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Functionalized Surface Arrays for Spatial Targeting of Immune Cell Signaling

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An important problem in cell biology with broad implications is how cellular responses are affected by the chemistry and topography of interfacing surfaces. Fundamental to normal physiology, these interactions are also highly relevant for any situation where living systems encounter device surfaces such as in medical implants and cell-based sensors. Because of widespread interest in understanding and regulating such cellular processes, significant efforts are being made to tailor surface-active materials and develop effective surface chemistry.[1]

Self-assembled monolayers (SAMs) formed by adsorption of alkanethiols on a gold layer provide well-packed, chemically stable surfaces.[2] Surfaces patterned with modified SAMs can be used to present specific ligands with controlled surface densities within well-defined regions. Thus, for ligands that bind specifically to cell surface receptors, this provides a tunable means for investigating spatial control of cellular responses. For example, optical or electron microscopy can be utilized for direct visualization and quantitative analysis of subcellular reorganization that occurs in response to these spatially defined stimuli. These observations can in turn provide valuable mechanistic insights about the biochemical pathways that regulate and target these processes. As described in this report, we employed high-resolution lithography to pattern SAMs with submicrometer feature size. We evaluated specific binding by anti-DNP immunoglobulin E (IgE), both for the soluble form of this antibody and when it is associated with its high affinity receptor (FcϵRI) on the surface of rat basophilic leukemia (RBL) mast cells. These ligand-SAM substrates were also tested for their capacity to investigate early signaling processes that are stimulated by the engaged IgE−FcϵRI.

Clustering of cell surface IgE−FcϵRI by multivalent ligands (antigens) initiates intracellular signaling pathways associated with mast cell effector functions in allergic and inflammatory responses.[3] Recently, micrometer-size patterned lipid bilayers containing 2,4-dinitrophenyl-caproate (DNP-cap)-modified lipids were used to control the location and size of IgE−FcϵRI clusters, and intracellular components were shown to redistribute selectively with the patterns of receptors.[4] To visualize the earliest molecular interactions associated with receptor-mediated signaling requires even finer resolution. Patterning of biological molecules at the submicrometer scale has been achieved with various techniques, including dip-pen, block copolymer micelles, and supramolecular stamping.[5] A useful alternative to these selective bottom-up approaches is top-down, electron beam lithography, which creates ~10 nm to 1 μm features with high accuracy and reproducibility and offers easy access to submicrometer resolution of cellular redistributions.[6,7] Our approach further incorporates SAMs with specific binding groups and other desired, quantifiable properties. We used a lift-off process to generate gold arrays of submicrometer features on a silicon chip (Supporting Information, Figure S2). The gold/titanium posts (50 nm in height, 1 μm to 45 nm in width) were then selectively chemically modified using ligand-SAMS that were designed to interact specifically with antibodies and cells.

The DNP-cap-PEG4C16 molecules synthesized for these SAMs contain a long alkyl chain, $(CH_2)_n$ ($n = 16$), with a disulfide terminal group and an oligoethylene glycol (OEG) spacer terminated by a DNP-cap (Scheme S1). The hydrocarbon segment ($n > 10$) served to enhance molecular organization and packing,[8] while the OEG linker was incorporated into the system to introduce an interfacial water layer that would orient the headgroup away from the surface as well as minimize nonspecific interactions of the proteins with the underlying surface.[9] The terminal DNP-cap group binds specifically to anti-DNP antibodies, including anti-DNP IgE.[10] SAM-modified, patterned surfaces were incubated with Alexa-488-labeled anti-DNP IgE at 37 °C for 1 h. Fluorescence images reveal that this antibody binds to the patterned patches of DNP-cap-PEG4C16-SAMs with high specificity (Figure 1a, 1 μm features; Figure S3, 600 nm features). A conservative estimate of the specific/nonspecific ratio is ~5 (see Supporting Information). An immunoassay of these ligand-modified gold chips showed saturation binding of anti-DNP antibodies (Figure S4) with coverage corresponding to more than 10^{11} antibody molecules/cm^2. These results demonstrate that patterned surfaces of this type can provide well-defined arrays of antibodies or other proteins with submicrometer features for potential applications in biosensors.

To examine specifically stimulated cellular responses, the patterned substrates were incubated with a suspension of RBL-2H3 cells that had been sensitized with AlexaFluor-488-labeled anti-DNP IgE bound to cell surface FcϵRI receptors. The cells settled on the silicon chip and became adherent within 2−3 min at room temperature (RT). The fluorescently labeled IgE receptor complexes that were originally dispersed over the cell surface became clustered after binding to the DNP-cap-PEG4C16-SAM modified features (Figure 1b,c). We confirmed that the gold structures do not interfere with the fluorescence signal (Figures 1b, S3, and S5). Receptor clustering is due to ligand binding because the cell-bound fluorescent IgE remains dispersed and does not concentrate on the features when the chips are preincubated with soluble anti-DNP IgE or when the chips are modified instead with methoxy-PEG4C16-SAMs (Figure S5). Thus, DNP-cap PEG4C16-SAMs presented on submicrometer surface features engage specific cell surface receptors and cluster them in the same defined pattern.

We used these patterned surfaces to extend our investigation of spatially regulated, transmembrane signaling events. It is known that clustering of IgE−FcϵRI causes intracellular Lyn kinase to co-redistribute and phosphorylate tyrosine residues in the receptors' cytoplasmic segments. RBL cells sensitized with unlabeled anti-DNP IgE were incubated with the DNP-SAM chips for 15 min at RT, then fixed and labeled with monoclonal anti-phosphotyrosine

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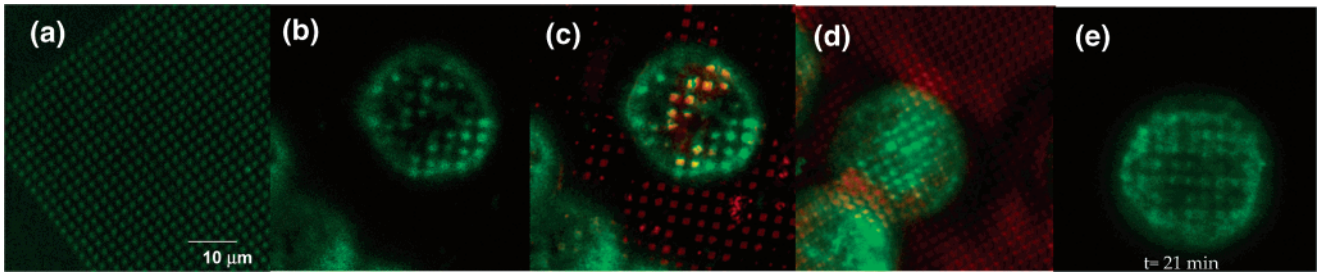

Figure 1. Confocal fluorescence images of surfaces patterned with DNP-cap-PEG4-C16 SAMs of 1 μm (a−c) or 600 nm (d, e) feature sizes. (a) Specific binding of soluble anti-DNP IgE labeled with Alexa488. (b−e) Adherent RBL cells sensitized with Alexa488 labeled (b, c) or unlabeled (d, e) anti-DNP IgE which binds to cell surface FcϵRI receptors. (b, c) IgE−FcϵRI clusters (green) on cell surface within 2−3 min in a pattern that coincides with the DNP-SAM-modified gold (red). (d) Antibody 4G10 (green) marking Lyn-mediated tyrosine phosphorylation that co-clusters with IgE−FcϵRI on DNP-SAM-modified gold (red) after 15 min. (e) LAT-EGFP (green) co-clusters with IgE−FcϵRI on DNP-SAM-modified gold after 15−20 min.

antibody (4G10) and Alexa 488-labeled secondary antibody. As shown in Figure 1d, anti-phosphotyrosine concentrates on the patterned features corresponding to the clustered IgE−FcϵRI, demonstrating that initiation of cell activation occurs with these chips similarly to the patterned lipid bilayers used previously.[4] In a control experiment, the secondary antibody showed no concentration on the patterns in the absence of 4G10 (Figure S6).

We then evaluated spatial redistribution of a subsequent signaling component, not previously reported for patterned surfaces. Linker for activation of T cells (LAT) is an important adaptor molecule in immune cells. In mast cells, LAT recruits other protein participants in activation pathways leading to release of chemical mediators of inflammation.[10] RBL cells were transfected with a LAT-EGFP construct,[11] and live cells expressing this endogenous fluorescent protein were incubated with the DNP-SAM chips and examined with microscopy in real time. Concentration of LAT-EGFP over the patterned features was observed in more than 60% of the adherent cells within 15−20 min of incubation with the modified surfaces (Figure 1e). Interestingly, the LAT clusters appear to become more diffuse over time, although they remain concentrated in the region of clustered receptors (data not shown). The resolution of fluorescence microscopy cannot distinguish overlapping from proximal clusters that are on the order of 100 nm. Similar patterns with electron microscopy visualization will allow testing of the hypothesis that LAT clusters separate from receptor clusters, possibly representing secondary signaling domains.[12] A major advantage of the patterned presentation is that co-localization within an array is readily determined and quantified using, for example, Fourier transform analysis. Our results with cells confirm that receptor-mediated signaling can be stimulated with submicrometer spatial definition using this approach.

In summary, we demonstrate that E-beam lithography and designed SAMs can be combined to create arrays for spatially defined presentation of specific ligands (on feature sizes 1 μm to 600 nm), which in turn bind specifically to receptor proteins in solution or on cells in the same pattern. In addition to their potential for protein arrays for diagnostic or detector applications, these are proving to be valuable for evaluating spatial regulation and targeting of receptor-mediated cellular signaling. The ligand specificity can be generalized; for example, succinimidated or hydroxylated PEG4-C16-S-S- derivatives could be used to attach specific ligands containing amine or caproate groups, respectively. Biotin−avidin sandwiches also could be readily incorporated within this scheme. With fluorescence and electron microscopy visualization, patterned SAMs provide a powerful tool for systematic examination of the earliest receptor-mediated cellular signaling interactions that occur with spatial resolution on the nano and longer length scales.

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Supporting Information Available: Materials, syntheses, fabrication, and experimental procedures including control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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