

Allergen Arrays for Antibody Screening and Immune Cell Activation Profiling Generated by Parallel Lipid Dip-Pen Nanolithography

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Multiple-allergen testing for high throughput and high sensitivity requires the development of miniaturized immunoassays that allow for a large test area and require only a small volume of the test analyte, which is often available only in limited amounts. Developing such miniaturized biochips containing arrays of test allergens needs application of a technique able to deposit molecules at high resolution and speed while preserving its functionality. Lipid dip-pen nanolithography (L-DPN) is an ideal technique to create such biologically active surfaces, and it has already been successfully applied for the direct, nanoscale deposition of functional proteins, as well as for the fabrication of biochemical templates for selective adsorption. The work presented here shows the application of L-DPN for the generation of arrays of the ligand 2,4-dinitrophenyl[1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[6-[(2,4-dinitrophenyl)amino]hexanoyl] (DNP)] onto glass surfaces as a model system for detection of allergen-specific Immunoglobulin E (IgE) antibodies and for mast cell activation profiling.

1. Introduction

Hundreds of common allergens that exert hazardous effects in allergic disorders remain to be identified. To achieve this, an assay that provides multiple-allergen testing in a timely and cost-effective manner is required. Miniaturized immunoassays

that utilize protein microarray technology now offer the possibility of circumventing most of the current limitations of this technology in the serodiagnosis of allergic disease; these limitations include processing time and large quantities of both sample and reagents, which have, to date, limited their application for mass screening. In this context, the heterogeneous nature of allergens presents many challenges in all aspects of developing such miniaturized arrays, from immobilization of the allergen, to detection of the bound ligand. However, allergen biochips are a promising new technology for the in vitro measurement of allergen-specific antibodies in allergic patients. With recombinant and purified allergens and small haptens currently available for determining specific antibodies targeted at different allergenic components,^[1] the utilization of microarrays for diagnostics becomes possible with the advantage to determine the detailed sensitization profile of each individual patient. Technological advancements have improved molecule deposition over the past ten years,^[2–4] but the patterning of multiple compounds still relies on spotting techniques.^[5,6]

The aim of this work is to design a platform towards combinatorial allergen arrays generated with L-DPN^[7] that can

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be used as biochips for detection of allergen-specific immunoglobulin E (IgE) antibodies in allergic patients and for live mast cell sensitization tests. In order to generate arrays of biologically active molecules, we have applied L-DPN using headgroup-modified phospholipids as ink. DPN is a direct patterning method that uses the tip of an atomic force microscope as an ultrasharp pen to locally deliver molecular inks onto surfaces. Also, as it allows high-throughput and high-resolution generation of arrays on the millimeter scale, DPN is a perfect technique to create biologically active chips. It has so far been successfully used for the direct deposition of functional proteins,^[8–12] as well as for the fabrication of biochemical templates for selective adsorption.^[13–15] The work presented here applies the DNP ligand admixed with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) phospholipid for the chemical control of the generated patterns under humidity-controlled lithography conditions. This set-up can be extended to multiplexing various lipids with modified headgroups in order to simultaneously pattern multiple allergens for large-scale screening. DNP–allergen arrays are used as a model system for binding DNP-specific IgE antibodies and IgE-driven mast cell activation. Mast cells are present in almost every organ of the body, particularly in tissues interacting with the environment, and their role in allergy and IgE-mediated atopic disorders has been well established.^[16,17] They bind IgE antibodies that are produced after the exposure to an allergen (antigen) through FcεRI, which is the receptor for IgE that is expressed on their surface. The FcεRI receptor is cross-linked upon contact with an allergen, leading to the recruitment of intracellular signaling molecules at the site of the clustered receptors. Finally multiple cellular responses are initiated to release pro-inflammatory mediators.^[17,18] In in-vitro studies, mast cells serve as a robust read-out system in allergen activity screens; hence, they are used to test the biological activity of DNP–allergen chips and show potential in receptor-mediated mast cell activation profiling.

2. Results and Discussion

In order to generate arrays of allergen microfluidic inkwells capable of simultaneously delivering different lipid mixtures to tips were used (**Figure 1a**). Tip arrays were coated with ink mixtures containing increasing amounts of DNP headgroups (2.5 to 10 mol%) admixed to the carrier lipid DOPC with 1 mol% content of rhodamine-labeled lipid (for the chemical structure of lipids, see the Supporting Information). Using an M-type 12-tip array (DPN 5000 system, NanoInk Inc.) for the lithography process presented in **Figure 1**, one tip from the array can generate dot structures within a $60\text{ }\mu\text{m} \times 80\text{ }\mu\text{m}$ area in one lithography step. This area allows for 6 dots $\times$ 8 dots with $10\text{ }\mu\text{m}$ spacing. If the lithography is repeated ten times, one tip in the array can cover an area of $60\text{ }\mu\text{m} \times 800\text{ }\mu\text{m}$ making 480 dots. Tips with $66\text{ }\mu\text{m}$ spacing in one array can cover an area of $780\text{ }\mu\text{m} \times 800\text{ }\mu\text{m}$, giving 5760 dots in total, in less than 5 min of the lithography process. This number of features in the array is sufficient to gain statistical information on antibody-recognition of the allergen on the surface or cell response to the allergen pattern.

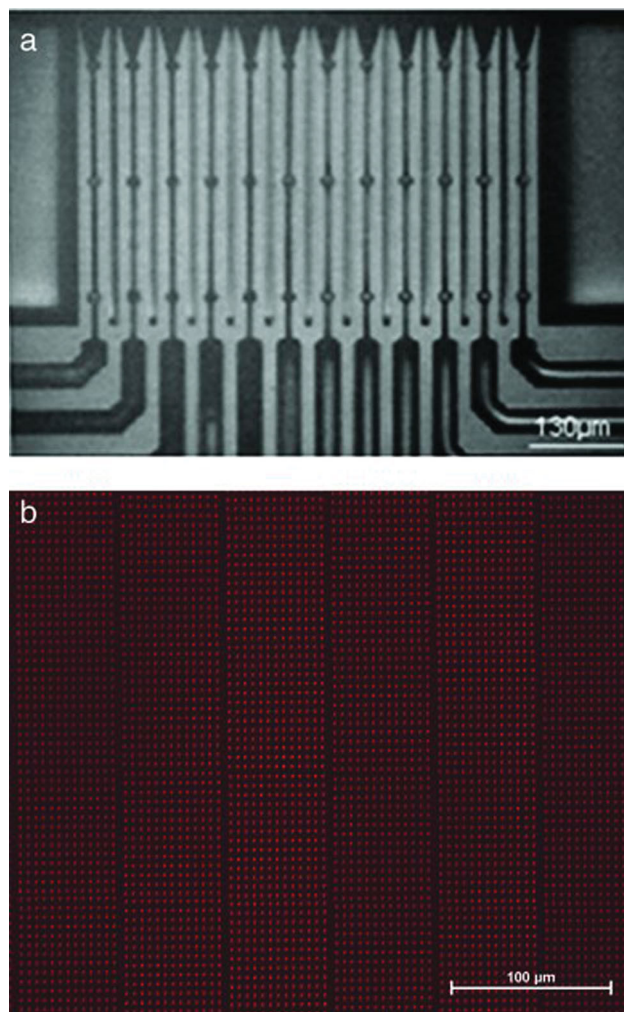


Figure 1. DPN of phospholipids with allergenic headgroups. a) An optical image of M-type pen array over microfluidic-inkwell channels. b) Fluorescence image of an array of 10 mol% DNP patterned on a glass substrate.

To evaluate the influence of DNP admixing with the DOPC carrier lipid, we performed measurements of the pressure–area (p – A) isotherms of pure DOPC, DNP, and a 10 mol% DNP admixture in DOPC in a Langmuir–Blodgett (LB) trough. Solutions of the pure compounds in chloroform were prepared (concentration $\sim 1\text{ mg mL}^{-1}$) and mixed appropriately to gain a ratio of 10 mol% DNP in DOPC chloroform solution. These solutions were then spread at the air–water interface in a LB trough, and the surface pressure versus molecular area was recorded (**Figure 2**). In large areas, the molecules are widely spaced, similar to gas molecules in 3D space. Upon compression, a point is reached where the surface pressure begins to rise. At this point, the molecules—or domains of molecules—are pressed against each other, and they start to experience restrictions in their freedom of movement. The area per molecule can be calculated by dividing the confinement area by the number of molecules deposited. The overall appearance of the DOPC isotherm, as expected,^[19] shows a rise in lateral pressure from when the area is about $110\text{ }\text{\AA}^2$ and reaches a plateau at about 47 mN m^{-1} of lateral

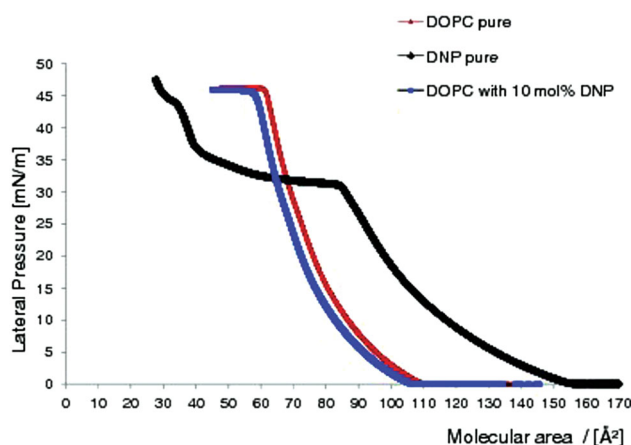


Figure 2. Surface p - A isotherms for pure DOPC, pure DNP, and DOPC with 10 mol% DNP lipid mixture recorded at 25 °C.

pressure. The rise in lateral pressure sets in at a significantly higher molecular area for pure DNP ($\sim 155 \text{ Å}^2$), with the isotherm showing a phase transition at a lateral pressure of 32 mN m^{-1} . The earlier rise in lateral pressure for DNP comes from the bulky headgroup of this phospholipid, which takes up more space at the air–water interface. The isotherm of the mixed solution (DOPC with 10 mol% DNP) is almost identical to pure DOPC, showing a good compatibility of these two compounds. The DNP does not seem to significantly disturb the organization of the DOPC monolayer, indicating that DNP is well integrated into the DOPC monolayer.

Assuming small quantities of DNP in DOPC membranes are homogeneously mixed, we can estimate the number of accessible DNP molecules (i.e., molecules that are positioned in the outermost membrane layer) in one dot of the patterned array. This is done by considering the dot area A (in μm^2) and the molecular area a (in Å^2) of a DOPC molecule within a floating monolayer. Admixing of 10 mol% DNP yields 180 000 molecules (i.e., 0.30 amol) of DNP potentially accessible in the outermost surface membrane layer of one dot in a typical array (see the Supporting Information for calculation). This presupposes that cells contacting the pattern will encounter only DNP molecules available in the top layer of the patterned membranes, and in each dot 180 000 molecules (i.e., 0.30 amol) of DNP have the potential to trigger IgE–Fc ϵ RI clustering.

Allergen detection was assessed by the binding of DNP-specific IgE antibodies onto the patterned ligand. An array of dots with DNP admixtures ranging from 10 to 2.5 mol% DNP was prepared. Dots were spaced $10 \mu\text{m}$ apart and, overall, 4000 dot features of each DNP concentration covered an area of $50 \mu\text{m} \times 800 \mu\text{m}$. Arrays were incubated with anti-DNP IgE antibody solution, washed, and incubated with anti-IgE fluorescein isothiocyanate (FITC) antibody. **Figure 3a,b** show DNP gradient arrays before and after binding of anti-DNP IgE. The chart presented in Figure 3c shows quantification of the anti-IgE FITC binding to the DNP gradient based on fluorescence signal intensity measurements. Binding of anti-DNP IgE detected with anti-IgE FITC was highest on the 10 mol% DNP arrays and was still detectable with wide-field fluorescence on 2.5 mol% DNP dots (Figure 3c).

The number of molecules calculated from p - A isotherms (Figure 2) in 10 mol% DNP content was estimated to be 180 000 (0.30 amol) of DNP in each dot, and the concentration of IgE antibody used for DNP detection allows visible identification of DNP headgroups present in the dots written with the 2.5 mol% DNP admixtures at concentrations as low as 0.075 amol (Figure 3c).

The biological activity of the allergen arrays was assessed by applying and analyzing the response of sensitized, fluorescently labelled mast cells used as sensors on the DNP arrays. Application of live cells as sensors is an emerging field, with

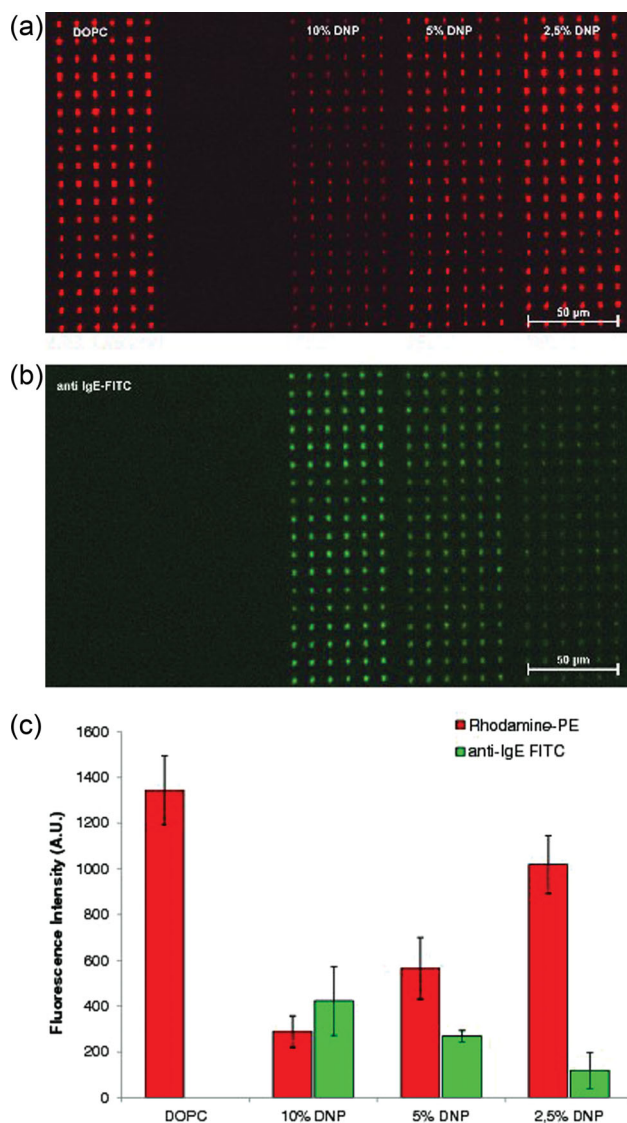


Figure 3. IgE reactivity profile on DNP-gradient allergen arrays. a) Fluorescence image of an allergen array containing 0, 10, 5, and 2.5 mol% DNP. The decrease in red channel fluorescence at high DNP concentration is due to the DNP molecule affecting the rhodamine fluorescence.^[20] b) Binding of anti-DNP IgE to the DNP arrays was detected with anti-IgE FITC antibody. c) Quantification of an antibody binding from (b), based on changes in mean fluorescence intensity after background extraction, is presented on the graph. Red bars correspond to the rhodamine-dye signal from the lipid pattern; green bars correspond to the anti-IgE FITC antibody signal detected on the DNP pattern.

potential in numerous applications including pharmaceutical screening or clinical diagnosis.^[21–23] The main advantage of using mast cells, over direct detection of specific IgE binding on allergen arrays (e.g., as shown in Figure 3) is the added information on actual biological cross-linking of proteins occurring within the cell. For live-cell imaging, arrays of DNP allergen were deposited in such a way that they were clearly spaced from one another, allowing visualization of clustering of the fluorescently labeled receptors, which is triggered at the cell–ligand contact points. RBL-2H3 mast cells were sensitized for 2 h with anti-DNP IgE antibody labeled with Alexa 488 dye, then washed, and loaded on the sample. Adherent cells could be viewed at the sample surface with bright-field and fluorescence microscopy 2 to 3 min after their addition. Upon contact with the DNP ligand, sensitized cells show co-localization of the labeled IgE receptor over the DNP dots. These can be visualized as IgE–FcεRI clusters through the Alexa 488 staining (Figure 4a), and on a merged image of the Alexa-488-stained cells adhered onto the rhodamine-labeled DNP dots (Figure 4c). This co-localization was observed as soon as the cells adhered to the glass surface and persisted over 15 min. No IgE–FcεRI clusters were formed on the DOPC patterns, where cells showed only homogeneous Alexa 488 staining around their edges (Figure 4b,d).

Clustering of the IgE receptors on mast cells initiates signal-transduction cascades that lead to degranulation and release of chemical mediators.^[17,18] The median number of IgE receptors equally distributed on the cell surface before activation is estimated to be 100 000 per cell.^[23] Upon surface contact, an average of 25 000 receptors on the semi-spherical interface would potentially come into contact with the allergen array. It is reported that cross-linking of a minimum of 200 receptors is necessary for initiation of a signaling event.^[22] With the calculated value of 180 000 molecules of DNP per dot (0.3 amol DNP), allergen arrays prepared via L-DPN should deposit sufficient numbers of ligand molecules for cell activation to be observed. To confirm that receptor-clustering, observed via live-cell imaging on DNP arrays, indeed triggers activation of intracellular signaling, we analyzed the total tyrosine phosphorylation at the DNP–FcεRI clusters. The first observable events after activation of mast cells are tyrosine phosphorylation of FcεRI clusters and receptor-proximal kinases,^[24] which can be monitored by immunofluorescence with an anti-phosphotyrosine antibody staining. Therefore, we sensitized RBL-2H3 mast cells for 2 h with anti-DNP IgE, loaded them onto the array, allowed them to settle on the dots for different periods of time, and then fixed and stained these cells with anti-phosphotyrosine antibody. A clear phosphotyrosine staining was detected on the glass surface at the dots containing the DNP headgroups (Figure 5a); however, this was not the case at DOPC dots (Figure 5b), where a homogeneous antibody staining was detected around the cells. Control experiments—carried out where the cells were fixed and stained with the secondary anti-IgE FITC antibody, but in the absence of the primary phosphotyrosine antibody—did not show a difference in the cells at the DNP when compared with the DOPC dots. This demonstrates the specificity of the action of the phosphotyrosine antibody (Figure S1, Supporting Information). The

results of the phosphotyrosine staining were quantified over 15 min, and are presented as bar charts in Figure 5c. On the whole, increasing numbers of mast cells adhere to the glass

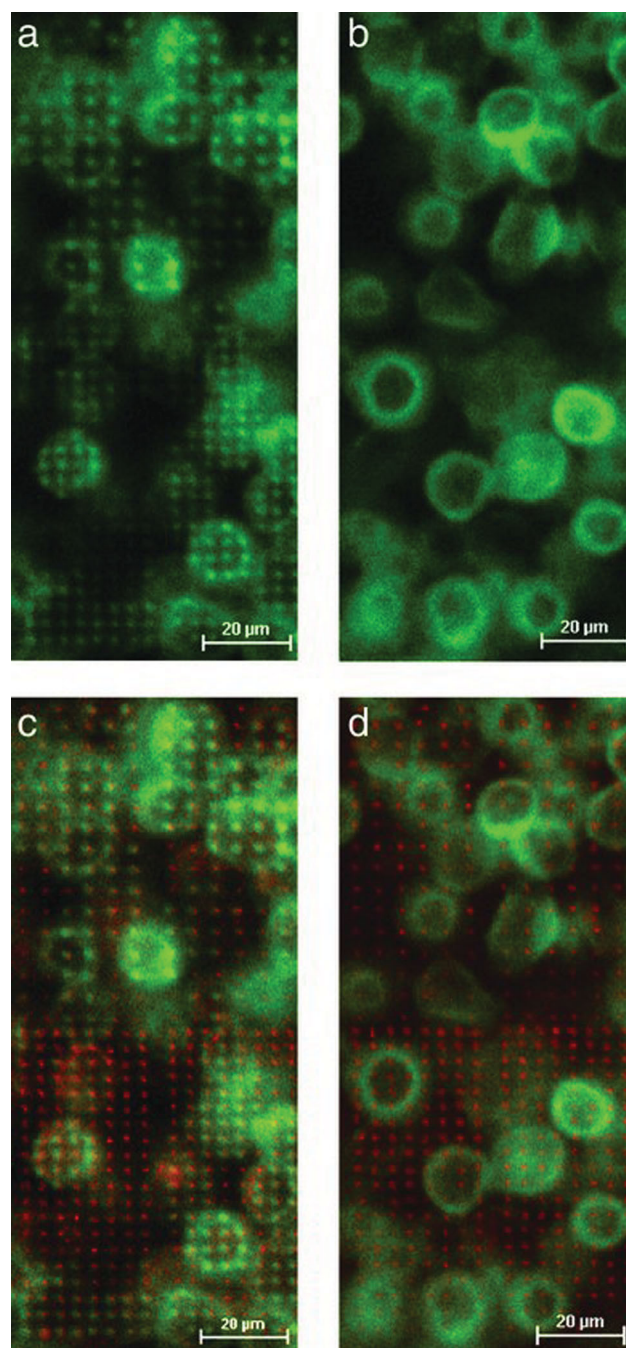


Figure 4. IgE–FcεRI co-localization over allergen arrays. Live RBL-2H3 cells, sensitized for 2 h with anti-DNP IgE–Alexa-488 antibody, were loaded onto the samples and imaged by fluorescence microscopy: FITC channel to see the cell labeling with Alexa 488 (a,b) and Texas Red channel to see the lipid pattern doped with rhodamine dye (c,d). a) At the presented time point (15 min), sensitized cells show co-localization of the labeled IgE receptor over the DNP dots. b) No IgE–FcεRI clusters were formed on the DOPC patterns, where cells showed only homogeneous Alexa 488 staining around their edges. Merged image of the Alexa-488-stained cells adhered onto: c) the rhodamine-labeled DNP pattern and d) the DOPC pattern.

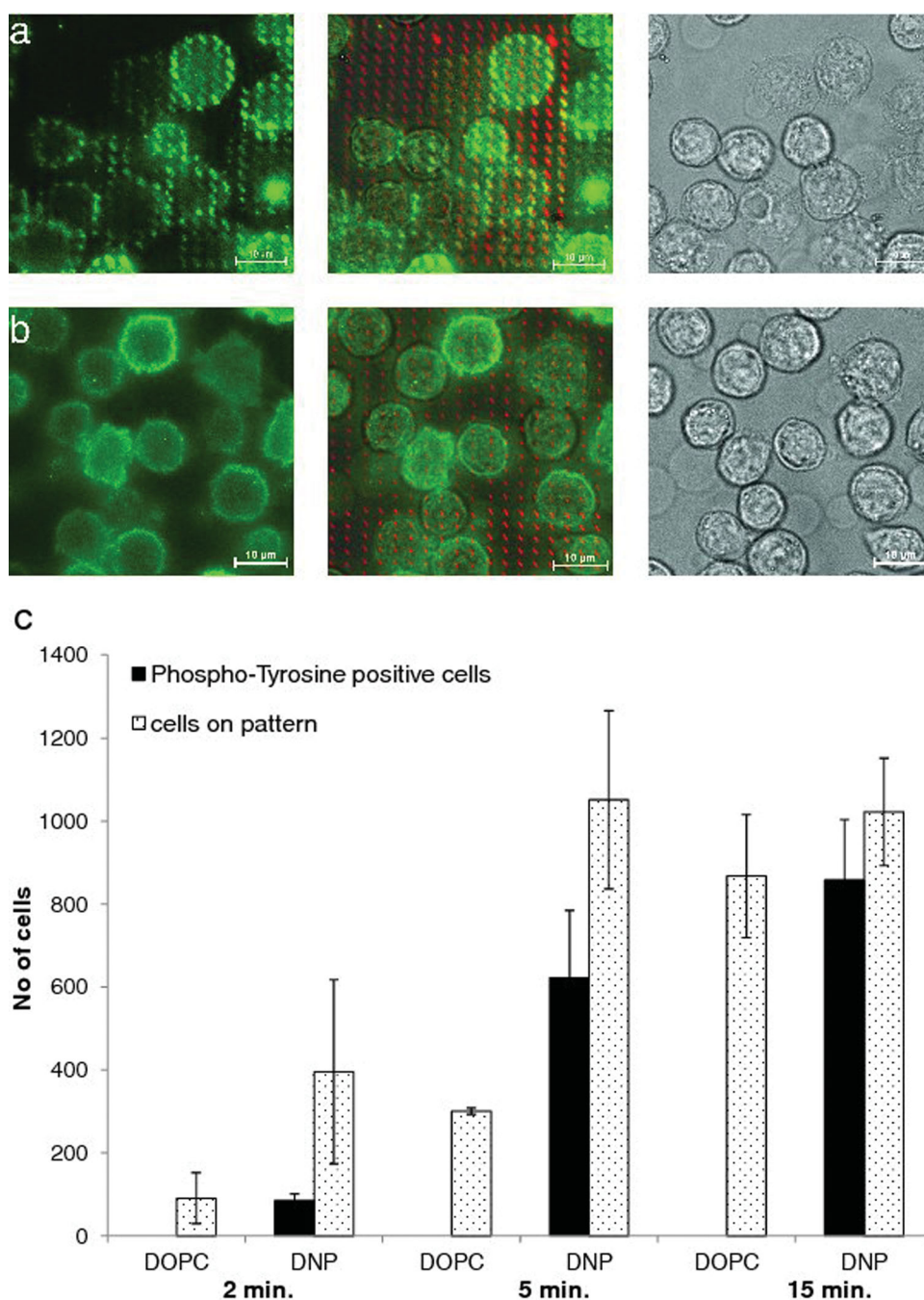


Figure 5. Activation of mast cells on DNP arrays. RBL-2H3 mast cells, sensitized for 2 h with anti-DNP IgE antibody, were loaded onto the samples, allowed to adhere, then fixed and stained with anti-phosphotyrosine antibody. a) Concentration of phosphorylated tyrosine signal is visible on cells exposed to DNP allergen. b) No phosphorylated tyrosine signal is visible in cells adhered onto DOPC array; only homogeneous antibody staining around the cells is detected after 15 min. c) Quantification of the number of activated cells on 10 mol% DNP and DOPC arrays followed over 15 min. Empty bars show the number of cells scored on the DOPC and DNP samples at the indicated time point. Filled bars show the number of activated cells where co-localization of the phosphorylated tyrosine signal over the DNP dot was detected.

surface with time (from 2 to 15 min), independent of whether the surfaces were patterned with DNP ligand or DOPC alone. The kinetics of adherence was faster for the cells on the DNP dots than for those on the DOPC dots (Figure 5c, empty bars). It should be noted; however, that at 15 min, although almost equal numbers of cells adhere to both surfaces containing DNP and DOPC patterns, co-localization of the phosphorylated tyrosine signal over the dots was only

observed in the case of the DNP pattern. The tyrosine phosphorylation at the dot was monitored over time from 2 to 15 min (Figure 5c, filled bars). These results confirm that contact of the mast cells with the allergenic ligand triggers a biological response evidenced by the tyrosine phosphorylation of intracellular molecules. Thus, other allergens that bind to mast cell surfaces could be similarly analyzed by L-DNP in multi-allergen testing, and the strengths of the activation of

the individual allergens could be quantified and compared with one another.

3. Conclusion

The aim of this work was to apply L-DPN to create a platform towards the generation of combinatorial allergen arrays, which could be used as biochips in antibody detection and as tools for live mast cell sensitization tests. L-DPN is a suitable technique to create biologically active allergen chips because it allows high-throughput and high-resolution generation of arrays on the millimeter scale. We have successfully patterned an allergenic hapten linked to a phospholipid showing the potential of L-DPN as a lithography technique in bio-research. Specific and sensitive recognition of the various doses of allergen by dedicated IgE molecules, as well as the quick response of living mast cells, shows the potential of allergen arrays in diagnostic research. These technical developments demonstrate the feasibility of chip-based approaches for allergen screening. Additionally, complex and dynamic studies on the co-operation between membrane receptors and partner proteins involved in allergic responses, which are impossible to reveal using other techniques such as enzyme-linked immunosorbent assays (ELISA) or flow cytometry, could be studied using microscopy techniques via this approach.^[25–27]

Moreover, keeping in mind previous applications of the DPN technique^[7–15,28,20] the proposed application could be extended to manufacture microarrays containing various antigens, such as proteins, peptides, DNA, and enzymatic complexes with novel inks such as hydrogels.^[29] Such allergen chips can be used to perform simple, low-sample-volume, fluorescent-based characterization of antibody responses. In contrast to conventional *in vitro* tools, chips consisting of multiple-allergen components patterned on subcellular scales would allow multiple analyses to be performed in a single measurement. In the present application of L-DPN, the spots were placed close enough for the round mast cells, with a diameter of up to 25 μm ,^[29] to make contact with several spots, each of which contains more than 200 DNP molecules for activation. Future studies where the spacing of the spots is altered to exhibit only one dot per cell would allow comparison of the response of the individual cells in a multiple-allergen array. Alternatively the spacing could be manipulated so that different allergens are allowed to interact with one cell, demonstrating the combinatorial action of the allergens. The possibility of combinatorial and multiple direct deposition of allergens on subcellular levels, when using L-DPN, shows the advantage of this method over other techniques such as spotting or micro-contact printing.

The application of fluorescently labeled secondary antibodies to detect specific auto-antibody binding to microarrays has been shown to be sensitive at nanogram per milliliter concentrations, and its sensitivity has been shown to be greater (four- to eight-fold) than conventional ELISA assays.^[5] In future studies a wider range of different allergens should be tested for use in microarrays. By addressing issues

such as preservation of 3D structures, steric interferences, and electrostatic repulsion, and by analyzing the strength of the cellular activation in response to pattern dimensions and spacing, the method described here provides great potential for upcoming work in allergology.

4. Experimental Section

Materials: The phospholipids DOPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[6-[(2,4-dinitrophenyl) amino] hexanoyl] (DNP-cap-DPPE), and fluorophore-labeled lipids 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (18:1 lissamine rhodamine/PE) were purchased from Avanti Polar Lipids, Alabaster, AL, USA.

Glass cover slips (18 mm $\times$ 18 mm) were purchased from VWR Scientific.

Antibodies: Mouse monoclonal anti-2,4-dinitrophenyl (DNP) IgE (clone7) was purchased from Sigma Aldrich and fluorescently modified with Alexa Fluor 488 antibody labelling kit according to the manufacturer's instructions (Molecular Probes); anti-phosphotyrosine, clone 4G10 was purchased from Millipore.

Cell Culture: Cell-culture medium and supplements were purchased from Invitrogen Gibco, Germany. RBL-2H3 cell line was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL Penicillin, and 100 mg/mL Streptomycin under standard cell-culture conditions: 37 °C and 5% CO₂.

For sensitization, RBL-2H3 cells treated for 2 h with anti-dinitrophenyl (DNP) IgE (Sigma-Aldrich, Germany), harvested, washed, and suspended in Tyrode's buffer with 0.1% bovine serum albumin (BSA) 10⁷ cells/mL were added onto the samples in each experiment.

For immunofluorescence, cells suspended in pre-warmed Tyrode's buffer with 0.1% BSA, were loaded onto and allowed to settle on the samples, fixed after indicated time points with 3.7% paraformaldehyde, washed, blocked with 10% BSA, and stained with anti-phosphotyrosine 4G10 antibody (1 h, 1:100 dilution phosphate buffered saline (PBS)) The signal was detected with goat anti-mouse-FITC-conjugated secondary antibody (45 min, 1:10 dilution in PBS). Control experiments were carried out with the secondary antibody goat anti-mouse-FITC without prior anti-phosphotyrosine 4G10 antibody incubation.

DPN Patterning: Tip coating and lithography was performed with commercially available instrumentation (DPN 5000/ NLP 2000, Nanolnk Inc., Chicago, IL, USA) and the following accessories: 1D tip arrays of the M-type (M-12) and M-12MW inkwells. The inkwells were filled with a chloroform solution of the phospholipid ink (1 mL, 1 μm , doped with 1 mol% of the dye-labeled lipid). The solvent was allowed to evaporate for 1 h before coating the tips. Tips were inked by placing them in contact with the inkwell and increasing the humidity to 70% for 30 min. Excess ink was removed from freshly coated tips by writing at high humidity (>55%) on a sacrificial substrate for 10 min.

Fluorescence Microscopy and Quantification of Signal: Fluorescence microscopy was carried out on an inverted TE 2000 microscope (Nikon Instruments Inc.). Fluorescence images were taken using 20 $\times$ or 63 $\times$ (oil immersion) objectives. Images were recorded with a digital charge coupled device (CCD) camera Orca

(Hamamatsu Photonics). Patterns were aligned for imaging using alignment markers scratched onto the glass surface. Bleaching of fluorophores was minimized (especially for quantitative measurements) by first focusing on the alignment mark before exposing the patterned area to light, and the lamp intensity was kept at a minimum.

Fluorescence intensity quantification of IgE reactivity profile experiment was performed with NIS-Elements AR software installed on the microscope PC (Nikon Instruments Inc.). For measurements, patterned areas of interest was marked, and fluorescence intensity was automatically recorded. Mean fluorescence intensity after background extraction was automatically calculated.

Quantification of the phosphotyrosine positive cells in immunofluorescence experiments was performed manually. Samples from each time point were imaged in bright-field channel to see the cells: a Texas Red channel to see the DNP pattern and an FITC channel to see the anti-phosphotyrosine antibody staining of the cells (Figure 5). The graph shows the number of all cells scored at indicated time points (empty bars), and the number of phosphotyrosine positive cells at indicated time points (filled bars). Cells that showed co-localization of the anti-phosphotyrosine antibody signal over the DNP dot pattern are considered activated and phosphotyrosine positive. Cells that did not show the co-localization of the two fluorescent signals were not considered as activated.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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