Cell Patterning

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Mass Spectrometry Assisted Lithography for the Patterning of Cell Adhesion Ligands on Self-Assembled Monolayers**

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Control over the spatial arrangement of cells on a solid support is important for research in various fields, including fundamental biology and applied medicine.[1] In fact, fabricating gradients of cell adhesion ligands is essential for studying cell adhesion, migration, and polarization. To date, various fabrication techniques, such as photolithography,[2] soft lithography,[3] microfluidics,[4] and dip-pen lithography[5] have been developed to pattern cell adhesion ligands on two-dimensional surfaces. In particular, microfluidics and photochemical methods have been actively used to engineer gradients of ligands on surfaces.[6] Most of the patterning technologies, however, require complicated procedures or elaborate chemical synthesis, and thus demands are growing for relatively simple, easy to use, and flexible techniques to engineer the ligand gradient patterns.

Herein, we report a simple and flexible method for patterning cell adhesion ligands on a self-assembled monolayer (SAM) which uses matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Previously, Su and Mrksich reported that molecules of SAMs on a gold substrate can be readily desorbed and ionized, and give a mass/charge ratio in a commercial MALDI-TOF mass spectrometer.[7] Mrksich and co-workers applied this principle to characterize SAMs, and further applied it as a signal readout tool in SAM-based biochips engineered for enzyme assays, protein-protein interactions, and chemical screening.[8]

In this current study, SAMs on a gold substrate were selectively desorbed and removed at designated regions by MALDI-TOF MS, and the exposed bare gold regions were then backfilled with a second alkanethiol.[9] For the patterning of ligands, we first selectively desorbed and removed SAMs with tri(ethyleneglycol) (EG_3) groups at designated regions on a gold substrate by using a MALDI-TOF MS nitrogen laser.[10] The ethyleneglycol groups prevent nonspecific adsorptions of cells to the surfaces.[11] We then backfilled the exposed bare gold surface with an alkanethiol presenting carboxylic acid groups, which act as molecular handles for the sequential immobilization of maleimide and the cell adhesion peptide GRGDSC on the surface.[12] In this way, patterns of cell adhesion ligands were created among the background ethyleneglycol groups. We termed the patterning method mass spectrometry-assisted lithography (MASSAL; Figure 1).

We first prepared SAMs presenting tri(ethyleneglycol) groups on a gold-coated glass coverslip, which was rinsed with ethanol and distilled water, and an organic matrix solution

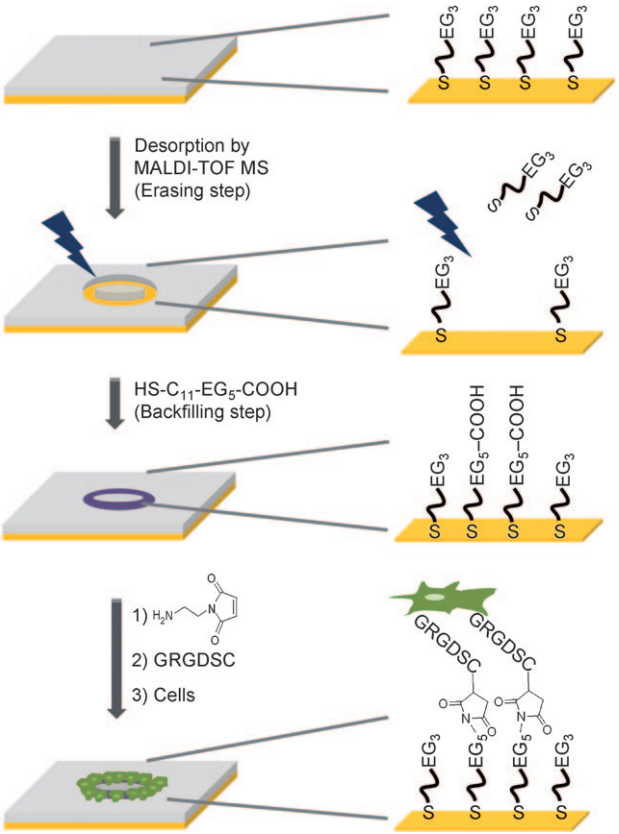

Figure 1. Strategy for patterning cell adhesion ligands on SAMs by using MALDI-TOF MS assisted lithography. A monolayer presenting tri(ethyleneglycol) groups is irradiated with a mass spectrometer laser at designated regions for local desorption (erasing step). The gold surface is then backfilled with alkanethiols terminated with carboxylic acids, which are next sequentially conjugated to a maleimide molecule and to the ligand peptide GRGDSC. Cells are then cultured on the ligand-patterned substrate, thereby creating patterns of cells.

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(2,4,6-trihydroxyacetophenone (THAP), 5 mg mL^{-1} in anhydrous acetonitrile) was then applied to the SAM surface. Analysis of the surface by MALDI-TOF MS showed signals corresponding to disulfide adducts of the tri(ethyleneglycol)-terminated alkanethiol (**M1**) at *m/z* 671 [**M1**+H]$^+$, 693 [**M1**+Na]$^+$, and 709 [**M1**+K]$^+$ (Figure S1 in the Supporting Information). Next, the matrix-coated SAMs were desorbed by a repeated laser irradiation process at pulse repetition rates of 3 Hz and an increasing number of laser scans at constant laser power while manually moving the laser spot along an arbitrary line at a rate of 60 μm s^{-1}. As expected, the signal intensities corresponding to the disulfide **M1** decreased as the number of laser scans increased. In this experiment, MALDI-TOF MS was exploited for three purposes: to characterize the EG_3-terminated SAMs, desorb the SAMs, and monitor the degree of desorption. We could visually check pattern formation during desorption of the SAM on a monitor that was attached to the MALDI-TOF mass spectrometer; since the removal of the matrix coating along with the SAMs resulted in brighter reflecting light from the surface than from the matrix-coated region, the monitor displayed a magnified image of a sample plate in real time (Figure S1 in the Supporting Information). In addition, visual inspection confirmed that the gold layer itself was not damaged by laser irradiation under the experimental conditions used.

Next, we demonstrated that four different basic and complicated patterns of cells could be produced by using MALDI-TOF MS. The EG_3-presenting SAMs were first prepared, coated with the matrix, and subjected to desorption inside the MALDI-TOF mass spectrometer. The surface was repeatedly irradiated manually with the laser to remove the SAMs in stripes as well as in triangle, square, and circle shapes. Alternatively, software-guided automatic laser movement based on three predesigned "smiley" patterns was used. The substrate was then rinsed thoroughly with ethanol and distilled water to remove any matrix coating from the surface. The bare gold regions on the substrate were then backfilled by immersing the substrate in a mixed solution of a thiol terminated with a carboxylic acid (0.25 mM) and a thiol terminated with EG_3 groups (0.75 mM). Serial surface conjugation reactions on the substrate were carried out to introduce the maleimide group and then the cell adhesion peptide GRGDSC. The surface reactions were characterized by MALDI-TOF MS (Figure S2 in the Supporting Information). HeLa cells transfected with the enhanced green fluorescent protein (eGFP) were then cultured on the substrate.[13] Microscopy analyses showed four different HeLa cell patterns (stripes, triangle, square, and circle) and three smileys of different sizes and appearance (Figure 2). The patterns of cells were clearly recognized by bright-field and fluorescent imaging. To verify that cell attachment was due to specific interactions between the RGD peptide and cell surface receptor, we carried out a control experiment in which cells were preincubated with excess RGD peptide before plating. As expected, no recognizable cell attachment was observed. This result indicated that the attachment of the cells to the surface was mediated by the specific interaction of cell surface receptor integrins with RGD peptides displayed on the substrate (data not shown).

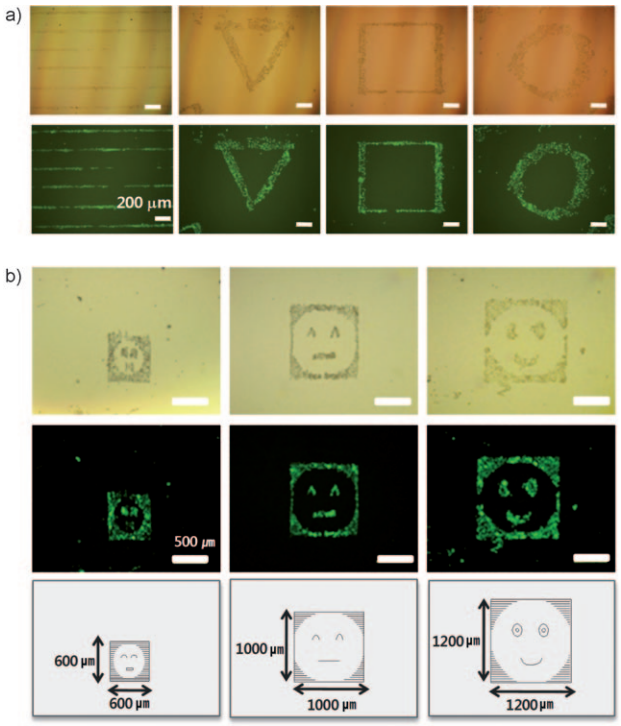

Figure 2. Various patterns of HeLa cells were produced by MASSAL. The upper rows correspond to bright field and the lower/middle rows to fluorescence images. a) HeLa cells were patterned in stripes and triangle, square, and circle shapes by the desorption of SAMs by manual operation of the laser movement, followed by backfilling and immobilization of the GRGDSC peptide. b) Three different patterns of HeLa cells were fabricated by using software-guided desorption. Templates to guide the desorption were first designed (bottom) and then the desorption was carried by following the templates.

We next showed that the current MASSAL method is applicable to fabricating surfaces with various RGD densities on a single substrate. The degree of SAM removal was controlled during desorption by applying a different number of laser shots, thus resulting in different extents of backfilling. The degree of desorption can be conveniently adjusted by monitoring the signal intensities in the mass spectra recorded during the desorption process. In this way, the surface density of the cell adhesion ligand can be simply controlled during the desorption step. To verify this, we first desorbed EG_3-presenting SAMs from five separate rectangular regions on the SAM surface with a varying number of laser shots (10, 50, 100, 150, and 200) at constant laser power, guided by MALDI-TOF MS software. The substrate was rinsed and backfilled with an alkanethiol terminated with a carboxylic acid by immersing the substrate in a mixed solution of an alkanethiol terminated with carboxylic acid and one terminated with EG_3. Then, the five backfilled regions on the substrate were characterized by MALDI-TOF MS. Signals corresponding to cation adducts and oxidized cation adducts of disulfide **M1** (*m/z* 693, 709, 725), and cation adducts of disulfide **M2** (*m/z* 840, 856, 862) were observed in each rectangle at varying relative intensities.[14] The mass spectra confirmed that increasing the number of laser shots used for desorption during the erasing step increased the amount of alkanethiol terminated with

carboxylic acid after backfilling (Figure 3). Signal intensity ratios (I_2/I_1, where I_2, and I_1 represent relative signal intensities corresponding to **M2** and **M1**, respectively) were

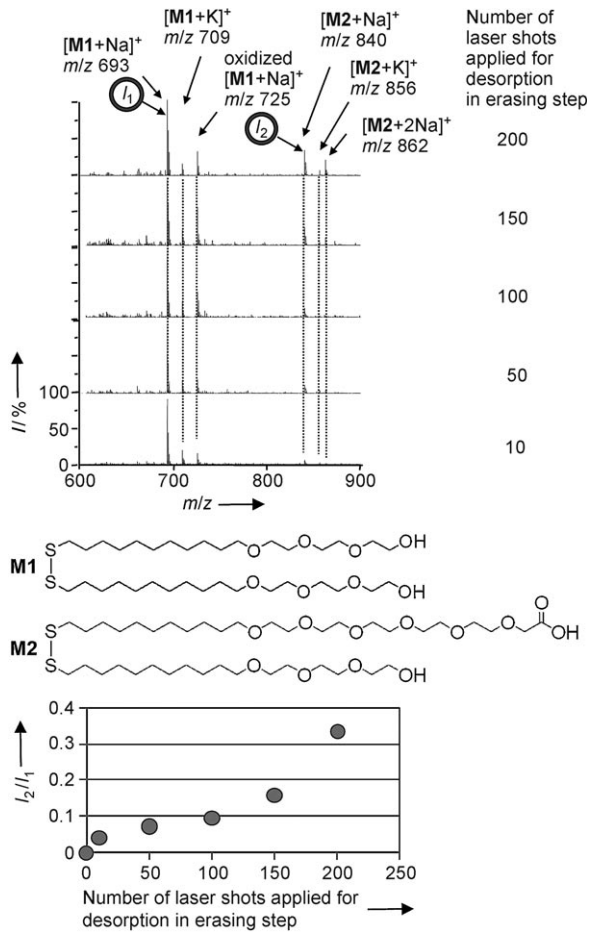

Figure 3. MALDI-TOF MS analyses of the surfaces that were desorbed by using MALDI-TOF MS and backfilled with an alkanethiol terminated with a carboxylic acid group. The EG_3-terminated SAM substrate was divided into five rectangles and the number of laser shots applied to each rectangle varied from 10 to 200 during desorption. Each rectangle on the substrate was characterized after backfilling with the alkanethiol terminated with a carboxylic acid group. Top: The mass spectra show signals corresponding to EG_3-terminated disulfide (**M1**) and a mixed disulfide of the alkanethiol presenting EG_3 groups and one presenting carboxylic acids (**M2**). Bottom: The ratio of the signal intensities corresponding to the sodium adducts of M2 and **M1** (I_2/I_1) is plotted against the number of laser shots applied for desorption of the EG_3-presenting SAMs during the erasing step.

plotted against the number of laser shots applied to remove the SAM by desorption (Figure 3, bottom). The data showed that the surface density of the carboxylic acid increased with the number of laser shots applied during the desorption of the EG_3-terminated SAMs. Thus, in this experiment, MALDI-TOF MS was utilized as a tool to desorb the SAMs and as a method to analyze the SAM surface after backfilling.

After immobilizing maleimide and the GRGDSC peptide onto the substrate, we plated eGFP-transfected HeLa cells on the backfilled substrate. After briefly rinsing the substrate, we observed that the HeLa cells became attached inside the rectangular patterns; the number of attached cells in each rectangle varied according to the degree of desorption. The plot showed that the number of cells increased as the number of laser shots increased (Figure 4). This result shows that patterns of ligand gradients can be fabricated by controlling

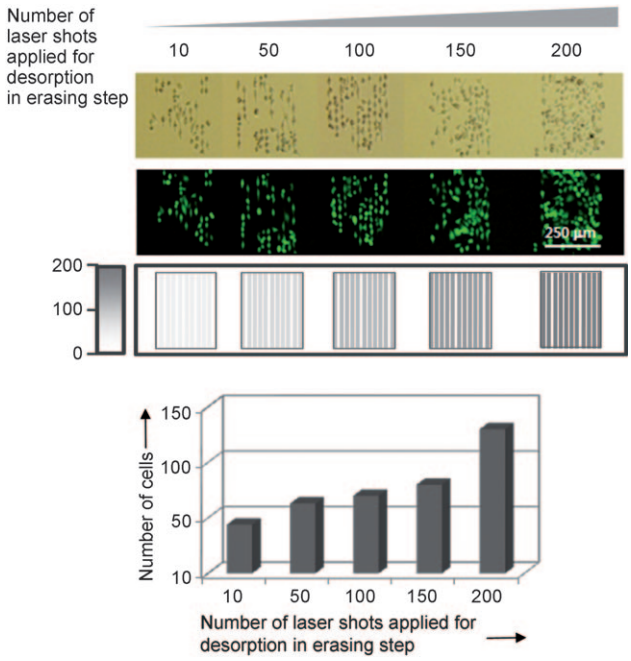

Figure 4. HeLa cells were plated on the RGD-patterned substrate prepared as described in Figure 3. Top: After a brief rinsing, bright field and fluorescent images of the cell patterns were obtained. Middle: The numbers of laser shots applied for desorption in each rectangle are displayed as color intensities. Bottom: The number of cells attached to each rectangle is plotted versus the number of laser shots applied during desorption.

the surface ligand density through the number of laser shots applied during desorption by MALDI-TOF MS. Note that the graph in Figure 4 displays a trend similar to the plot correlating the relative signal intensities of the disulfide terminated with carboxylic acid (**M2**) with the number of laser shots fired during the erasing step (Figure 3). The similarity in the trends of the two graphs implies that the number of attached cells directly correlates with the number of cell adhesion ligands on the surface.[15]

Our strategy possesses several notable features. First, the degree of SAM removal can be monitored by examining the MALDI-TOF mass spectra in real time. As SAMs are desorbed, the number of molecules at a specific region decreases and, as a result, signals corresponding to the SAM molecule show a gradual decrease and finally disappear. Therefore, the extent of SAM removal can be controlled and adjusted by constantly checking the signal intensities in real time. Second, any pattern shapes can be engineered within minutes without any restrictions since the desorption process is guided by MALDI-TOF MS software. Third, this patterning method can be extended to patterning any thiol-contain-

ing molecules. Fourth, the current method is technically simple and easy to use compared to other patterning methods, including a photochemical method. Finally, the only equipment the MASSAL method requires is a MALDI-TOF mass spectrometer, an instrument that is widely available in research institutes. The latter feature is important because the current method has the potential to create patterns of any molecule on a gold substrate, and thus has applications in broad research areas including chemistry, biology, and medicine. In this respect, the accessibility of the patterning technique to researchers is one of the important features of this technology.

In conclusion, a new method of patterning cell adhesion ligands has been developed. A MALDI-TOF mass spectrometer was used to selectively remove cell-repulsive SAMs at designated regions on a gold substrate, and this was followed by backfilling the exposed gold regions with desired molecules for immobilization of the ligands. MALDI-TOF MS plays multiple roles in this approach—it is used as a tool to characterize EG_3-terminated SAMs, desorb SAMs, and analyze the SAM surface after backfilling and immobilization of the ligand, as well as acting as a guide to decide when to stop the desorption process. The simplicity of the method and the wide availability of the required equipment will make this surface-patterning method readily applicable to tissue engineering and cell arrays.

To the best of our knowledge, the present study is the first to use MALDI-TOF mass spectrometry to create patterns of cell adhesion ligands on SAMs with simple control over the ligand density. We believe that this new patterning technique will be one of the most convenient, inexpensive, and flexible methods for engineering patterns of molecules of interest on SAMs. In addition, the MASSAL technique is applicable to fabricating surfaces with patterns of different ligand types on a single surface through sequential, repeated desorption, and backfilling. We are currently examining the utility of this multiligand-patterning strategy and its applications for the study of cell-cell interactions and cell-protein interactions, as well as interactions between nanomaterials and surfaces with varying physical and chemical properties.

Experimental Section

Self-assembled monolayers were prepared according to previously reported procedures.[16] For EG_3-presenting SAMs, gold-coated substrates were immersed in an ethanolic solution of EG_3-terminated alkanethiol (1 mM) for 6 h. The substrates were then rinsed thoroughly with ethanol and dried. After desorption of the SAMs by MALDI-TOF MS, the substrates were rinsed with ethanol and distilled water to completely remove the matrix. For backfilling, the substrates were immersed in a mixed solution of the alkanethiol terminated with carboxylic acid (0.25 mM) and one terminated with EG_3 groups (0.75 mM) in ethanol. Maleimide-presenting SAMs were prepared by treating SAMs presenting carboxylic acid groups with an aqueous solution of aminoethylmaleimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in phosphate-buffered saline (PBS) for 3 h at room temperature. The peptide GRGDSC was immobilized on maleimide-presenting SAMs by treating maleimide-presenting SAMs with an aqueous solution of GRGDSC (1 mM in PBS) for 3 h at 37 °C.

Mass analyses and SAM desorption were carried out with a Voyager DE STR mass spectrometer (Applied Biosystems, USA) equipped with a nitrogen laser operating at 337 nm.

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