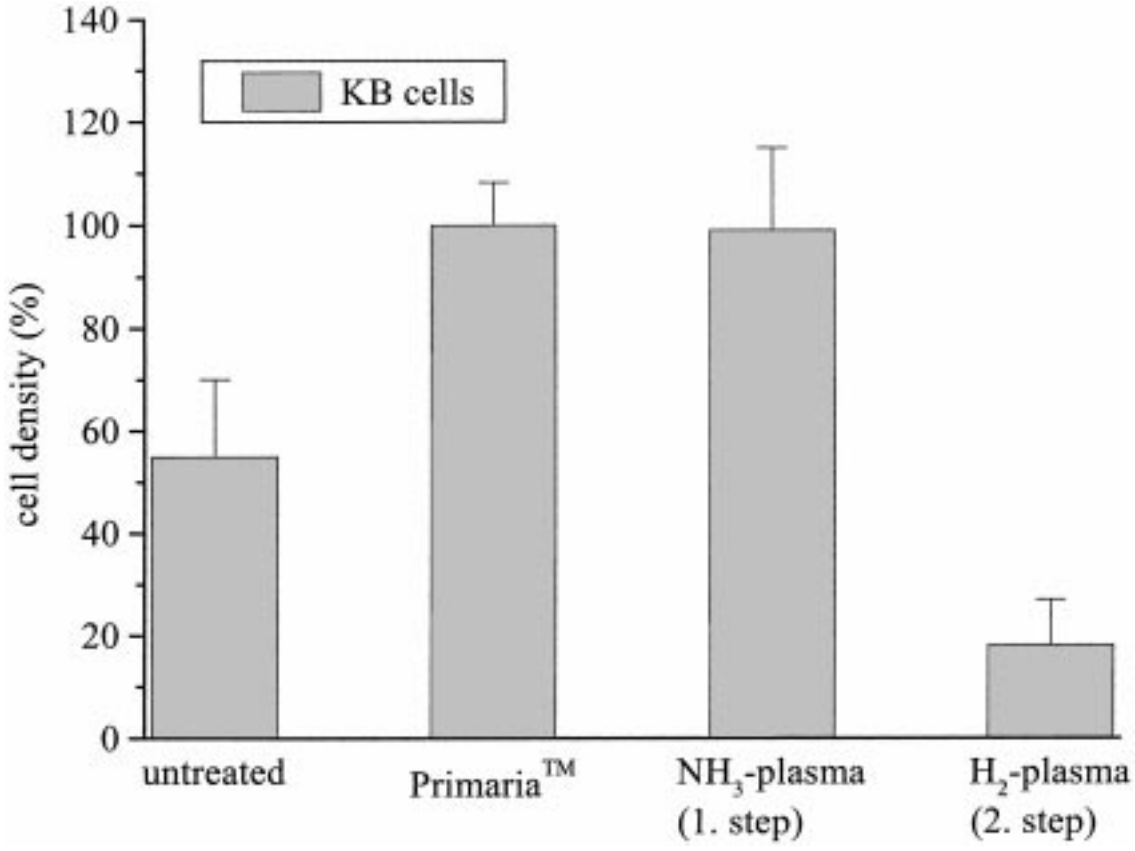


(b)

Figure 3 (a) Characteristic surface properties (chemical composition, measured by XPS, and H_2O contact angle) and (b) cell densities of untreated PEEK, functionalized PEEK after the first processing step (with NH_3-plasma) and etched PEEK after the second processing step (H_2-plasma modification), compared to Primaria™. The plasma processes have comparable effects on surface properties and cell densities like PS (see Fig. 2). Especially, the introduction of nitrogen containing (amino) groups after NH_3-plasma functionalization lead to cell density comparable to Primaria™ and after H_2-plasma modification the cell density is drastically reduced.

microwave plasma in Ar/H_2 (30 Pa pressure, $[Ar]/[H_2] = 3/1$ partial pressure ratio, $1\,W\,cm^{-3}$ plasma power density). This leaves a very low energetic surface which is characterized by reduced densities of oxygen and nitrogen (see Figs 2a and 3a); the aromatic ring contents are reduced to about 30% of the untreated bulk material.

A substrate, chemically structured in this way, induces micropatterned cell culture growth. The results of the cell culture experiments, shown in Fig. 4, exhibit a

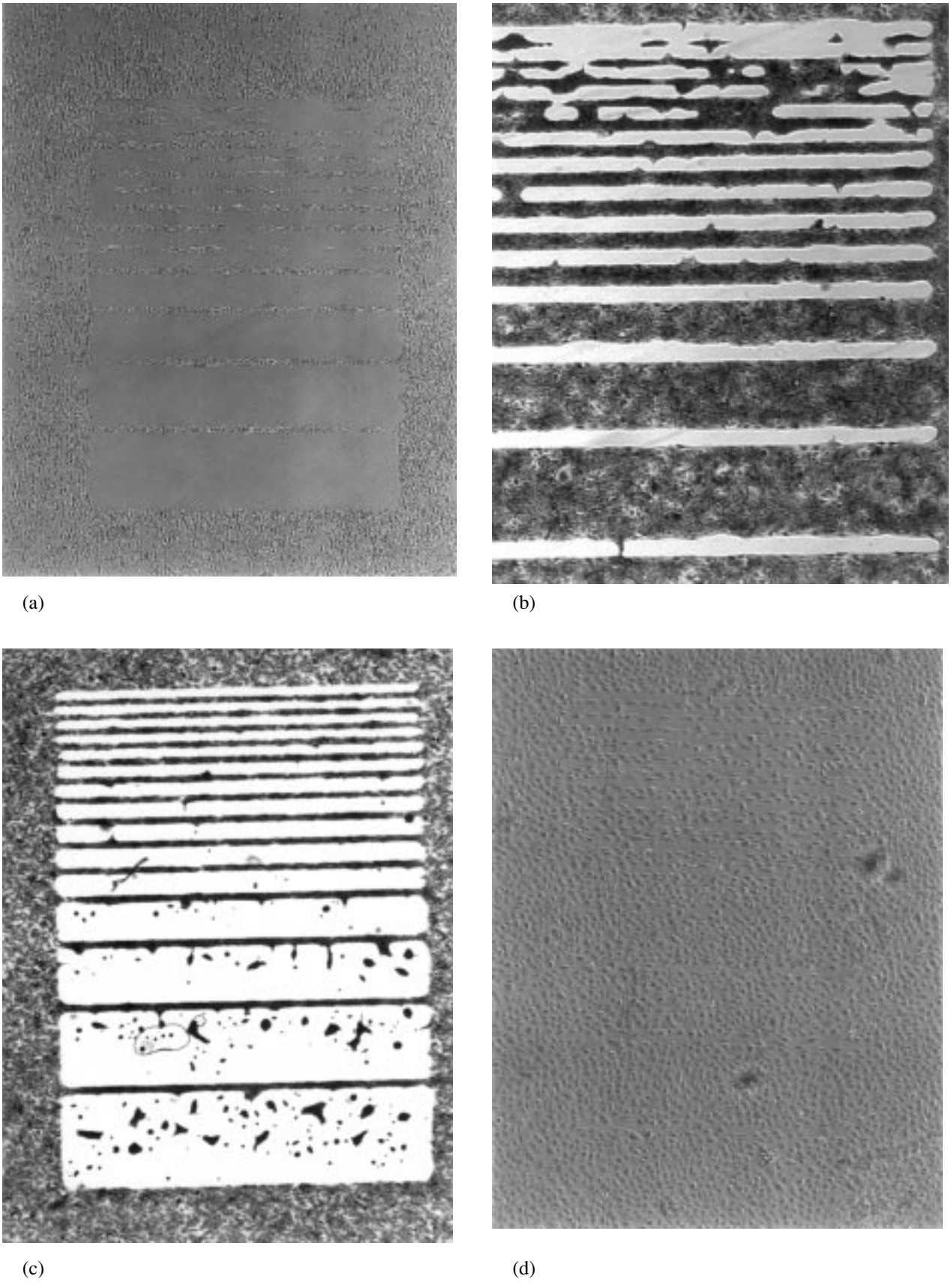
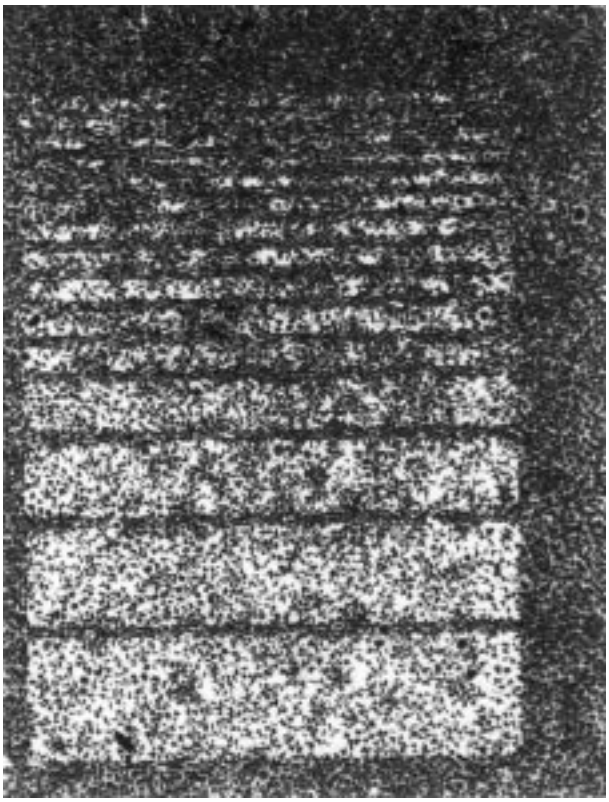


Figure 4 Microphotography of cell cultures of the following cell types: cell lines: (a) KB, (b) RT112, (c) HaCaT, (d) 3T3, (e) L929; primary cells: (f) HBF, (g) HUVEC.



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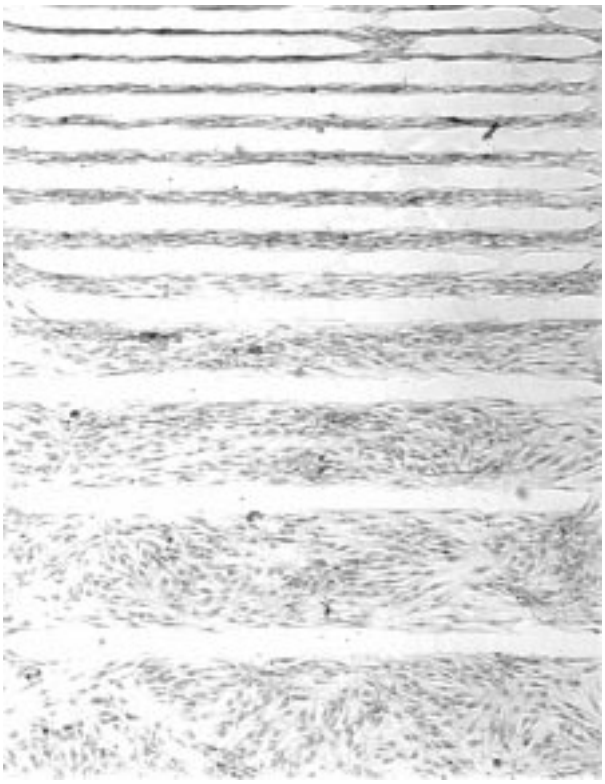

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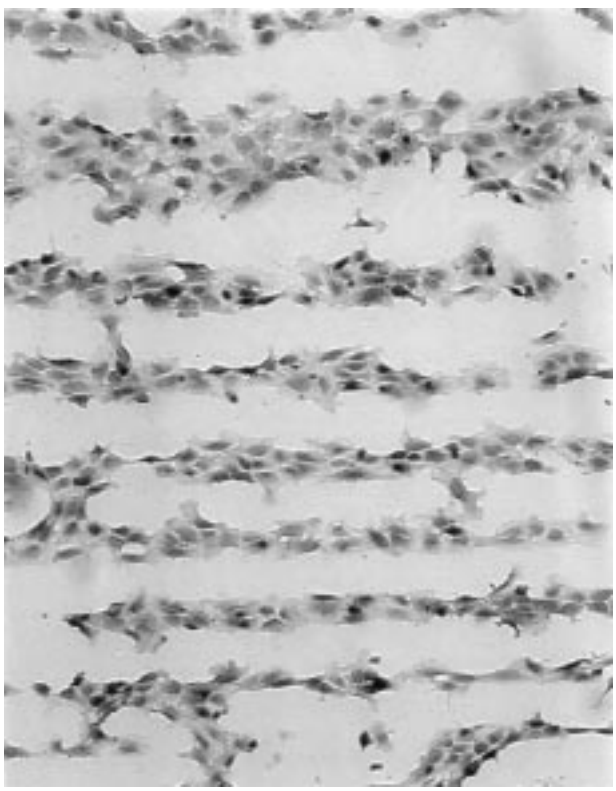


(g)

Figure 4 (Continued)

considerable contrast in cell density between plasma-functionalized and plasma-etched areas of the substrate. As Figs 2b and 3b show, the resulting contrasts in cell density on PS and PEEK are comparable.

Analysis of cell morphology by phase contrast microscopy, of cell proliferation by fluorescent BrdU staining and of membrane integrity after fluorescence staining with fluorescein diacetate and ethidium bromide showed no impairment of physiological cell characteristics. Especially, the chemical transitions on the surface by plasma modification did not have any toxic effects on cells or membrane integrity. The temporal development of cell patterns is characterized by a fast initial pattern recognition process due to oriented migration followed

by proliferation and migration processes on the adhesive surface domains (for an example see Fig. 5). Differences in pattern recognition quality can be explained by the size and the type of the cells. While epithelial cells nicely reproduce the underlying chemical contrast, fibroblast patterns are characterized by more diffuse borders (see Fig. 4). In order to study this effect in a micropatterned culture of 3T3 fibroblasts F-actin (element of the cytoskeleton) was visualized with fluoroscein isothio cyanate (FITC)–phalloidin while vinculin (for focal

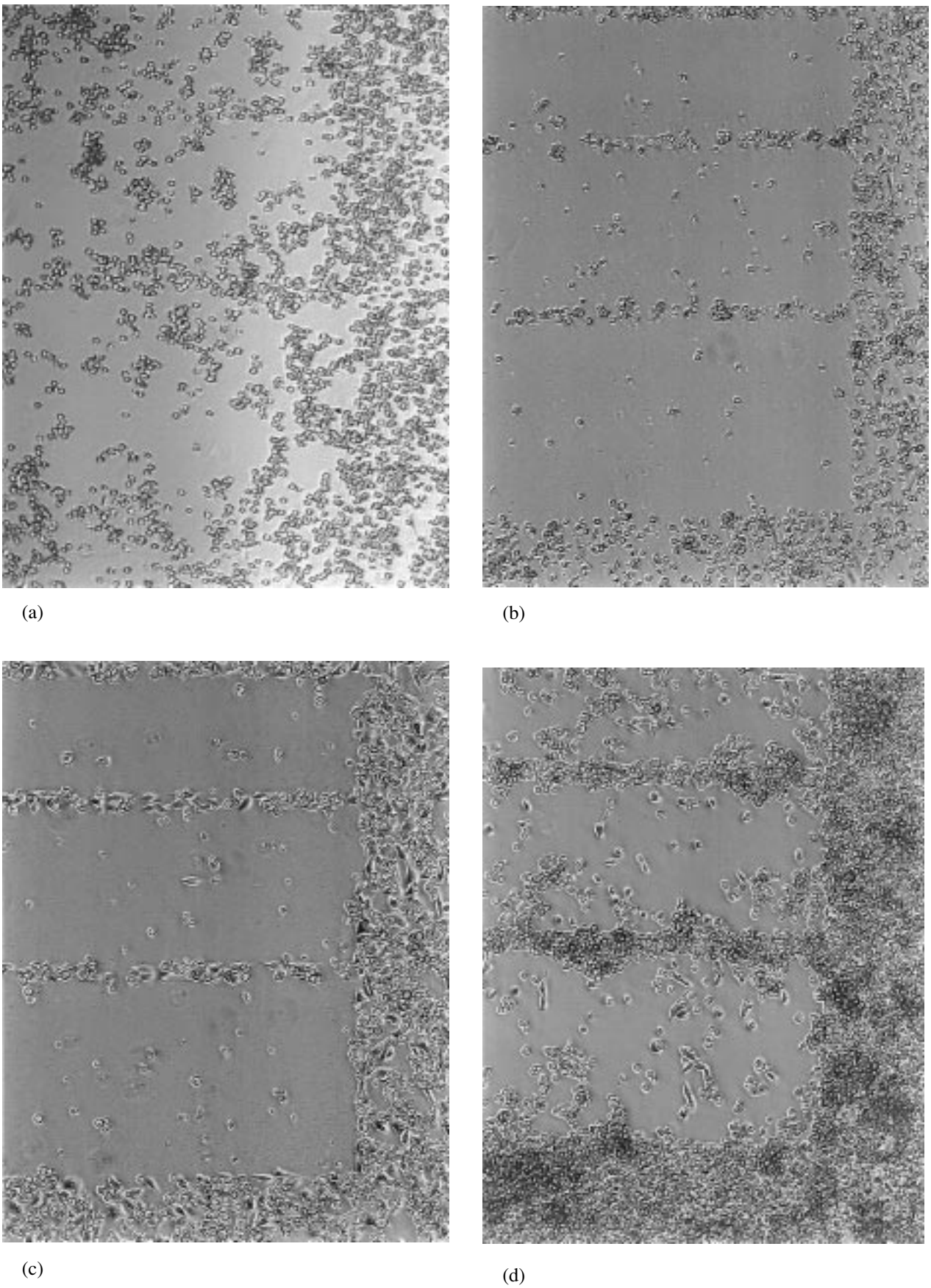
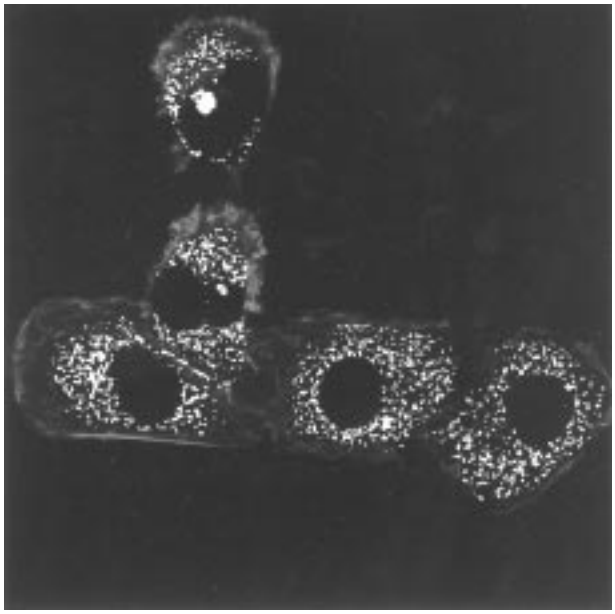
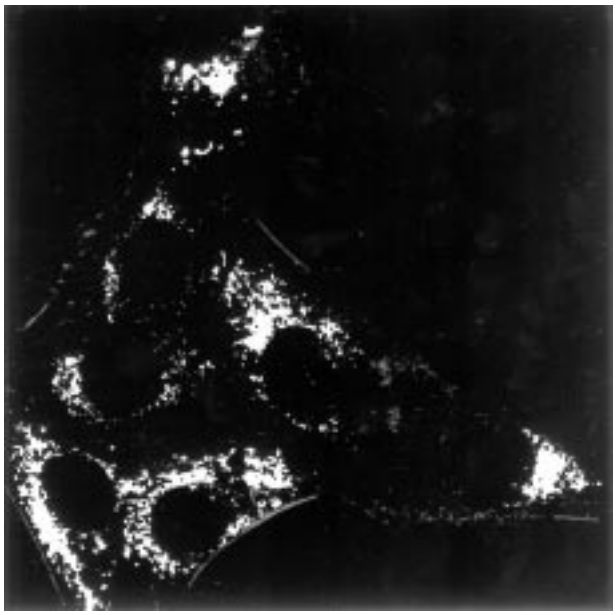


Figure 5 Temporal development of pattern-guided growth of KB cell cultures: (a) 3 h, (b) 6 h, (c) 48 h, (d) 197 h after seeding.



(a)



(b)

Figure 6 Distribution of F-actin (gray structures at the edges of the cells) and laminin (white, preferably dotted structures inside the cell bodies) after double staining (see text) in a culture of 3T3 fibroblasts on patterned substrates: (a) after 4 h of cell culture; (b) after 36 h of cell culture.

contacts), laminin and fibronectin (both components of the extracellular matrix; ECM) were visualized by immunofluorescence staining with specific antibodies. During the first 4 h of cell culturing the deposition of ECM components is restricted to the adhesive areas of the substrate (see Fig. 6a). Afterwards, ECM can also be found outside these areas (see Fig. 6b). Fibroblasts are known to produce ECM components very efficiently. Long-term observations of 3T3 fibroblasts and KB cells on chemical micropatterns with different geometry showed that cell patterns typically remained dynamic, but well-expressed, for 7–14 days.

4. Conclusions

In this paper an approach to chemical micropatterning of polymer substrates is presented, which is completely based on low-pressure gas discharge plasma processes. It allows the induction of well-expressed, micropatterned cell cultures on polystyrene and poly(ether ether ketone) with different cell types. The results indicate that this processing strategy may be an approach of rather universal applicability.

Acknowledgments

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