POLYMER PEN LITHOGRAPHY FOR BIOSENSING AND BIOMEDICAL APPLICATIONS

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Abstract

Polymer pen lithography (PPL) is a powerful method to generate patterns of bioactive substances with features in the nano- to microrange and over large surface areas (square centimeters). Especially, it allows for the generation of multiplexed pattern arrays (i.e. more than one component within near vicinity or within one sub-pattern). This makes the technology very interest for applications in the biomedical field ranging from sensing, to screening purposes and cell culture experiments. The multiplexing capability allows to present more than one component within an underlying pattern to a single cell, offering many options for polarization, differentiation and activation experiments. This paper will present recent progress in the application of multiplexed PPL in regard to covalent immobilization by click-chemistry approaches and introduction of versatile binding motifs in form of oligo-nucleotides for DNA-directed immobilization (DDI). The generated patterns can be encapsulated within microfluidic chips for easier handling and enhanced stability and reproducibility in use. Two examples of biomedical applications are presented in the form of mast cell activation studies and the capture of circulating tumor cells (CTCs) in such systems. As example for applications in biosensing, the use of PPL generated ink pads for the multiplexed functionalization of arrays of goblet shaped whispering gallery mode (WGM) sensors is demonstrated.

Keywords: Polymer pen lithography, allergy, mast cells, circulating tumor cells, sensors

1. INTRODUCTION

Polymer pen lithography (PPL) [1] is a technique combining aspects of dip-pen nanolithography (DPN) [2] in regard of high resolution, spatial control and multiplexing capability and microcontact printing (µCP) [3] in respect to large area printing, parallelization and inexpensive consumables. The mild process parameters (ambient conditions, no need for resist layer lift-off steps, no high energy irradiation) make PPL a great tool for the generation of bioactive surfaces as well as patterned, multiplexed functionalization of surfaces and sensor devices with delicate, sensitive compounds not compatible with more conventional lithography methods as photo- or electron beam lithography. Recently, we developed a novel inking and printing strategy for PPL (**Figure 1**) that allows for

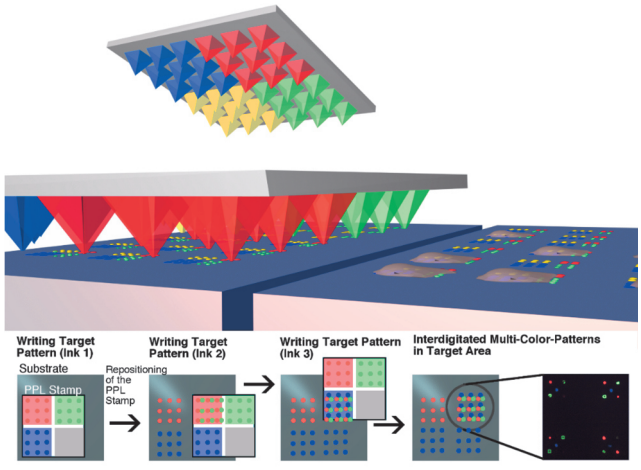

Figure 1 Multiplexed PPL. With permission from [4]

interdigitated and fully multiplexed patterns in sub-cellular dimensions without the need for complicated inking procedures by microfluidic networks or inkjet printing [4]. Here, the high precision positioning capability of a sample stage in the PPL setup is used to print one color / component of the patterns after the other subsequently with high registry. The obtained patterns allow for the presentation of several chemical ques to a single cell, e.g. for cell polarization, guidance or selective capture experiments. At the same time the pattern is repeated over a large area (~cm²), enabling good statistics in experiments with cells (**Figure 2**). To further improve the applicability of PPL in the field of bioactive patterning and functionalization, we coupled the PPL-generated surfaces with microfluidics and for easier handling and incubation. Another challenge addressed in our work is the multiplexed immobilization of proteins in PPL generated micropatterns. Here, the introduction of DNA-directed immobilization (DDI) [5] for PPL generated oligonucleotide arrays offers virtually unlimited orthogonal binding tags for self-assembly of proteins onto such patterns.

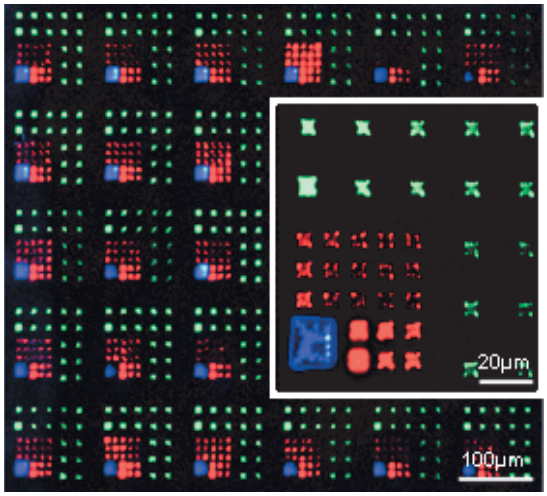

Figure 2 PPL-generated multiplexed micropattern consisting of different types of fluorescently labelled phospholipids. With permission from [4]

2. APPLICATIONS OF PPL-GENERATED PATTERNS

In this section, we present some recent examples for applications of the PPL generated patterns.

2.1. Cell Activation Studies

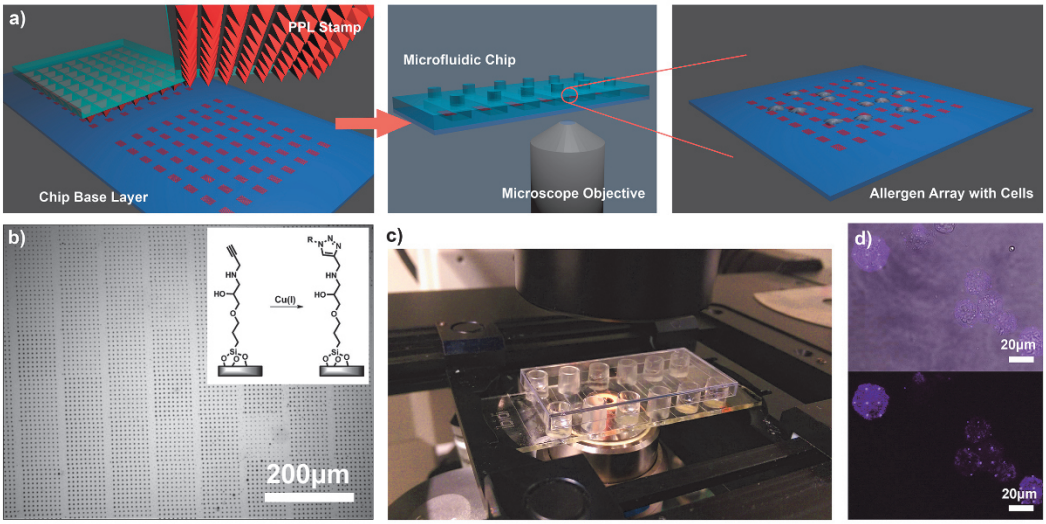

Figure 3 Microfluidic chip for mast cell activation studies. a) The base layer of the chip is functionalized by PPL with an allergen presenting array (left) and incorporated into a microfluidic chip allowing easy incubation and observation by fluorescence microscopy (middle). Mast cells were seeded onto the pattern and co-localization and activation was monitored (right). b) Allergen containing microdroplets after lithography. The inset exemplifies the immobilization chemistry. c) A picture of a six channel microfluidic chip on a microscope table. d) Combined bright field and fluorescence image (top) and fluorescence channel only (bottom) of mast cells showing co-localization of IgE receptor with the allergen array. With permission from [6]

As allergies have a profound impact on the well-being of a substantial percentage of the world's population, an enormous interest is invested into research, diagnostics and treatment [7, 8]. Profiling allergic responses is a powerful tool in biomedical research and in judging therapeutic outcome in patients suffering from allergy. Here, a shift to the single cell level instead of bulk analysis of immune response will yield novel insights into the signaling cascades involved in mast cell activation. To this end, we developed a microfluidic encapsulated allergen array (**Figure 3**). First, a base layer carrying several allergen arrays - in our model dinitrophenol (DNP), which we also used in previous activation studies [9, 10] - is printed by PPL, the allergen being immobilized on the substrate via click-chemistry. The base layer is then encapsulated in a microfluidic chip with several channels. Mast cells sensitive to DNP can be incubated on the allergen arrays in parallel and easily monitored by standard fluorescent microscopy. The mast cells show receptor co-localization with the allergen features and get activated, while non-sensitized cells show no response to the pattern. We observed an enrichment of sensitized cells when mixed populations are incubated, showing the potential of the system for identification and even capture of sensitized cells [6].

2.2. Capture of Circulating Tumor Cells

Cancer is the second most frequent cause of death in developed societies and causing immense personal sorrow and societal costs [11-13]. In recent years, the "liquid biopsy" aimed at recovering circulating tumor cells (CTCs) from patient blood has gained increased interest, as it can be used as prognostic tool and source for individual tumor cells without need for access to the primary tumor via classical biopsy, enables monitoring of patients even after removal of the primary tumor, and is much less invasive for the patient [14]. A key challenge in recovering CTCs is their low abundance in blood of about 1 in a billion of normal healthy cells. Therefore, CTC capture devices need very high specificity while also retaining at best every available cancer cell in a sample.

We established a versatile microarray-based platform able to capture single target cells from large background populations (**Figure 4**). Or platform is especially versatile, as cells can be incubated with an antibody cocktail of free choice and all targeted cells are captured on the microarray in a microfluidic chip. The accessibility of the arrays (that offers

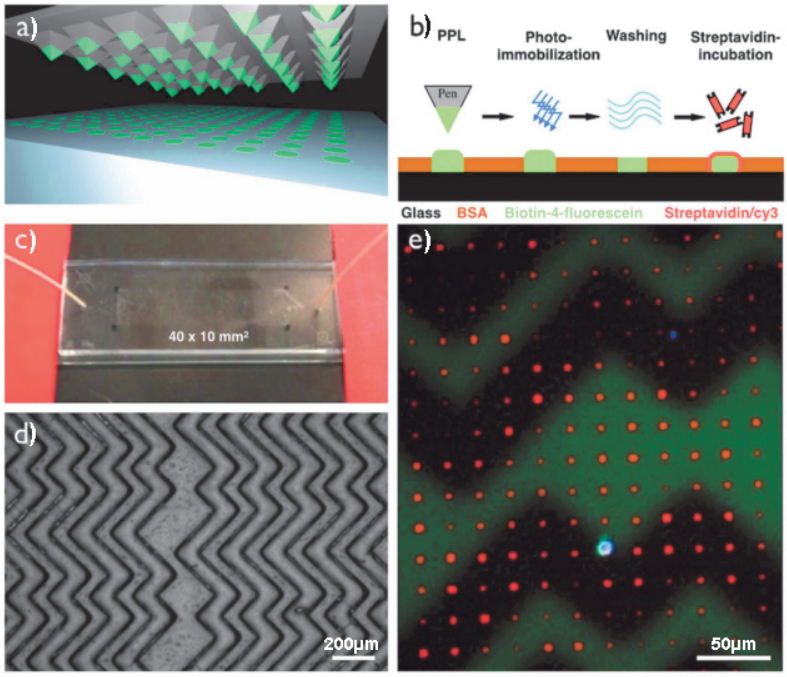

Figure 4 Capturing and immobilization of biotin-labeled CTCs by the micropattern platform. a) The micropattern is fabricated PPL. b) The general steps in generating the capture array. A BSA coated microscopy glass allows covalent binding of the fluorescein-tag by photobleaching. A topographically flat biotin-array is achieved after washing with PBS. After mounting the fluidics, the micropattern is functionalized with streptavidin/cy3. c) The microfluidic system on a hot plate. Black dots mark the patterned 4 cm² area capture array. d) Herringbone mixer in channels top wall. e) A captured cancer cell trapped on the streptavidin micropattern. The herringbone structure in the channel ceiling is visible as shadow. With permission from [15]

an essential flat capture area instead of porous or pillar structures) allows for subsequent recovery of captured cells for further analysis. The microarray facilitates exclusion of false positive capture events by co-localization allowing for detection without fluorescent labelling. When analyzing blood samples from actual cancer patients our platform reached and partly outreached gold standard performance, demonstrating feasibility for clinical application. The clinical researchers' free choice of antibody cocktail, where changes do not require reconsiderations in chip manufacturing or incubation protocol, allows virtual arbitrary targeting of capture species and therefore wide spread applications in biomedical sciences also for targets other than CTCs [15].

2.3. Sensor Functionalization

PPL is intrinsically a surface functionalization technique and not ideal for the generation of complex 3D structures. However, classical lithography methods struggle with the deposition of soft materials typically used in biosensor applications, and multiplexing is usually not readily available without several rounds of lithography that in itself may remove or destroy previous functionalizations. Here, we present an approach combining classical photolithography and etching to provide high quality and precision 3D pre-structures with PPL to deliver a lipid-ink-based functionalization enabling high sensitivity, high specificity biosensors (**Figure 5**). We previously demonstrated the functionalization of highly sensitive whispering gallery mode (WGM) structures by L-DPN [16]. The lipid ink fulfils three functions in one: (i) act as optical active material to enable lasing of the goblet sensor structure, (ii) provide specific binding sites for the targeted protein, and (iii) inhibit unspecific binding by repelling non-target proteins. By utilizing PPL instead of DPN, highly parallel and multiplexed functionalization of the goblet structures could be achieved [17,18]. For this, multiplexed PPL is used to generate a multi-color lipid stamp pad that is then in turn stamped upon an array of goblet structures. A typical result of such procedure is show in **Figure 6**. Three different lipid ink mixtures were used for parallel functionalization of three WGM sensor goblet structures in a subset of each four, while the forth remains unfunctionalized as a reference. When proteins attach to the rim of the WGM structure, the refractive index and hence the exact resonance frequency supporting the sensors lasing mode shifts, which can be used for detection. Due to the lipid functionalization suppressing unspecific binding, the sensor can work in highly complex media as e.g. serum. Using sensor structures located close together and with different sizes (as in **Figure 6c**) enables parallel readout, as the different sized WGM structures can be

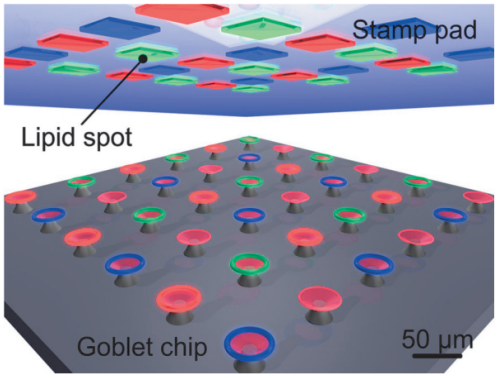

Figure 5 Scheme of multiplexed WGM goblet functionalization by PPL generated stamp pad. With permission from [17]

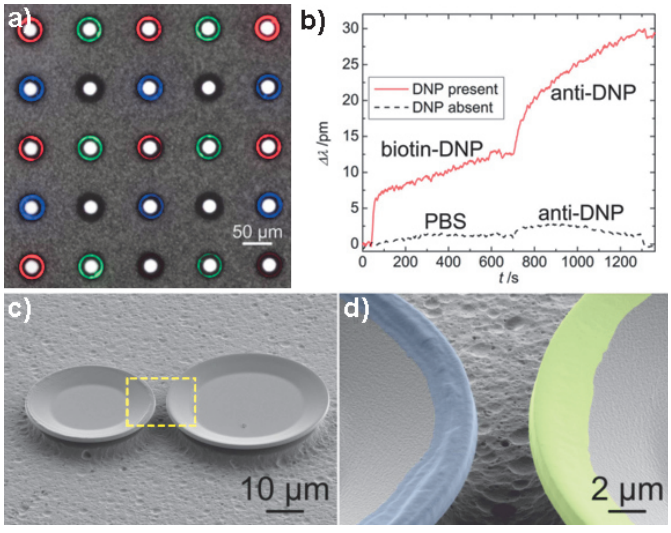

Figure 6 a) Multiplex-functionalized goblet struc-tures. b) Sensing of DNP antibodies. c) Electron mi-croscopy image of two functionalized WGM structures and d) close-up with false colored lipid. Adapted with permission from [17, 18]

distinguished by their respective lasing mode patterns. This approach enables even easier referencing and additional checks and filters against signal disturbances caused by local temperature or concentrations changes in the analyte medium [18].

3. CONCLUSION

PPL offers great opportunities for patterning sensitive and bioactive compounds not compatible with classic lithography processes in high precision and a multiplexed fashion on subcellular scales. We presented as examples applications in cell activation, cell capture and bio-sensing. As PPL offers large area patterning (~cm²), functional arrays are easily incorporated with microfluidics, opening up the route for parallel experimentation and easy and reproducible handling, e.g. for biomedical and diagnostic devices. By combining different lithographic approaches, PPL can be used for the functionalization of pre-existing complex 3D structures to enable even more sophisticated devices e.g. for sensor applications.

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