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Biomaterials 27 (2006) 3044–3063
Biomaterials
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Review

Surface engineering approaches to micropattern surfaces for cell-based assays

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Received 17 October 2005; accepted 30 December 2005
Available online 3 February 2006

Abstract

The ability to produce patterns of single or multiple cells through precise surface engineering of cell culture substrates has promoted the development of cellular bioassays that provide entirely new insights into the factors that control cell adhesion to material surfaces, cell proliferation, differentiation and molecular signaling pathways. The ability to control shape and spreading of attached cells and cell–cell contacts through the form and dimension of the cell-adhesive patches with high precision is important. Commitment of stem cells to different specific lineages depends strongly on cell shape, implying that controlled microenvironments through engineered surfaces may not only be a valuable approach towards fundamental cell-biological studies, but also of great importance for the design of cell culture substrates for tissue engineering. Furthermore, cell patterning is an important tool for organizing cells on transducers for cell-based sensing and cell-based drug discovery concepts. From a material engineering standpoint, patterning approaches have greatly profited by combining microfabrication technologies, such as photolithography, with biochemical functionalization to present to the cells biological cues in spatially controlled regions where the background is rendered non-adhesive ("non-fouling") by suitable chemical modification. The focus of this review is on the surface engineering aspects of biologically motivated *micro*patterning of two-dimensional (flat) surfaces with the aim to provide an introductory overview and critical assessment of the many techniques described in the literature. In particular, the importance of non-fouling surface chemistries, the combination of hard and soft lithography with molecular assembly techniques as well as a number of less well known, but useful patterning approaches, including direct cell writing, are discussed.
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Keywords: Microfabrication; Lithography; Surface modification; Cell patterns; Protein adsorption

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doi:10.1016/j.biomaterials.2005.12.024

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1. Introduction

Microfabrication techniques combined with surface chemistry and material science knowledge has provided new tools to further explore, in vitro, the interactions of anchorage-dependent cells with their environment. Cellular developments such as proliferation, differentiation, migration or apoptosis are guided by multiple surface cues that are potentially remodeled during cell culture assays. The cell responses are controlled by intra-cellular signaling pathways that are originally triggered by transmembrane proteins interacting with the engineered surface [1]. The surface chemistry characterized by the type of cell-binding ligands (peptides, proteins, etc.), their surface density [2–4] and spatial distribution [1,5,6] as well as their conformation [7], have been demonstrated to be important surface cues. In order to be viable, anchorage-dependent cells require an adhesive surface to exert forces and consequently spread. The ability to constrain the spreading to a specific cell-surface contact area has been shown to dramatically affect cellular development [8–10]. Mechanical compliance of cell-adhering substrates can also substantially affect the cellular response and development [11]. The ability to spatially and temporally control the chemistry, the pattern geometry and the local substrate stiffness will continue to provide new insights into the fundamental aspects of cell–surface interactions [12,13].

Apart from its use in fundamental cell-surface investigations, arrays of living cells (individual or multiple) have found major applications in both cell-based sensors and drug discovery. Cell-based sensor devices contain living cells that monitor perturbations of the environment such as toxic or pathogenic agents [14–16]. Cell-based assays in drug discovery are considered promising screening approaches, intermediate between gene- or protein-based studies and whole animal models [15,17]. The use of cell-based assays that mimic specific in vivo behavior is believed to decrease costs while leading to more accurate prediction in the drug discovery process [18].

The different engineering approaches aiming at a precise control of cell adhesion and spreading, through chemically and spatially designed surfaces, are the main focus of this review. It primarily addresses engineers new in the field of cell patterning/cell-based sensors as well as cell biologists interested in exploiting new tools to solve specific questions related to cell–surface interaction. Therefore, the concept, the required infrastructure, and the limitations of each technique are presented, along with selected scientific results, while highlighting the novelty or the originality of each approach.

Note that this review exclusively presents two-dimensional chemical micropatterning techniques (down to 1 μm pattern dimensions) but does not include the engineering of surface topographies. Several reviews on topographical structuring have been recently published [19–22]. Chemical nanopatterns have recently gained significant attention because they provide tools to study fundamental aspects of cell-adhesion at the level of single protein/receptor molecules [23]. Nanopatterned surfaces are not included in this review due to space limitation; however, several excellent reviews on nanopatterns are available [24–28].

2. The basis of cellular patterning—non-fouling surface chemistries

In any surface-coupled cellular patterning, the ability to suppress non-specific interactions between the surface and the protein-containing media is crucial in order to generate unbiased experimental outcomes. Advances in surface chemistry have made possible the synthesis of so-called non-fouling surfaces that significantly reduce or eliminate the non-specific adsorption of proteins and other biomolecules from biological fluids such as cell culture media. Several types of native molecules have the ability to reduce the adsorption of proteins at surfaces, e.g., carbohydrates such as agarose and mannitol as well as albumin [7,29]. Due to their limited efficiency and stability, a number of synthetic materials have been developed [30]. The most widely used system is poly(ethylene glycol) or PEG with the monomeric repeat unit [–CH_2–CH_2–O–]– (also known as poly(ethylene oxide) or PEO). The factors governing protein resistance of a PEG-graft co-polymer

(PLL-*g*-PEG) were recently investigated in detail, with quantitative information provided on the interfacial architecture of PEG chains and their influence on protein resistance [31–33]. Many different PEG surface-immobilization strategies have been successfully applied. One example is the use of triblock co-polymers PEG–PPO–PEG [34] that assemble spontaneously on hydrophobic surfaces through hydrophobic-hydrophobic interactions. This polymer class is also known as poloxamers or Pluronics® and has been extensively studied [35–39].

Another widely used PEG-chemistry-based approach relies on oligo-EG or PEG-modified alkanethiolate self-assembled monolayers (SAMs) [40–43]. Although these PEG-modified SAMs show substantial reduction of protein adsorption, they still adsorb significant amounts of serum proteins. Moreover, they tend to oxidize under ambient conditions restricting their use to short-term cell culture studies [44]. Bearinger et al. proposed an attractive alternative for modification of gold surfaces [45] based on PPS–PEG diblock or PEG–PPS–PEG triblock copolymers with poly(propylene sulfide) (PPS) as the central block (that binds to gold surfaces) and PEG grafted chains. Other polymer architectures such as gels and polymeric SAMs have also been successfully utilized to link PEG-chains to surfaces. For example, Healy and coworkers developed a gel-like interpenetrating polymeric network (IPN) of poly(acrylamide) and poly(ethylene glycol) [P(AAm-*co*-EG)] [46,47], while Toner et al. used a poly(ethylene glycol) diacrylate (PEG-DA) hydrogel [48].

Polycationic poly(L-lysine)-*g*-poly(ethylene glycol) (PLL-*g*-PEG), has proven to be a particularly attractive system for the modification of negatively charged surfaces (metal–oxides, tissue culture polystyrene—TCPS) by electrostatically driven self-assembly [49]. Dalsin et al. used a biomimetic linker based on dihydroxyphenylalanine (DOPA), a key component of mussel adhesive proteins shown to strongly bind to titanium oxide as well as other types of surfaces [50,51]. Ma et al. modified alkanethiolates by polymerizing oligo(ethylene glycol) methyl methacrylate monomers by surface-initiated atom transfer radical polymerization (SIATRP) [52]. This living polymerization technique provides control over chain length (coating thickness) and surface density of the growing polymeric "bottle" brushes. Silanes are commonly used as a surface-linker for PEG; however, they are nowadays less popular in view of their hygroscopic behavior and tendency to polymerize and form island-like domains leading to heterogeneous coatings.

Lipid bilayers constitute another class of cell/protein repellent adlayers [53]. The lipid composition can easily be tailored with differently charged lipids [54]. Peptides and proteins can be embedded and thus provide interesting possibilities for patterning [55,56]. Surface-immobilized lipid bilayers suffer one major drawback: they cannot be dried and must therefore always be stored in aqueous solution. Surface-immobilized polyelectrolyte multilayers (deposited by the layer-by-layer (LbL) technique) have also been reported to resist the adsorption of cells under specific pH conditions [57–59].

A novel generation of polymers, able to switch between interactive and non-interactive properties upon a change of temperature are emerging [60,61]. Layers of such "smart polymers" have been used by Yamato et al. in the context of cell-sheet engineering [62]: a thermoresponsive polymer based on poly(*N*-isopropylacrylamide) (PIPAAm) backbone with *n*-butyl methacrylate (BMA) grafted side chains was shown to have a temperature-induced phase transition (hydrophobic–hydrophilic) allowing the removal of entire cell-sheets upon lowering the temperature by a few degrees [63]. Electrochemistry has been used by Mrksich and coworkers to dynamically control the surface properties for cell studies [64]. This class of smart polymers is believed to be highly promising for future applications as enabling surface techniques to achieve not only spatial control over the chemistry (patterns), but also with the ability to change "on demand" local chemical properties.

The choice of protein/cell resistant chemistry is often dictated by the type of substrate material to be used. Some chemistries are highly versatile and can be applied to different surfaces (e.g., PLL-*g*-PEG), while others require specific substrates as for example in the case of the gold–thiol system. However, each approach has its specific strengths and weaknesses. Complexity of the synthesis, long-term stability and compatibility with a specific patterning technique are typical aspects to be considered. Covalently bound molecules have higher binding strength than physisorbed adlayers; however, non-covalent immobilization offers many attractive ways to modify surfaces. It should be pointed out that cell-patterning investigations frequently use serum-free or serum-depleted cell culture media. Some groups completely exclude serum from the media while others initially plate the cells in serum-free conditions and later add adequate amounts of serum to keep cells alive. These measures are sometimes necessary in order to prevent the cells to attach to the background, which would result in poor cell pattern quality. We note that such protocols have been frequently used in publications reporting the use of EG_3 or EG_6-modified alkanethiols. This is not so surprising since it is known that such coatings are not highly resistant to the adsorption of proteins. Also, when nearly defect-free arrays of single cells are reported, they have been usually achieved with non-adherent cells [65]. Moreover, the challenges in patterning cells vary with cell type; some cells produce large amounts of ECM proteins and require a highly effective non-fouling background, while others are less delicate (in particular non-adherent cell types). Studies of neuronal cells and networks have significantly benefited from chemical micropatterns; however, neurons are relatively easy to pattern since they often require a specific protein to attach to (e.g., laminin) and are unlikely to grow out of such geometrically defined protein patterns, even if the background is not highly protein resistant [66–68].

3. Soft lithography

"Soft lithography" is commonly used to create chemical structures on surfaces for controlling cell–substrate interactions [69,70]. The name "soft lithography" does not cover one specific method but rather a group of techniques with the common feature that at some stage of the process an elastomeric ("soft") material is used to create the chemical structures. In this review we concentrate on two related techniques of this family: microcontact printing (μCP) and microfluidic patterning (μFLP).

3.1. Microcontact printing

Among the soft lithographic techniques, μCP is the most widely used. Originally developed for creating patterns for microelectronics applications [71], μCP was soon adapted to produce substrates for cellular patterning [72]. Since that time there have been numerous publications on biological applications and many useful methodological developments/variants were introduced. The popularity of the technique originates from its simplicity, cost-effectiveness and flexibility, with regards to both the choice of substrate and the material to be transferred during imprinting. The process of μCP as used in the majority of cell-patterning applications is illustrated in Fig. 1. First, an elastomeric stamp is formed by casting a liquid-phase polymer over a microstructured master (e.g., silicon). After curing/hardening, the stamp is removed from the master. The next step is the "inking" of the stamp, which is followed by the actual stamping procedure upon which the "ink" is transferred to the substrate. The final step is generally backfilling of the non-stamped areas with a second molecule. Optionally the stamped-backfilled surface can be further modified (e.g., through adsorption of further molecules). In the next sections, the individual steps of the stamping process are discussed in more detail.

3.1.1. Creating the master

The starting point of the technique is a topographically structured master. This master is traditionally created by photolithography (for technical details see Section 4) although recently other methods such as mechanical scribing [73] or acoustic micromachining [74] have also been proposed. The stamps are formed by casting the elastomer over this topographic master and producing the corresponding replica. The correct topological design of the stamp master is one of the critical points of the technique since the mechanical properties of the stamp material limit the structures that can be reproduced [75].

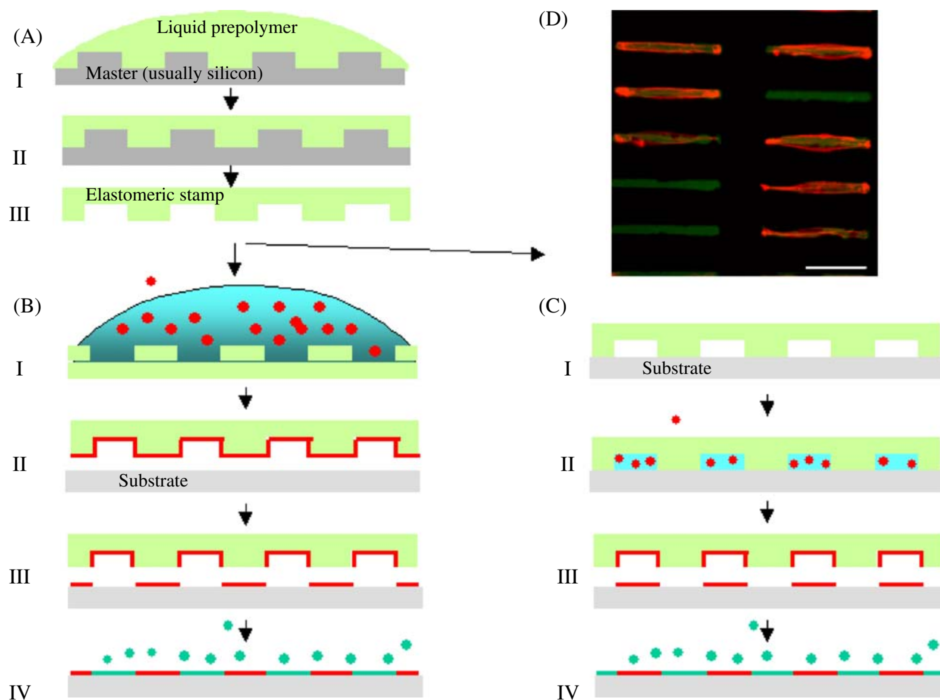

Fig. 1. Schematic representation of the microcontact printing (μCP) and microfluidic patterning (μFLP) techniques. The two methods share the common procedure of creating a stamp of suitable structural dimensions. (A) A liquid pre-polymer is cast on the structured master surface (I.) After curing (II.) the elastomeric stamp is ready for use (III.) (B) In μCP the stamp is inked with the solution containing the (bio)molecules to be printed (I.). The biomolecules are transferred by printing onto the substrate (II.). Following the removal of the stamp (III.) the surface is backfilled with the second (passivating) molecular solution (IV.) (C) In μFLP the stamp is first brought into tight contact with the substrate (I.). The patterning solution is afterwards introduced into the channels (II.), usually using capillary forces. After adsorption of the molecules of interest the stamp is removed (III.) and the remaining area backfilled with a passivating solution (IV.). (D) A543-Phalloidine stained fibroblasts adhering onto a pattern of A488-fibronectin; the pattern was produced by μCP of the labeled fibronectin onto glass and backfilling (passivating) with the copolymer PLL-*g*-PEG. Pattern size was $10 \times 90\,\mu m^2$. Scale bar: 50 μm.

3.1.2. The stamp material

The most widely used stamp material is poly(dimethyl)-siloxane (PDMS) (in ~95% of the cases Sylgard 184 from Dow Corning is used); however, other siloxanes have also been successfully used as stamp material [76], as well as alternative materials such as poly-olefin-plastomers (POP) [77] or agarose gels [78]. In most of the applications the PDMS stamps are used after an oxygen/air plasma treatment to facilitate the adsorption of the ink to the stamp surface. Specific functionalization of the stamp surface offers additional possibilities. As an example, Bernard et al. activated the surface of PDMS stamps by means of silanization and subsequently, covalently immobilized antibodies specific to the adhesion-molecule NgCAM on to the stamp surface. These modified stamps were used to extract the NgCAM molecules from tissue homogenates and the "affinity purified" molecules were subsequently printed on to clean surfaces [79].

3.1.3. Type of ink

The ink is the molecular layer that is transferred during the printing process from the stamp surface to the substrate. The transferred pattern can then be used for cellular patterning, when the printed ink needs to be bio-functionalized in a subsequent step, or directly, when cell adhesion promoting molecules are used as the ink. Although the stamp is inked in solution (aqueous or organic solvent), the printing itself happens in the dried state.

3.1.4. Indirect cellular patterning

Cells have been successfully attached to patterns on gold (and silver)-coated samples that were fabricated by μCP of thiol-based molecules to produce geometrically controlled patches of SAMs. In the "conventional" approach, hydrophobic alkanethiolates are printed on to gold substrates thereby creating hydrophobic patterns [72,80,81]. In a second step the non-stamped areas are passivated (rendered protein resistant) through the adsorption of ethylene–glycol-terminated thiols. Subsequently, the hydrophobic regions are functionalized through the adsorption of fibronectin (optionally other proteins of interest may be also used). In this process, the passivation step is essential to limit the bio-functionalization to the printed areas only. In another application, Patel et al. [82] formed biotinylated poly-lactid–poly(ethylene glycol) (PLA–PEG) films on a polystyrene surface and printed avidin on these modified substrates. The avidin pattern can then be functionalized with any biotinylated protein. Biotin printing has also been used to pattern activated polymer surfaces such as carboxylic acid derivatized poly(ethylene terephthalate) [83]. In order to ensure the full functionality of the patterned molecule (extra-cellular domain of the cell adhesion molecule L1) Oliva et al. have chosen another approach. They patterned protein A through conventional μCP on glass substrates. In a second step the patterns were functionalized with a chimeric protein construct consisting of the extracellular domain of the axonal guidance molecule L1 recombinantly linked to the Fc fragment of immunoglobulins (IgG) via the selective binding of protein A to the Fc fragment of IgG. Upon backfilling the surface with poly-L-lysine and plating neurons on to the surface, they observed a selective axon growth over the patterns while the dendrites were found over the background [84]. Reactive polymer coatings deposited by chemical vapor deposition may also provide an essentially substrate-independent platform for surface modification and subsequent cellular patterning as demonstrated by Lahann et al. [85].

3.1.5. Direct patterning

There is a wide range of approaches to directly pattern the substrates with functional (cell adhesion promoting) molecules. In the simplest case, the molecules are only physisorbed on the surface. The list of applicable adhesion promoting molecules is long. In most cases different extracellular matrix (ECM) proteins or synthetic peptide constructs with ECM binding sites have been printed. It is desired that the functionality of the proteins be conserved during the printing, and the long list of different proteins and peptides which have been successfully printed and used for biological experiments indicates that a large percentage of the stamped molecules do remain functional [86]. The type of the printed ECM molecules needs to be matched to the cellular system used in the experiments. In the very first protein-printing reports, Bernard et al. stamped different model proteins (different immunoglobulins, BSA, and NgCAM) [87] and James et al. used poly-L-lysine [88]. Endothelial and several other cell types have been patterned using fibronectin as the ink [89–91].

Neurons were successfully patterned by using laminin or synthetic polypeptides containing the cell binding sequences of laminin [66,92], polylysine-conjugated laminin [93] or synaptogenic molecules such as agrin [94]. Patterning (and detection) of bacteria (*Escherichia coli*) has been achieved through stamping of specific antibodies [95]. Another interesting approach is the printing of supported lipid bilayers as demonstrated by Hovis et al. [96] since this offers the possibility (at least theoretically) to print both cell-repellent and cell-adhesive structures depending on the lipid composition.

The stability of the molecular patterns that are not covalently bound to the underlying substrate may not be sufficient for longer-term cell-biological studies, creating a need for stronger, e.g., covalent linkage of the stamped molecule to the surface. On gold surfaces the same thiol-based SAM chemistry can be used as in the indirect patterning case. In one example synthetic oligopeptides containing a cell adhesion motif were coupled to the thiol residues and patterned to substrates [97]. Silanization of glass or silicon oxide-coated surface may also offer a way to form covalently linked patterns [94,98,99]. Chemical activation of different polymeric surfaces is also a promising approach to create stable patterns [85,100].

3.1.6. Backfill procedures

The "empty space" between the patterned regions is generally backfilled with a second molecular system. In most cases this backfill is used to passivate those background areas to ensure that the cells adhere only on the patterns. Since the introduction of μCP a number of passivation strategies have been implemented. Depending on the substrate, almost all passivation chemistries/methods summarized in Section 3 can be applied. For some applications, passivation of the substrates may not be mandatory because either the cells (e.g., neurons) show selective preference for the stamped regions [93] or the surface itself: hydrophobic polystyrene (PS) [66,67,101], poly(methyl methacrylate) (PMMA) [102] or PDMS [103] were found to inhibit cell adhesion to a certian extent. Different molecular constructs utilizing PEG as the protein/cell resistant moiety have also been successfully used for passivation. On gold surfaces, thiolated PEGs have been used in several reports [72,80,81]. For other surfaces (glass, metal–oxides, hydrophilic polystyrene) poly-L-lysine grafted PEG (PLL-*g*-PEG) is a useful backfill system [90]. Unmodified PEG has been used to passivate silanized glass surfaces [98]. Pluronics® offers an alternative way for passivation if the background is hydrophobic [104,105]. Supported planar phospholipid bilayers have also been used to block the adhesion of endothelial cells [89]. Bovine Serum Albumin is frequently reported to act as a passivation agent [106], however, other studies report a rather poor and very short-term passivation effect of BSA [29].

3.1.7. Negative patterning

Although in the majority of the published studies, the printing is used to form the cell adhesive regions (and the subsequent backfill to passivate the non-stamped areas), there are exceptions. One example is to stamp cell-repellent octadecyltricholorosilane (OTS) onto silicon wafers to create cell-free areas, while a backfill with N^1[3-(trimethoxysilyl) propyl]diethylenetriamine (DETA) is used to form the cell adhesive patches [107,108]. Negative patterning approaches are rather rare, possibly since the transfer efficiency of the ink is less than 100%; therefore, even though the passivating ink is protein (and cell) resistant on the stamp surface, this is usually not the case for the substrate after printing [90].

3.1.8. Choice of substrate

One of the strengths of μCP is its flexibility to pattern different substrates. Virtually any surface commonly used in cell-surface studies can be patterned by μCP. As listed in the previous sections ("*Ink*" and the "*Backfill*"), gold, silver, various metal or metal–oxide surfaces, glass, and different plastic substrates including biodegradable polyurethane films [109] have all been patterned successfully. Even "exotic" materials such as human eye lens capsules (obtained during cataract surgery) have been printed with inhibitory factors for cellular patterning [110].

3.1.9. Limitations of the method

Structural constraints: The success of a μCP process highly depends on the mechanical properties of the stamp material. On one hand, the stamp must be soft enough to enable conformal contact with the substrate, which means that it must adapt elastically without leaving voids created by the natural roughness of the substrate. On the other hand, a precise geometric definition of micropatterns requires a rigid material. These two opposing requirements limit not only the resolution of the technique (smallest possible structure size) but also the possible geometries, the largest possible pitch being equal to the distance between two patches of a given shape [75,111]. In biological applications the smallest reported structure size is ~100 nm [77,91]. However, this was achievable only either using special stamp material or mechanically supported stamps. In practice, with "normal" PDMS stamps, the smallest reasonable structure size is generally ~1 μm. A further limitation of the method is that under routine laboratory circumstances it is difficult to reproducibly and homogeneously pattern larger areas than $\sim 2 \times 2\,cm^2$.

Stamping soft materials: Although the exact molecular mechanism of μCP is still not entirely understood, a general practical rule is that for a successful printing the substrate needs to have a higher affinity towards the ink than the stamp. Furthermore, the substrate should not be much softer than the stamp material. Therefore stamping of very soft substrates is possible only through liquid-phase printing (see next chapter).

Creating complex structures: Upon stamping and subsequent backfilling it is straightforward to create substrates with two different molecular species. Creating surfaces where three or more molecular species are involved is a more challenging task. One way to achieve this goal is to ink the stamp in a patterned manner, meaning that the stamp itself needs to be chemically patterned (using different inks). For example, Bernard et al. used μFLP (see the following section) to ink a stamp with 16 different proteins, which were afterwards stamped to a polystyrene substrate [112]. As the same substrate can be printed several times others used repeated and micro-aligned μCP with different proteins to create multi-component patterns in several consecutive steps [98,113]. An alternative approach to create aligned, multi component surfaces through μCP was suggested by Tien et al. In their experiments a stamp with complex topography was formed and in a sequential inking/stamping procedure they inked the different topographical features with different proteins. In the final printing step they exploited the collapse of the stamp topography (due to increased pressure) to transfer complex features to a substrate [104].

Controlling ligand density: In some biological applications it may be essential to control the density of the adhesive ligands in the pattern. In principle, it should be straightforward to control ligand density at the stamp surface by adjusting the concentration of the inking solution. If the transfer efficiency is ~100% as reported

in some cases [87], one should be able to vary the ligand density in the printed patches quantitatively. However, in practice, the transfer efficiency is likely to be below 100% and may show large variations from one different experiment to the other. Furthermore, during the stamping process a certain (uncontrolled) fraction of sensitive molecules is likely to loose its biological activity [86]. An additional problem is that in most μCP applications the molecules are only physisorbed on the surface; thus, exchange and degradation processes are likely to occur when the substrate is in contact with the cell-culture medium components (especially if it contains serum) [29].

3.2. Microfluidic patterning and liquid-phase printing

Apart from many other useful applications [114], microfluidics can also be used to chemically pattern surfaces. In μFLP, the microchannels are used to deliver fluids to selected areas of a substrate. As demonstrated in Fig. 1, the "patterning material" (i.e., the solution in the microchannels) is transferred to sites where the soft-polymer does not contact (and thus does not protect) the surface. The applicable chemistry and the possible substrates are basically identical to the ones used in μCP. μFLP has advantages and disadvantages. Among all patterning methods reported in this review, μFLP offers the easiest approach to produce, in a parallel fashion, patterns consisting of many different molecules [115,116]. It is also possible to produce and exploit patterns without the need for drying the surfaces; this is of particular importance for applications where the functionality relies on the use of surface-immobilized, sensitive biomolecules such as labile proteins or enzymes in biosensing. Compared to μCP, improved control over surface-ligand density is another advantage. On the other hand, the pattern geometries are limited to open network structures, although by repeated deposition combined with rotation of the microchannel device, grid-like structures can be produced [115].

Filling the microchannels is achieved either by using capillary forces, pressure or electro-osmotically driven flows [114]. The use of soft microchannels (PDMS) offers a further advantage: μCP and μFLP can be performed in a parallel manner by using the surface contact sites for stamping and the channels for solution based delivery [117].

A special (although rarely used) case of μFLP is the liquid-phase μCP. Here the surface of the stamp material is modified such that the inking solution is trapped in the grooves of the stamp while the rest of the stamp remains free of ink. Upon contact with the surface (similar to μFLP) the ink is transferred to the substrate [12,118].

4. Patterning with photolithography

In the photolithography process, geometric features drawn on a mask are transferred via UV illumination onto a substrate. A mask is generally made of a quartz (glass) plate coated with a thin layer of non-transparent chromium, and presents the desired geometric features. The design of the mask can be created with any computer-aided design (CAD) software and can be sent to a mask manufacturing company. Such quartz/chromium masks routinely allow feature resolution down to 1–2 μm. When poorer resolution is acceptable, e.g., with a tolerance of 10 μm, one can easily produce a mask by printing the drawn features on a transparent foil using a commercial inkjet printer. The foil can then be taped onto a glass plate serving as the mask.

Photolithography has originally been developed for the fabrication of semiconductor devices. Silicon wafers are widely used as the substrate material, having finely tunable semiconductor properties and being atomically flat. For biological applications one has the possibility to use transparent wafers (quartz, Pyrex, glass) that are better suited for optical microscopy. Prior to transferring the patterns from the mask onto the substrate one needs to spin coat the wafer with a thin layer of UV-sensitive polymer, named photoresist (PR). After spin coating, the PR-coated wafer is placed on a hot plate for baking, i.e., hardening of the PR by solvent degassing. The PR-coated wafer is then brought in close contact with the mask (in certain cases a gap between the two surfaces is preferred) in a mask-aligner device. The latter is equipped with a UV source and is activated when the wafer, protected by the mask, is aligned below the lamp. A positive-tone PR is altered by UV light in a way that the irradiated regions become soluble and are removed in the subsequent

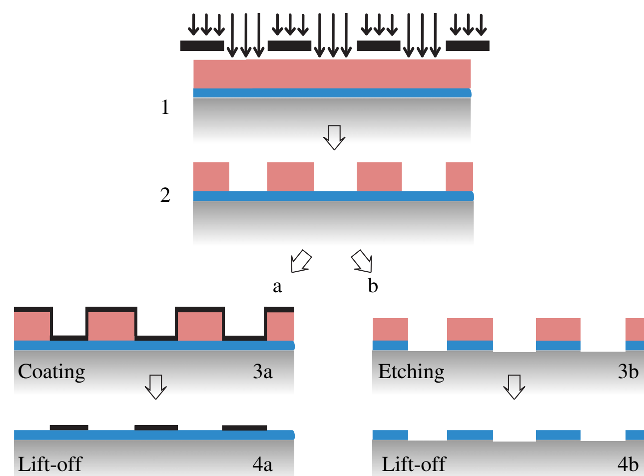

Fig. 2. The photolithography process: 1. A spin-coated photoresist (PR) is locally exposed with UV light through a mask; 2. PR development to provide local access to the underlying substrate. One of two routes is generally followed from that stage. Route a: Deposition (3a.) of a thin layer of either metal or bioactive molecules (peptides, proteins, polymers) and lift-off in an organic solvent (4a). Alternative route 3b: utilizing the patterned PR layer as a mask for local dry etching of the metal (oxide) layer (which was deposited prior to PR spin coating) down into the underlying substrate and 4b lift-off the residual PR.

development process. This two-step process is summarized in steps 1 and 2 of Fig. 2. Negative-tone PRs become insoluble when irradiated locally, thus generating a negative image with respect to the mask. Upon development the surface is composed of patterns with ‘‘windows’’ providing access to the substrate (wafer) and a background protected with PR. At this point, any further processing steps will mainly be dictated by the type of chemical pattern one needs (Fig. 2 route a or b).

For example, Scotchford and coworkers created micropatterns simply composed of an inorganic contrast between two metals (covered by their respective native oxide layers) achieved by vapor depositing (CVD process) of the desired metal 1 prior to spin-coating PR onto the whole wafer [119]. Subsequent to PR patterning, the wafer was again coated, this time with metal 2. As a final step the PR is simply removed by dissolution in an organic solvent with sonication (so-called ‘‘lift-off ’’ process). The patterned surface is finally composed of two metals: metal 1 = background; metal 2 = pattern and corresponds to route a. of Fig. 2. Interestingly, cells are able to sense differences between different metal–oxides, particularly when aluminum in the background was combined with niobium, titanium or vanadium as patches (tens of micrometers sizes). Shortly after cell plating the cells start to sense the surface and migrate. After 24 h, the aluminum surface is significantly depleted with cells and the more cytophilic patches exhibit an increased cell population. The preference of the cells for the metal–oxide surfaces composed of TiO_2, Nb_2O_5 and V_2O_5 was correlated with the observation of significantly increased surface concentration of the cell binding protein fibronectin (and correspondingly decreased albumin concentration) on those oxides in comparison to Al_2O_3, thus providing a more favorable surface for cell attachment and immobilization. Such patterns are simple to produce but the pattern recognition by the cells is far from perfect since the aluminum oxide surface is not intrinsically cytophobic and does not inhibit the adsorption of proteins. However, it is a clear demonstration that cells react sensitively to local variations in composition and surface density of proteins, although cell attachment and spreading is not significantly different for the four metal–oxides, when cells are cultured on the corresponding homogeneous samples.

Healy and coworkers produced line patterns of aminosilane/alkylsilane via photolithography and PR lift-off technique as presented earlier by Kleinfeld et al. [4,68]. The patterned PR and the substrate were coated with a hydrophobic alkylsilane, followed by PR lift-off (Fig. 2, route a.). The previously PR-protected background was subsequently coated with a hydrophilic (and charged) aminosilane. In the presence of serum, cells (rat calvaria osteoblasts) showed a clear preference (higher cell number) for the aminosilane surface (charged and hydrophilic) compared to the alkylsilane (uncharged, hydrophobic). Interestingly, bone-derived cells would only recognize the patterns if plated in serum-containing media or if the pattern was previously incubated with serum proteins and then exposed to cells in serum-free media. It is again very likely that the different affinity of the two silane-terminated surfaces for (specific) serum proteins is the driving force for the preferential cell attachment and organization on the aminosilane surface. Mineralization after 20 days of cell culture was again almost exclusively restricted to the aminosilane line pattern. It is interesting to note that the same experiment performed with human osteosarcoma cells did not show the same outcome. In this case the cells would organize on the aminosilane surface in the absence of serum proteins. Bhatia et al. used a similar approach but directly immobilized the proteins in the ‘‘open windows’’ prior to PR removal [120]. The major drawback with this approach (Fig. 2, route a) is that the immobilized proteins are exposed to an organic solvent (acetone is usually used to dissolve the PR) and is known to denature proteins to a certain degree. On the other hand, it was reported that the overall bioactivity of the immobilized collagen I, after acetone PR lift-off, remained sufficient for supporting hepatocytes growth. Since the aim was the production of a two-dimensional co-culture of two different cell types (hepatocytes and 3T3 fibroblasts) no passivating chemistry was used after lift-off requiring initial culture to be performed under serum free conditions. The second cell type was then plated and allowed to attach to the glass substrate.

In an effort to create patterns with greater cytophilicity/cytophobicity contrast, the combination of metal–oxide pre-patterns (for example patterns of two different metals or oxides) and advanced surface chemistry has proven to be a promising approach. The underlying idea is to exploit the specific surface affinity of two differently functionalized molecules and thus selectively adsorb molecule A on one oxide (or metal) and molecule B on the other oxide. Veiseh et al. specifically immobilized thiolated self-assembled monolayers on gold patches and further functionalized them with NHS and EDAC chemistry for covalent attachment of fibronectin. The SiO_2 background was modified with PEG-silanes in order to reduce protein adsorption and limit cell adhesion [121]. After 24 h of culture and upon rinsing, the $20 \times 20\,\mu m^2$ squares of fibronectin were occupied by typically 4–5 macrophage cells and no cell attached to the PEG background. Using gold surfaces as pre-patterns allows the use of thiolated molecules, a class of molecules extensively studied since the 1980s in the context of producing SAMs and μCP applications (see Section 2). A disadvantage of alkanethiolate SAMs is their limited stability to oxidation, a process that can take place even under ambient environmental conditions in the dark after only a few hours [44,45]. Moreover, high-resolution fluorescence microscopy with an inverted objective setup requires transparent samples such as thin microscopy glass cover slips. Nanometric gold coatings have not only limited transparency but when working with fluorescently labeled molecules in close proximity to the surface or interface, fluorescent energy

transfer to metals reduces the fluorescence intensity by quenching, unless special measures are taken to spatially separate the fluorophore from the metal surface as proposed by Biebricher et al. [122].

4.1. Selective molecular assembly patterning (SMAP)

In order to circumvent the above-mentioned limitations, Michel et al. proposed a similar approach based on pre-patterns of two different, transparent metal–oxides [123]. With this technique, named "SMAP", the wafer is initially coated with two thin layers of oxides (titanium oxide and silicon oxide) prior to PR spin coating (Fig. 3) [124]. Upon PR development the wafer is placed into a reactive ion-etching device in order to locally etch the silicon oxide layer (outer coating) down to the underlying titania layer. After PR removal the resulting wafer is composed of patches of TiO_2 with a SiO_2 background with a step height of typically 10–20 nm. Two types of molecules are used to locally modify the surface, both based on simple dip and rinse processes. First, the sample is dipped into a solution of ammonium dodecyl phosphate (DDP) which spontaneously forms a dense and ordered SAM on the TiO_2 islands [125,126]. This selective adsorption exclusively occurs on the TiO_2 since the phosphate group has no affinity to the SiO_2 surface. The SiO_2 background is then rendered non-fouling by dipping the sample into a solution of PLL-*g*-PEG for 10–30 min. This polymer spontaneously adsorbs out of aqueous solutions onto negatively charged surfaces such as oxides of silicon, niobium, titanium and tantalum as well as tissue culture polystyrene [49,127]. This polymer has shown to efficiently reduce the protein adsorption from serum down to $<2\,ng/cm^2$. Therefore, it is also non-adhesive for cells whereas, on the hydrophobic DDP-modified TiO_2 ($-CH_3$ terminated SAM) proteins adsorb strongly via hydrophobic exclusion. Consequently the cells only attached and spread on the TiO_2-modified patterns (Fig. 3 (g) and (h)). The protein layer adsorbed on the TiO_2-DDP originating from the serum forms a heterogeneous and partially denatured layer of proteins. Such chemically patterned surfaces benefit from the good spatial resolution achieved by photolithography as shown by the 1 μm fine structures presented by Lussi et al. [124] (Fig. 3f–h).

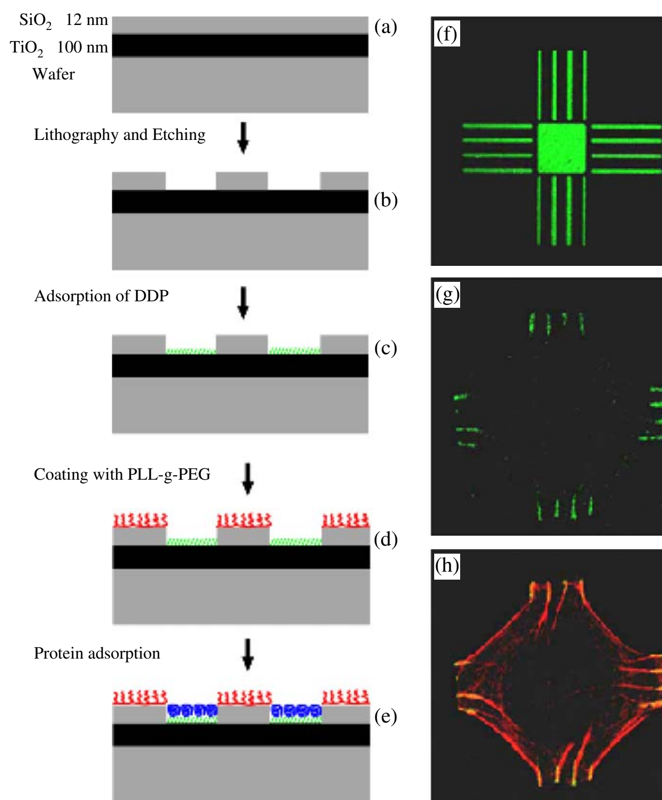

Fig. 3. The SMAP process converts an inorganic pattern contrast (produced by sputter coating, photolighography and etching) into a chemical pattern (schematics a and b). The TiO_2/SiO_2 patterned surface is dipped into an aqueous solution of methyl-terminated dodecyl phosphate (DDP). The DDP molecules form an oriented self-assembled monolayer on TiO_2, rendering it hydrophobic (c). There is no interaction between DDP and the SiO_2 surface, which is left completely bare. After rinsing with water, PLL-*g*-PEG adsorbs from a buffered solution to the bare SiO_2 and making the background resistant to the adsorption of proteins (d). The chemical contrast between hydrophobic and protein-resistant areas can then be converted into an adhesive/biofunctional contrast by simply exposing the surface to proteins (e). The fibronectin contrast was visualized by immunofluorescence (f). Single cells were shown to form focal adhesions (vinculin stain) co-localized with f-actin fibers (rhodamine-phalloidin stain) (g and h) [127]. The cross diameter is 50 μm. [Reproduced from Lussi JW, et al., A novel generic platform for chemical patterning of surfaces. Prog Surf Sci 2004; 76(3–5): 55–69 by permission of Elsevier B.V.].

The main disadvantage of the SMAP chemical-patterning technique is the lack of control over the composition and conformational activity of the protein layer. Besides projected cell shape, it is known that the type of ligand, their accessibility by the cells and their conformation influence the cellular response [81,128]. Even in the case of a protein pre-coating (e.g., fibronectin), dynamic events such as cell-surface remodeling by enzymes, cell-induced mechanical tensions as well as synthesized ECM proteins will together alter the interfacial protein layer [129,130]. Protein exchange between surface and solution (Vroman effect) will further contribute to modifying the initially immobilized protein layer [131,132]. These natural events can be limited or inhibited by covalently immobilizing the protein on the surface as proposed by Veiseh et al. [121]; however, this strategy still lacks control over the surface density and activity of the protein layer.

4.2. Molecular assembly patterning by lift-off (MAPL)

Driven by the increasing need to access model surfaces that allow an independent and quantitative control over pattern geometry and type/density of bioligands, Falconnet et al. developed a patterning technique termed "MAPL" (Fig. 4) [133,134]. MAPL combines a top-down approach based on photolithography and a bottom-up strategy through the self-organization of multifunctional molecules:

a PR pattern is transferred into the desired biochemical pattern by means of spontaneous adsorption of a biologically functionalized, polycationic PEG-graft copolymer, followed by PR lift-off. The background surface, between the biointeractive patches, is rendered non-fouling by a simple dip & rinse process to form a monolayer of the corresponding non-functionalized PEG-graft polymer. Biointeractivness is achieved by functionalizing the PLL-*g*-PEG copolymer with a linear 12 amino acids peptide containing the Arg–Gly–Asp (RGD) epitope (known to specifically interact with the integrin cell receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [135]). This approach has the advantage of eliciting exclusively specific interactions with the biological environment since the functionalized polymer retains its resistance towards non-specific adsorption of serum proteins. The bioligand surface density can be tuned precisely by simply mixing, in controlled molar ratios, functionalized and non-functionalized polymer (Fig. 4, inserts A–D). This quantitative approach enables an independent study of the influence of the ligand surface density and the cell-surface contact area. Also, the PEG-arms can easily be functionalized with other peptides and thus produce patches composed of multiple types of cell-binding sites. This approach could be highly interesting for studying the influence of a peptide containing the synergy site of Fibronectin [136].

Currently the greatest limitation to the MAPL process is the inability to immobilize large, fragile proteins. While short peptides (e.g., 15 amino acids) or biotin groups do not suffer any damage during the lift-off step in the organic solvent, this would not hold for larger, more complex and thus more delicate biomolecules. With the aim to immobilize complex proteins (or biomolecules in general) and to benefit of the advantages of the MAPL technique, simplicity and control over ligand density, NTA-functionalized PLL-*g*-PEG (NTA = nitrilotriacetic acid) has been synthesized. Microarrays with islands of PLL-*g*-PEG/PEG–NTA surrounded by a non-interactive background are an attractive way to immobilize, in a reversible manner, proteins tagged with six histidines. In the presence of nickel ions the six histag proteins are known to bind to the NTA/Ni^{2+} complex with a predetermined orientation (depending on whether the histag is on the C- or N-terminus of the protein) [137–139].

In certain applications, patterns of proteins directly adsorbed on to the substrate are sufficient, even though the surface chemistry may not be well controlled. For such needs researchers have investigated the possibility to

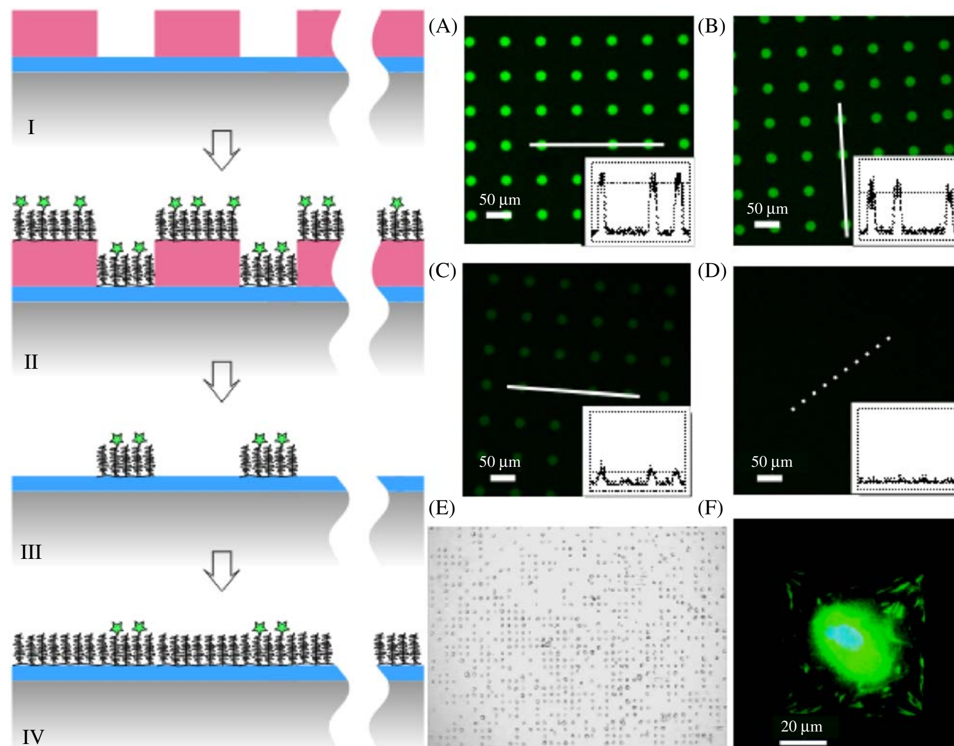

Fig. 4. The MAPL process converts a Nb_2O_5/photoresist (PR) pattern into a surface with well-controlled biointeractive patches in a non-interactive background. The sample composed of a PR pattern (stage I) is dipped into a solution of functionalized (biotin or peptide (RGD)) PLL-*g*-PEG copolymer (stage II). The PR is removed with an organic solvent in a sonicated bath, without altering or desorbing the functionalized polymer that is immobilized on the transparent Nb_2O_5 patches (stage III). In a last dip and rinse step the sample is incubated in a solution of non-functionalized PLL-*g*-PEG in order to render the background, between the adhesive regions, resistant to the adsorption of proteins/cells (stage IV). The ligand surface density can be precisely tuned by mixing, in controlled molar ratio, two solutions of functionalized and non-functionalized PLL-*g*-PEG. Inserts A, B, C and D are surfaces with decreasing biotin surface densities and revealed by decreased streptavidin fluorescence intensity. E is an array of single cells (MG63 cell line) on round islands of 20 μm diameter. F shows a single fibroblastic cell constrained on a $60 \times 60\,\mu m^2$ square pattern [136]. [Reproduced from Falconnet D, et al., Adv Funct Mater 2004; 14(8): 749–56 by permission of Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim].

immobilize the active biomolecules prior to PR spin coating. This strategy was faced with the incompatibility between the biomolecules and the PR developer (generally an alkaline solution). Bates and others proposed to protect the proteins with a thin layer of sucrose prior to PR spin coating. This avoids the contact of the developer with the bioactive molecules during PR development [140,141]. The major problem experienced with such concepts is the wafer processing temperature. The PR needs to be baked (often between 90 and 120 °C) upon coating for hardening. Such temperatures are likely to compromise protein pattern quality. Insufficient PR baking, on the other hand, will result in poor pattern resolution. As an alternative approach a number of groups have recently proposed the synthesis of novel "biocompatible" PR formulations that only require mild developing solutions rather than the traditional alkaline developer and the organic lift-off solvent [142,143]. In brief, the biocompatible PR is locally altered by UV illumination through a mask and developed. Subsequently, the whole surface (substrate and PR) is coated with the desired biomolecules. Finally the PR remaining in the background is rendered soluble by a second UV illumination step applied across the whole surface. Again, a mild aqueous solution is used to remove the resist while preserving the already immobilized bioactive molecules. A major conflict in this approach is the need for a second UV irradiation step and the authors have not specifically addressed the influence of UV light on the immobilized molecules. It is known that UV light alters, in a dose-dependent manner, organic moieties and might thus affect their activity. However, small molecules such as biotin are less likely to loose their specificity upon short UV illumination than more complex biomolecules. Therefore this process is probably better suited for small and stable biomolecules rather than for more delicate entities. Despite this limitation, such innovative photosensitive polymers have a great potential because they can be used to coat, develop and lift-off resists on a larger palette of substrates. For example, these resists can be lifted-off in solutions that are compatible with polymeric surfaces such as polystyrene for tissue culture (TCPS) or PET.

Ilic and Craighead proposed a way to get around the problem of the second UV illumination; instead of spin coating the PR directly on the wafer they first spin coated a thin layer of parylene on the surface and then added a layer of PR [144]. The overlying PR was then patterned and developed generating patches of parylene in a PR background. Oxygen reactive ion etching was subsequently used to remove the parylene layer not protected by the PR. The PR can then be lifted-off in an organic solvent. Finally the surface is composed of oxide patches (substrate) surrounded by parylene. The surface is finally coated with the desired protein solution and dried. Ilic elegantly proposed to remove the parylene template by mechanical peeling. In addition to avoiding contact between the biomolecules and an organic solvent, the dry lift-off process allowed for the patterning of cells on 2 μm wide lines with high fidelity. Orth et al. applied this technique for patterning cells on lipid bilayers down to 1.5 μm pattern size [55].

Combining the MAPL technique with a dry lift-off as presented by Ilic et al. would potentially allow the patterning of complex proteins (with controlled surface density) on a PEG layer. However, performing mechanical sucrose lift-off implies a number of additional steps in the sample preparation. The reactive oxygen ion etching requires specialized equipment and also great care in the etching parameters in order to prevent structure enlargement. Such procedures are greatly simplified by the use of polymeric stencils.

Regular arrays of single cells (no cell–cell contacts) have become increasingly attractive. For example, it is known that physical cell–cell contacts upregulate proliferation in endothelial and smooth muscle cells [104]. Aiming at studying the specific influence of substrate chemistry on cell survival, growth and differentiation requires the use of isolated cell arrays. If individual cells are to be studied by optical methods, measuring for example size and shape of focal adhesions, perfect cell arrays are not required, meaning that selected islands containing single cell can be visually selected. Such approaches have proven in the past to provide important basic insights into specific cell mechanisms. The approach, however, is time consuming, as a large number of single cells have to be investigated in order to reach statistically meaningful data. Such approaches are believed to be very promising, but great efforts will be needed in analysis automation of such samples. Producing larger arrays (thousands) of single cells confined to identical geometrical islands will then become essential, ensuring an identical microenvironment for each cell of the population. Such cellular arrays would enable the investigation of the influence of cell-shape, toxicity of drugs, or the role of surface chemistry in a more accurate manner through the use of massively parallel analysis techniques such as real-time PCR, particularly if combined with microfluidic devices. Generating defect-free large arrays of single cells remains a challenging task. The emerging field of cell-based sensors would greatly benefit from such capabilities because the ability to address individual cells in an automated way would enable the recording of cell responses upon stimuli (electrical or pharmacological) [16,18,145]. Decreasing the size of the adhesive spot in order to physically accommodate only one cell is one approach towards this goal. We have observed that producing round patterns of 20 μm diameter enables the immobilization of single osteoblast-like MG63 cells (cultured in DMEM with 10% FBS). This was achieved on $1 \times 1\,cm^2$ arrays containing approximately 6000 islands (Fig. 4E). This approach poses a number of restrictions in terms of pattern size. Already adhesive islands with diameters as small as 30 μm are often occupied by two or more cells. Furthermore, traditional in vitro culture (on homogeneous TCPS) of mammalian cells generally shows projected cell areas (several thousands of μm^2) much

greater than the area defined by a 20 μm diameter pattern (314 μm^2). Therefore small patterns do not allow spreading and initiation of cytoskeleton tension and organization and may therefore not be suitable to investigate fundamental aspect of single cell–surface interactions.

Recently dielectrophoresis has been presented as an interesting strategy for generating arrays of one or two cells per island with relatively large adhesive areas. Gray et al. fabricated a photolithographically based device able to generate local electric forces for capturing cells on specific regions of the surface [146]. Although the approach proposed requires some know-how in microfabrication, each processing step can be easily carried out in any conventional clean room. The idea is to generate one or two local electrical field sources centered below each adhesive island and actively attract the cells to the patterned surface. The cells in the electric field are polarized and directed towards the electrical field maxima, located at the site of the adhesive pattern. The cells then attach and spread on the fibronectin islands, constrained by a Pluronics modified background. This flexible technology combining photolithography and surface chemistry enables the active immobilization of single (or few) cells on individual adhesive patterns that are larger than 100 μm^2. The advantage of this dielectrophoretic cell patterning technique is the active guidance of cells towards the patterns rather than relying on random cell seeding. Other techniques based on the active placement of individual cells are discussed in the section ‘‘Laser-guided patterning and cell printing’’.

5. Plasma polymerization combined with photolithography or laser ablation

Photolithography combined with plasma thin-film polymerization has been exploited over the past years for the patterning of biomaterials dedicated to two-dimensional in vitro cell cultures [147,148]. Plasma is an ionized gas where some or all the electrons of the outer atomic orbitals have become separated from the atoms or the molecules. Artificial plasmas are often generated by radio-frequency (MHz) glow discharge setups. Provided that plasma parameters are adequate they allow deposition of polymeric films with thicknesses ranging from several nanometers up to micrometers. In the work by Hoffman and coworkers it is demonstrated that plasma lithography can be a valuable alternative to other techniques [149]. Polyethylene terephthalate (PET) substrates are first coated with a non-fouling plasma polymer of tetraglyme (tetraethylene glycol dimethyl ether). Standard photolithography procedure is then applied as described in the previous paragraph (PR spin coating, UV illumination through a mask and development of the structures). The resulting surface composed of tetraglyme polymer patches surrounded by PR is then coated by a fluorocarbon (FC) plasma-deposited polymer. Subsequently, the PR is lifted-off and the final surface is composed of hydrophobic cell-adhesive patches (FC) in a non-interacting hydrophilic background. Smooth muscle cells (SMC) are seeded on these surfaces initially without serum in order to avoid cell spreading on the tetraglyme background. For longer time culture of SMC on patterns ($10 \times 100\,\mu m^2$; $40 \times 40\,\mu m^2$; $20 \times 20\,\mu m^2$), 10% CS is added to the media 24 h after seeding. Cells are shown to maintain pattern fidelity for at least 14 days.

An alternative approach to the photolithographically based patterning of plasma polymers was proposed by Thiessen et al. [34]. Here a cell-resistant PEO-based plasma polymerized surface is locally ablated via laser and thus rendered protein/cell adhesive. Collagen I was pre-adsorbed on the ablated patterns and bovine corneal epithelial cells were shown to attach and spread exclusively on the protein patterns. This laser ablation technique allows for the creation of sub-cellular features down to 1 μm in size. Yamato et al. applied laser ablation on electron beam-grafted cell-repellent polymers (PIPAAm) [62]. It was reported that hepatocytes could be cultured on $50 \times 50\,\mu m^2$ patterns of fibronectin pre-coated TCPS up to two weeks.

Plasma lithography is a very versatile and useful technique in the sense that a great variety of substrates can be used to deposit plasma polymer films; moreover, plasma polymerization is compatible with large-area surface treatment, provided the equipment used has been designed accordingly. The use of photolithography (PR and UV) makes the process efficient and reproducible. As for every technique there are specific limitations, too. It is usually difficult to produce highly stable plasma polymer layers. Finding the optimal conditions to prevent delamination is considered to be relatively time consuming compared to simple dip and rinse processes. We believe that plasma lithography is not (yet) a standard technique to produce reliably surfaces with well-defined surface chemistries in terms of biofunctional groups.

6. Photoimmobilization and photochemically generated patterns

Surface-immobilized photoreactive molecules have been used for generating chemical micropatterns for cell attachment. Hu and coworkers immobilized oligopeptides containing the Arg–Gly–Asp (RGD) sequence on top of ethylene glycol modified-alkanethiols via UV or laser activation of benzophenone groups [150]. They showed that a linear correlation exists between the exposure time and the amount of immobilized oligopeptide (in the range of 0–5 $pmol/cm^2$) [151]. By scanning the laser at different velocities (different illumination times) they successfully created peptide gradients inside the micropatterns. After 24 h in serum-free media more fibroblasts attached to the high peptide-density patterns compared to the more sparsely immobilized peptide patterns. More recently Dillmore et al. used a similar approach where a hydroquinone group (linked to tri(ethylene glycol)-alkanethiol

SAM) was rendered accessible by photochemical deprotection of its NVOC entity [152]. Subsequently the hydroquinone group was reversibly oxidized to benzoquinone by applying an electric potential. Finally, peptides were covalently immobilized to the activated areas via Diels-Alder reaction thus providing cell-binding sites. 3T3's Swiss fibroblasts were seeded in serum-free media, after 4 h the media was exchanged for serum-containing media. The cells were shown (24 h after cell seeding) to follow the RGD peptide patterns. It is reported that the cells bind specifically to the peptides as demonstrated by cell detachment upon addition of soluble, competitive RGD peptides to the media.

Different methods were used to locally photoactivate the surface. In [151] a photolithography mask was used, potentially allowing downsizing to 1 μm features. Laser light photoactivation was also reported by using a beam of 20 μm in diameter. The advantage of the laser-beam activation is the ability to create heterogeneous patterns by varying the exposure time, as well as complex 3D structures as shown by Shea and coworkers [153]. However, this process is much slower in comparison to the massively parallel production capabilities of photolithography. In [152] microfiche masks were used to demonstrate the feasibility of the parallel processing method. The second strategy was a scanning type of lithography where the surface was directly activated through a microscope objective and a mercury lamp. The authors conclude that such serial photoactivation could produce high-resolution patterns when activated with techniques such as scanning near-field optical microscopy (SNOM). These techniques have the merit of flexibility with regard to the type and density of the immobilized ligands. However, they are probably not well suited for long term (>24 h) cell culture since the thiols are likely to oxidize under ambient conditions [45].

7. Stencil-assisted patterning

A stencil is a membrane (stiff or flexible) that is structured with through-holes of the desired size and geometry. When the stencil is brought in close contact with the substrate it can be used as a template to locally modify the surface while the areas outside the holes remain protected by the stencil (Fig. 5). Processes such as deposition or ablation of molecules for chemical patterning as well as cell seeding (without any chemical surface treatment) will therefore only occur on those surface areas where holes in the stencil provide access to the surface. Stencil materials range from soft membranes (e.g., PDMS) to highly stiff grids (e.g., metal-based). For example, Folch et al. [154] used elastomeric stencils (PDMS membranes structured by photolithography) to directly pattern cells without any need for chemical modification of the surface. This simple process offers the advantage of not requiring specialized tools or chemistry once the master template is available. The stencil is sealed onto the surface prior to cell seeding. Upon cell attachment the stencil can be manually peeled-off. With such patterns each local cell population will rapidly grow out of the pre-defined areas if no cell-resistant chemistry is present between the patterns. Therefore model surfaces prepared in this manner are potentially interesting to study the migratory behavior of cells as well as creating co-cultures of two (or more) cell types where the first cell type attaches and spreads in the holes delimited by the stencil, while the second type spreads on the background once the stencil has been removed. Tourovskaia and coworkers [155] exploited the convenience of the PDMS stencil in conjunction with a non-fouling substrate. A homogeneous layer of grafted interpenetrating network (IPN) of poly(acrylamide-*co*-ethyleneglycol) (PAAm-*co*-EG) [156] was polymerized and cross-linked on a glass surface. The stencil applied onto the polymer layer enabled the local etching (O_2 plasma), and hence removal of the protein/cell resistant coating in islands defined by the stencil features (Fig. 5b). This highly swellable IPN resists the adsorption of proteins and confers stability to the cell patterns up to 60 days [156].

More recently Kim et al. proposed an elegant procedure to produce soft, low residual stress shadow-masks (stencils) with modified SU-8 PR [157]. The stencils are composed of a photolithographically patterned thin membrane (5 μm) and a frame membrane of 150 μm for mechanical stability. They created patterns composed of different deposited metals through multistep evaporation and stencil realignment. After the first evaporation the shadow-mask is displaced and realigned with a precision of 2 μm with the help of jigs and alignment pins before the next evaporation is performed with another metal. This process can be carried-out virtually indefinitely and is, therefore, very interesting for patterning different materials on the same substrate with a high spatial resolution. This concept has also been applied to the production of a new generation of stencils that combines micro and nano features (100 nm) produced by focused ion beam milling [158]. We believe that such stencils have a great potential for biological applications, such as protein immobilization and cell patterning. Although it requires some technical skills to align the flexible stencil on the surface (with respect to electrodes or underlying chemical patterns) and ensure an adequate sealing, the technique is a simple, large scale and cost-effective replication technique. It is reasonable to envision combining chemical patterns and stencils for creating large arrays of single cells. For example aligning a stencil composed of holes of 5–20 μm diameter, that would accommodate only one cell, on top of protein/peptide patterns, calibrated for any desired cell spreading (for example 70 μm). The cells could be seeded on a patterned surface–stencil construct; each hole would be filled by maximum one cell that is attached to the underlying protein/peptides. The stencil could then be peeled-off and the cell allowed to spread on the constrained adhesive pattern. The stencil could easily be coated with non-fouling chemistry in order avoid cell-disruption upon stencil peeling.

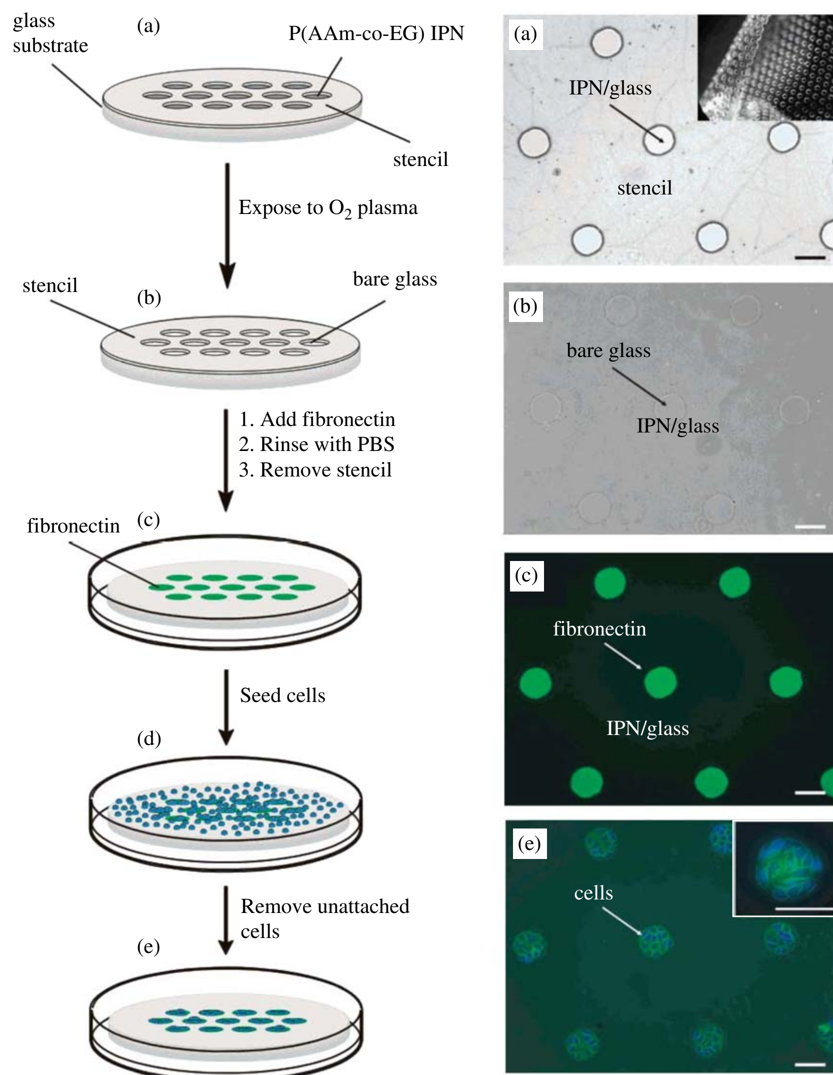

Fig. 5. Schematic illustration (left column) and select applications (right column) of the procedure for cellular micropatterning based on the use of PDMS stencils. (a) The stencil is placed onto a glass substrate homogeneously grafted with a polymeric interpenetrated network (IPN). A picture of the stencil before (inset) and after application to the surface is shown on the right. (b) The stencil serves as a mask for selective etching of the IPN in oxygen plasma. The plasma etching produces islands of IPN-free glass surrounded by IPN (as shown in the corresponding phase-contrast image on the right). (c) Fluorescently tagged fibronectin (Fn) is adsorbed onto the plasma-etched islands of glass, and the stencil is removed. (d) The fibronectin/IPN-patterned substrate is incubated with a cell suspension. (e) Unattached cells are removed by exchanging the medium. The inset shows a fluorescence image of C2C12 myoblasts (blue) attached to the fibronectin islands (green). Scale bars are 100 μm [160]. [Reproduced from Tourovskaia A, et al., Langmuir 2003; 19(11): 4754–64 by permission of American Chemical Society].

Additionally two significant advantages of the stencil technique are the suitability to virtually any substrate material (metals, oxides and stiff/soft polymers) as well as curved surfaces. The absence of organic solvents also makes it an interesting candidate for the immobilization of delicate molecules.

8. Jet patterning (ink-jet technology)

Ink-jet printing is another example where tissue-engineers have adapted existing technologies for biological research and applications. For example, it is possible by minor modification of a commercial printer to create chemical patterns by directly jetting alkanethiols or proteins onto surfaces [159–162]. Wilson et al. reported direct printing of cells onto gel membranes with a reported dead-cell percentage of 25% after 72 h. Cell death was primarily attributed to dehydration of the cell microenvironment (drying of the drop) rather than the passage through the needles [163]. The ink-jet technology has the advantages of being inexpensive, flexible in terms of substrate choice and fast. Furthermore, multifunctional surfaces can be created by using a multiple nozzle device for printing proteins for example. It is also sufficiently precise to print multiple compounds on the same spot. However the spot size and resolution is greatly limited by

the printer resolution, the nozzle diameter and the liquid/solid interfacial tension. The smallest reported spot by ink-jet technique is approximately 100 μm. We believe that this technique is potentially interesting to create large arrays of single cells if combined with surfaces patterns composed of cell-adhesive and cell-repellent areas. The challenge would be to precisely spot each cell on a single adhesive island (or sufficiently close to it) to be able to generate, in a high throughput manner, arrays of single cells.

9. Laser-guided writing with cells

Optical force is another way to direct living cells to specific locations on surfaces. Objects such as cells or particles, with a higher refractive index than the surrounding environment, and will be confined into a so-called optical trap when submitted to focused laser light. Such ''lock-ins'' can be punctual and thus allow the displacement of one cell at a time (laser tweezers) [164] or they can be linear. A linear ''optical trap'' is defined by the laser beam and the low numerical aperture lens. Odde and Renn have shown that it is possible to continuously propel cells along a light path of ~300 μm length and to create clusters of cells on a target surface [165]. The laser beam continuously captured cells as they drift into the light path by natural convection in the fluid medium. The optical forces exerted on the cells originate from the interfaces between two different refractive indexes. Each time the light is redirected at the interface, the cell is redirected in response and the total momentum is conserved. Forces in the order of a few piconewtons have been reported. The further away from the light source the more the beam will be divergent resulting in smaller optical forces. Above ~300 μm the natural convection force of the media dominates over the optical force, thus no longer allowing for control over cell position. This limitation has been overcome by guiding the cells inside a hollow optical fiber which allows for total internal reflection of light. Control of cell placement across distances as large as 7 mm have been reported with traveling speeds of 50 μm/s when using optical fibers with an inner diameter of 100 μm [165].

With the recent development of an advanced spatial light modulator, Glückstad and coworkers were able to micromanipulate in real-time multiple particles and living cells [166]. Given an adequate set-up, they could arbitrarily move each optical trap with the ''mouse-cursor'' of the computer. The parallel and real-time working capabilities of such devices are believed to have a substantial potential for future cell patterning and tissue engineering applications.

As for the ink-jet cell-printing technique, the laser-guided method has the advantage to potentially create patterns composed of different cell types and thus engineer heterogeneous tissues. Furthermore the laser-guided writing benefits from a resolution (~1 μm) much better than for the ink-jet method (~100 μm) and thus allows for a higher degree of spatial organization. This non-contact patterning method is potentially suited for building up successive layers of cells to create three-dimensional networks or tissue. However, one important aspect of this technique that remains poorly investigated is to what extent cells become damaged by the laser light. Odde et al. reported that using near-infrared light the patterned cells were viable but it is not clear whether functional biomolecules were altered during the process. It has also been reported [167] that higher input power levels are required when large arrays of cells are to be manipulated (in parallel) because of the low-differential index contrast to the surrounding medium and the irregularity of cell morphology. The need for relatively high laser power might again induce uncontrolled modification in the cell. Also, once the cells start to spread on the substrate, they will do so randomly and thus rapidly transform the originally written pattern unless specifically designed surface chemistry is combined with this cell-deposition technique.

10. General conclusions

A number of techniques utilized for cell patterning have been reviewed; some are still at their early stage of development (e.g., laser-guided cell deposition), while others benefit from several decades of dedicated development and application (e.g., photolithography). The reviewed techniques can be separated in two categories; those where the cells are passively patterned by random seeding on surfaces modified with cytophilic and cytophobic regions, and those where the cells are actively deposited on the surfaces via optical or electrical forces or even directly printed. We believe that there is a great potential for the future in combining specific techniques with the aim of solving a number of problems inherent to long-time culturing of single cell arrays. Engineering chemical patterns large enough for cell spreading and cytoskeleton organization (1000–3000 μm^2) and then actively depositing single cells, one by one, on each adhesive pattern via optical, electrical or stencil methods is one example.

Each of the reviewed techniques presents specific advantages and limitations that have been discussed in some detail. Additionally, there remain a number of more general issues to be addressed (1) Photolithography is a parallel process allowing the production of homogeneous patterns across large surface areas. Today, wafers with diameters up to 30 cm are commercially available and the efficiency of parallel process is obviously a major prerequisite for industrial large-scale production. Also, the robustness of chromium masks enables virtually an unlimited number of reproductions. On the other hand, access to specialized clean-room labs and facilities is still a limiting factor for many academic research groups; therefore, soft techniques that can be applied in standard labs (requiring simply a laminar flow box) remain very attractive, including their use for screening of larger pattern matrices and for rapid prototyping.

(2) Sample transparency is a key issue for cell culture because it allows for the visualization of living cells and for studying the dynamics of interfacial processes such as protein-surface interactions using inverted microscopes (frequently used in cell biology). Metal or metallized substrates are generally not transparent, but glass, metal–oxides (often used as thin coatings on standard substrates) and tissue culture polymers such as TCPS are. The choice of surface modification and patterning techniques is often intimately linked to the type of substrate to be used; it is therefore essential to consider all these aspects for the production of desired patterned surfaces.

Another challenge for the future will be the systematic exploitation of cell culturing in patterns on compliant substrates such as high elasticity PDMS or poly(acryl amide), that will be likely to provide more detailed insights into the interplay between cell shape, substrate compliance and forces exerted by the cell. In addition, flexible substrates also provide opportunities to study the effect of external forces on cell development and function.

Acknowledgements

This work was supported by the EC Sixth Framework Programme (Project ''NANOCUES''), and as part of the European Science Foundation EUROCORES Programme 'Self-Organized NanoStructures (SONS)'', by funds from the Swiss National Science Foundation and the EC Sixth Framework Programme. Financial support by ETH Zurich and EPF Lausanne is also acknowledged.

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