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The use of glass substrates with bi-functional silanes for designing micropatterned cell-secreted cytokine immunoassays

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ABSTRACT

It is often desirable to sequester cells in specific locations on the surface and to integrate sensing elements next to the cells. In the present study, surfaces were fabricated so as to position cytokine sensing domains inside non-fouling poly(ethylene glycol) (PEG) hydrogel microwells. Our aim was to increase sensitivity of micropatterned cytokine immunoassays through covalent attachment of bio-recognition molecules. To achieve this, glass substrates were functionalized with a binary mixture of acrylate- and thiol-terminated methoxysilanes. During subsequent hydrogel photopatterning steps, acrylate moieties served to anchor hydrogel microwells to glass substrates. Importantly, glass attachment sites within the microwells contained thiol groups that could be activated with a hetero-bifunctional cross-linker for covalent immobilization of proteins. After incubation with fluorescently-labeled avidin, microwells fabricated on a mixed acryl/thiol silane layer emitted ~ 6 times more fluorescence compared to microwells fabricated on an acryl silane alone. This result highlighted the advantages of covalent attachment of avidin inside the microwells. To create cytokine immunoassays, micropatterned surfaces were incubated with biotinylated IFN-γ or TNF-α antibodies (Abs). Micropatterned immunoassays prepared in this manner were sensitive down to 1 ng/ml or 60 pM IFN-γ. To further prove utility of this biointerface design, macrophages were seeded into 30 μm diameter microwells fabricated on either bi-functional (acryl/thiol) or mono-functional silane layers. Both types of microwells were coated with avidin and biotin-anti-TNF-α prior to cell seeding. Short mitogenic activation followed by immunostaining for TNF-α revealed that microwells created on bi-functional silane layer had 3 times higher signal due to macrophage-secreted TNF-α compared to microwells fabricated on mono-functional silane. The rational design of cytokine-sensing surfaces described here, will be leveraged in the future for rapid detection of multiple cytokines secreted by individual immune cells.

1. Introduction

Cytokines are proteins secreted by mammalian cells in the process of endocrine communication. These proteins may be released in response to injury, causing inflammation or cell death, [1,2], or on the contrary, protecting against tissue injury [3]. Cytokine production in leukocytes is an important means of monitoring immune competency or disease progression in patients. For example, detection of inflammatory cytokines such as IFN-γ is used for diagnosing latent form of tuberculosis [4]. In an attempt to get more detailed and nuanced understanding of the roles played by leukocyte subsets in the immune response, immunologists are becoming increasingly interested in connecting cytokine secretion profiles to specific leukocyte subsets and to specific single cells [5–9]. This goal is complicated by the fact that certain cytokines like TNF-α and IFN-γ may be secreted by multiple leukocyte subsets. Therefore, standard immunoassays of blood serum are insufficient to discern which leukocyte subsets secreted cytokines in question.

The main immunology tools used for cytokine profiling are flow cytometry and enzyme-linked immunospot (ELISpot) assays [10–12]. These technologies are robust and have been adapted by the immunology community; however, there is a need to develop new tools that are less expensive and more suited for detecting cytokine release from live cells. Microfabrication and micro-patterning approaches are particularly well-suited for tackling the challenge of connecting specific cells with specific secreted signals [13,14]. Several groups have been developing microdevices [15–18],

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micropatterned surfaces [19–21] and microarrays [22–25] for leukocyte analysis. Previously, our group described the use of microarrays for capturing groups of T-cells on anti-CD4 or anti-CD8 T-cells spots (150 cells per spot) and detecting secreted cytokines on adjacent anti-cytokine antibody spots [25]. More recently, we demonstrated patterning hydrogel microwells on top of a mixed layer of anti-cell/anti-cytokine Abs to enable capturing individual CD4 T-cells from human blood and detecting IFN-γ secreted by single cells [21]. However, in these past studies, Ab molecules were immobilized by physical adsorption on the glass attachment sites inside the microwells. We therefore hypothesized that orienting Ab molecules inside the microwell will improve sensitivity of micropatterned cytokine-sensing surfaces.

Surfaces functionalized with NH_2, SH, COOH, NHS ester or epoxide end groups are commonly used for covalent immobilization of proteins. Avidin-biotin and protein A-Ab interactions provide additional routes for oriented attachment of Ab molecules [26]. Because we are interested in using micropatterned surfaces for capturing single cells and detecting cytokines or other proteins released by the cells, our desired surface needed to have a periodic pattern of non-fouling regions and cell/cytokine adhesive domains. Micropatterning strategies described in the literature include microcontact printing, photoresist lithography and direct write/spot approaches [27–30]. Another strategy, used extensively in our previous studies, is poly(ethylene glycol) (PEG) photolithography whereby PEG prepolymer is photopatterned to create hydrogel microwells that are used to confine attachment of cells or proteins to certain regions of the glass substrate surface [31,32]. This is a simple micropatterning strategy and can be used to populate large surface area with microwells for cell analysis [20,32]. Attachment of hydrogel microstructures to glass or other oxide containing surfaces is typically promoted using an acrylated silane coupling agent [20,32]

Moving beyond simple mono-functional silane layers, we wanted to design a coupling layer that would not only anchor hydrogel microstructures but could also be used for oriented attachment of cytokine-specific Ab molecules. Several strategies for creating multi-functional silane layers have been proposed. For example, Stenger and Dulcey developed an approach of laser desorption/patterning of silane molecules and backfilling with another silane type to create periodic bi-functional silane surfaces [33]. We favored a simpler approach of defining composition of functional groups on the surface by random co-assembly of two different silanes [34–36]. This method was recently employed by Lee et al. to modify glass substrates with a silane layer comprised of ally- and amine-terminated silanes for covalent attachment of both hydrogel structures and avidin molecules [37]. In this paper, co-assembly of acrylate- and thiol-terminated silanes was used to create a bi-functional layer suitable for anchoring hydrogel microstructures and for oriented attachment of antibodies. These micropatterned surfaces were employed in construction of immunoassays for detection of exogenous and endogenous (cell-secreted) cytokines.

2. Materials and methods

2.1. Materials

Glass slides (75 × 25 mm^2) were obtained from VWR (West Chester, PA). (3-acryloxypropyl) trimethoxysilane (MW 234) and 3-mercaptopropyl trimethoxysilane (MW 196) were purchased from Gelest, Inc. (Morisville, PA).

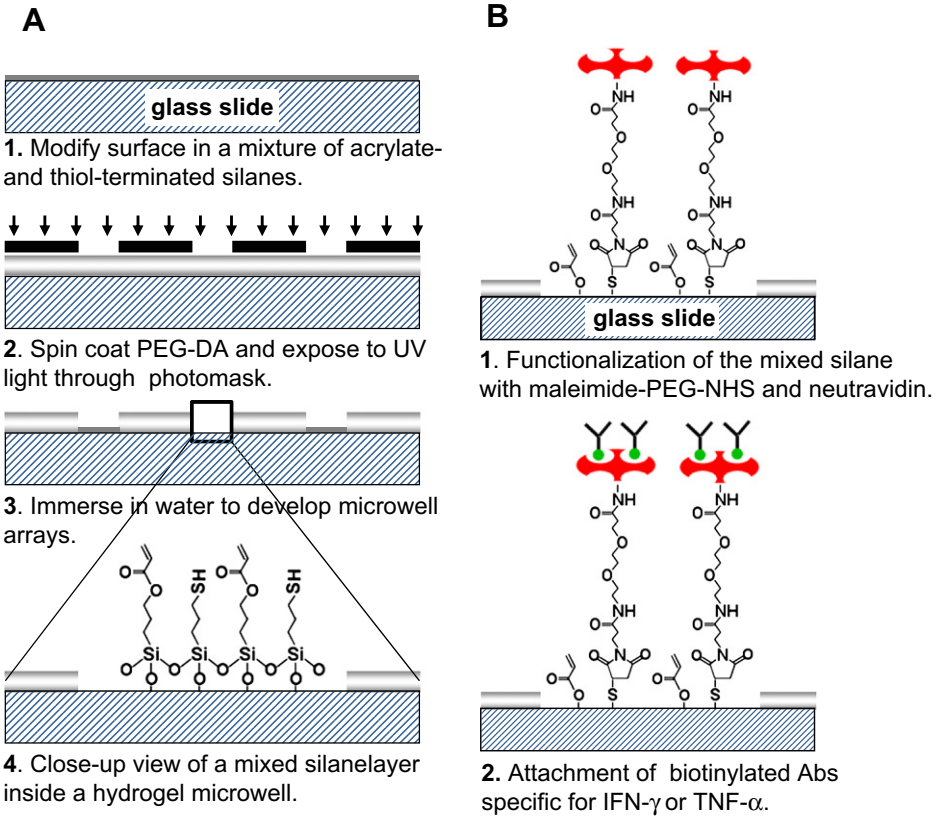

Fig. 1. (A) A process flow diagram for micorpatterning hydrogel microwells on glass. In addition to acrylate moieties used to couple hydrogel microstructures to glass, silane layer also contains thiol groups for covalent linking of proteins. (B) Strategy for immobilizing biomolecules inside microwells. Mixed silane layer was activated using a hetero-bifunctional cross-linker, and then incubated with avidin and biotin-antibody. Throughout this paper the sensitivity of immunoassays constructed on mixed silanes was compared to immunoassays created by physical adsorption of avidin on mono-functional acrylated silanes followed by immobilization of biotin-antibody.