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Controlling cell behavior with peptide nano-patterns

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ABSTRACT

Proper cell polarization and division are critical for a developing organism and a number of downstream biological processes including cancer metastasis, cell migration, and organelle organization. Both cell behaviors are complex and influenced by a number of external factors including, the extracellular matrix (ECM), physical–mechanical and hydrodynamic forces. In particular, the ECM functions as a dynamic 3-dimensional scaffold support for tissue segregation and cell adhesion. Although cells are microns in size, they sense and respond to dynamic nanoarchitecture changes of the ECM. To further understand these complex processes model substrates have been developed to recapitulate the spatial presentation of ligands as gradients and single cell patterns. However, until now, the interplay between ligand affinity, ligand density and ligand area at the nanoscale on cell behavior has received little attention due to the lack of synergistic surface chemistry, microscopy, cell biology, and nanopatterning technologies. In this report, we develop biospecific nanopatterned peptide array substrates to examine how the nano-environment controls cell behavior utilizing parallel dip-pen nanolithography.

1. Introduction

The extracellular matrix is a very complex, heterogeneous mixture of proteins, peptides, and hormones, which has proven essential to cell survival. Mammalian cells adhered to the dynamic extracellular matrix interact with a range of proteins, growth factors and soluble signals that then shape and modulate internal cell machinery [1–6]. This outside-in signaling process is generally mediated by cell surface adhesion receptors (integrins) that recognize and bind ligands on large ECM proteins [7]. Additionally, spatial and temporal changes within the structure of the dynamic ECM, which reveal and hide ligands on extracellular matrix proteins, modulate cell behavior by altering integrin-ligand affinity, spatial distribution of ligands, and temporal availability of ligands [8]. Currently, a number of model substrates and systems have been developed utilizing polymers, layer-by-layer methods, and self-assembled monolayers (SAMs) [9–15]. SAMs of alkanethiolates on gold in particular, have proven to be useful model substrates with a number of key advantages [16]; SAMs are chemically well defined, synthetically flexible, conductive, compatible with live cell high resolution fluorescence microscopy techniques, can be patterned at the micro- and nanoscale, and most importantly they can be made to resist non-specific protein adsorption. These advantages allow for fabrication of complex, flexible substrates for studies of cell phenomena at the molecular level. To tailor SAMs on gold with precise spatial control with quantification of ligand density, smart SAM surfaces have been developed to immobilize a variety of ligands by convergent synthetic approaches [17–19]. By installing the peptide ligand sequence RGD (an epitope for the ECM protein fibronectin) cells have been biospecifically adhered to SAMs to study cell behavior based on specific ligand-receptor interactions [20]. Additionally, SAMs on gold are amenable to a number of different surface patterning techniques to allow for spatial control over the surface, although only a few allow for nanometer scale control and are biocompatible [21].

Dip pen nanolithography (DPN) has revolutionized nanoscience and is based on a scanning probe technique in which an atomic force microscopy (AFM) tip is used to pattern molecules on a surface with precise nanometer scale features [22–26]. A major under developed area of research in which DPN nanoarray technology will make a significant impact is in cell biology, only a small number of patterning methodologies can achieve nanometer control over cell adhesive areas. In particular, the number and size of biospecific interactions between extracellular ligands and cell surface receptors is critical for cell adhesion and migration. For example, the spatial presentation of cell adhesive ligands influence the

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sub-cellular nanoarchitecture of adherent cells and affect their behavior [27]. These phenomena remain poorly understood and elusive due to the lack of easily available molecularly defined nanopatterned model substrates.

Previous DPN approaches to study cell behavior were performed on a standard AFM (i.e. designed specifically for imaging, not lithography), limiting the types of cell studies to serial pattern production using a single AFM tip, which restricted substrate throughput, pattern design, and pattern quantity [28,29]. These limitations have recently been overcome with the advent of parallel tip arrays and instrumentation designed specifically for large area, parallel nanolithography, instead of relying on AFM metrology tools that were designed with patterning secondary to image acquisition. Parallel DPN allows for printing multiple unique patterns onto a single substrate in a fraction of the time it would take with a single tip, which permits almost unlimited pattern design and feature sizes to combinatorially study cellular behavior [30].

The polarity of a cell can be experimentally measured through the systematic reorientation and alignment of several organelles in the cell including the nucleus, centrosome and golgi apparatus, which can be visualized using fluorescent dyes to map the direction of polarity [28,31]. The orientation of cell division can be determined by observing the plane between the two resulting daughter cells immediately after division or where the chromosomes position themselves at the metaphase plate before the metaphase to anaphase transition followed by cytokinesis. The experiments described in this report are important to the nanoscience and cell motility research fields because previously no method to separate the dynamic processes of cell adhesion, cell polarity, and cell migration from each other without the use of genetic or small molecule manipulations. However, by using single cell nanoarrays, cells may first adhere to the nanoarray pattern, determine if the conditions are met for establishing polarity, then polarize but not migrate. These surfaces would allow for the analysis of cell polarity and cell division orientation and determine how the underlying adhesive environment influences cell behavior.

Herein, we utilize parallel DPN to generate single cell patterns with control of spot size and distribution within each pattern to study their role in determining cell polarization direction and cell division orientation. Five asymmetric patterns were generated with different ligand densities in different regions. Due to advantages of parallel fabrication, substrates were generated with many different copies of each pattern on the surface (Fig. 1). The decreased fabrication time was critical for allowing multiplexed analysis of the asymmetric patterns as well as ligand effects on cell behavior. When combined with SAMs of alkanethiolates on gold and an electroactive immobilization strategy, surfaces can be rapidly generated that are capable of presenting many different ligands with the unique ability to resist non-specific protein adsorption. These surfaces provided a versatile platform to study cell adhesion with nanoscale control over the length of a single cell (approximately 2500 μm^2). Following activation of the substrate and peptide immobilization, peptide nanoarrays were generated and characterized by lateral force microscopy (LFM). Finally, we observed the effects of ligand affinity, ligand area and ligand density on cell polarization direction and cell division orientation.

2. Experimental methods

2.1. Peptide and alkanethiol synthesis

The RGD and PHSRN peptides, tetra (ethylene glycol), and hydroquinone terminated alkanethiols were synthesized as previously reported [18].

2.2. Gold preparation

Glass coverslips were cleaned by sonication in ethanol for 30 min. Then, an adhesion layer of titanium (4 nm) and a transparent layer of gold (10 nm) were thermally evaporated on them.

2.3. DPN patterning

All DPN experiments were carried out using DPN 5000 system (NanoInk Inc., Skokie, IL) at 25 °C and 30% humidity. A 1-dimensional (1-D) tip array (6-tip, 280 μm pitch, Nanoink Inc., Skokie, IL) was used for patterning. This silicon nitride 1-D pen array was immersed in a hydroquinone-terminated alkanethiol ($HQEG_4C_{11}SH$, 5 mM in acetonitrile) for 10 s and then air-dried. The patterns of

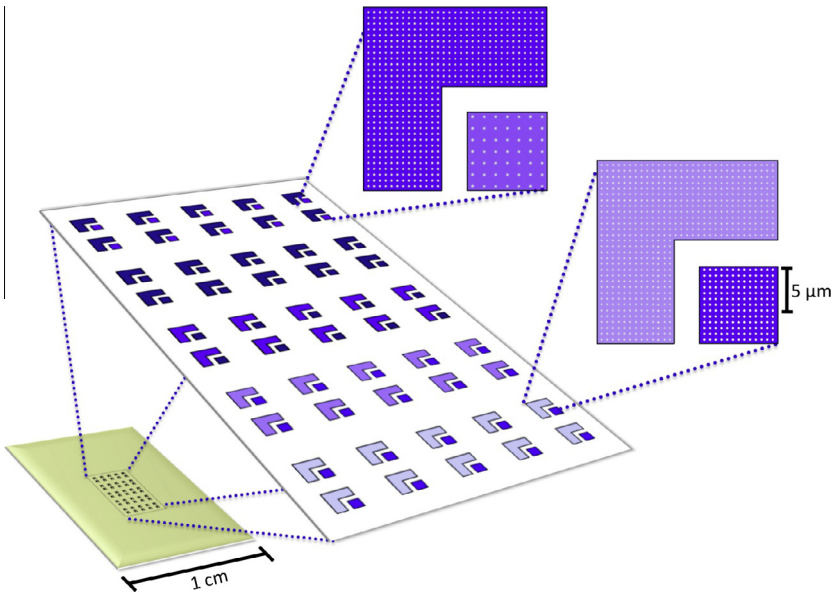

Fig. 1. Schematic of the cell biochips and patterns used for evaluating cell behavior on dip-pen nanolithography (DPN) patterned peptide nanoarrays. Gold surfaces (1 cm × 1 cm) contained around a hundred single cell patterned arrays with each single cell pattern consisting of different distributions of nanometer sized cell adhesive peptide spots.