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Patterning neuronal and glia cells on light-assisted functionalised photoresists

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Abstract

A common photosensitive polymeric material used in semiconductor microlithography (diazo-naphto-quinone/novolak resist) was pattern-exposed with near-UV light to create carboxylic-rich areas on the polymer surface. The patterned surfaces were further functionalised via: (1) the anchorage of peptides for specific cell-attachment or cell-detachment functions; or (2) the diffusion of silicon rich chemical species to achieve the cell detachment. Pairs of antagonistic surface characteristics controlled the cell attachment: (1) amino-rich or carboxylic-rich surfaces; and (2) hydrophilic or hydrophobic surfaces; in which the former promoted the adhesion. It was found that common microlithographic materials and techniques can be upgraded to allow an effective control of the lateral organisation of the artificial arrays of neuronal and glia cells.

Keywords: Cell adhesion; Neuronal cells; Surface functionalisation; Microlithography

1. Introduction

The interaction of the living cells with artificial materials has relevance to several bioengineering fields. For instance, vertically structured cell engineering found applications in biosensors based on plant and animal tissues, (Wijeseriya and Rechnitz, 1993), in creating electronically modulated biological functions (Aizawa et al., 1994), and in devising appropriate biomaterials for stable three-dimensional—but random in organisation—scaffolds capable of supporting axonal regeneration for the treatment of severe neurological injuries (Fields et al., 1989; Plant et al., 1995). Laterally structured cell engineering pioneered by Klebe's work (Klebe, 1988) builds on the understanding of the specific interactions between cell and artificial surface, with the aim of reaching an effective two-dimensional control of these interactions on the same substrate. Cell patterning was applied to research on cell apoptosis (Chen et al., 1997), cell guidance (Britland et al., 1992a; Clark et al., 1992; Clark 1994; Clark et al., 1997), neuronal cell differentiation triggered by specific substrates (Clark et al., 1993), and the fabrication of artificial neuronal cell-on-electronic devices (Connolly et al., 1990; Fromherz et al., 1991, 1993; Fromherz and Muller, 1994; Gross et al., 1995). In view of the fact that photoresists are purposefully designed as patterning materials, we recently assessed (Nicolau et al., 1996) several microlithographic materials and techniques as candidates for patterning artificial arrays of neuronal cells.

In a parallel development, the laterally structured biomolecular engineering (Bhatia et al., 1989; Matsuda and Sugawara, 1995; Pritchard et al., 1995a; Mrksich et al., 1996) found applications in biosensor fabrication (Pritchard et al., 1995b), and combinatorial chemistry (Fodor et al., 1991). Regarding the latter, techniques using light-directed, spatially addressable, step-wise chemical synthesis of bioactive peptides have been reported (for recent reviews, see Ellman and Gallop, 1998; and Gallop and Fitch, 1997). Again, in the light of the proven printing capabilities of the common semiconductor microlithographic materials, we assessed

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their potential for biomolecular patterning (Nicolau et al., 1998a; Nicolau et al., 1998b). The potential synergism between the patterning of biomolecules and the patterning of cells was explored via the chemical linkage of the bioactive proteins (Aplin and Hughes, 1981; Britland et al., 1992a; Clark et al., 1993; Chen et al., 1997; Mrksich et al., 1997) or via the wettability manipulation through silanazation (Kleinfeld et al., 1988; Clark et al., 1992; Britland et al., 1992b; Clark, 1994).

In a previous study (Nicolau et al., 1996), we illustrated how the surface photochemistry of the common microlithographic materials can be used to control the specificity of the neuronal cell attachment on the polymer surfaces. To this end, the present study augments our previous work, aiming at using upgraded microlithographic materials and techniques to print patterns of proteins, peptides and silylated surfaces as scaffolds for artificial arrays of neuronal and glia cells.

2. Materials and methods

2.1. Materials

The photosensitive material used was a common, positive tone, commercial photoresist, (S1400-17, purchased from Shipley, Co.) doped with a contrast enhancer (imidazole at approximate 1% in solid content). The photoresist is a mixture of a photoactive compound based on diazo-naphto-quinone (DNQ) and a novolak based resin. A common, metal-free developer (MF-319, purchased from Shipley Co.), diluted to reach 0.237 N, was used to obtain the selective dissolution of the patterned-exposed features.

Two peptides with high and low nitrogen content were used for the peptide-functionalisation of the polymer surface. The nitrogen-rich peptide (SFP4) is a neuropeptide synthesized in-house according to a procedure described previously (Taguchi et al., 1995) with the sequence Gly–Ala–Cys–Gly–Arg–Gly–Asp–Ser–Pro–Cys–Gly–Ala and an intramolecular linkage between the two Cys. The peptide with low nitrogen content is poly-glutamic acid (pGA) with a molecular weight higher than 8000 (purchased from Peptide Institute Inc., Japan). The crosslinker used for anchoring the protein or peptide amino ends to the carboxylic sites created on the photoresist surface was (1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide)/HCl (EDC, purchased from Pierce Co.). Finally, SigmaCoat (purchased from Aldrich Co.) was used for the silylation of the photoresist surface.

2.2. Experimental procedures

2.2.1. Substrate preparation for cell patterning

The procedures for coating, exposure and functionalisation of the photosensitive material were described in detail elsewhere (Nicolau et al., 1998a). Briefly, 15 mm glass substrates were spin coated with photoresist, dried for 1 h at 85°C in a convection oven, patterned-exposed using an exposure aligner (Mikasa manipulator MA-8, Mikasa Co.) and e-beam patterned chromium masks at exposure energies of ~ 100 mJ/cm^2. The exposed areas are rich in carboxylic groups due to the photolysis of DNQ and because we used high exposure energies to fully photolise the DNQ. The chromium masks were designed to (1) reproduce the average geometry of the neuronal cell used in experiments, i.e. rectangles of 5×5 μm; (2) the average geometry of the neurite, i.e. lines 50 μm long and 2 μm thick; and (3) the connectivity of an array of neuronal cells. Furthermore, the peptide functionalisation of the exposed areas consisted of (1) placing the exposed slides on the bottom of the wells of a Clostar cluster dish; (2) adding 400 μl/well of a solution of the neuropeptide with a concentration of 25 μg/ml; (3) flooding the wells with 100 μl/well solution of EDC at a concentration of 2 mg/ml; (4) leaving the dish for incubation for 2 h at 37°C; and (5) washing thoroughly with deionized water followed by soft drying. To promote a higher selectivity of the neuronal cell attachment on the patterned-exposed areas functionalised with neuropeptides, against the areas surrounding the pattern, the presented procedure was followed by (6) exposing the whole surface of the photoresist; (7) the functionalisation of the previously unexposed areas with pGA using the same procedure as above; and (8) washing of the surface followed by soft drying.

A different procedure was used to assess the attachment of the neuronal cells in grooves developed in the photoresist layer. After coating, soft bake and patterning exposure, the printed features were developed via selective dissolution with the developer. The profiled features were blanket exposed, immersed in saturated HCl solution at 90°C for 15 min, and baked for 1 h at 100°C in a convection oven. This procedure induces both the decarboxylation of the inden-carboxylic acid as well as the crosslinking of the novolak resin.

Finally, the procedure for selective silylation comprised in the patterning exposure as previously presented, followed by a selective process of liquid diffusion of the silylating agent (SigmaCoat) in the hydrophobic, unexposed areas. For this process to occur, one drop of SigmaCoat solution was deposited on the surface of the patterned photoresist surface followed by a slow spinning of the substrate for 1 min.

2.2.2. Neuronal cell culture

Cells for the long-term culture were prepared from cerebral hemispheres of chick embryos as previously described (Takioka et al., 1993). Briefly, the dissociated cerebral neurons (5×10^4/cm^2) were plated on each

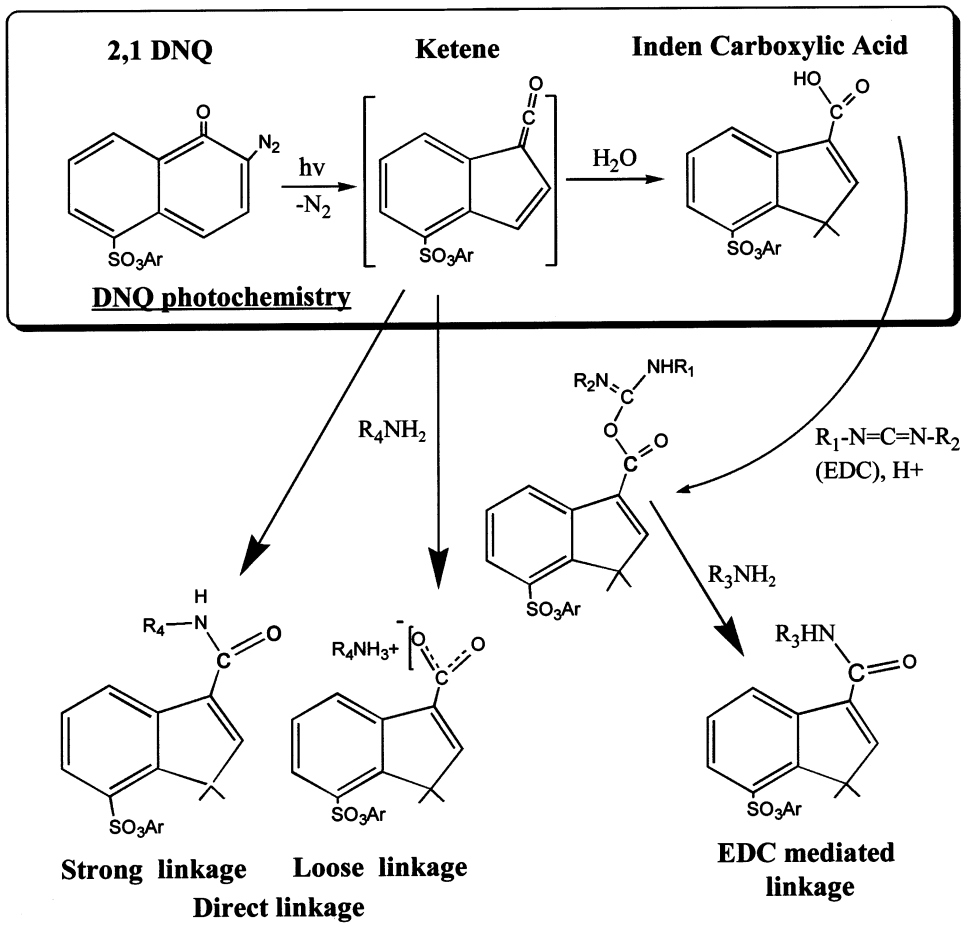

Fig. 1. The photochemistry of the DNQ/novolak system and its subsequent use for the peptide-specific functionalisation of the photoresist surface.

control or modified substrate settled in a well of 24 well cluster dish containing 50% of modified Earl's minimum essential medium, 10% fetal calf serum (FCS) and 40% GIT medium (Nihon Seiyaku Co. Ltd.). Cells were cultured for 7 days at 35°C in 5% CO_2/95% air and saturating humidity. Half of the culture medium was changed every two days. The objective of this study was to achieve the selectivity of the patterning of the neuronal cells and glia cells with respect to the surface-patterned chemical functionalities, and with respect to each other. Hence no attempt has been made to separate the neuronal cells from glia cells in the long-term culture.

3. Results and discussion

The neuronal cell adhesion was studied in view of the light-assisted chemical functionalisation of the DNQ/novolak polymeric surface. This functionalisation was induced after exposure through the attachment of peptides with specific functions on the exposed surface and through the selective silylation of the unexposed surface. The adhesion of the neuronal/glia cells on flat but functionalised surfaces was compared with the confinement of the neuronal/glia cells in profiled 'grooves' produced via the dissolution of the patterned photoresist.

3.1. The photochemistry and surface functionalisation of the DNQ/novolak

The DNQ/novolak system is the most common photosensitive, patternable material used in today's semiconductor microlithography. The merits of the DNQ/novolak system (i.e. relatively high sensitivity, high optical contrast, and aqueous based dissolution of the exposed areas) have been upgraded over the years via several technological improvements, e.g. Image Reversal and silylation-based Surface Imaging techniques

This classical system benefited from extensive reviews on DNQ/novolak-based microlithographic techniques and materials and DNQ photochemistry (Ersov et al., 1981; Dammel, 1993).

We presented (Nicolau et al., 1996) the photo- and thermo-chemistry of the DNQ/novolak system in the context of the patterning of the neuronal cells directly on the photoresist surface without any further function alisation (as opposed to the present study, where the

photo-assisted peptide functionalisation is used). The functionalisation of the exposed patterns was achieved via the chemical linkage of the peptide amino groups to the photolised-induced carboxylic groups on the polymer surface (Nicolau et al., 1998a,b). The chemistry of the DNQ/novolak system used for printing peptides on the photoresist surface is presented in Fig. 1. The silylation of the unexposed areas has been achieved via the selective diffusion of a liquid silylating agent in the hydrophobic, unexposed areas. This procedure is similar to the surface imaging techniques available in semiconductor microlithography (Nicolau et al., 1992; Dammel, 1993).

3.2. Cell attachment on the photoresist surface

In a previous study (Nicolau et al., 1996), we studied the control of the attachment of the neuronal cells directly on the photoresist surface using only the chemical transformations induced by light or temperature, without any further functionalisation. The high selectivity of the cell attachment targeted in this study was achieved incrementally, based on the understanding of the interaction between the cell and the photopolymer surface. This understanding, with regard to the attachment of the neuronal and glia cells on the photo- and thermal-only functionalised photoresist surface, is summarised below:

- Neuronal cells have different attachment behaviour than glia cells.
- Neuronal cells are more sensitive to surface functionality than surface hydrophilicity, preferring hydrophilic, amino-rich and positively charged surfaces.
- Glia cells are more sensitive to surface hydrophilicity than surface chemical functionality. Moreover, glia cells seem to adapt on a larger range of surface hydrophobicity. Subsequently, the neuronal cells attach on a 'blanket' of glia cells.
- Of the photoresist formulations and microlithographic techniques available in semiconductor microlithography, it was found that the DNQ/novolak polymer doped with a basic additive (imidazole) processed in the positive tone assured the highest selectivity. Therefore, this material and technique were used as the starting point for the printing the scaffolds for the neuronal cells patterning.
- Photoresist profiled features could not prevent the spreading of the glia cells across the patterned features. Subsequently, the outgrowth of the neuronal cells is not confined in the channels etched in the resist layer.
- The non-adhesiveness was more difficulty attained than the specific attachment. It was observed that the non-adhesiveness of the neuronal cells is favoured by (1) very hydrophobic surfaces containing predominantly silicon functionalities; (2) very high concentration of carboxylic groups, possibly ionised (the surface of neuronal cells is also negatively charged, Yavin and Yavin, 1974); (3) mildly hydrophobic surfaces of crosslinked novolak (via thermal processes or UV radiation); and (4) mildly hydrophobic surfaces of novolak doped with the by-products of basic catalysed decarboxylation of the indene carboxylic acid (i.e. indene).

These results are summarised the first two rows in Fig. 2. The neuronal cell is represented with the negatively charged membrane facing down toward the photoresist surface. Each surface is described in terms of its hydrophobicity/hydrophilicity and the nature of the chemical functionalities on the surface. The surfaces are also represented with the probable type of the surface charges. The rectangle at the left of each surface represents a gauge-like indicator (not to scale) of the effect of the surface on the neuronal cell non-adhesiveness (the higher the rectangle the lower the propensity for cell attachment).

3.3. Selectivity of the cell attachment on peptide-functionalised surfaces

A sample of the neuronal and glia cell culture on the peptide-specific functionalised surface of the resist film is presented in Fig. 3. The 2 μm lines and the 5×5 μm rectangles at the intersections of the lines were functionalised with the SFP4 neuropeptide, whereas the 50×50 μm areas adjacent to the patterns were functionalised with pGA. The patterned features suffered residual dissolution (due to contact with the SFP4 solution with slightly higher than neutral pH).

In this study, we aimed at obtaining a higher selectivity of the attachment via (1) the increase of the adhesion of the neuronal cells on the patterns designed to mimic a neuronal network; and (2) the increase of the non-adhesiveness of the neuronal cells (and to a lesser extent of the glia cells) on the areas adjacent to the patterned features. A previous study using the same procedure for neuronal cells culture (Takioka et al., 1993) proved that SFP4 peptide has an important and favourable effect on the growth of the neuronal cells as compared with, e.g. poly-lysine functionalised surfaces. Because both poly-lysine and SFP4 have a large content of amino groups, we concluded that the adhesion of the proteins present in solution (e.g. albumin) did not reach a level that would obstruct the cell-peptide specific interaction.

The features functionalised with the SFP4 neuropeptide (i.e. 2 μm lines and 5×5 μm rectangles) promote the attachment of the neuronal cells, whereas the 50×50 μm areas functionalised with pGA (i.e. adjacent to the patterns) induce the relative non-adhesiveness of the neuronal cells. The spatial selectivity of the patterning of the neuronal cells is similar to the spatial selectivity

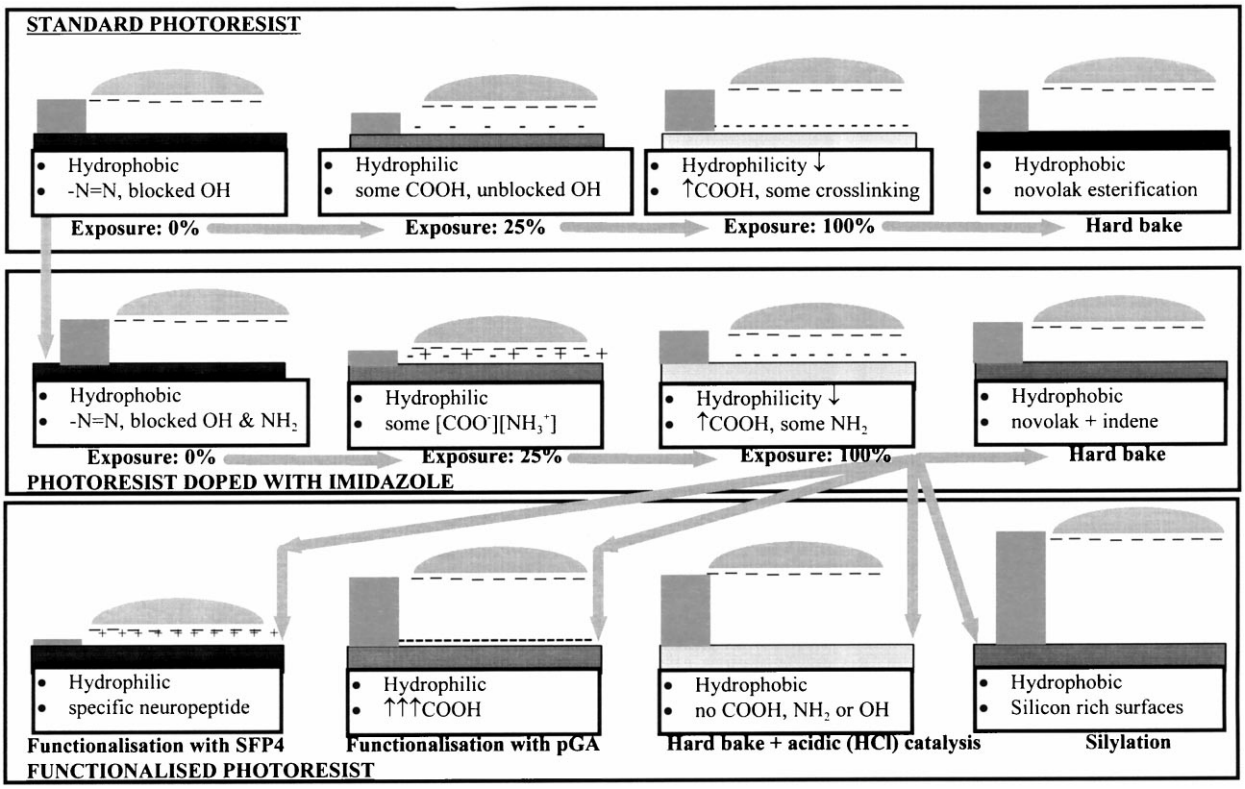

Fig. 2. The effect of the surface functionalities on the selectivity of the attachment of the neuronal cells. The bar at the left of each cell-substrate group represents an indicative measure of the non-adhesiveness of the neuronal cell to the functionalised surface.

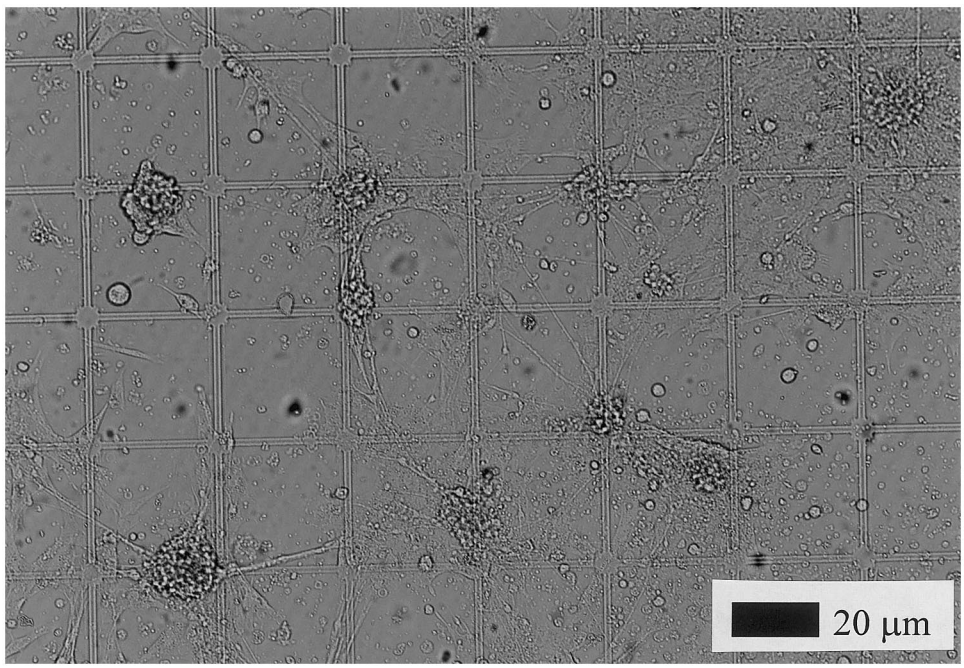

Fig. 3. Selective attachment of the neuronal and glia cells on the peptide-specific patterns on the photoresist surface.

obtained confining the glia cells in the 'channels' etched in photoresist layer (Fig. 5). However, the patterning on peptide-functionalised surfaces is superior with regard to the neuronal-to-glia selectivity, as the culture in Fig. 3 contains glia and neuronal cells, whereas the culture in Fig. 5 is almost free of neuronal cells.

As outlined above, the non-adhesiveness of the glia cells is less specific. An inspection of the Fig. 3 shows

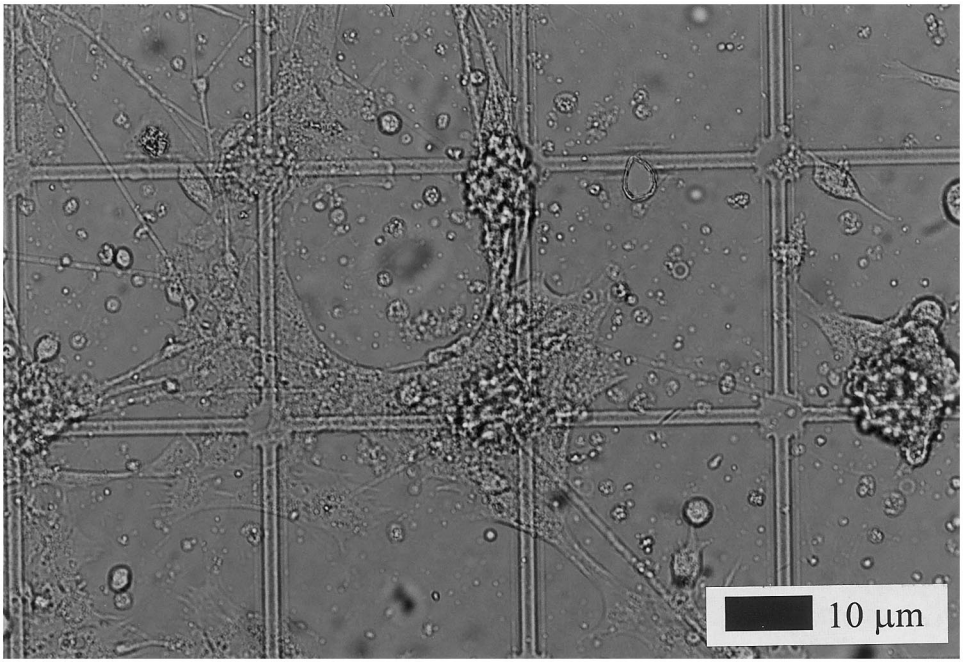

Fig. 4. Close-up view of an artificial network of neuronal and glia cells on the peptide-patterned photoresist. The patterns (lines and rectangles) are SFP4-functionalised and the adjacent areas are pGA-functionalised.

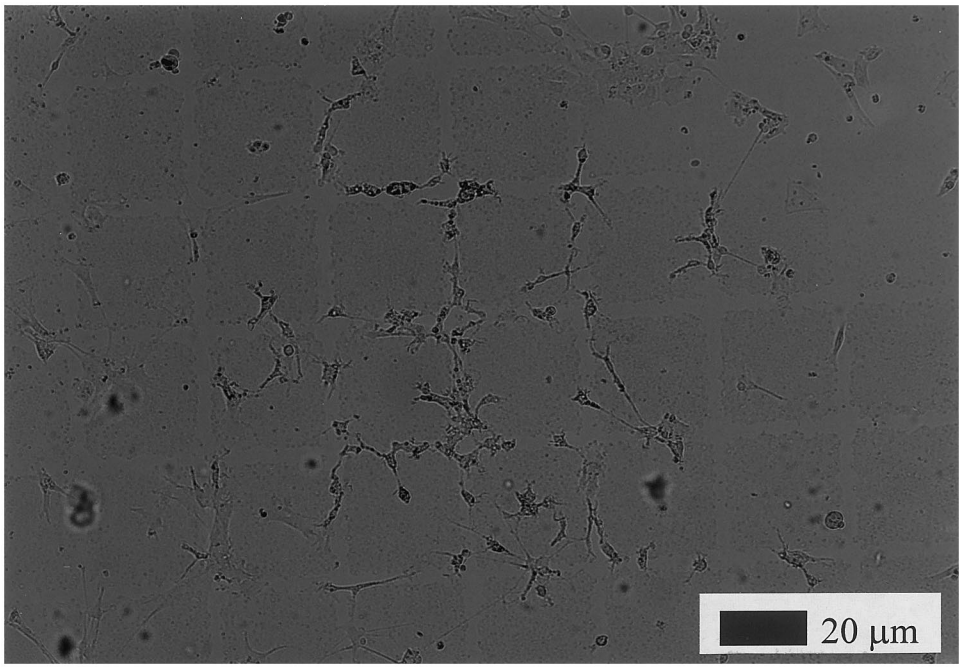

Fig. 5. Network of cells (mainly glia cells) in the channels selectively etched in the photoresist layer. The depth of the channels is ~ 0.5 μm.

that glia cells tend to aggregate on the 2 μm narrow lines and near the intersection of the lines. On the other hand, clusters of neuronal cells are almost invariably placed on the neuropeptide functionalised lines. A provisional statistics shows that (1) more half of the centres of the neuronal clusters are placed within a distance less than 10 μm from the rectangle; and (2) an important proportion of the centres of the neuronal clusters are centred near the middle of the line.

Moreover, a close-up of the artificial networks of neuronal cells (in Fig. 4) shows that:

- The neuronal cells have a tendency to adhere to the rectangles designed to mimic a neuronal cell—'seed' (dimensions 5 μm). When this occurs, the neuronal cells cluster has the tendency to grow laterally along the SFP4-functionalised lines. On the other hand, when the initial attachment occurs on a feature with smaller dimensions (e.g. on a line with a width of

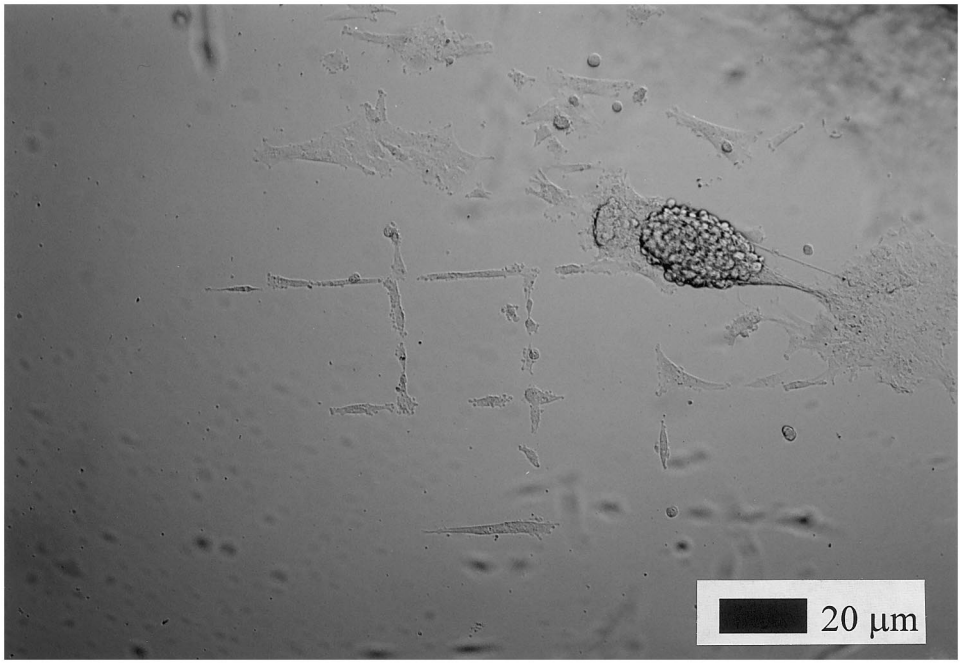

Fig. 6. Network of cells on silicon-rich patterns on the photoresist surface. The areas adjacent to the patterns are silicon-rich.

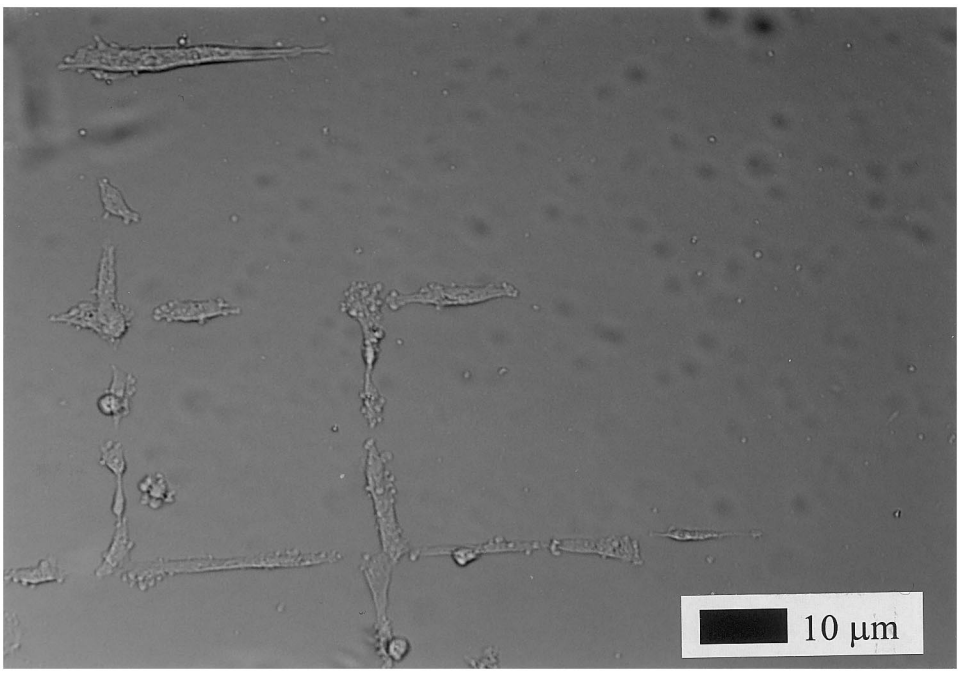

Fig. 7. Close-up picture of an artificial network of cells outside the silylated patterns on the photoresist surface. Most of the cells on patterns are glia cells.

2 μm, as on the right side of Fig. 6) the neuronal cells cluster remains confined to the area previously covered by a glia cells cluster, growing vertically in a stacking manner.

- The glia cells have the tendency to aggregate around the patterned features and to leave the rest of the surface uncovered. However, this tendency seems to be less pronounced than that of the neuronal cells.
- The patterned features have a minimal effect on the guidance of the neurite outgrowth. More importantly, the coverage with glia cells seems to have a more favourable effect on the guidance of neuronal cells growth.

This behaviour can be understood in the framework of the advantages offered by the small SFP4-functionalised rectangles (with four preferred directions for growth) versus the large rectangles functionalised with neuronal cell—repellent peptide (i.e. pGA). This advantage decreases for the position in the middle of the line (only two available directions for growth). The coverage with glia cells (less sensitive to the functionalities on the surface) decreases the specificity of the neuronal cells clustering.

Finally, the evident preference of the placement of the centre of the neuronal cells cluster on the neuropeptide—functionalised line (or on the small rectangle) can be understood if we consider the dynamics of the attachment of cells. It is reasonably to infer from this almost perfect match that at the beginning of the culture (i.e. when the density of the cells on the surface is small) the first neuronal cells will attach precisely on the neuropeptide—functionalised areas, whereas the glia cells will attach with less selectivity on and around neuropeptide—functionalised areas. Because glia cells spread quicker around these areas, the subsequent process of growth of the neuronal cells is less selective because of the presence of a glia cells blanket that will further confine the growth of the neuronal cells cluster.

3.4. Cell attachment in patterned grooves

A sample of the cell culture on the profiled areas developed in the resist film is presented in Fig. 5. The lines and the rectangles at the intersections of the lines have a depth of ~ 0.5 μm compared with the surface of the photoresist. The bottom of the grooves presents a poly—lysine rich glass surface. The adjacent areas are mildly hydrophobic following the thermally induced generation of hydrophobic species (indene and crosslinked novolak). The 'mechanical' confinement has a distinct effect in restricting the cells' growth on the expense of repelling almost entirely the neuronal cells. In the case of peptide—patterned functionalised surfaces this effect is superimposed on the functionality—induced selective attachment (the patterned features in Figs. 3 and 4 are shallower because of the partial dissolution). Still this effect is secondary for the patterning on peptide—functionalised surfaces, as the depth of the grooves is small (~ 0.1–0.2 μm).

3.5. Neuronal cell selective attachment on silylated surfaces

A sample of the cell culture on the patterned—silylated photoresist surface is presented in Fig. 6, and a close—up is presented in Fig. 7. The lines and the rectangles at the intersections of the lines were functionalised with the SFP4 neuropeptide, whereas the areas adjacent to the patterns were silylated via the functionalisation with SigmaCoat.

The attachment of the neuronal cells on the silylated areas (i.e. on areas between the lines/rectangles) seems to be almost entirely suppressed. The attachment of the glia cells is restricted to the patterned lines and rectangles functionalised with the SFP4 neuropeptide. In these conditions, the neuronal cells cannot grow properly forming unstructured spherical clusters on small areas covered by the glia cells (right hand side in Fig. 6).

3.6. Selectivity of neuronal cell attachment on functionalised polymer surfaces

The 'contrast' of the patterning of the neuronal cells increases considerably after the pattern—functionalisation of the photoresist surface (as presented in the bottom row in Fig. 2). The appropriate selection of the photoresist formulation (i.e. DNQ/novolak/imidazole mixture) combined with the appropriate lithographic techniques and the peptide/silyation functionalisation increases the selectivity of the neuronal cells attachment up to a level where the patterning of the neuronal cells becomes feasible. Moreover, the artificial networks of neuronal cells mimic the natural neuronal ones (i.e. the neuronal cells grow on a 'blanket' of glia cells). Further improvement can be achieved in the future via more specific pattern—functionalisation. For example, a specific peptide might be appropriate for guiding the neurite growth and a different one might be appropriate for assuring a tight attachment of the neuronal cell. Finally, the proposed techniques can be used in a combinatorial approach for 'screening' the libraries of neuropeptides against the response of the cell. These potential capabilities will be explored in the future.

4. Conclusion

Microlithographic techniques in conjunction with peptide surface chemistry and selective diffusion of a silylating agent were used to print neuropeptide features and silylated—rich areas, respectively, on the surface of a diazo-naptho-quinone/novolak photoresist. The features were designed to mimic an artificial network of neuronal cells. The patterning of the neuropeptide features promoting cell adhesion, coupled with the functionalisation of the photoresist areas adjacent to the patterned features with cell-repelling peptide or silylation, can control the selective attachment of the neuronal cells at a level sufficient for an effective cell engineering on specifically functionalised, flat polymer surfaces.

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