University of Alberta

Cell Array Biochip

by

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Your file Votre référence
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Our file Notre référence
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To my parents

Abstract

Two fabrication methods have been developed to deposit *E. coli* on an ordered dense array of cell adhesion spots. In one method, gold regions are patterned and then coated with various thiols, and the other uses photolithography to pattern a self-assembled monolayer of thiols through photo-oxidation and subsequent thiol exchange. The challenge associated with growing cells on these surfaces was addressed by a variety of approaches, including the liquid LB method in which patterned cells are incubated in LB media, the top agarose method that uses agarose gels to grow cells, and the nitrocellulose (N/C) membrane method in which cell patterns are transferred to N/C membranes for culturing. The last was identified to be suitable for on-chip cell incubation. The SAM patterning method was also utilized to pattern Hela cells, one kind of animal cells on flat surfaces. Cell patterns were observed on the regions with large sized adhesion spots.

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List of Abbreviations

μCP	Microcontact Printing
AFM	Atomic force microscopy
CCD	Charge coupled device
DI	Deionized
DMEM	Dulbecco's Modified Eagle's Medium
DPN	Dip-pen nanolithography
DRIE	Deep reactive ion etching
ECM	Extracellular matrix
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal bovine serum
FTIR	Fourier transform infrared
GFP	Green fluorescent protein
IR	Infrared
IRRAS	Infrared reflectance absorbance spectroscopy
LB	Luria-Bertani
MW	Molecular Weight
N/C	Nitrocellulose
PBS	Phosphate buffered saline
PDMS	Poly(dimethylsiloxane)
PEI	Polyethylenimine
PLL	Poly-L-lysine
RPMI	Roswell Park Memorial Institute Medium
SAM	Self-assembled monolayer
STM	Scanning tunneling microscopy
Thiol-EG-OMe	1-mercaptoundec-11-yl-triethylene glycol methylether
Thiol-OH	6-mercapto-1-hexanol
UV	Ultraviolet

Chapter 1 Introduction

1.1 High-density Array Systems

High-density cell array systems, analogous to DNA and protein microarrays, allow thousands of analyses to be performed automatically on a compact chip. Arrays of genetically engineered live cells can serve as platforms for high-throughput screening of drug candidates and provide useful information about gene expression.[1-3] The isolation of cells on a surface makes it possible to investigate events occurring in individual cells repeatedly because of their addressable positions, which is promising for monitoring and controlling cell behavior and studies of cell-cell communication and cell-surface interactions.[4-7]

The objective of this thesis was to develop useful and convenient methods for patterning bacteria as well as animal cells on a grid pattern. The cell array formed was then cultured to create a colony array that could be used to construct complementary DNA (cDNA) or other genomic libraries. In small scale library construction, highly diluted cells are spread on agar plates to ensure there is one cell per spot, making colonies a single clone. In highly automated library production, as is done at pharmaceutical companies, microtitre plates are used. Many hundreds of plates may be required to achieve the needed level of dilution, which can be costly. Cell array chips could provide an alternative, lower cost platform. Self-assembled monolayer (SAM) chemistry, the primary tool employed in our work, will be described in this introductory chapter. A brief review of commonly-used cell patterning techniques will also be presented. This chapter will also discuss some principles of cell adhesion, which play an important role in the formation of cell arrays.

1.2 Self-Assembled Monolayer Chemistry

Self-assembled monolayers (SAMs) are assemblies of ordered molecules organized spontaneously on a substrate surface. SAMs are typically prepared by exposure of a surface to molecules with chemical groups that possess strong affinities for the substrate. The quality of SAMs formed is determined by the interaction between substrate and

adsorbate, as well as the intermolecular interactions between the adsorbates. The interaction of substrate and adsorbate can be described as chemisorption or as physisorption, according to the enthalpy of this interaction. Examples of chemisorbing systems are alkylsiloxanes on silicon dioxide surfaces[8], carboxylic acids on aluminum oxide and silver surfaces[9, 10], and alkanethiols on gold surfaces[11, 12]. Due to their ease of preparation and controllable chemical functionalities, SAMs have received significant interest in fundamental studies of wetting, adhesion, lubrication, and corrosion[13-16]. In this thesis, SAMs of alkanethiolates were used to make surfaces selectively adsorb cells.

1.2.1 Formation of Alkanethiolate SAMs on Gold

Nuzzo and Allara first demonstrated that alkanethiolate SAMs on gold, prepared by adsorption of di-n-alkyl disulfides from dilute solution, were structurally ordered and crystalline[11]. Shortly after, it was discovered that SAMs could also be prepared from alkanethiols as well as other organosulfurs[12]. Since then, a tremendous number of investigations have focused on alkanethiolate SAMs. They provide suitable model systems in the majority of studies of cell behavior and biological interfacial interactions for several reasons[17]. First, gold is a chemically inert metal, it does not form a stable oxide and it resists atmospheric contamination. Therefore, SAMs can easily be prepared in ambient conditions. Second, long chain alkanethiols form densely packed and stable monolayers on gold. Third, gold has a strong specific interaction with sulfur that allows SAMs to be formed in the presence of many other functional groups. Moreover, different chemical functional groups can be incorporated into alkanethiols, rendering various surface properties.

Alkanethiolate SAMs are formed by immersing a clean gold substrate into an alkanethiol ethanol solution. The time required for formation of SAMs depends on solution concentration and alkanethiol species. The surface active group is sulfur. The bonding between sulfur and gold is usually considered to be oxidative addition of S-H to the gold surface and reductive elimination of hydrogen:

$$R\text{-}S\text{-}H + Au = R\text{-}S^{-}\text{-}Au^{+} + 1/2\ H_2$$

This step is fast, typically within seconds to minutes[18]. The next several hours involve the self-assembly of an ordered adsorbate phase aided by exchange of adsorbates with the

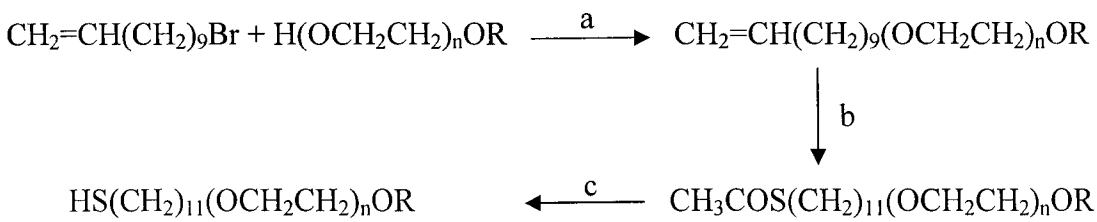
solution phase[19]. On a gold surface, the hydrocarbon tails generally tilt approximately 30° from the surface normal and adopt the all-trans configuration to maximize van der Waals interactions[20].

The structure of alkanethiolates has three parts: a sulfur head group, an alkyl chain, and a tail group. Alkanethiolate SAMs with more than 10 methylene (CH_2) units are essentially impermeable to water, and the properties of their surfaces are determined by terminal groups[18, 21]. Because the terminal group can easily be modified, alkanethiolate SAMs offer the ability to generate surfaces with a wide range of chemical and physical properties.

1.2.2 Oligo(ethylene glycol)-terminated Alkanethiolate SAMs

Oligo(ethylene glycol) (OEG)-terminated alkanethiols have the structure of $HS(CH_2)_{11}(OCH_2CH_2)_nOR$, where n = 3-7, R is H or Me. OEG-terminated thiols are important in surface patterning techniques because of their strong resistance to protein adhesion, which was discovered by Prime and Whitesides[22-24]. They compared and correlated the surface composition of different functionalized alkanethiolate SAMs with the amount of protein adsorbed from a single component protein solution and found that both the hydroxyl- and the methoxy-terminated oligo(ethylene glycol) SAMs showed protein resistance. Later work indicated that these alkanethiols are also very effective in preventing cell adsorption[25]. The bonding of interfacial water by the OEG moieties may play an important role in their strong ability to resist the adsorption of both proteins and cells[26]. However, the detailed mechanisms are still not well understood.

The general procedure of $HS(CH_2)_{11}(OCH_2CH_2)_nOR$ synthesis is described below[24]:



(a) Aqueous NaOH, 100 °C; (b) CH_3COSH, AIBN, THF and (c) HCl, MeOH.

In our work, $HS(CH_2)_{11}(OCH_2CH_2)_3OCH_3$ was synthesized according to this

procedure and used to prevent non-specific cell adhesion.

1.2.3 Methods for SAM Characterization

A variety of experimental methods have been used to investigate the physical structures and chemical properties of SAMs on solid substrates. These techniques range from ensemble average measurements which typically characterize SAMs on the macroscopic level, to local probes which examine the structures of SAMs down to atomic resolution. Ensemble average techniques include ellipsometry to measure film thickness[12], contact angle goniometry (CAG) to determine the hydrophobicity of monolayers[17], X-ray photoelectron spectroscopy (XPS) to evaluate the composition of the bound species[18, 27], Fourier transform infrared spectroscopy (FT-IR) to identify the presence of particular functional groups and examine tilt, order, and gauche defects[12, 18, 28], and X-ray diffraction (XRD) to investigate the physical structures of assemblies[29]. Fourier transform infrared reflectance absorbance spectroscopy (FT-IRRAS), one type of FT-IR, is a well established technique for studying thin films or monolayers on metal substrates[30]. The specificity of IRRAS is that the infrared light is reflected off a reflective surface rather than transmitted through the sample. In this thesis, FT-IRRAS and CAG were used to investigate the different surfaces formed during patterning of SAMs. Scanning probe spectroscopy, including atomic force microscopy (AFM)[31], scanning tunneling microscopy (STM)[32], and lateral force microscopy (LFM)[33], can provide information about local compositions and structures of SAMs.

1.2.4 Patterning of SAMs

Patterned SAMs have important applications in both materials science[34] and biological sciences[35]. Particularly, they provide suitable platforms for DNA, protein, and cell arrays, which can be used for high-throughput screening, development of biosensors, as well as the fundamental studies of cell biology. Patterning of SAMs can be achieved by soft lithography, scanning probe lithography, photolithography, electron-beam irradiation[36], and X-ray irradiation[37] etc. The former three methods are most widely used and will be briefly described in this section.

Soft lithography was first introduced by Whitesides' group[38]. Since then, it has found

vast usage in patterning SAMs. Soft lithography techniques include microcontact printing (μCP)[38], replica molding (REM)[39], microtransfer molding (μTM)[40], micromolding in capillaries (MIMIC)[41], and solvent assisted micromolding (SAMIM)[42]. Microcontact printing (μCP) is broadly used in patterning alkanethiolate SAMs on gold and silver substrates because of the ease of fabricating the printing tools, high spatial resolution of features achieved, large parallel printing capacity, as well as relatively low costs. The general process includes the following steps[43]. In the first step, a polished silicon wafer coated with a thin layer of photoresist is exposed to UV light through a photomask and developed. The second step is the fabrication of an elastomeric polymer stamp by casting polydimethylsiloxane (PDMS) against the silicon master. Then the stamp is inked with a solution of the alkanethiol or other organic molecules. After it dries, the stamp is pressed on a gold or silver surface. The alkanethiol is transferred only to the regions where the stamp contacts the surface, and self-assembles into patterns which are determined by the patterns of the stamp. Finally the substrate is immersed into a second alkanethiol. This method can typically give features having hundreds of nanometer to micrometer dimensions[44]. A recent report described the generation of simple nanostructures with critical dimensions down to 30 nm over cm^{2-}-sized regions using μCP[45]. However, the resulting patterns are limited by the elastomeric stamp and not all patterns on the stamp are stable or accurately reproduced during molding or printing processes[46]. This method only works well with methyl-terminated alkanethiolate SAMs.

Scanning probe lithography is a technique that patterns a surface using scanning probe microscopes, including AFM and STM. Generally, placement of a probe tip on a surface leads to a change in that location, such as the direct placement of molecules or the desorption of surface-adsorbed molecules. Therefore, moving the location of the tips on the surface can generate diverse patterns. Using this method, resolution close to the molecular scale can be obtained. It also allows the patterning and characterization of surfaces to be performed at the same time. As an example of scanning probe lithography, Mirkin and coworkers developed a nanopatterning technique named dip-pen nanolithography (DPN)[47]. In this technique, an AFM tip is used to deliver molecules from that tip to a surface via a solvent meniscus, which naturally forms in the ambient atmosphere. By inking the end of an AFM tip with alkanethiol molecules one can write

patterns on gold surfaces with 30-nanometer linewidth resolution[47]. However, one main drawback of this technique is the relatively slow speed of the writing process. Massive parallel writing using multiple probe tips[48, 49] have been developed to compensate for this disadvantage. A 26-tip array has been reported to generate as many as 10^5 nanostructures per hour, while still maintaining sub-100 nm resolution[50]. Patterned SAMs can also be formed by moving an STM tip on a SAM-coated surface to desorb the molecules the tip approaches[51].

Photolithography employs UV light irradiation to pattern alkanethiolate SAMs. The process can be divided into two steps. First, a SAM-coated gold substrate is exposed to UV light in the presence of air through a photomask. The irradiated alkanethiolate adsorbates ($R\text{-}S^-$) oxidize to the corresponding alkanesulfonates ($R\text{-}SO_3^-$)[52]. The rate of photooxidation strongly depends on the alkyl chain length[53, 54], with a fast rate for short-chain SAMs and slow rate for long-chain SAMs. Second, alkanesulfonate species formed are displaced by immersing into another thiol solution, due to the weak interaction between $R\text{-}SO_3^-$ and gold.[55] Therefore, a binary-component pattern is generated. Tarlov *et al.*[56] were the first to show well-patterned monolayers of decanethiol and mercaptoundecanoic acid prepared using an electron microscopic grid as the mask and a high-pressure mercury lamp as the light source. Later studies demonstrated that 254 nm UV can lead to the rapid and complete photochemical oxidization of alkanethiolate SAMs[57]. With this method, micrometer scale spatial resolution can be attained[58]. However, the resolution is limited by light diffraction effects for submicron mask features[59]. A new photolithography process, referred to as scanning near-field photolithography (SNP)[60, 61], was developed using a UV laser coupled to a scanning near-field optical microscope as the light resource. SNP can produce feature sizes as small as 40 nm.

Compared to soft lithography and scanning probe lithography, the procedure for photopatterning SAMs is relatively simple and straightforward. Micrometer scale resolution satisfies the goal of cell array patterning. Photolithography was applied to pattern two different alkanethiols on the same substrate. With the correct choice of the adherent and non-adherent thiols and polymers, the surface was able to adsorb cells in the spatially selective manner.

1.3 Cell Patterning Techniques

Patterning of cells is an experimental tool that has the ability to precisely control the position of cells on a substrate surface. Cell patterning techniques are increasingly used by researchers for biosensors, high-throughput screening, tissue engineering, as well as fundamental studies of cell biology.[4, 62-64]. Various methods have been developed, which can be generally divided into two categories[65], topographical patterning and physiochemical patterning.

Topographical patterning employs surfaces with topographical features used to induce the selective adhesion of cells. Surface topography is normally generated using lithography to pattern photoresist on a substrate, then selectively etching the unprotected substrate. Reactive ion etching (RIE) fabricates anisotropic features in a wide range of patterns and shapes, and therefore broadly used. Craighead and coworkers[66] applied photolithography and RIE to fabricate arrays of micron-sized columns on silicon substrates. They observed that certain kinds of cells preferred to adhere to the column array surfaces rather than to the smooth surfaces. Textured patterns can also be duplicated on polymers using microfabricated silicon as templates by either casting or injection molding[67]. Because of their biocompatibility and diversity, these selectively patterned 3-dimensional polymers have more advantages in cell patterning compared to other solid substrates.

Most investigations have concentrated on using physiochemical patterning to achieve cell patterns. For this type of patterning, surfaces are chemically modified to adsorb cells selectively. Different patterning techniques are available in this category, including microcontact printing[7, 38, 68], membrane-based patterning[69, 70], and photolithography[71-74].

Microcontact printing (μCP) uses a PDMS stamp to transfer a desired pattern to a substrate. The procedure involves fabrication of an elastomeric polymer (PDMS) stamp, inking the stamp with an organic solution, and pressing the stamp on a substrate. The combination of microcontact printing with SAMs provides a convenient and versatile method for patterning cells. In a typical experiment, one kind of alkanethiol is printed onto a gold surface to generate patterns that promote cell adhesion followed by immersing the substrate into an oligo(ethylene glycol)-terminated thiol solution to render the uncovered surface inert to the adsorption of cells.[35] The patterned substrates can be

first dipped into a solution of extracellular matrix (ECM) protein to ensure the efficient attachment of cells. Using μCP, SAMs patterned into 15 × 40 μm rectangular adhesion islands resulted in the formation of individual adherent cell arrays.[7] Varying the size of the islands could control cell shape, and thereby influence cell function. A recent report by Lee *et al.*[68] demonstrated the ability to generate single cell arrays by patterning streptavidin on a surface using μCP and biotinylating cells. The small dimensions of patterns (square-shaped with 10 μm in width) and the biospecific interaction of biotin-streptavidin led to the generation of single cell arrays. Cells were well-patterned over a large area, but some imperfections were observed, such as empty spots and the aggregation of cells in interstitial spaces. This method can be applied to any arbitrary cell type, especially useful for weakly adherent and non-adherent cells that normally have low binding affinity to the surface. Generally, microcontact printing has sufficient resolution to allow the patterning of cells. Multiple stamps can be cast from a single master and each stamp can produce many patterned substrates. However, the fabrication of a master still requires some form of lithography, which is expensive and time-consuming. Moreover, some patterns can not be accurately reproduced during printing.

Membrane-based patterning[69, 70] provides an alternative method for cell patterning by selectively blocking the access of a surface using physical barriers. An elastomeric membrane, which contains through holes, is fabricated by pouring PDMS on a master of photoresist posts and curing.[70] This membrane is placed on a substrate surface that is then exposed to the solution of proteins and cells through the holes. After the membrane is peeled away, a cell array is obtained. The arrays of individual cells can be achieved by choosing suitable size of the holes. Membrane-based patterning is able to arrange cells on a wide range of substrates, including glass, Petri dishes, PDMS, polyurethane, silicon, and even nonplanar surfaces. This method also allows the cells to be grown on chip without constraints, and thus can be employed to study cell spreading and immigration. The drawback of this method is that removal of the PDMS membrane will damage cells which simultaneously attach to both the membrane and the substrate. Great care is also needed to manipulate the relatively thin PDMS membrane.

Photolithography is a technique initially developed for fabricating microelectronic circuits and can also be used for patterning cells on flat substrates. In early work[71], cells

were directly deposited onto a photoresist pattern that prevented the attachment of cells. So the cells preferentially attached to the bare surface. Rohr *et al.*[72] improved this method by first coating a surface with agar. They then used photolithography to generate a photoresist pattern on the agar coated substrate followed by coating it with a layer of attachment factors. After the photoresist was lift-off, the substrate was incubated with a cell solution. Because agar is resistant to the cell adhesion, a cell pattern was achieved according to the pattern of attachment factors. But some chemicals used in normal photolithography processes are not biocompatible and can denature the adsorbed proteins. Methods without use of photoresists are demanded. Deep UV irradiation of polymers, such as polystyrene, polymethylmethacrylate, and polycarbonate, can alter their chemical composition and strongly favor the adsorption of cells.[73] Therefore, masked irradiation of polymeric substrates is a simple and economical route for structuring cell patterns and eliminates the further chemical treatment. Photolithography can also be used to pattern alkanethiolate SAMs on a surface, as described in section 1.2.4. Briefly, UV exposure oxidized thiolates into sulfonates, which can be replaced with another alkanethiol. Copper and coworkers[74] fabricated a binary-component pattern of methyl-terminated and carboxylic acid-terminated SAMs on gold using UV photopatterning. They found that cells exclusively attached to the acid-functionalized regions, leaving the methyl-functionalized areas bare. This patterning method is easier to operate and less laborious compared with microcontact printing and membrane-based patterning because it does not require the fabrication of PDMS stamps or membranes. However, long-time exposure with UV in the air and possible exchange of two thiols may degrade the quality of patterned SAMs, causing poorly-defined cell patterns.

Besides, physiochemical patterning methods, using microfluidic channels[75, 76], poly(ethylene glycol) hydrogels[77, 78], PDMS microwells[79], polyelectrolyte multilayers[80], colloids arrays[78, 81], plasma lithography[5], and electroactive substrates[82], have also been developed. One thing worth mentioning is that most of these patterning techniques are primarily employed to pattern and culture animal cells, such as hepatocytes, fibroblasts, and endothelial cells. Relatively few reports were published about patterning bacteria (e.g. *E. coli*) on a surface. As bacteria are typically one order of magnitude smaller than most animal cells, smaller patterns are required. Rowan *et al.*[83] used a soft lithography process

based on microcontact printing and subsequent hyperbranched polymer grafting to fabricate an array of corrals that consist of poly(acrylic acid)/poly(ethylene glycol) walls and n-hexadecanethiol interiors. As *E. coli* preferentially adhered to the SAM region, a bacterium array formed. They showed that corrals that are 12 μm square contained 2 ± 1 bacteria. Spatially controlled positioning of bacteria was also achieved using arrays of the microwells composed of poly(ethylene glycol) hydrogel walls.[84] Walt's group[85] developed a method using etched optical imaging fibers to generate single bacterium arrays, which could be integrated with an automatic detection system. *E. coli* adsorbing on fibers containing 50,000 microwells (each with a diameter of 2.5 μm) were reported to give a high single cell probability at overall occupancy of 50%. However, the spot sizes, shapes, and inter-spot spacings are limited by the commercially available imaging fibers. Bacterial culture is difficult on this system.

In our lab, two methods for patterning bacterium arrays were developed. One method[86] was to pattern an alkanethiolate SAM and a polymer on the same substrate, which would selectively adsorb bacterial cell. The gold patterns on silicon were created by standard microfabrication process and then modified by a thiol that is inert to cell adhesion. The remaining bare silicon was treated with a polymer solution. The other method was to pattern two different SAMs using photolithography. As mentioned above, photolithography is simpler and more straightforward than most patterning techniques. This method was applied to pattern both bacteria and animal cells.

1.4 Cell Adhesion

Cellular adhesion onto solid surfaces is a critical step in cell array formation. Bacteria and anchorage-dependent cells (referring to those requiring a solid substratum for growth) have a strong tendency to attach to some surfaces, such as the solid glass or plastic surface of a culture dish. The process of cell adhesion has been extensively studied. It has now become clear that it involves multiple kinetic steps[87, 88].

First, cells are transported from the bulk solution to the vicinity of an adhesion surface. In general, this transport process is a combination of fluid and diffusion mechanisms[89]. Meanwhile, water, ions, and proteins present in the solution adsorb to the surface.

In the second step, the cells are loosely and reversibly bound by the adhesion surface. This step is the result of the action of many long-range forces, including the van der Waals force and the electrostatic force. Van der Waals force is generally attractive, due to the induced dipole interaction between molecules in bacteria and molecules on substrate. The electrostatic force results from the interaction of the electrical layers existing surrounding the cells and those on the adhesion surface. This force is repulsive if they have like charges and attractive if they have opposite charges. Many animal cells are coated with a hydrated layer of long-chain protein and hydrocarbon molecules. As cells attach to a surface, these molecules will be compressed, which leads to a steric force preventing the cell adhesion.

Finally, the initially reversible adsorption becomes irreversible as a result of specific ligand-receptor interactions and non-specific hydrophobic and electrostatic interactions. For example, most bacteria bear adhesins that interact specifically with certain molecules (receptors) on the adhesion surface. Short-range forces dominate the attachment process, such as hydrogen and covalent boding between extracellular moieties of cells and the substrate surface. Some cells adsorbed on the surface have the ability to continuously secrete proteins and other molecules to the surrounding areas (referred to as biofilms or bioslimes), which enables them to multiply to colonies in a suitable growth environment[90]. The irreversible attachment stage is relatively slow compared to the initial adsorption step[91].

Overall, cell adhesion is still not well understood, because of its complexity and diversity. The process is influenced by many factors, including the surface characteristics of cells and the components of a suspension media. The properties of the substrate surface[92], such as chemical composition, hydrophobicity, surface charge and roughness also affect cell adhesion. In general, hydrophobic cells prefer a hydrophobic surface, while hydrophilic cells are more likely to attach to a hydrophilic surface. Surfaces are more adhesive to cells with opposite charges than to those with like charges. In our work, cell adhesion was controlled by surface modification. Our previous group member, Ni Yang, examined the ability of different synthetic surfaces to adsorb bacteria (*E. coli*). Her results[86] showed that *E. coli* had the greatest affinity to NH_2 and OH derivatized surfaces in PBS (phosphate buffered saline) buffer, while surfaces coated with oligo(ethylene

glycol)-terminated alkanethiolate SAM ($HS(CH_2)_{11}(OCH_2CH_2)_3OCH_3$) had a strong resistance to cell adhesion. These results were utilized in this thesis.

1.5 Scope of This Thesis

DNA chips and protein chips have proven very useful for biochemical and bioanalytical applications, in large part due to their highly parallel analysis capacity in a small volume. High-density cell array systems would have the similar benefit. This thesis is focused on developing a whole procedure for depositing bacterial as well as animal cells on an ordered dense grid pattern and culturing them to a colony array, which has promising implications in recombinant DNA technologies, cell biology, high-throughput screening of drug candidates and biomarkers, and the development of biosensors.

Chapter 2 describes two strategies for fabricating cell array chips developed in our lab, referred to as the gold patterning method and the SAM (self-assembled monolayer) patterning method. SAM chemistry was employed in both methods to modify a substrate surface into cytophilic and cytophobic regions. The feasibility of the SAM patterning method was examined by infrared reflectance absorbance spectroscopy (IRRAS), contact angle goniometry (CAG), a half-half test, and a $CuSO_4$ crystal test. The results of *E. coli* patterns on variously sized features generated by these two methods were demonstrated and compared.

In Chapter 3, efforts to address the challenges associated with culturing the bacteria *E. coli* on chip were presented. Different growth methods were proposed and tested on the chips fabrication by both strategies described in Chapter 2. By comparing the incubation results, an appropriate method for creating high-density, ordered colony arrays was identified.

In Chapter 4, chips made by the SAM patterning method were applied to pattern animal cells on flat surfaces. Hela and Jurkat cells were used as the model cells to represent two kinds of different-natured animal cells, anchorage-dependent cells and suspension-cultured cells. Cell distributions on the regions with differently sized adhesion spots were investigated.

Chapter 5 presents future work and the conclusions from the previous chapters.

1.6 References

1. Bhatia, S. N.; Chen, C. S., *Biomedical Microdevices* **1999,** 2, 131-144.

2. Kapur, R.; Giuliano, K. A.; Campana, M.; Adams, T.; Olson, K.; Jung, D.; Mrksich, M.; Vasudevan, C.; Taylor, D. L., *Biomedical Microdevices* **1999,** 2, 99-109.

3. Beske, O. E.; Goldbard, S., *Drug Discov. Today* **2002,** 7, 131-135.

4. Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E., *Science* **1997,** 276, 1425-1428.

5. Goessl, A.; Bowen-Pope, D. F.; Hoffman, A. S., *J. Biomed. Meter. Res.* **2001,** 57, 15-24.

6. Jiang, X. Y.; Bruzewicz, D. A.; Wong, A. P.; Piel, M.; Whitesides, G. M., *Proc. Natl. Acad. Sci.* **2005,** 102, 975-978.

7. Singhvi, R.; Kumar, A.; Lopez, G. P.; Stephanopoulos, G. N.; Wang, D. L. C.; Whitesides, G. M.; Ingber, D. E., *Science* **1994,** 264, 696-699.

8. Parikh, A. N.; Schivley, M. A.; Koo, E.; Seshadri, K.; Aurentz, D.; Mueller, K.; Allara, D. L., *J. Am. Chem. Soc.* **1997,** 119, 3135-3143.

9. Allara, D. L.; Nuzzo, R. G., *Langmuir* **1985,** 1, 45-52.

10. Laibinis, P. E.; Whitesides, G. M., *J. Am. Chem. Soc.* **1992,** (114), 1990-1995.

11. Nuzzo, R. G.; Allara, D. L., *J. Am. Chem. Soc.* **1983,** 105, 4481-4483.

12. Porter, M. D.; Bright, T. B.; Allara, D. L.; Chidsey, C. E. D., *J. Am. Chem. Soc.* **1987,** 109, 3559-3568.

13. Brain, C. D.; Whitesides, G. M., *J. Am. Chem. Soc.* **1989,** 111, 7164-1765.

14. McDermott, M. T.; Green, J. B. D.; Porter, M. D., *Langmuir* **1997,** 13, 2504-2510.

15. Zamborini, F. P.; Crooks, R. M., *Langmuir* **1998,** 14, 3279-3286.

16. Clear S. C.; Nealey, P. F., *J. Colloid Interface Sci.* **1999,** 213, 238-250.

17. Bain, C. D.; Troughton, E. B.; Tao, Y.-T.; Evall, J.; Whitesides, G. M.; Nuzzo, R. G., *J. Am. Chem. Soc.* **1989,** 111, 321-335.

18. Nuzzo, R. G.; Dubois, L. H.; Allara, D. L., *J. Am. Chem. Soc.* **1990,** 112, 558-569.

19. O'Dwyer, C.; Gay, G.; Viaris de Lesegno, B.; Weiner, J., *Langmuir* **2004,** 20, 8172-8182.

20. Poirier, G. E.; Pylant, E. D., *Science* **1996,** 272, 1145-1148.

21. Whitesides, G. M.; Laibinis, P. E., *Langmuir* **1990,** 6, 87-96.

22. Pale-Grosdemange, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M., *J. Am. Chem. Soc.* **1991,** 113, 12-20.

23. Prime, K. L.; Whitesides, G. M., *Science* **1991,** 252, 1164-1167.

24. Prime, K. L.; Whitesides, G. M., *J. Am. Chem. Soc.* **1993,** (115), 10714-10721.

25. Lopez, G. P.; Albers, M. W.; Schreiber, S. L.; Carroll, R.; Peralta, E.; Whitesides, G. M., *J. Am. Chem. Soc.* **1993,** 115, 5877-5878.

26. Harder, P.; Grunze, M.; Dahint, R.; Whitesides, G. M.; Laibinis, P. E., *J. Phys. Chem. B* **1998,** 12, 426-436.

27. Yam, C. M.; Zheng, L.; Salmain, M.; Pradier, C. M.; Marcus, P.; Jaouen, G., *Colloid Surf. B* **2001,** 21, 317-327.

28. Laibinis, P. E.; Whitesides, G. M.; Allara, D. L.; Tao, Y.-T.; Parikh, A. N.; Nuzzo, R. G., *J. Am. Chem. Soc.* **1991,** 113, 7152-7167.

29. Camillone, N., III; Eisenberger, P.; Leung, T. Y. B.; Schwartz, P.; Scoles, G.; G.E., P.; Tarlov, M. J., *J. Am. Chem. Soc.* **1994,** 101, 11031-11036.

30. Frey, B. L.; Corn, R. M., *Anal. chem.* **1996,** 68, 3187-3193.

31. Dürig, U.; Zuger, O.; Michel, B.; Häussling, L.; Ringsdorf, H., *Phys. Rev. B* **1993,** 48, 1711-1717.

32. Poirier, G. E., *Chem. Rev.* **1997,** 97, 1117 - 1128.

33. Wilbur, J. L.; Biebuyck, H. A.; Macdonald, J. C.; Whitesides, G. M., *Langmuir* **1995,** 11, 825-831.

34. Kumar, A.; Biebuyck, H. A.; Whitesides, G. M., *Langmuir* **1994,** 10, 1498-1511.

35. Mrksich, M.; Whitesides, G. M., *TIBTECH* **1995,** 13, 228-235.

36. Lercel, M. J.; Redinbo, G. F.; Pardo, F. D.; Rooks, M.; Tiberio, R. C.; Simpson, P.; Sheen, C. W.; Parikh, A. N.; Allara, D. L., *J. Vac. Sci. Technol.* **1994,** 12, 3663-3667.

37. Zharnikov, M.; Grunze, M., *J. Vac. Sci. Technol.* **2002,** 20, 1793-1807.

38. Kumar, A.; Whitesides, G. M., *Appl. Phys. Lett.* **1993,** 63, 2002-2004.

39. Xia, Y.; Kim, E.; Zhao, X. M.; Rogers, J. A.; Prentiss, M.; Whitesides, G. M., *Science* **1996,** 273, 347-49.

40. Zhao, X. M.; Xia, Y.; Whitesides, G. M., *Adv. Mater.* **1996,** 8, 837-840.

41. Kim, E.; Xia, Y.; Whitesides, G. M., *Nature* **1995,** 376, 581-584.

42. Kim, E.; Xia, Y.; Zhao, X. M.; Whitesides, G. M., *Adv. Mater.* **1997,** 9, 651-654.

43. Smith, R. K.; Lewis, P. A.; Weiss, P. S., *Progress in Surface Science* **2004,** 75, 1-68.

44. Xia, Y.; Whitesides, G. M., *Angew. Chem. Int. Engl.* **1998,** 37, 550-575.

45. Odom, T. W.; Thalladi, V. R.; Love, J. C.; Whitesides, G. M., *J. Am. Chem. Soc.* **2002,** 124, 12112-12113.

46. Bietsch, A.; Michel, B., *J. Appl. Phys.* **2000,** 88, 4310-4318.

47. Piner, R. D.; Zhu, J.; Xu, F.; Hong, S. H.; Mirkin., C. A., *Science* **1999,** 283, 661-663.

48. Hang, S. H.; Zhu, J.; Mirkin, C. A., *Science* **1999,** 286, 523-525.

49. Hang, S. H.; Mirkin, C. A., *Science* **2000,** 288, 1808-1811.

50. Salaita, K.; Lee, S. W.; Wang, X.; Huang, L.; Dellinger, T. M.; Liu, C.; Mirkin, C. A., *Small* **2005,** 1, 940-945.

51. Gorman, C. B.; Carroll, L.; Fuierer, R. R., *Langmuir* **2001,** 17, 6923-6930.

52. Huang, J.; Hemminger, J. C., *J. Am. Chem. Soc.* **1993,** 115, 3342-3343.

53. Hutt, A. D.; Leggett, G. J., *J. Phys. Chem.* **1996,** 100, 6657-6662.

54. Hutt, A. D.; Leggett, G. J., *J. Phys. Chem. B* **1998,** 102, 174-184.

55. Tarlov, M. J.; Newman, J. G., *Langmuir* **1992,** 8, 1398-1405.

56. Tarlov, M. J.; Burgess, D. R. F. Jr.; Gillen G., *J. Am. Chem. Soc.* **1993,** 115, 5305-5306.

57. Brewer, N. J.; Rawsterne, R. E.; Kothari, S.; Leggett, G. J., *J. Am. Chem. Soc.* **2001,** 123, 4089-1090.

58. Huang, J.; Dahlgren, D. A.; Hemminger, J. C., *Langmuir* **1994,** 10, 626-628.

59. Mendes, P. M.; Preece, J. A., *Current Opion in Colloid & Interface Science* 9, 236-248.

60. Sun, S.; Chong, K. S. L.; Leggett, G. J., *J. Am. Chem. Soc.* **2002,** 124, 435-441.

61. Sun, S.; Leggett, G. J., *Nano Lett.* **2002,** 2, 1223-1227.

62. Bhatia, S. N.; Balis, U. J.; Yarmush, M. L.; Toner, M., *Biotechnol. Prog.* **1998,** 14, 378-387.

63. You, A. J.; Jackman, R. J.; Whitesides, G. M.; Schreiber, S. L., *Chem. Biol.* **1997,** 4, 969-975.

64. Pancrazio, J. J.; Bey, P. P.; Cuttino, D. S.; Kusel, J. K.; Borkholder, D. A.; Shaffer, K. M.; Kovacs, G. T. A.; Stenger, D. A., *Sens. Actuators, B-Chem.* **1998,** 53, 179-185.

65. Jung, D.; Kapur, R.; Adams, T.; Giuliano, K. A.; Mrksich, M.; Craighead, H. G.; Talor, D. L., *Critical Reviews in Biotechnology* **2001,** 21, 111-154.

66. Craighead, H. G.; Turner, S. W.; Davis, R. C.; James, C.; Perez, A.; St. John, P. M.; Isaacson, M. S.; Kam, L.; Shain, W.; Turner, J. N.; Banker, G., *J. Biomed. Microdevices* **1998,** 1, 49-64.

67. Kapur, R.; Spargo, B. J.; Chen, M. S.; Calvert, J. M.; Rudolph, A. S., *J. Biomed. Mater. Res.: Appl.Biomater.* **1996,** 33, 205-216.

68. Lee, Z.-W.; Lee, K.-B.; Hong, J.-H.; Kim, J.-H.; Choi, I.; Choi, I., *Chem. Lett.* **2005,** 34, 648-649.

69. Folch, A.; Jo, B. H.; Hurtado, O.; Beebe, D. J.; Toner, M. J., *J. Biomed. Mater. Res.* **2000,** 52, 346-353.

70. Ostuni, E.; Kane, R.; Chen, C. S.; Ingber, D. E.; Whitesides, G. M., *Langmuir* **2000,** 16, 7811-7819.

71. Rohr, S.; Scholly, D. M.; Kleber, A. G., *Circ. Res.* **1991,** 68, 114-130.

72. Rohr, S.; Fluckiger-Labrada, R.; Kucera, J., *Eur. J. Physiol.* **2003,** 446, 125-132.

73. Welle, A.; Gottwald, E., *Biomedical Microdevices* **2002,** 4, 33-41.

74. Copper, E.; Wiggs, R.; Hutt, D. A.; Parker, L.; Leggett, G. J.; Parker, T. L., *J. Mater. Chem.* **1997,** 7, 435-441.

75. Takayama, S.; McDonald, J. C.; Ostuni, E.; Liang, M. N.; Kenis, P. J. A.; Ismagilov, R. F.; Whitesides, G. M., *Proc. Natl. Acad. Sci.* **1999,** 96, 5545-5548.

76. Chiu, D. T.; Jeon, N. L.; Huang, S.; Kane, R. S.; Wargo, C. J.; Choi, I. S.; Ingber, D. E.; Whitesides, G. M., *Proc. Natl. Acad. Sci.* **2000,** 97, 2408-2413.

77. Revzin, A.; Tompkins, R. G.; Toner, M., *Langmuir* **2003,** 19, 9855-9862.

78. Liu, V. A.; Bhatia, S. N., *Biomedical Microdevices* **2002,** 4, 257-266.

79. Ostuni, E.; Chen, C. S.; Ingber, D. E.; Whitesides, G. M., *Langmuir* **2001,** 17, 2828-2834.

80. Berg, M. C.; Yang, S. Y.; Hammond, P. T.; Rubner, M. F., *Langmuir* **2004,** 20, 1362-1368.

81. Gleason, N. J.; Nodes, C. J.; Higham, E. M.; Guckert, N.; Aksay, I. A.; Schwarzbauer, J. E.; Carbeck, J. D., *Langmuir* **2003,** 19, 513-518.

82. Yousaf, M. N.; Mrksich, M., *J. Am. Chem. Soc.* **1999,** 121, 4286-4287.

83. Rowan, B.; Wheeler, M. A.; Crooks, R. M., *Langmuir* **2002,** 18, 9914-9917.

84. Koh, W.-G.; Revzin, A.; Simonian, A.; Reeves, T.; Pishko, M., *Biomedical Microdevices* **2003,** 5, 11-19.

85. Biran, I.; Walt, D. R., *Anal. Chem.* **2002,** 74, 3046-3054.

86. Yang, N., Cell Arrays on Chemically Modified Chip Surfaces, University of Alberta, Edmonton, 2004.

87. An, Y. H.; Friedman, R. J., *Handbook of bacterial adhesion*, Human Press, 2000.

88. Ofek, I.; Doyle, R. J., *Bacterial Adhesion to Cells and Tissues*, Chapman and Hall, 1994.

89. Hjortso, M. A.; Roos, J. W., *Cell Adhesion: Fundamentals and Biotechnological Applications*, Marcel Dekker, Inc., 1994.

90. Aquilar, B.; Amorena, B.; Iturralde, M., *Vet. Microbiol.* **2001,** 78, 183-191.

91. Cunliffe, D.; Smart, C. A.; Alexander, C.; Vulfson, E. N., *Appl. Environ. Microbiol.* **1999,** 65, 4995-5002.

92. Kiaie, D.; Holfman, A. S.; Jorbett, T. A.; Lew, K. R., *J. Biomed. Mater. Res.* **1995,** 29, 729-739.

Chapter 2 Chip Fabrication and Bacterial Deposition

2.1 Introduction

High-density cell array systems have recently received increasing interest from researchers because of promising applications in fundamental studies of cell biology, such as gene expression patterns and metabolism in live cells[2-5]. A set of techniques, such as photolithography[6], micro-contact printing[7], microfluidic patterning[8], and membrane patterning[9] are available to fabricate patterned substrates for cell adhesion.

The bacterium *E. coli* is broadly used in biological sciences as a host organism for recombinant DNA technologies, because it is easy to culture, and its biochemistry is well understood. Patterning of *E. coli* arrays would facilitate the construction of complementary DNA (cDNA) libraries and the high-throughput screening of gene products. Therefore, our lab focused on developing methods for patterning *E. coli* on flat surfaces.

Two strategies have been developed, which we refer to as the gold patterning method and the self-assembled monolayer (SAM) patterning method. Both methods employ alkanethiolate SAMs on gold, due to their great flexibility for the control of surface properties. The gold patterning method was developed by our former group member, Ni Yang, and was described in detail in her thesis "Cell Arrays on Chemically Modified Chip Surfaces."[1] Briefly, gold was deposited on the wafer and patterned using photoresist lift-off. The chip was immersed into an ethanolic solution of a thiol which is inert to cell adsorption, then into an aqueous polymer solution. Since SAMs only formed on the gold surface, the polymer layer coated the bare surface, which defined cell adhesion spots. In the SAM patterning method, adhesion spots are defined by exchanging photooxidized SAMs on a glass surface with another SAM. With the correct choice of modified thiols, this gives adhesive and non-adhesive regions. A polymer layer is coated to promote the cell adsorption. In this chapter, infrared reflectance absorbance spectroscopy (IRRAS) and water contact angles were used to characterize the properties of different surfaces for the SAM patterning method. *E. coli* patterns generated by both methods were demonstrated and compared with each other.

2.2 Experimental

2.2.1 Gold Patterning Method

2.2.1.1 Chip Design

The photomask of the cell array chip was fabricated on a 5" × 5" × 0.09" soda lime glass substrate by Adtek (photomask, Montreal, QC, Canada), based on the device layout in Figure 2.1, which was designed with L-Edit software. Four device designs with various spot spacings were made on this photomask. Each device is composed of four arrays with spots of various diameters (4, 6, 10, and 20 μm). The spot size and spacing are given in Table 2.1.

2.2.1.2 Chip Fabrication

The devices were fabricated on a silicon wafer (100 mm diameter, 500 μm thick, Silicon Valley Microelectronics, San Jose, CA, USA) at the University of Alberta Nanofab, through the process illustrated in Figure 2.2. Prior to lithography, every substrate was coated with HMDS (hexamethyldisilazane) to form an adherent layer. The HPR 504 was then deposited on the HMDS-coated surface. After baking, the substrate was exposed to UV light for 4 seconds through the cell array photomask in a mask aligner (ABM, San Jose, CA, USA). A dilute developer (3:2 microposit 354 developer/deionized H_2O) was used to remove the exposed photoresist to define adhesion spots. Deep reactive ion etching (DRIE) was performed on an Oxford Instruments series 100 DRIE (Bristol, UK). About 15 cycles of SF_6 etching (10 seconds) and polymer deposition (5 seconds) were required to etch a depth of 9 μm. Layers of Cr (50 nm) and Au (100 nm) were sequentially sputtered on the etched Si wafer. A lift-off process was then performed by sonicating the wafer in acetone.

2.2.1.3 Surface Modification

The devices were cut to suitable sizes (2.5 cm × 4 cm) with a Disco DAD 321 dicing saw and flushed thoroughly with acetone (ACS grade, Fisher Scientific) to remove remaining photoresist. These chips were then immersed into cold piranha solution (3:1 98% H_2SO_4/30% H_2O_2, caution: "piranha solution" aggressively reacts with many organic materials and should be handled with great care) overnight, followed by flushing

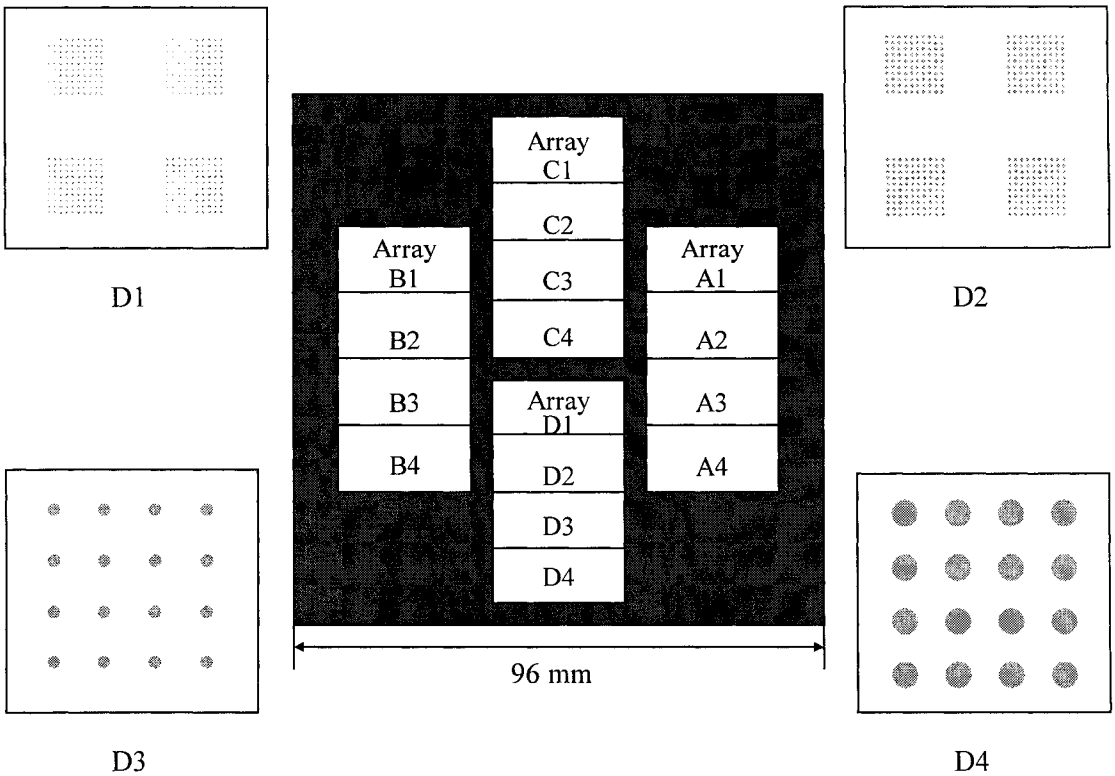


Figure 2.1 Photomask design: the typical pattern of each array type is shown for D1-4, for which inter-spot spacing is the smallest. Duplicated from Ni Yang's thesis[1].

Array	Centre to Centre Spacing (μm)	Spot diameter (μm)	Array	Centre to Centre Spacing (μm)	Spot diameter (μm)
A1	2000	4	C1	125	4
A2	2000	6	C2	125	6
A2	2000	10	C3	125	10
A4	2000	20	C4	125	20
B1	500	4	D1	10	4
B2	500	6	D2	10	6
B3	500	10	D3	40	10
B4	500	20	D4	40	20

Table 2.1 Spacing and diameter of spots on arrays. Duplicated from Ni Yang's thesis[1].

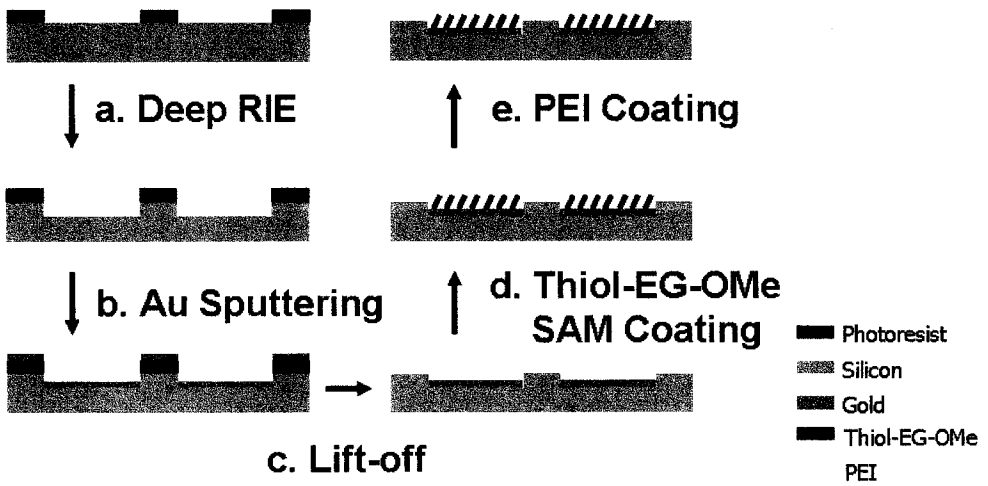


Figure 2.2 Process of the gold patterning method.

with autoclaved deionized H_2O (DI H_2O, from an Ultra-pure water system, Millipore, France), then blown dry with a stream of nitrogen gas. Devices were rinsed with absolute ethanol (200 proof, Commercial Alcohols Inc.) and immersed into 1 mM ethanolic thiol-EG-OMe (1-mercaptoundec-11-yl-triethylene glycol methylether, synthesized according to the method described by Ni Yang[1]) solution overnight. Upon removal, devices were rinsed thoroughly with ethanol and blown dry with nitrogen gas. A few milliliters of 0.1% PEI (polyethyleneimine, MW 423, Aldrich) aqueous solution were then placed on the top of the chips to cover the surfaces. After 1 hour, these chips were flushed with DI H_2O and blown dry with nitrogen. These chips were ready for cell deposition study.

2.2.2 SAM Patterning Method

2.2.2.1 Chip Design

A 5" × 5" × 0.09" quartz substrate was a kind gift from the Nanofilm Company (CA, USA). The photomask of the cell array chip was produced by the Heidelberg DWL-200 laser pattern generator at the University of Alberta Nanofab. The design of this photomask is illustrated in Figure 2.3, with the spacing for each device being given in Table 2.2. The photomask contains four devices in one half, with their corresponding inverse designs in the other half. Each device is composed of seven arrays with variously

sized spots. The layout of device D is shown in Figure 2.4 and the spot size and spacing for each array are given in Table 2.3.

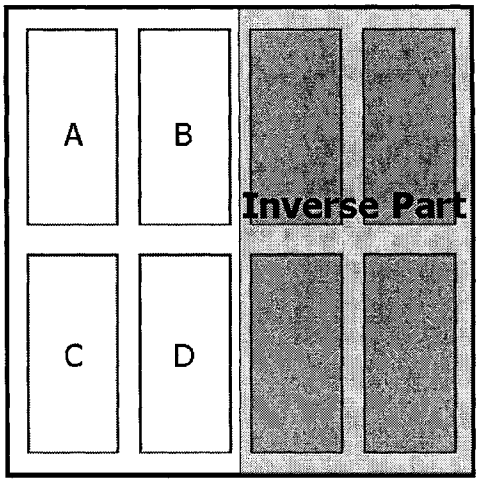


Figure 2.3 Photomask design: the photomask is divided into two halves, one half is the inverse part of the other.

Device	A	B	C	D
Center to Center Spacing (μm)	1000	750	500	Specified in Table 2.3

Table 2.2 Spacing for each device.

2.2.2.2 Chip Fabrication and Surface Modification

Cr (10 nm) and Au films (100 nm) were deposited on a 4" × 4" × 540 μm 0211 glass substrate (Corning, NY, USA). The substrate was cut to suitable sizes (2.5 cm × 4 cm) by the same dicing saw used for the gold patterning method. After cold piranha cleaning, each device was immersed into 1 mM thiol-EG-OMe ethanol solution overnight. The device was rinsed with absolute ethanol, blown dry with a stream of nitrogen gas, and irradiated with 254 nm UV light through the cell array photomask for 3 hours using a 254 nm UV transilluminator (Spectronics Corporation, NY, USA). The autoclaved DI H_2O was used to rinse away the alkanesulfonate generated during the irradiation process that is illustrated in Figure 2.5. This device was then immersed into 1 mM thiol-OH (6-mercapto-1-hexanol, 97%, Aldrich) ethanol solution for 1 hour. Before use, the chip was flushed with absolute ethanol, blown dry with nitrogen, and treated with 0.1% PEI (MW 423) solution for 20 seconds.

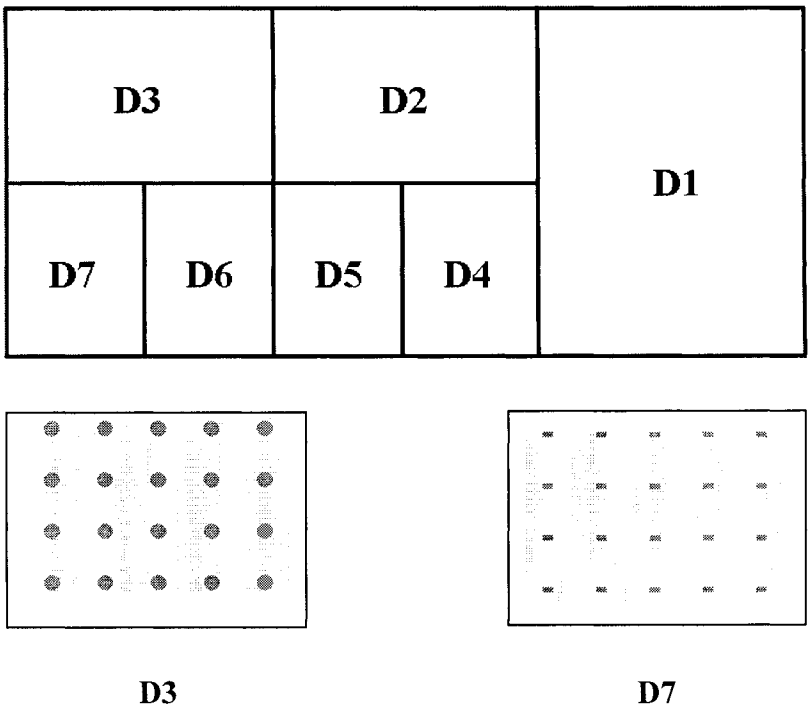


Figure 2.4 Device D layout: the typical pattern of each array type is shown for D3 and D7, for which the inter-spot spacing is the smallest.

Array	Shape	Centre to Centre Spacing (μm)	Spot Dimension/Diameter (μm)
D1	Circle	80	20
D2		40	10
D3		20	6
D4	Rectangle	20	3 × 6
D5		20	3 × 4
D6		20	2 × 6
D7		20	2 × 4

Table 2.3 Shape, spacing, and dimension of spots on arrays of the device D.

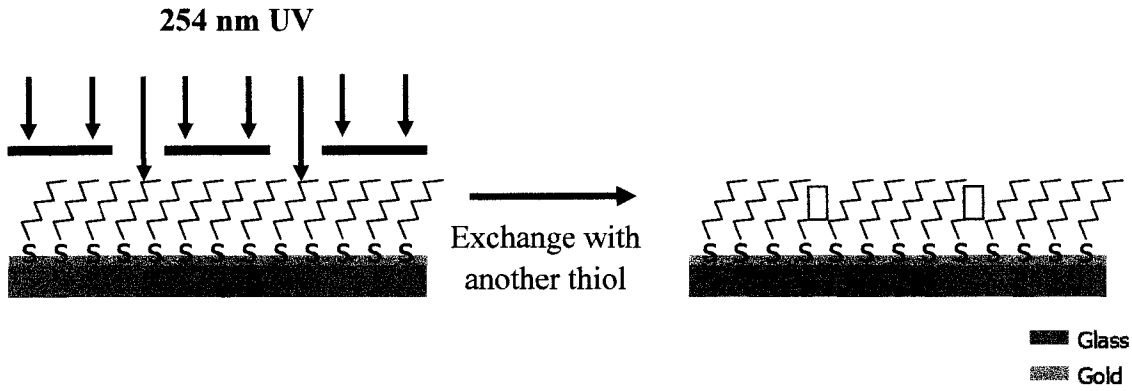


Figure 2.5 Process of the SAM patterning method.

2.2.2.3 Infrared Spectroscopy for Surface Characterization

2.2.2.3.1 Sample Preparation

Glass microscope slides (3" × 1", Fisher Scientific) were precleaned in hot piranha for 20 minutes, followed by being thoroughly flushed with DI H_2O. Gold substrates were prepared by thermal vacuum evaporation of a layer of Cr (10 nm) and then a layer of gold (100 nm) on microscope slides. After cold piranha cleaning, gold-coated slides were immersed into 1 mM thiol-EG-OMe ethanol solution overnight or into 1 mM thiol-OH ethanol solution for 1 hour. One thiol-OMe-EG-SAM-coated slide was immersed into 1 mM ethanolic thiol-OH solution for 1 hour, while two other slides of this coating were irradiated with 254 nm UV light for 3 hours, with one of them being further immersed into 1 mM ethanolic thiol-OH solution for 1 hour.

2.2.2.3.2 Operation of Infrared Spectroscopy

FT-IRRAS (Fourier Transform - Infrared Reflectance Absorbance Spectroscopy) spectra were recorded with a Mattson Infinity FTIR Spectrometer (Madison, WI) equipped with a liquid nitrogen-cooled and low-noise MCT (mercury-cadmium-tellurium) detector and purged with dry nitrogen. A reflection accessory and a homebuilt sample holder housed in an external auxiliary bench were employed. Spectra were taken by accumulating five hundred scans at a resolution of 2 cm^{-1} with a glancing angle of 86°. The IR spectrum of n-$C_{18}D_{35}SH$ SAM on a gold-coated microscope slide was taken as a reference spectrum. Negative absorption bands in all the spectra at 2050-2250 cm^{-1} are due to the C-D absorptions for the perdeuterated reference sample[10].

2.2.2.4 Water Contact Angles for Surface Characterization

2.2.2.4.1 Sample Preparation

Sample substrates were gold-coated glass microscope slides (3" × 1", Fisher Scientific), which were made as described in Section 2.2.3.1. Prior to use, substrates were washed with cold piranha solution and flushed with DI H_2O. Thiol-EG-OMe and thiol-OH-SAM-coated samples were prepared by either dipping gold-coated substrates into 1 mM thiol-EG-OMe ethanol solution overnight or into 1 mM thiol-OH ethanol solution for 1 hour. After contact angle measurement, two thiol-EG-OMe-SAM-coated substrates were irradiated with 254 nm UV light for 3 hours with or without being covered by chrome-coated area of a mask, followed by immersing into 1 mM ethanolic thiol-OH solution for 1 hour. The contact angles for these surfaces were compared with that obtained for the gold surface directly coated with thiol-OH or thiol-EG-OMe SAM. Each of these two substrates was then treated with 0.1% PEI (MW 423) solution for 20 seconds, and immersed into 70% ethanol solution for 5 minutes. Contact angles were measured after each treatment step to test how angles changed during surface modification.

2.2.2.4.2 Contact Angle Measurements

Water contact angles were determined on a First 10 Angtroms Contact Angle Measurement System at room temperature without control of the relative humidity. Water droplets were dispensed from a micropipette onto a horizontal substrate and imaged with a CCD (charge coupled device) camera. The angles were then measured directly on the video monitor, taking the average of five measurements as the contact angle value for that surface.

2.2.2.5 Half-half Test

The half-half chip refers to a chip that was coated with a SAM of thiol-OMe-EG on one half and a SAM of thiol-OH on the other. To fabricate this chip, a gold-coated glass microscope slide was first immersed into 1 mM ethanolic thiol-OMe-EG solution overnight. Half of the thiol-EG-OMe-SAM-coated chip was exposed to 254 nm UV light using a 254 nm UV transilluminator for 3 hours, and dipped into 1 mM thiol-OH ethanol solution for 1 hour. After being flushed with absolute ethanol, the chip was blown dry and

exposed to bacterial solution in PBS (phosphate buffered saline) buffer. This chip was then washed with PBS buffer three times and investigated using a reflecting light microscope (BH-2 Olympus, Japan). Pictures were taken with a CCD camera (JVC, Model TK-1280U). The details about bacterial solution preparation will be described in Section 2.2.4. This test was to evaluate the different abilities of these two alkanethiolates for adsorbing cells.

2.2.2.6 $CuSO_4$ Crystal Test

Before the chip fabricated by the SAM patterning method was used for cell deposition, a $CuSO_4$ crystal test[11, 12] was performed to analyze the hydrophobicity of the array pattern formed. A chip was made according to the procedures described in Section 2.2.2.2 except that PEI treatment was not performed. The chip was immersed into 1 M $CuSO_4$ aqueous solution for 5 minutes and then slowly taken out vertically. It was left air dry in one vial while still being kept vertical. After the water evaporated, this chip was investigated under the microscope.

2.2.3 Bacterial Culture and Maintenance

E. coli DH5α (pGFPuv, Plasmid green fluorescent protein-uv, Invitrogen) were used for cell patterning. One liter of the autoclaved fresh LB media (10 g tryptone, 5 g yeast, and 10 g NaCl dissolved to 1 liter H_2O and adjusted to pH 7.2 using 1 M NaOH), was supplemented with 100 μg/mL ampicillin (Sigma) at 30 °C and inoculated with the bacteria from a stock vial with an inoculating loop. Cultivation was carried out at 37 °C with shaking (220 rpm) in a shaker (Innova 4330, New Brunswick Scientific) overnight. OD (optical density) was measured using a UV-Vis spectrometer at 600 nm. Bacterial cells were then chilled on ice and transferred to a centrifuge tubes (Beckman, 250 mL) and centrifuged at 4 °C for 10 minutes at 3000 rpm with a Beckman centrifuge (model No. J2-21). Bacterial pellets were resuspended in 30 mL 10% sterilized glycol aqueous solution. Aliquots (400μL/tube) were transferred to centrifuge tubes on ice and stored at -70 °C in a freezer.

LB agar plates were prepared by pouring into Petri plates (100 × 15 mm, Fisher brand) about 20 mL aliquots of the autoclaved LB agar (10 g tryptone, 5 g yeast, and 10 g

NaCl, dissolved to 1 liter H_2O, adjusted to pH 7.2 using 1 M NaOH, and then added 15 g bacto agar) when cooled down to 50 °C. Prior to pouring, ampicillin was added to the liquid LB agar to give a final concentration of 100 μg/mL. After solidification, agar plates were stored in the refrigerator at 5 °C. For overnight culture, an inoculum (about 100 μL) of bacteria was scratched to an agar plate and then cultured in an incubator at 37 °C overnight. This agar plate could be stored in the refrigerator for up to one week and used for preparation of fresh bacterial solution.

2.2.4 Bacterial Deposition Study

Freshly cultured bacterial solution was used for every experiment in this chapter. Briefly, an inoculum (about 100 μL) from an overnight culture of bacteria on an agar plate was introduced into 20 mL autoclaved LB media. Cells were then grown at 37 °C with aeration and agitation at 220 rpm in a shaker overnight. The bacterial cells were centrifuged, washed with PBS buffer twice and resuspended into PBS buffer.

Before chips were immersed into prepared bacterial solution, they were disinfected by dipping into 70% ethanol for 5 minutes and thoroughly flushed with the autoclaved PBS buffer. After one hour incubation, these chips were removed and washed three times by dipping into PBS buffer. The chips were investigated using a reflecting light microscope.

2.2.5 Chip Regeneration

Chips could be regenerated by being immersed into 70% ethanol solution for 5 minutes and flushed with DI H_2O. Cold piranha solution was then used to clean the chip surface thoroughly. With this cleaning process, every chip could be reused more than five times. This is an advantage of the fabrication methods developed in our lab.

2.3 Results and Discussions

2.3.1 Feasibility Verification for SAM Patterning Method

2.3.1.1 IRRAS Test

The process of optical patterning of SAMs by photooxidation of adsorbed thiols, and adsorption of a new thiol is influenced by the chemistry of the individual thiols. Previous

studies have demonstrated that an exposure to deep UV light leads to effective oxidization of alkanethiols with different terminal groups, such as CH_3[13], OH[14], and COOH[15-17] terminated thiols, and these irradiated SAMs can be substituted by other thiols. However, no published paper has addressed the oxidization efficiency of thiol-EG-OMe and the completeness of subsequent replacement by thiol-OH. To test the feasibility of this fabrication method, the extent of the exchange between the two alkanethiolate SAMs:

$$HS(CH_2)_6OH + Au\text{-}S(CH_2)_{11}(OCH_2CH_2)_3OCH_3 = Au\text{-}S(CH_2)_6OH + HS(CH_2)_{11}(OCH_2CH_2)_3OCH_3$$

was also needed to be evaluated. Fourier transform infrared reflection absorption spectroscopy (FT-IRRAS) is widely used to examine thin films on metal surfaces[18]. In this study, IRRAS was used to characterize the properties of the gold surfaces coated with different SAMs to address these questions. Peak assignments are mainly based on references 10 and 19. Because the IR spectrum of n-$C_{18}D_{35}SH$ SAM on gold was taken as a reference spectrum, the strong C-D absorptions for the perdeuterated reference sample caused negative absorption bands at 2050-2250 cm^{-1} in all those spectra[10].

Figure 2.6 compares the FTIR spectra of the SAM of thiol-EG-OMe on gold before (A) and after (B) being immersed into 1 mM ethanolic thiol-OH solution. The strong absorption peak at about 1130 cm^{-1} is due to the C-O-C stretch, while the peak at 2919 cm^{-1} is assigned to the C-H stretch in the alkyl CH_2- group[10]. Spectrum B shows the results of an attempted exchange experiment with thiol-OH. No broad peak around 3300 cm^{-1}, which is typically the O-H stretching band, is found in spectrum B. Band shapes and positions in spectra A and B are nearly the same. The results indicate that thiol-OH does not exchange substantially with the SAM of thiol-EG-OMe while being immersed in the thiol-OH ethanol solution.

Figure 2.6 also shows the infra-red spectra of the SAM of thiol-EG-OMe on gold before (A) and after (C) UV irradiation. In spectrum C, the peak for the C-O-C stretching mode completely disappears. Although the small peaks at 2907 cm^{-1} and 1384 cm^{-1} may indicate some remaining alkyl CH_2- group on the gold surface, the results demonstrate that the SAM of thiol-EG-OMe can be oxidized and substantially removed under the experimental condition.

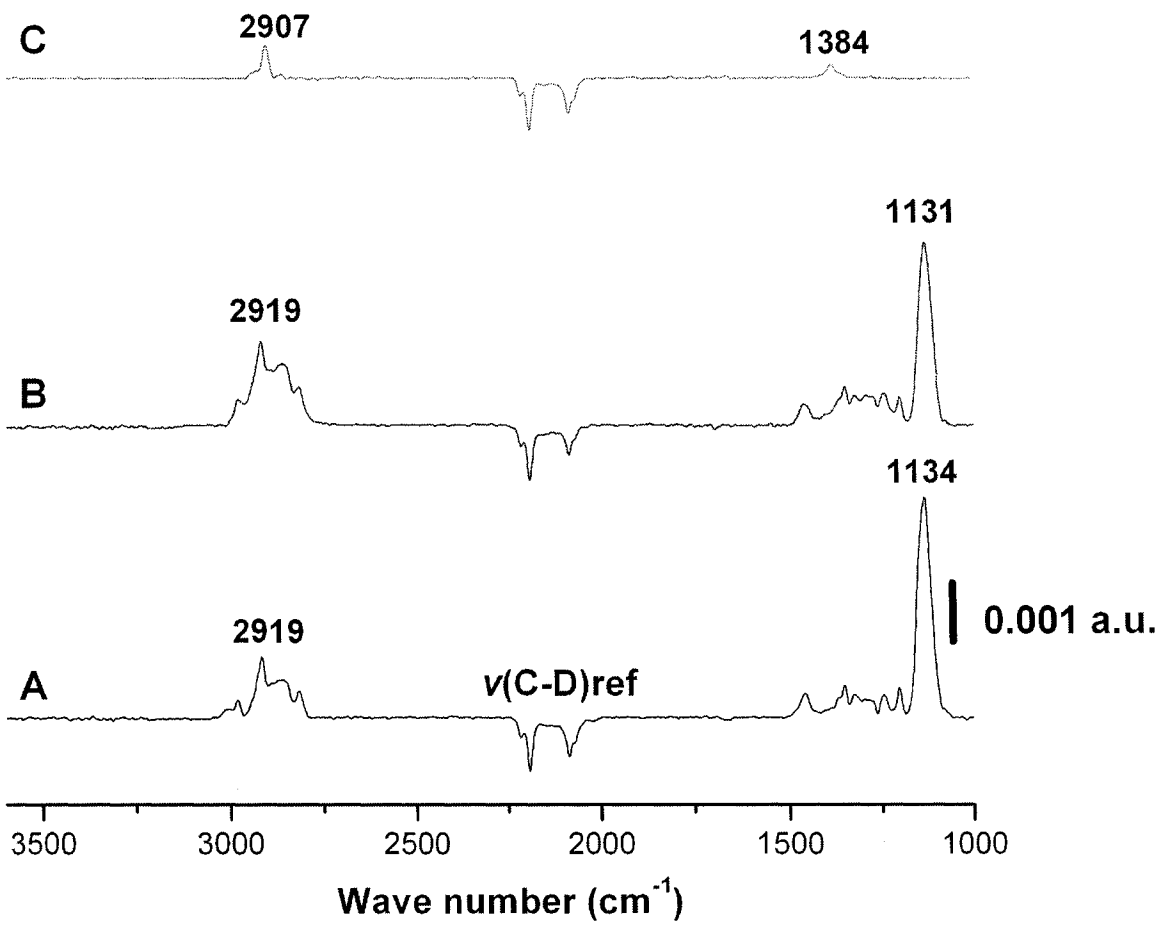


Figure 2.6 IR spectra of A) SAM of thiol-EG-OMe on gold, B) SAM of thiol-EG-OMe after being immersed into 1 mM thiol-OH ethanol solution, C) SAM of thiol-EG-OMe after being exposed to 254 nm UV light for 3 hours.

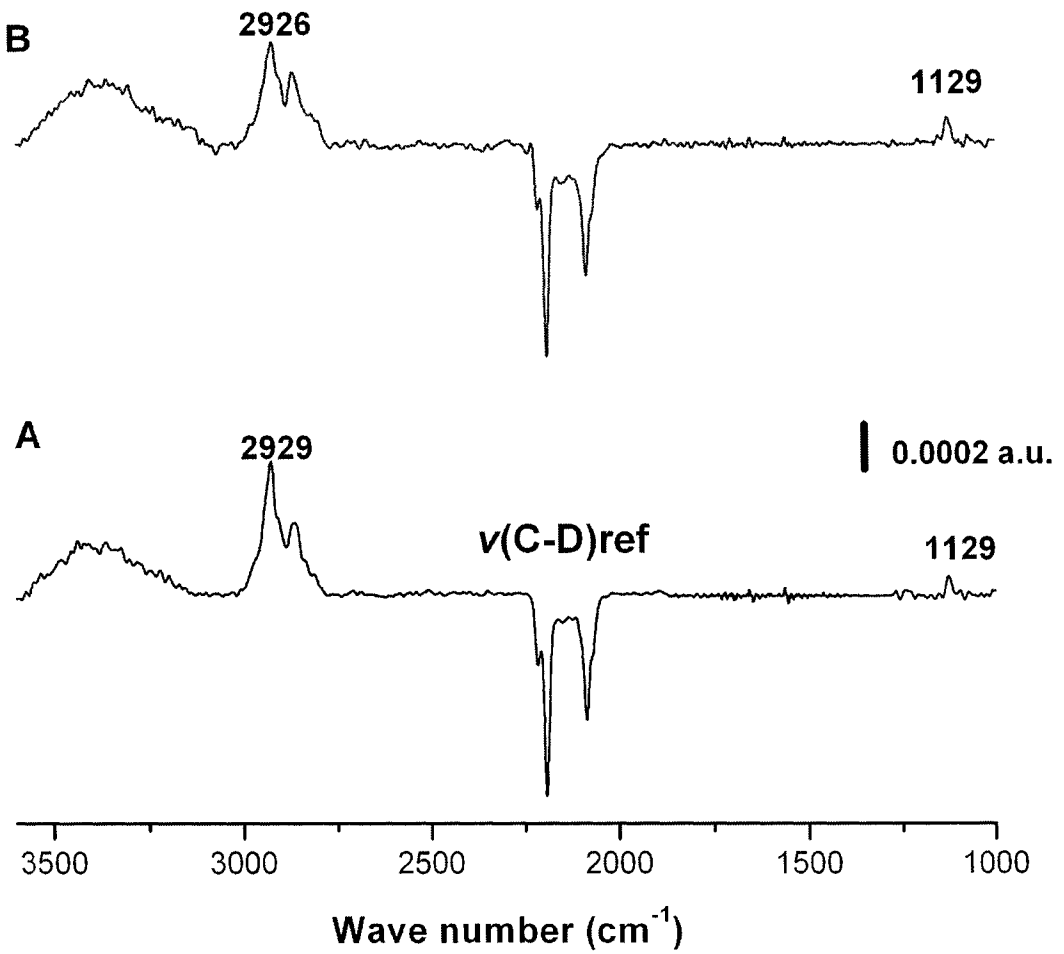


Figure 2.7 IR spectra of A) SAM of thiol-OH on gold, B) SAM of thiol-OH on a gold surface that was originally coated with the SAM of thiol-EG-OMe, then irradiated with 254 nm UV light, and exchanged with thiol-OH.

Figure 2.7 shows the FTIR spectra of a SAM of thiol-OH on gold (A) and the results obtained after exchanging thiol-OH with a UV-irradiated surface of thiol-EG-OMe (B). The peak at about 2927 cm^{-1} is due to the C-H stretch in the alkyl CH_2- group[19], and that at 1129 cm^{-1} is the C-O stretching band. The broad peak around 3300 cm^{-1} indicates the presence of OH group[10]. No strong peak due to the stretch of the C-O-C bond in thiol-EG-OMe is detected in spectrum B, and these two spectra have nearly the same band shapes and positions. The data show that the SAM of thiol-OH can completely replace the irradiated SAM of thiol-EG-OMe and the SAM formed is nearly the same as that formed on the bare gold.

2.3.1.2 Contact Angle Measurements

CAG (contact angle goniometry) is commonly used to study the wettability of chemically modified surfaces. Because of the different hydrophilicity of thiol-EG-OMe, thiol-OH, and PEI-coated surfaces, measuring the contact angles on gold substrates during surface modification can provide useful information for tracing the changes of coating layers after each step. For clarity, several abbreviations were adopted to represent different surfaces. The abbreviations and contact angles for each surface were listed in Table 2.4. The measurement system could give reproducible contact angles within about ±2°. The results show that the thiol-EG-OMe SAM on gold (contact angle: 55°) is more hydrophobic than the thiol-OH monolayer (contact angle: 38°).

For the thiol-EG-OMe-coated surfaces, the similar contact angles for the thiol-EG-OMe/Au (55°) and thiol-EG-OMe/UV/Au (54°) demonstrate that thiol-OH does not exchange substantially with the thiol-EG-OMe SAM on the gold substrate, which is consistent with the conclusion drawn from IR spectra. Further treatment of the thiol-EG-OMe/UV/Au surface with 0.1% PEI and 70% ethanol solutions under the experimental condition does not change contact angles (54°), implying that these steps do not significantly affect the thiol-EG-OMe monolayer.

For the thiol-OH-coated surfaces, the comparison between the contact angles for the thiol-OH (38°) and thiol-OH/UV/Au (39°) demonstrate that thiol-OH can replace the photooxidized thiol-EG-OMe and form a new SAM, which is also consistent with the

Abbreviations	Represented Surfaces	Contact Angles
Thiol-EG-OMe/Au	a gold surface directly coated with thiol-EG-OMe SAM	55° ± 2°
Thiol-OH/Au	a gold surface directly coated with thiol-OH SAM	38° ± 1°
Thiol-EG-OMe/UV/Au	a thiol-EG-OMe/Au covered by chrome-coated area of a mask, irradiated with 254 nm UV light for 3 hours, and immersed into 1 mM ethanolic thiol-OH solution for 1 hour	54° ± 2°
Thiol-OH/UV/Au	a thiol-EG-OMe/Au exposed to 254 nm UV light for 3 hours and immersed into 1 mM ethanolic thiol-OH solution for 1 hour.	39° ± 1°
PEI/thiol-EG-OMe/UV/ Au	a thiol-EG-OMe/UV/Au treated with 0.1% PEI for 20 seconds	54° ± 2°
PEI/thiol-OH/UV/Au	a thiol-EG-OH/UV/Au treated with 0.1% PEI for 20 seconds	36° ± 1°
Ethanol/thiol-EG-OMe/ UV/Au	a PEI/thiol-EG-OMe/UV/Au treated with 70% ethanol for 5 minutes	54° ± 1°
Ethanol/thiol-OH/UV/ Au	a PEI/thiol-OH/UV/Au treated with 70% ethanol for 5 minutes	36° ± 1°

Table 2.4 Abbreviations and contact angles for different surfaces.

results of IR spectra. Treating thiol-OH/UV/Au with PEI solution can lead to a decrease in the contact angle by roughly 3°. This result indicates that a layer of PEI formed on the thiol-OH-SAM-coated surface. The coating layers on the PEI/thiol-OH/UV/Au surface can withstand the 70% ethanol treatment step, demonstrated by the fact that the contact angles remain the same (36°) after being immersed in 70% ethanol.

It should be noted that both IRRAS and CAG belong to ensemble average techniques and can only characterize SAMs on the macroscopic level, as discussed in Chapter 1. Therefore, the fact that different surfaces have similar IR spectra or contact angles may not exclude the possibility that some changes in monolayer compositions on the microscopic level happen.

2.3.1.3 Half-half Test

Before the SAM patterning method was applied to cell array chip fabrication, the half-half test was performed to further test the feasibility of this fabrication method. For the half-half chip, one half was coated with a thiol-EG-OMe and the other with thiol-OH (without PEI treatment). As the thiol-EG-OMe layer on gold is relatively more hydrophobic than thiol-OH layer, a clear boundary between the water-covered (thiol-OH, upper) and non-water-covered (thiol-EG-OMe, bottom) regions formed when this chip was covered with water. The result is shown in Figure 2.8.

This chip was then immersed into a bacterial solution for 1 hour. The details of the bacterial solution preparation were mentioned in Section 2.2.4. After cell deposition, pictures were taken using a transmission light microscope. A clear boundary between the adhesive (thiol-OH, left) and the non-adhesive (thiol-EG-OMe, right) regions is shown in Figure 2.9. The results indicate a well-defined pattern formed by this method.

2.3.1.4 $CuSO_4$ Crystal Test

The chips used to in this study were fabricated by the SAM patterning method (without PEI treatment). On each chip, the adhesion spots were defined by the hydrophilic SAM of thiol-OH, while the inert surface was coated with the relatively hydrophobic SAM of thiol-EG-OMe. After the chips were removed from aqueous $CuSO_4$ solution, the water evaporated and $CuSO_4$ crystals were left. Due to the different wetting

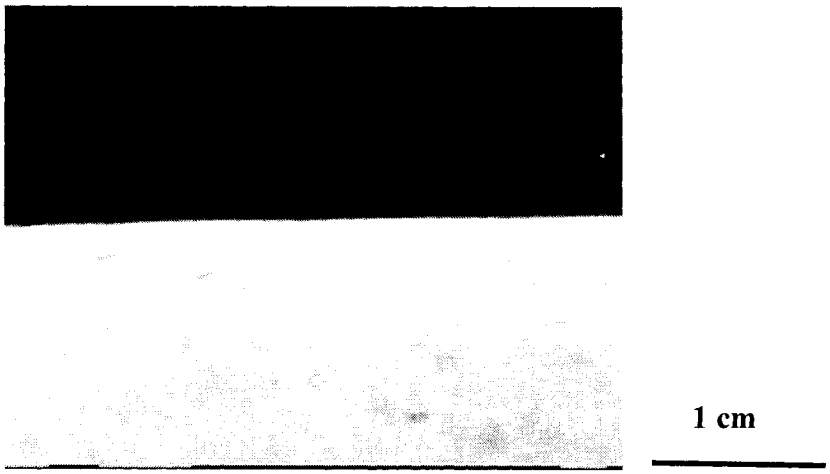


Figure 2.8 Water was used to visualize the boundary between the two SAMs of thiol-EG-OMe and thiol-OH (without PEI treatment) on the half-half chip. The upper half was coated with a SAM of thiol-OH, and thus covered with water; the bottom half was coated with a SAM of thiol-EG-OMe and as a result there is no water on it.

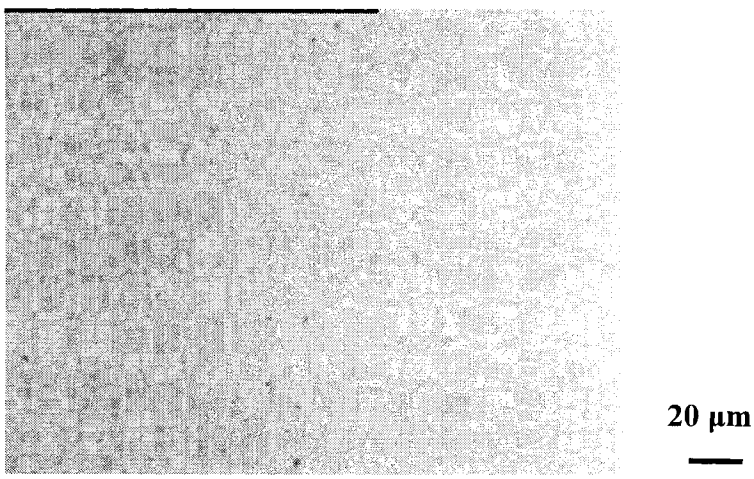


Figure 2.9 Microscopic image of *E. coli* on the half-half chip. The left side was coated with the SAM of thiol-OH (without PEI treatment) and the right side was coated with thiol-EG-OMe.

properties of the two SAMs, $CuSO_4$ crystals formed only on the thiol-OH region. Therefore, the adhesion spots could be visualized under the microscope by $CuSO_4$ crystals. Figure 2.10 illustrates the well-defined arrays of crystals on the 20 μm-circle and 10 μm-circle regions. However, for the other regions with smaller spot sizes, the patterns were no longer clear enough to be detected (data not shown). The volume of the liquid in each drop on the pattern of hydrophilic SAMs is effected by the size of the hydrophilic region[20]. At a fixed concentration of $CuSO_4$ solution, the volume of the solution in each drop decreases as the size of the pattern becomes smaller. Because of surface tension, very small droplets may not form. Instead, $CuSO_4$ solution droplets may bridge between several spots, which would look randomly distributed. Nevertheless, due to the clear patterns of $CuSO_4$ crystals on both 20 μm-circle and 10 μm-circle regions, we conclude that this fabrication process can be used for creating uniform arrays of adhesive spots, even for the smaller spot sizes.

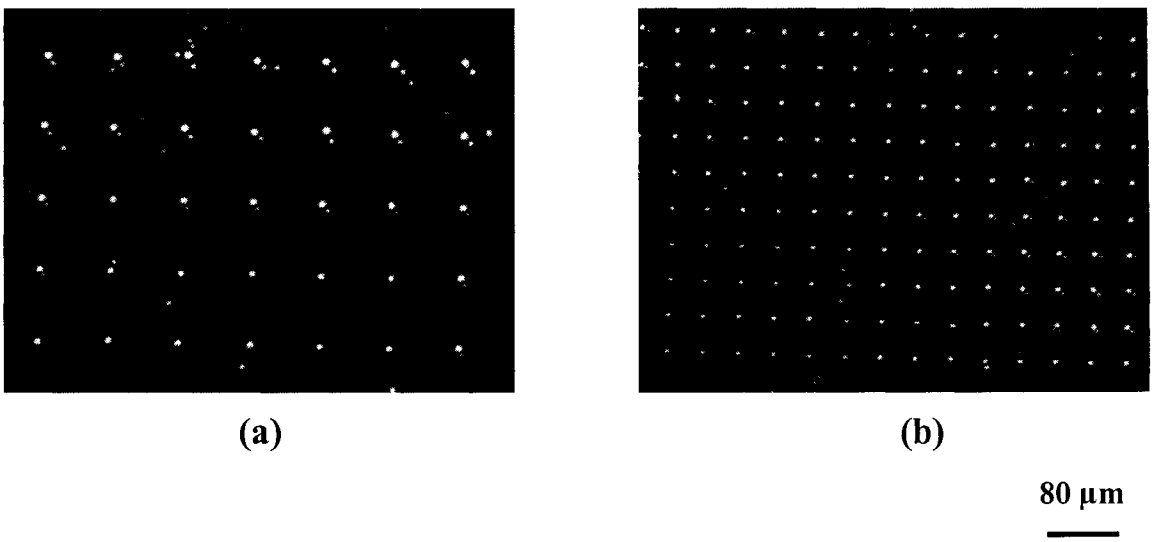


Figure 2.10 Microscopic images of $CuSO_4$ crystals on (a) 20 μm-circle region and (b) 10 μm-circle region of the chip fabricated by the SAM patterning method (without PEI treatment). Bar shows scale for both images.

2.3.2 Bacterial Deposition Study

Chips fabricated by both gold patterning and SAM patterning methods were used. Cell patterns were prepared as mentioned in the experimental part in this chapter and pictures were taken using the microscope and the CCD camera.

Images of cell patterns on the chips fabricated by the gold patterning method are shown in Figure 2.11. The chips with the smallest inter-spot spacings were used for study.

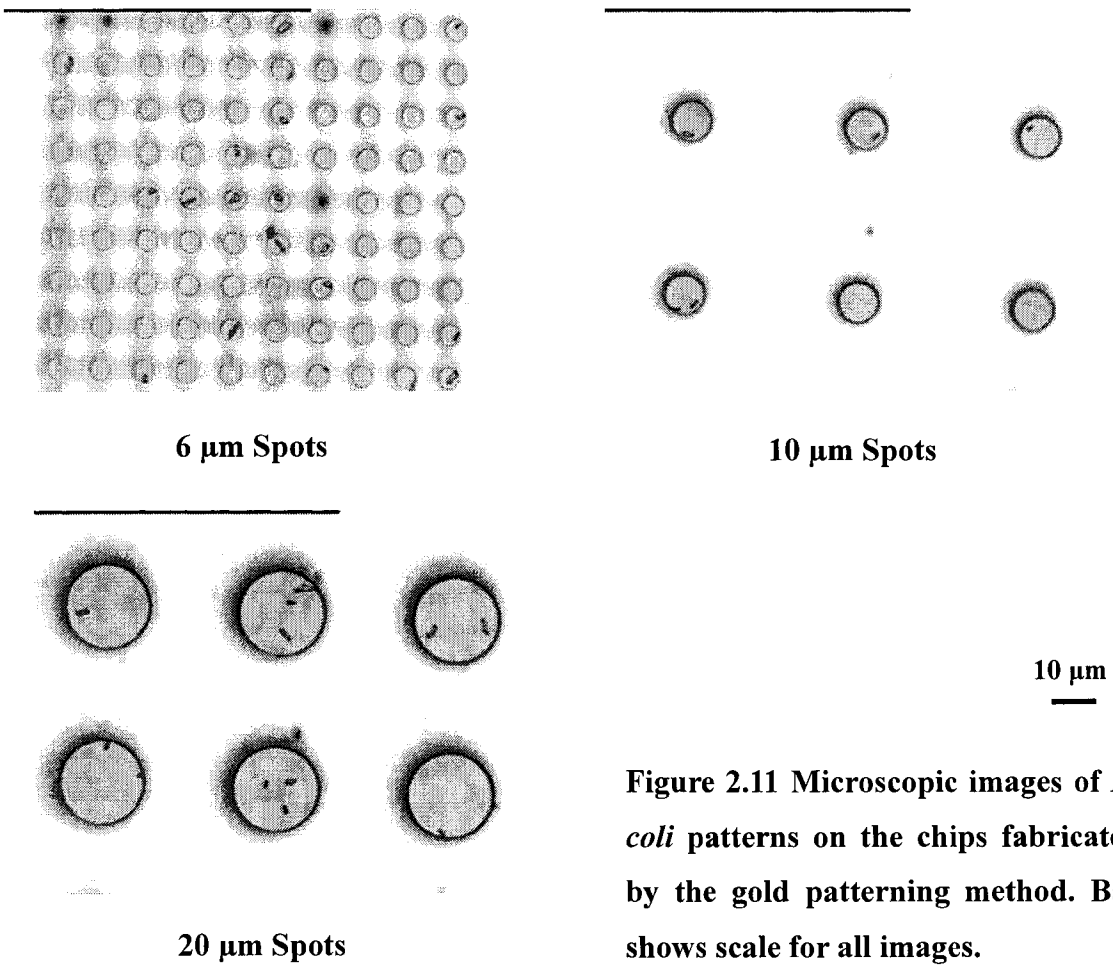


Figure 2.11 Microscopic images of *E. coli* patterns on the chips fabricated by the gold patterning method. Bar shows scale for all images.

Due to the technical limitations of DRIE, the 4 μm-circle region was not fabricated properly. Therefore, the results for the 4 μm-circle region were not studied. In this figure, the number of cells per adhesion spot increases with the spot size. This is consistent with expectations, in that the larger spot area can accommodate more bacteria. Ni Yang did statistical studies of the bacterial distributions on each spot size[1]. She employed the Langmuir isotherm to describe the relationship between the average numbers of cells per

spot as a function of bacterial concentration:

$$q = \frac{q_{\max} K_{ads} C}{1 + K_{ads} C}$$

where C is the bacterial concentration (cells/mL), q is the number of bacteria adsorbed per unit area, q_{max} is the maximum number of bacteria adhered per unit area, and K_{ads} is the adsorption equilibrium constant. Fitting the results to a Langmuir isotherm is shown in Figure 2.12 and the fitting parameters are given in Table 2.4.

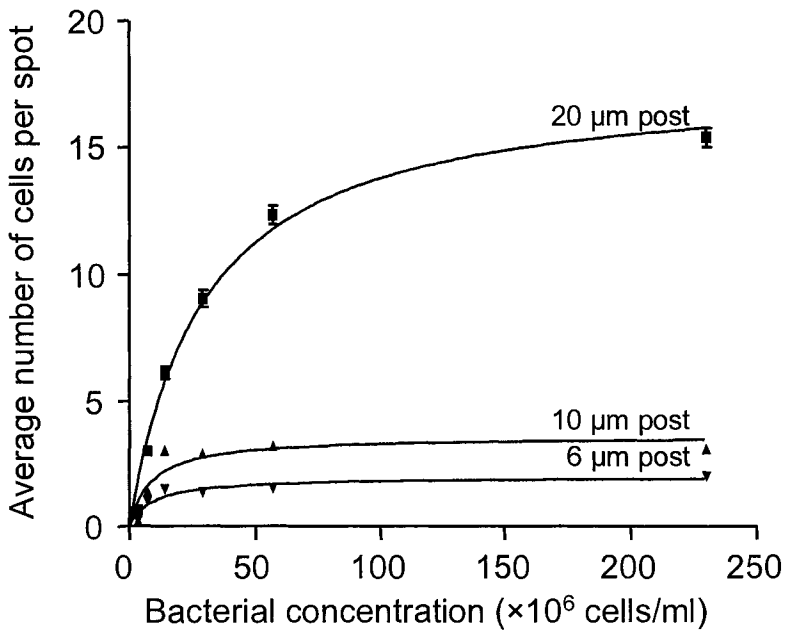


Figure 2.12 Average number of cells per spot as a function of cell concentration and size of spots. Smoothing lines are Langmuir isotherm fit to data. Duplicated from Ni Yang's thesis[1].

Spot Diameter (μm)	20 μm	10 μm	6 μm
q_{max} (cells/spot)	17.72 ± 1.08	3.59 ± 0.56	1.97 ± 0.23
K_{ads} ($\times 10^{-6}$ mL/cell)	0.035 ± 0.006	0.115 ± 0.068	0.110 ± 0.049
R^2 Goodness of Fit	0.984	0.786	0.850

Table 2.5 Langmuir fitting results for bacterial patterns on the chips fabricated by the gold patterning method. Duplicated from Ni Yang's thesis[1].

For chip fabrication, the SAM patterning method is simpler and faster than the gold patterning method, because it does not require the lift-off and DRIE processes. But adhesion spots can not be detected under the microscope, as no boundary exists. For imaging purposes, a high concentration of bacteria was used to visualize the adhesion spots. Images of cell patterns on chips fabricated by the SAM patterning method (with PEI treatment) are illustrated in Figure 2.13. The chips with the smallest inter-spot spacings were used for this study. Figure 2.13 shows that the bacterial arrays were clearly defined on the 20, 10, and 6 μm-circle regions and the 3 × 6 μm-rectangular region. Figure 2.14 shows enlarged pictures of 20 μm-circle and 3 × 6 μm-rectangular regions. The majority of bacterial cells attached to an adhesion spot and few cells were adsorbed on the area coated with thiol-EG-OMe. This indicates that thiol-EG-OMe is very effective in preventing cell adsorption. However, for the regions with smaller spot sizes, including 3 × 4 μm, 2 × 6 μm, and 2 × 4 μm-rectangular regions, no patterns were detected.

To reveal the reasons accounting for this problem, the bacterial patterns on the chips without PEI treatment, which is the last step for surface modification, were investigated and compared with those on the chips with PEI treatment. The result indicated that the array patterns could only be detected on the 20 and 10 μm-circle regions of the chips without PEI treatment. Therefore, a possible explanation is that the adhesion spots defined by the surface modification we applied may not have a strong ability to capture bacteria. This problem becomes more severe as the sizes of adhesion spots decreases. The PEI treatment appears necessary to increase the cell adhesion ability of thiol-OH surfaces.

Increasing the treatment time with PEI to increase the ability of adhesion spots to adsorb cells was not effective, when the treatment time is larger than 20 seconds the polymer solution can turn the hydrophobic surface coated with the SAM of thiol-EG-OMe to a hydrophilic surface. This conclusion is not consistent with the fact that 1 hour was used for PEI treatment in the gold patterning method without changing the thiol-EG-OMe surface. The difference of PEI treatment time may originate from the different surface modification procedures used in these two patterning methods. In the SAM patterning method, before PEI treatment, a thiol-EG-OMe-SAM-coated chip was

first irradiated with UV light for 3 hours in the air and dipped into a thiol-OH ethanolic solution for 1 hour. The long time exposure to oxygen and heat (generated by the transilluminator) during photooxidation and soak in the thiol-OH ethanol solution may degrade the thiol-EG-OMe monolayer. In the gold patterning method, PEI treatment immediately followed the formation of the thiol-EG-OMe SAM. It may be that this intact thiol-EG-OMe SAM can endure PEI treatment for a longer time than that in the SAM patterning method.

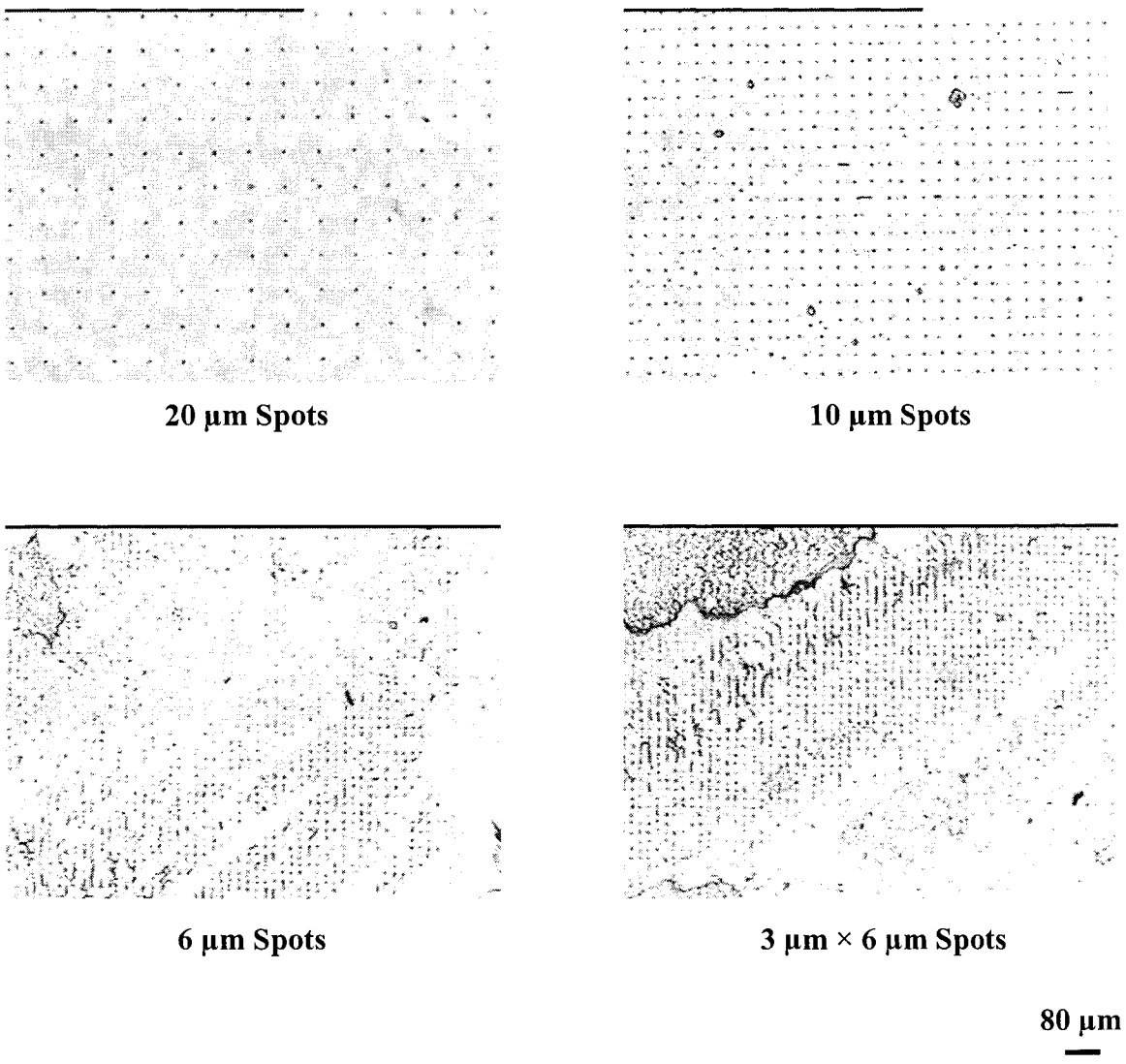
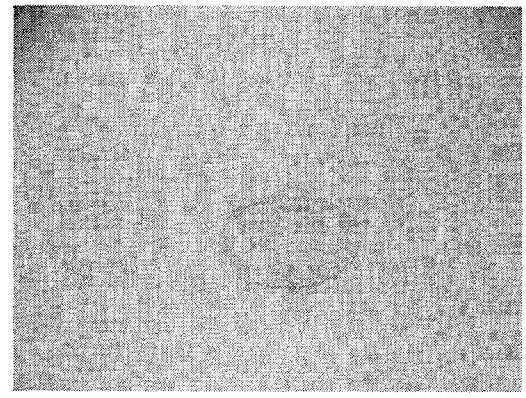
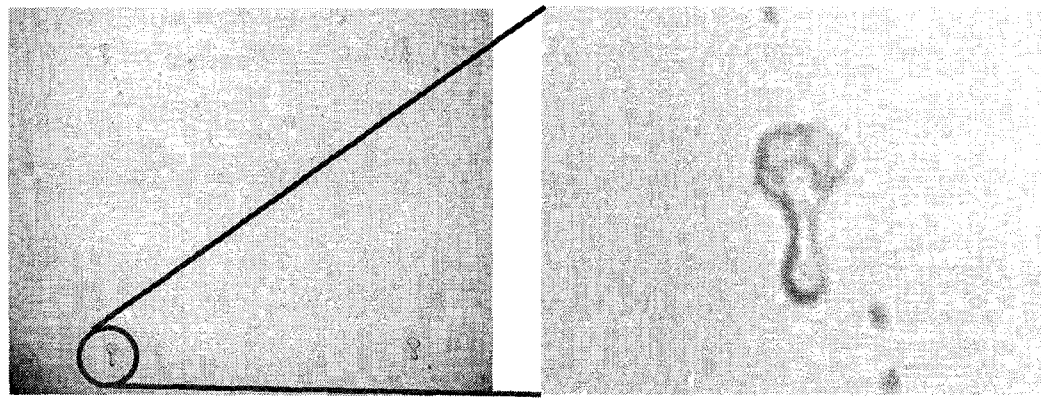


Figure 2.13 Microscopic images of *E. coli* patterns on the chips fabricated by the SAM patterning method (with PEI treatment). A high concentration of bacteria was used to visualize the adhesion spots. Bar shows scale for all images.



20 μm

(a)



10 μm

2 μm

(b)

Figure 2.14 Enlarged images of *E. coli* patterns on (a) the 20 μm-circle region and (b) the 3 × 6 μm-rectangular region of the chip fabricated by the SAM patterning method. A high concentration of bacteria was used to visualize the adhesion spots.

2.4 Conclusions

The objective of this study is to develop a convenient and useful method to pattern bacterial (*E. coli*) arrays on flat surfaces. Two fabrication methods were developed in our lab to demonstrate the ability to pattern bacteria in well-defined arrays.

The gold patterning method was previously developed by Ni Yang. In this method, a SAM pattern was obtained by solution deposition of thiol-EG-OMe on a patterned gold surface, fabricated using photolithography and DRIE. An adhesion coating of PEI was then deposited in open regions. Using the gold patterning method, the surface pattern can easily be located using a microscope. After bacterial deposition, clear cell patterns were obtained. The relationship between the average numbers of cells per spot with bacterial concentration can be modeled by the Langmuir isotherm.

The SAM patterning method was newly developed in our lab. A SAM pattern was generated by the displacement of UV-irradiated thiol-EG-OMe with thiol-OH. Surface characterization indicated that this method provided good surface chemistries for patterning bacteria. Compared to the gold patterning method, the fabrication process is simpler and faster, but adhesion spots can not be detected under a microscope. When the adhesion spots were coated with thiol-OH alone, bacteria were only patterned on the 20 and 10 μm-circle regions. For chips treated with PEI, array patterns could also be observed on the 10 μm-circle and 3 × 6 μm-rectangular regions, but still not on the regions with smaller sized spots. The results indicate that the surface with thiol-OH alone does not have the strong ability to adhere bacteria when the area of adhesion region is smaller than a 10 μm circle. A subsequent PEI treatment is then needed to increase the adhesion ability of the thiol-OH surface. However, too long a treatment time is detrimental to the hydrophobic surface of thiol-EG-OMe. Because small-sized spots are better for achieving single cell array, future work will involve the investigation and application of new materials that have the stronger interaction with bacteria *E. coli*.

2.5 References

1. Yang, N., Cell Arrays on Chemically Modified Chip Surfaces, University of Alberta, Edmonton, 2004.

2. Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E., *Science* **1997,** 276, 1425-1428.

3. Kapur, R.; Giuliano, K. A.; Campana, M.; Adams, T.; Olson, K.; Jung, D.; Mrksich, M.; Vasudevan, C.; Taylor, D. L., *Biomedical Microdevices* **1999,** 2, 99-109.

4. Beske, O. E.; Goldbard, S., *Drug Discovery Today* **2002,** 7, 131-136.

5. Stephan, J. P.; Schanz, S.; Wong, A..; Schow, P.; Wong, W. L. T., *Am. J Pathol.* **2002,** 161, 787-797.

6. Bhatia, S. N.; Yarmush, M. L.; Toner, M., *J. Biomed. Mater. Res.* **1997,** 34, 189-199.

7. Xia, Y.; Mrksich, M.; Whitesides, G. M., *J. Am. Chem. Soc.* **1995,** 117, 9576-9577.

8. Takayama, S.; McDonald, J. C.; Ostuni, E.; Liang, M. N.; Kenis, P. J. A.; Ismagilov, R. F.; Whitesides, G. M., *Proc. Natl. Acad. Sci.* **1999,** 96, 5545-5548.

9. Ostuni, E.; Kane, R.; Chen, C. S.; Ingber, D. E.; Whitesides, G. M., *Langmuir* **2000,** 16, 7811-7819.

10. Harder, P.; Grunze, M.; Dahint, R.; Whitesides, G. M.; Laibinis, P. E., *J. Phys. Chem. B* **1998,** 102, 426-436.

11. Kumar, A.; Biebuyck, H. A.; Whites, G. M., *Langmuir* **1994,** 10, 1498-1511.

12. Qin, D.; Xia, Y.; Xu, B.; Yang, H.; Zhu, C.; Whitesides, G. M., *J. Am. Chem. Soc.* **1999,** 11, 1433-1437.

13. Lewis, M.; Tarlov, M. J., *J. Am. Chem. Soc.* **1995,** 117, 9574-9575.

14. Hutt, D. A.; Cooper, E.; Leggett, G. J., *J. Phys. Chem. B* **1998,** 102, 174-184.

15. Brewer, N. J.; Rawsterne, R. E.; Kothari, S.; Leggett, G. J., *J. Am. Chem. Soc.* **2001,** 123, 4089-1090.

16. Tarlov, M. J.; Burgess, D. R. F. J.; Gillen, G., *J. Am. Chem. Soc.* **1993,** 115, 5305-5306.

17. Oh, S. Y.; Choi, H. S.; Jie, H. S.; Park, J. K., *Materials Science and Engineering* **2004,** 24, 91-94.

18. Dürig, U.; Zuger, O.; Michel, B.; Häussling, L.; Ringsdorf.H., *Phys. Rev. B* **1993,** 48, 1711-1717.

19. Frutos, A. G.; Brockman, J. M.; Corn, R. M., *Langmuir* **2000,** 16, 2192-2197.
20. Gorman, C. B.; Biebuyck, H. A.; Whitesides, G. M., *Chem. Mater.* **1995,** 7, 252-254.

Chapter 3 On-chip Cell Culture

3.1 Introduction

Recombinant DNA technologies are widely used to manipulate genetic materials for practical purposes. Generally, genes from different sources are combined and transferred into cells, where they can be replicated and expressed. The growing collection of cDNA libraries and other gene products demands the development of automatic and high-throughput characterization methods. A bacterial colony array developed from a single genetically-engineered bacterium array would be useful for these DNA techniques.

Most cell patterning techniques developed are mainly used for patterning and growing anchorage-dependent cells, which have a strong tendency to adsorb on a surface. Live cells will not detach from the substrate without special treatment. Culturing such anchorage-dependent cells on chip can be achieved simply by immersing the patterned substrate into fresh media[1, 2]. On-chip bacterial incubation seems much more complicated because of the main problem that bacteria can float free during growth in liquid. Kaya *et al.*[3, 4] developed a microbial chip fabricated by filling the micropores on a glass substrate with a mixed solution of collagen and *E. coli*. After gelation, cells were trapped in the micropores. This substrate was then exposed to the liquid media for incubation. An on-chip single bacterium cultivation system[5, 6], composed of microchamber arrays covered by semipermeable membranes, is also available. However, these methods are not compatible with our chips, on which cells are patterned on a flat surface. Two growth methods, which we have named the soft agar method and the solid agar method, were previously investigated in our lab.[7] In the soft agar method, growth agar solution (0.7% w.t. agar in LB media) was added on top of a patterned chip placed in a Petri dish. The Petri dish was then closed, sealed, and incubated at 37 °C. In the solid agar method, a chip was immediately covered with a film of solid agar (1.5% w.t. agar in LB media) after cell deposition, then incubated under the same conditions. Unfortunately, both methods were unsuccessful for bacterial growth on chip.

In this chapter, alternative growth methods are explored for bacteria on chips. They are referred to here as the liquid LB method, the top agarose method, and the nitrocellulose membrane transfer method. Cell array techniques, which were presented in

Chapter 2, were used to pattern bacteria on variously sized arrays for colony growth. The results help identify appropriate methods for creating high-density, ordered bacterial colony arrays.

3.2 Experimental

3.2.1 Substrate Modification

Both gold patterned and self-assembled monolayer (SAM) patterned chips were used in this chapter. Microchips were fabricated as described in Chapter 2.

For the gold patterning method, chips were immersed into cold piranha solution (3:1, 98% H_2SO_4/30% H_2O_2) overnight, flushed with autoclaved deionized H_2O (DI H_2O, from an Ultra-pure water system, Millipore, France) and blown dry with nitrogen. After being rinsed with absolute ethanol, these chips were immersed into 1 mM thiol-EG-OMe (1-mercaptoundec-11-yl-triethylene glycol methylether, synthesized according to the method described by Ni Yang[7]) ethanol solution overnight. The substrates were then covered with a few milliliters of 0.1% PEI (polyethyleneimine, MW 423, Aldrich) for 1 hr, followed by being flushed with DI H_2O and blown dry with a stream of nitrogen.

For the SAM patterning method, array B, C, and D on the photomask, shown in Figure 2.3, with 500, 750, and 1000 μm spacing each and variously sized spots, were used. After cold piranha cleaning, gold coated chips were immersed into 1 mM ethanolic thiol-EG-OMe solution overnight. The SAM was patterned by radiation with 254 nm UV light through the mask for 3 hours and then the chips were dipped into 1 mM ethanolic thiol-OH (6-mercapto-1-hexanol, Aldrich) solution for 1 hour. These chips were flushed with absolute ethanol, blown dry with nitrogen, and treated with 0.1% PEI (MW 423) for 20 seconds before use.

3.2.2 Bacterial Culture Preparation

E. coli DH5α (pGFPuv, Invitrogen) was used in this study. The details of bacterial culture and maintenance were described in Chapter 2. Freshly cultured bacterial solution was used for every experiment. Briefly, an inoculum (about 100 μL) from an overnight culture of bacteria was introduced into 20 mL autoclaved LB media. Bacteria were then grown at 37 °C with aeration and agitation at 220 rpm until the culture solution was

saturated. The bacterial cells were centrifuged, washed with phosphate buffered saline (PBS) buffer twice and resuspended into PBS buffer.

3.2.3 Cell Deposition

Prior to use, the chips were disinfected with 70% ethanol. After being rinsed with autoclaved PBS buffer, the chips were placed on Petri dishes (Fisher Scientific) and covered with the bacterial PBS solution for 1 hour. Gentle shaking was used to enhance bacterial adhesion and low temperature (Petri dishes were kept in ice) incubation was applied to increase the viability of cells. On-chip bacterial culture was sensitive to the challenge of non-adherent cells, because they caused either a high background of cell growth or cross contamination between adhesion spots. So an exhaustive washing step was crucial. For all experiments in this study, non-adherent cells were washed away according to the following protocol: each chip was immersed into PBS buffer in a Petri dish three times for 30 seconds each, while the Petri dish was gently agitated.

3.2.4 Liquid LB Method

Gold patterned chips were used for this growth method since the adhesion spots could be visualized under a microscope. After cell deposition and washing, the chips were placed in clean Petri dishes. A few mL of LB media was then added onto the surface of the chips. The Petri dishes were closed, sealed and incubated at 37 °C for various times of 6 hours, 18 hours, and 48 hours. When the culture was finished, the chips were investigated using a reflecting light microscope (BH-2 Olympus, Japan).

3.2.5 Top Agarose Method

A diluted growth agarose solution was made by dissolving 0.1% w.t. agarose in LB media. The patterned chips were placed on agar plates with the growth agarose solution poured on top. After about 1 hour, the top layers became gel and the agar plates were incubated at 37 °C overnight.

3.2.6 Nitrocellulose (N/C) Membrane Transfer Method

Both gold patterned and SAM patterned chips were tested using the N/C membrane transfer method. This method involved transferring the cell patterns on microchips to N/C

membranes for incubation. In the initially designed process, a N/C membrane was placed on top of an agar plate, then the cell-patterned chip was placed in contact with this membrane for 5 minutes. After the chip was removed, the agar plate with the membrane was incubated at 37 °C overnight. An improved process was developed, in which the cell patterned chip was first placed on a flat surface and covered with a slightly wet N/C membrane, with three pieces of dry filtration paper and a weight sequentially stacked on top. After 5 minute contact, the membrane was detached from the chip and placed on an agar plate for incubation at 37 °C overnight.

3.3 Results and Discussions

3.3.1 Liquid LB Method

Gold patterned chips with 6-μm, 10-μm, and 20-μm circle regions were used as the on-chip culture substrates for this method. Cell growth results were tested for various durations of 6, 18, and 48 hours. The Petri dishes were closed and sealed to minimize the evaporation of LB media during incubation.

Through two days of culture, similar growth trends[3] were observed for all differently sized adhesion spots. The biggest quantity and size of colony was observed after 18 hour growth, as shown in Figure 3.1, 3.2, 3.3. This time is much longer than that required for a normal solution culture. Due to the space constraints, lack of agitation and thus reduced oxygen transport, and the small amount of culture media, the microenvironment for cells on the microchips is different from that in the bulk solution, which may slow down the growth rate of bacteria. This situation is also demonstrated by the fact that few cells multiply within 6 hour culture. However, a culture time of 48 hours did not increase the size and quantity of colonies. Instead, smaller and fewer colonies were observed after 48 hours, compared to 18 hours. As only a small amount of media covered the chips during incubation, the nutrients in the media were quickly consumed, while the accumulation of wastes secreted during culture would be toxic to cell growth. Therefore, few cells would survive after 48 hour incubation. This suggests that the design of an on-line system for media exchange during incubation will be helpful in achieving better culture results.[6]

Using the protocol described in section 3.2.3, non-adherent cells could be effectively washed away. However, for the liquid LB method, there is an inherent disadvantage that

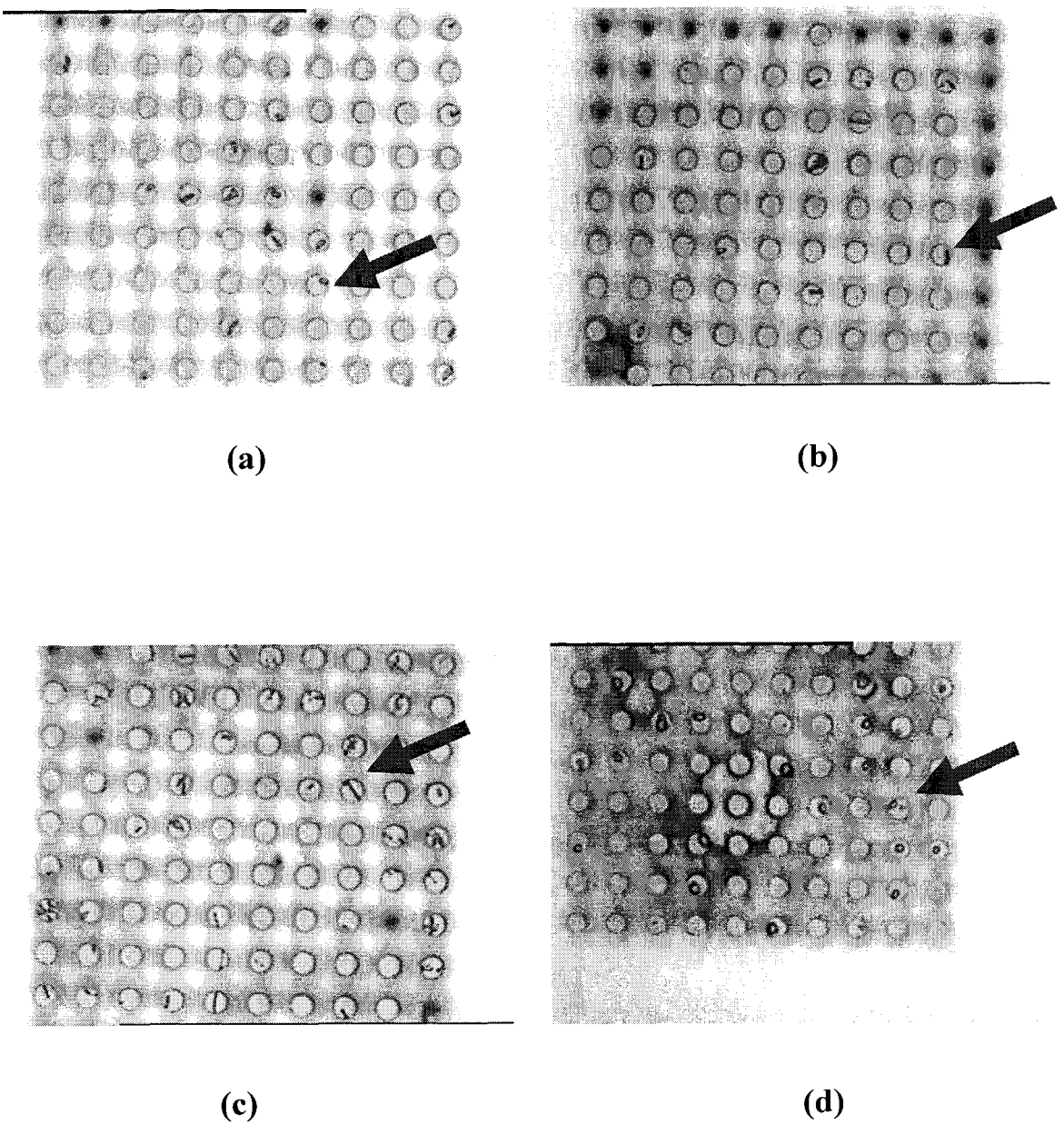


Figure 3.1 Microscopic images of *E. coli* colonies cultured on the 6-μm circle regions of gold-patterned chips using the Liquid LB method for (a) 0 hr, before culture (b) 6 hr (c) 18 hr (d) 48 hr. Arrows indicate the representative bacterial colonies. Bar shows scale for all images.

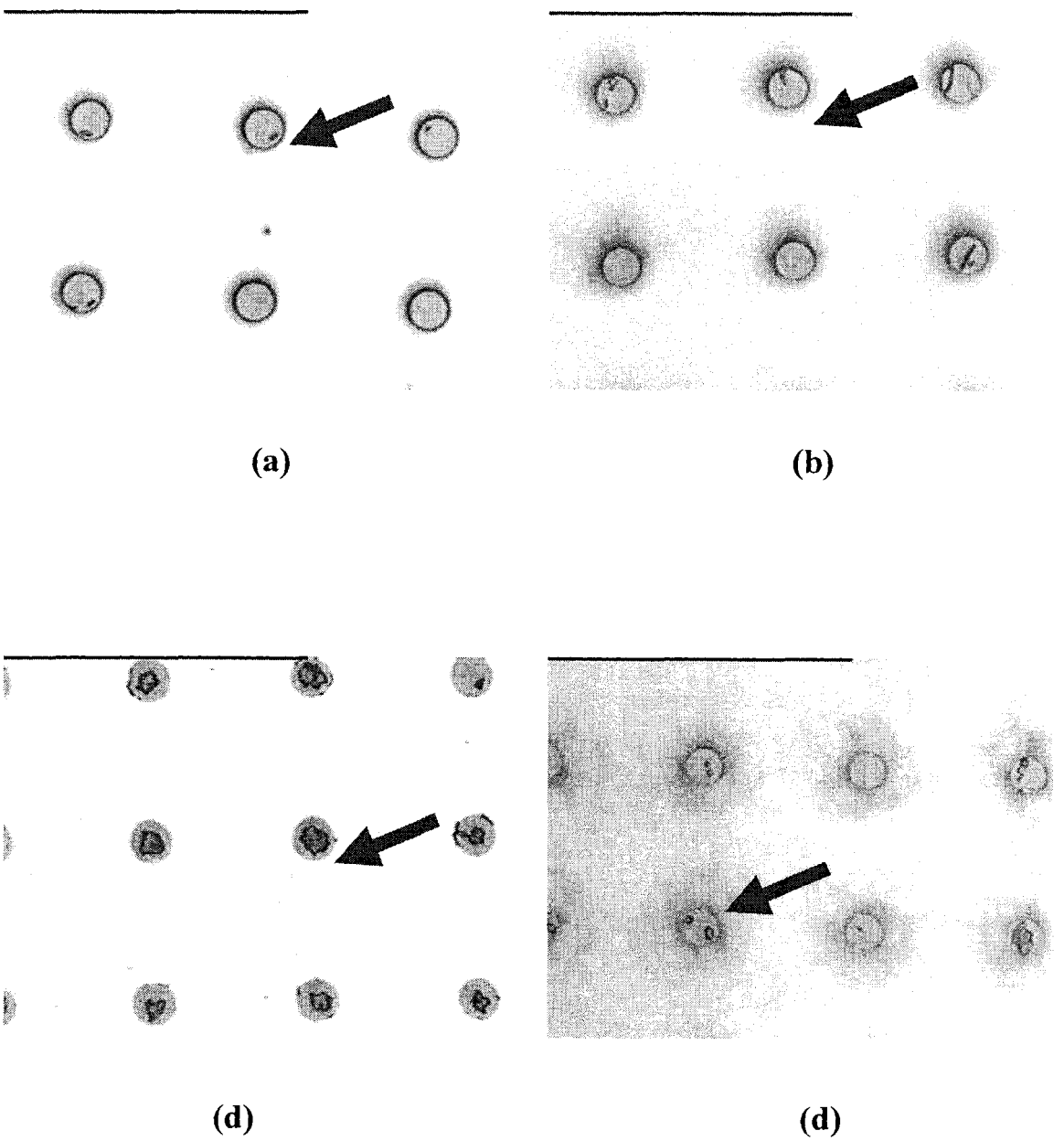


Figure 3.2 Microscopic images of *E. coli* colonies cultured on the 10-µm circle regions of gold-patterned chips using the Liquid LB method for (a) 0 hr, before culture (b) 6 hr (c) 18 hr (d) 48 hr. Arrows indicate the representative bacterial colonies. Bar shows scale for all images.

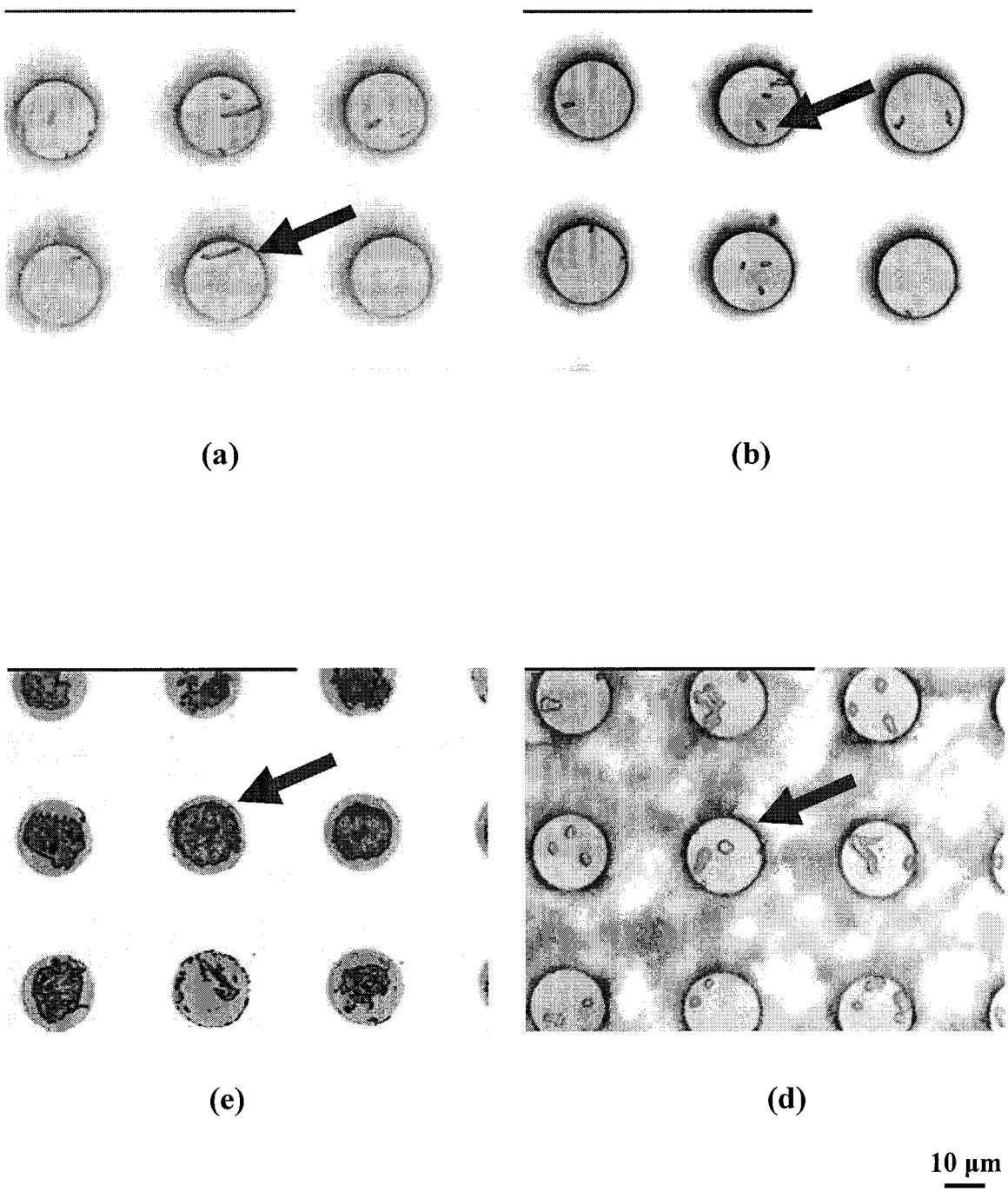


Figure 3.3 Microscopic images of *E. coli* colonies cultured on the 20-µm circle region of gold-patterned chips using the Liquid LB method for (a) 0 hr, before culture (b) 6 hr (c) 18 hr (d) 48 hr. Arrows indicate the representative bacterial colonies. Bar shows scale for all images.

the cells easily detach from the surface during incubation. These floating cells multiply in the media and deposit again onto other adhesion spots, causing cross contamination. This problem becomes more severe with time, as more and more cells divide into colonies, as demonstrated by the fact that the media becomes very murky over time. As a result, a colony on an adhesion spot is not necessarily a single clone, making the single cell patterning meaningless. This problem strongly limits the application of this method to very short term cell culturing.

3.3.2 Top Agarose Method

For the growth method using liquid media, the cell detachment problem is inevitable for mobile cells like *E. coli*. To address this problem, the top agarose method using a solid gel was proposed. Preliminary tests were performed before this method was applied to the microchips. An inoculum (about 100 μL) from an overnight culture of bacteria was scratched on an agar plate and the diluted growth agarose solution, made of 0.1% w.t. agarose and LB media, was added on top. After the solution became a gel, the agar plate was incubated at 37 °C overnight. The pore size of the gel formed from the diluted agarose solution allows diffusion of oxygen to the cells as well as adequate migration for

Figure 3.4 Preliminary result of *E. coli* culture using the top agarose method.

cell division. The result in Figure 3.4 shows that cells grew well under the top layer. Some colonies appeared on the top of the gel, which were probably from the cells that floated free during pouring of the agarose growth solution.

Culturing with the microchips led to two results (data not shown). First, most of colonies grew on the top agarose layer rather than on the chips. Second, a lot of colonies on the top layer were not in patterns. The first result is obvious since the top layer contains nutrients while the chips do not. For the second result, one possible explanation is that cells can also float away from the adhesion spots before the liquid gel becomes solid. Compared to the liquid LB method, which always uses the liquid media, the detachment problem here should be less severe. However, floating cells in the liquid media can either deposit again on the chips, or be flushed away during the washing step after incubation. In the top agarose method, a large portion of detached cells will remain in the top layer after the solid gel forms. These cells will multiply in the agarose layer, which causes the random distribution of colonies on the top layer. Accordingly, we conclude that this method is not suitable for our purpose.

3.3.3 N/C Membrane Transfer Method

Nitrocellulose (N/C) membrane is a negatively charged filtration membrane commonly used in biological science. For example, it can be used as the solid support in Southern blotting when DNA is transferred from a separation gel.[8] Since a bacterium has positively charged regions, it can adhere onto a N/C membrane. The mild strength of interaction between membranes and bacteria ensures that bacteria can move freely during multiplication, so the normal cell cycle is not disturbed. There are many kinds of N/C membranes with different average pore sizes. Membranes with a pore size of 0.22 μm were chosen because this size allows passage of nutrient and water molecules but traps cells. If the cell pattern on the microchip is transferred to a N/C membrane on an agar plate, nutrients and water from the agar plate should easily permeate the membrane to be absorbed by the bacteria. The waste secreted by the cells could diffuse away through the membrane. Therefore, the N/C membrane could be used as a platform for cell growth, which would certainly solve the cell detachment problem.

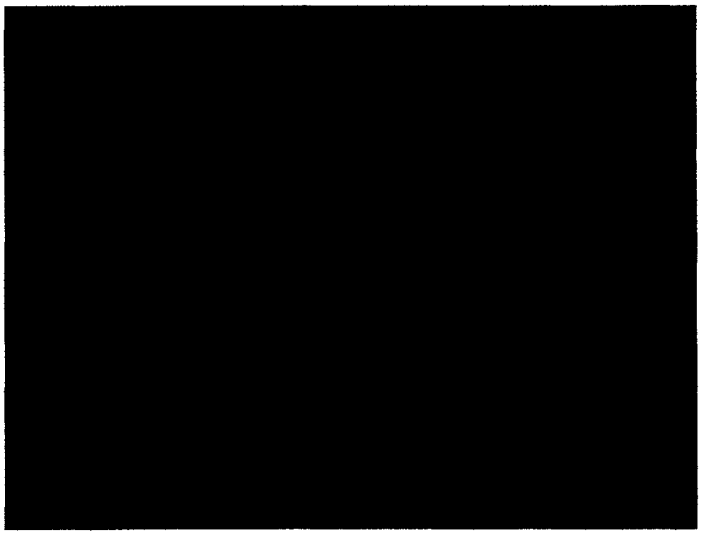


Figure 3.5 Result of *E. coli* culture on the surface of N/C membrane on an agar plate. A control experiment without bacteria was conducted for comparison.

Preliminary test was performed to prove the feasibility of this idea. An inoculum (about 100 μL) from an overnight culture of *E. coli* was scratched on the surface of a N/C membrane on an agar plate and then incubated at 37 °C overnight. Control experiments without bacteria were conducted for comparison. Colonies appeared and grew well only on the membranes scratched with bacteria (Figure 3.5), consistent with our expectation.

The "half-half" chip was used for further testing. For chip fabrication, half of one thiol-EG-OMe SAM-coated chip was radiated with 254 nm UV and exchanged by the thiol-OH SAM. As a result, one half of the chip was coated with thiol-EG-OMe SAM, which is strongly resistant to cell adhesion, while the other with thiol-OH has the opposite property. The chip was then dipped into bacterial solution for 1 hour, washed by immersion into PBS buffer three times, and contacted with a N/C membrane on an agar plate for 5 minutes to transfer the "half-half" cell

Figure 3.6 Result of *E. coli* culture using the N/C membrane transfer method for the "half-half" chip. The left half of the chip was coated with the thiol-EG-OMe SAM, while the right with the thiol-OH SAM.

pattern to the membrane. The result of an overnight culture is shown in Figure 3.6. A clear border is formed between the two halves, one with colonies, the other without, which suggested that the cell patterns on microchips could be faithfully transferred to membranes. A microscopic image taken of the thiol-OH part of the chip after transfer, is shown in Figure 3.7. There were still some cells remaining on the chip, which means that the cell transfer was not complete.

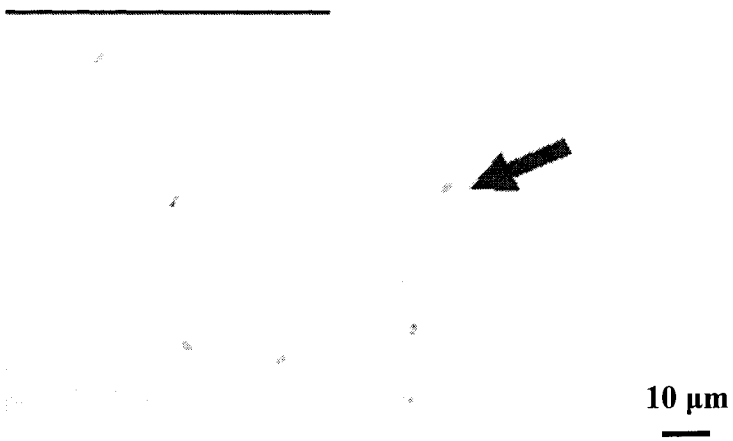


Figure 3.7 Microscopic image of the *E. coli* remaining on the chip after transfer to N/C membrane. The arrow indicates the representative cells.

For the experiments on SAM patterned chips with the initially developed procedure, some colony patterns were observed after overnight culture (Figure 3.8). But there were still many colonies not registered with the pattern, resulting in a high background of cell growth on the membrane. Some areas devoid of colonies may arise from air bubbles that were trapped between the membrane and the chip during the transfer process. A study was performed to reveal the source of these problems. Instead of transferring cell patterns to N/C membranes, microchips were contacted with the agar plates directly. The result of an overnight culture, shown in Figure 3.9, indicated that the high background and blank areas persisted on the agar plate alone, so these problems did not come from the N/C membrane. The main reason may be that water squeezed out of the agar gel when the chip was placed on the membrane, which moved the bacterial cells from the adhesion spots during the transfer process. Another problem with this initial method was that it suffered from trapping of air bubbles. Since the chips were laid on top of membranes,

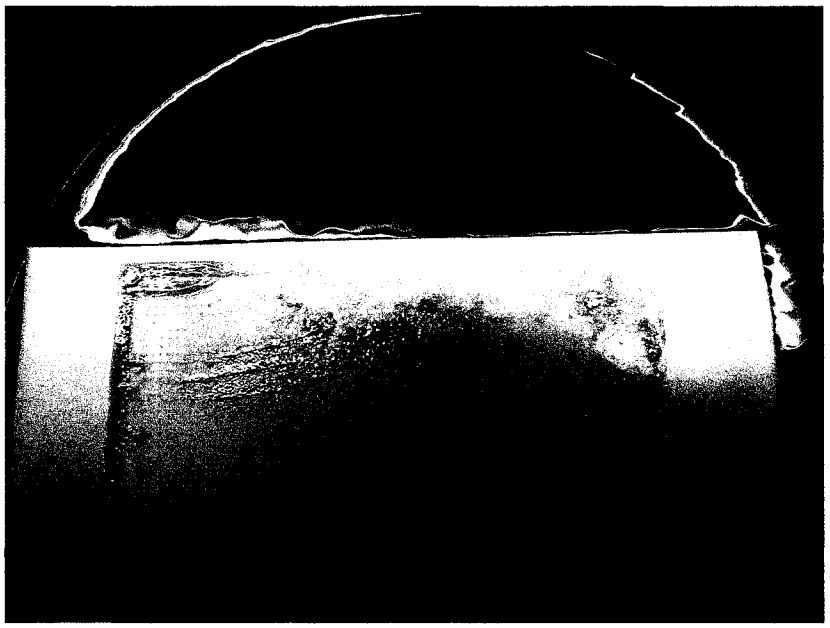


Figure 3.8 Image of *E. coli* culture on the N/C membrane using the initial N/C membrane transfer method. The SAM patterned chip with a spacing of 1000 μm was used.

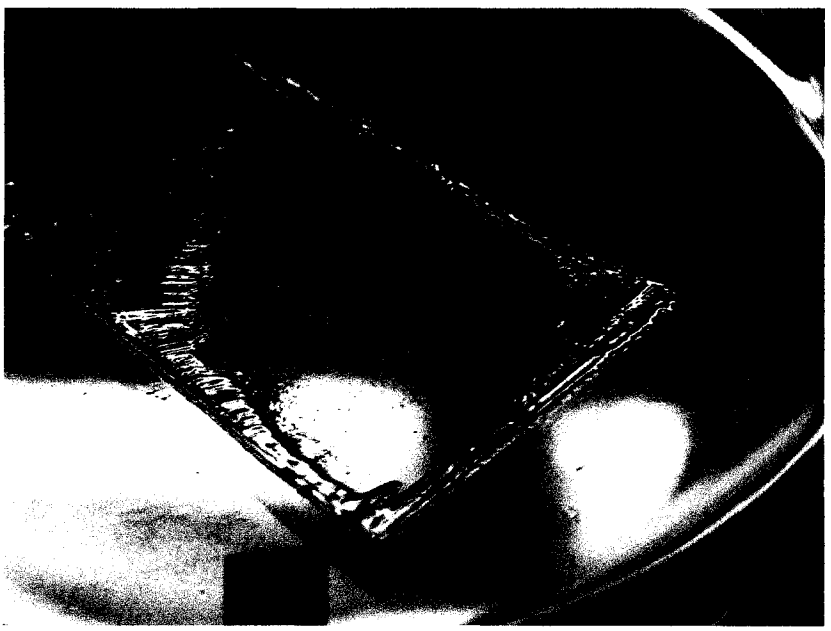
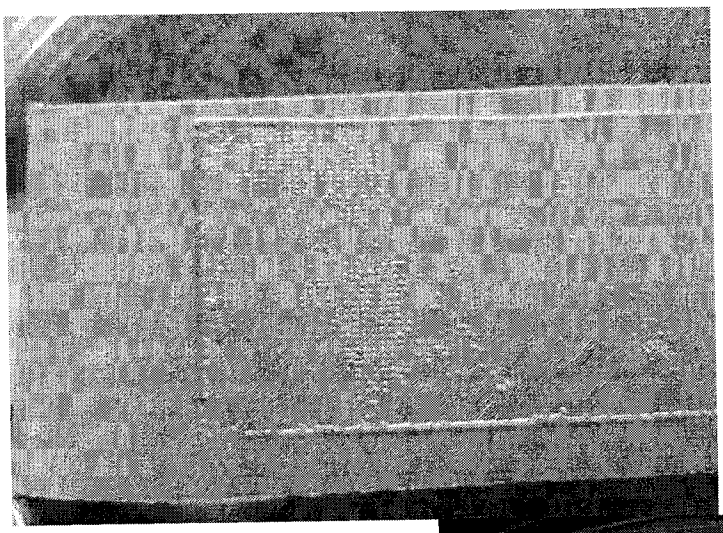


Figure 3.9 Results of *E. coli* culture by transferring the cell pattern on the chip directly to the agar plate. The inter-spot spacing is 1000 μm.

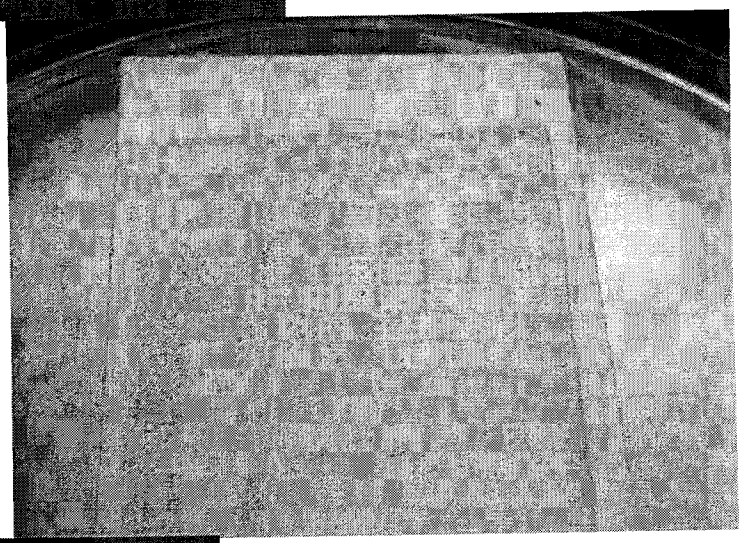
these air bubbles were difficult to remove. During the chip contact and removal process, movement of chips on the membranes was unavoidable, making the problem of smudged cell spots even worse.

An improved transfer process was necessary. The blotting procedure in Southern blotting was employed to transfer the cell patterns to membranes. In Southern blotting[8], after gel electrophoresis, a sheet of either nitrocellulose paper or nylon paper is laid over the gel with paper towels stacked on top. The gel is supported on a layer of sponge in a bath of alkali solution. As the buffer is sucked through the gel and the nitrocellulose paper by the paper towels, it transfers the single-stranded fragments from the gel to the surface of the nitrocellulose sheet, where they are adsorbed firmly and denatured by baking. Similarly, in the new procedure we developed, a piece of N/C membrane is placed on a chip with cells patterned on it. This membrane is slightly wetted by contact with an agar plate, while completely dry filtration paper is used to cover the membrane. As the water is sucked from the interface between the membrane and the chip to the filtration paper, the cells are also expected to adhere to the membrane. The overnight incubation results for the chips with various spacings of 500, 750, and 1000 μm are illustrated in Figure 3.10. (The bacterial agglomerates near the edge areas were from tweezers used during handling.) Colonies were well patterned in 20-μm circle regions on the three microchips, but for the other geometries, patterns were seldom observed. This is probably due to the low transfer efficiency between chips and membranes, as mentioned above. For the 20-μm circle there were usually many cells in an adhesion spot, so at least one cell per spot could be transferred to the membrane even if the transfer efficiency is not high. But for the regions with smaller features, the average number of cells per spot was lower; hence the transfer probability for each spot was much lower. Unfortunately, since the transfer efficiency problem is worse for smaller features, which are better for achieving the goal of single cell per spot, further improvement in transfer and growth is needed.

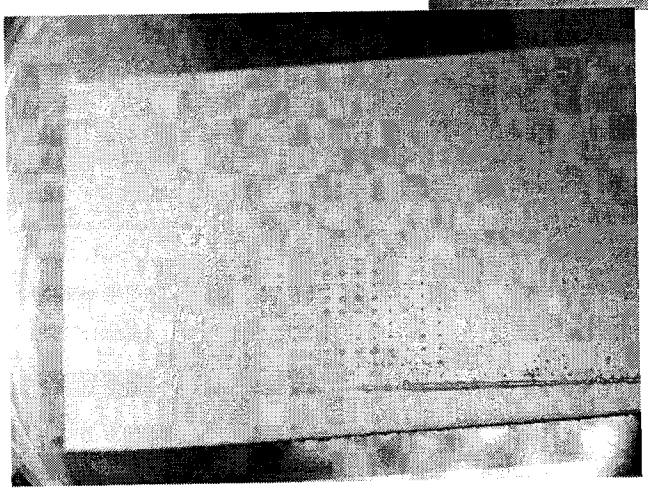
The results from Figure 3.10 also demonstrated that the spacing has little effect on colony patterns. As long as the spacing is big enough to prevent contact between adjacent colonies after incubation, smaller spacing will be able to achieve arrays with higher density.



(a) Spacing 500 μm



(b) Spacing 750 μm



(c) Spacing 1000 μm

Figure 3.10 Colony patterns of *E. coli* using the improved N/C membrane transfer method after overnight culture. The SAM patterned chips with various spacings of 500, 750, and 1000 μm were used.

The N/C membrane transfer method was also employed to transfer cell patterns from gold patterned chips. The chips with 20 μm (diameter) spots and 500 μm spacing were used for this test. Figure 3.11 showed well-patterned colonies on a N/C membrane, indicating the membrane transfer method can be applied to both types of chips.

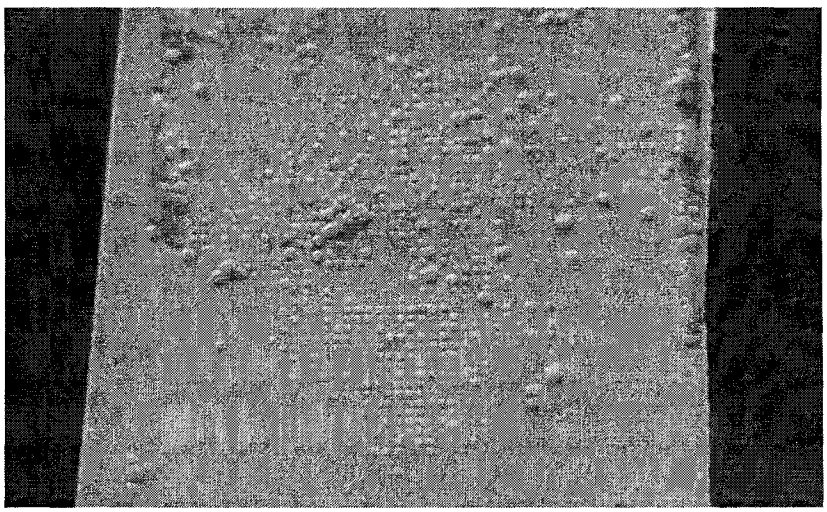
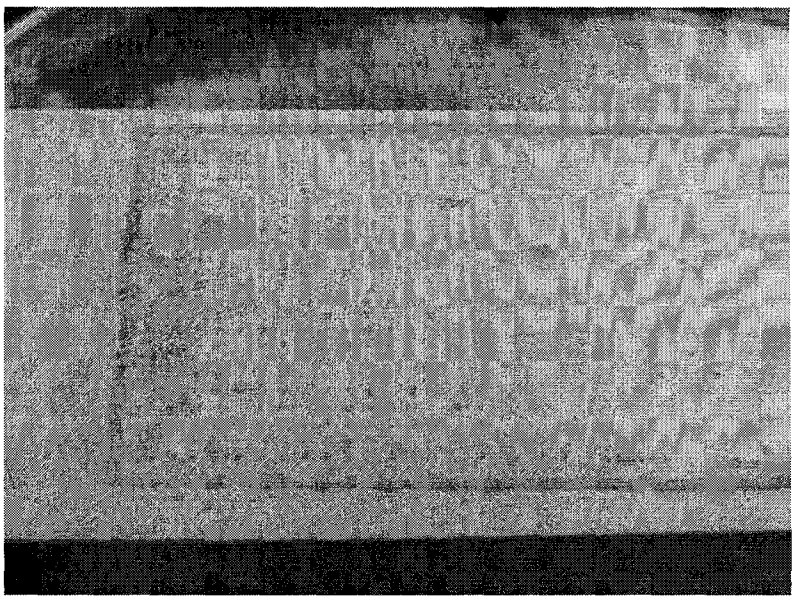
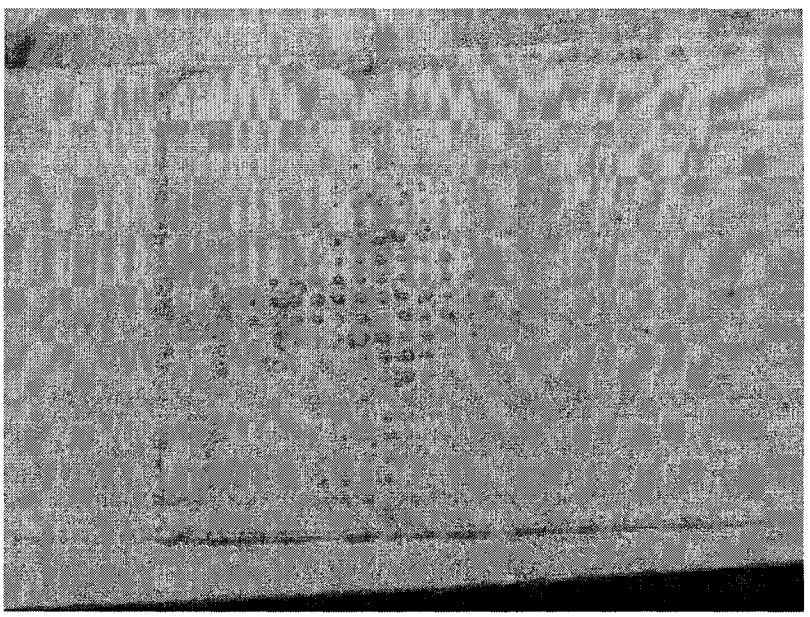


Figure 3.11 Image of *E. coli* colony pattern using the N/C membrane transfer method. The gold patterned chip with 20 μm (diameter) spots and 500 μm spacing was used.

Further experiments were conducted to analyze the stability of SAMs during the transfer process. After each transfer step, a SAM patterned chip with 20 μm (diameter) spots and 1000 μm spacing was thoroughly cleaned flushing with 70% ethanol, then autoclaving in DI H_2O. This chip was then immersed into a cell solution and subjected to the transfer process again. Using the same procedure, the chip was reused at least five times. Figure 3.12 compares the colony pattern from the first time the chip was used to that from the fifth time the chip was used. The colony array was still observed on the fifth use. It indicates that the SAMs are stable for multiple uses, which is an advantage of our chips.



(a)



(b)

Figure 3.12 Results of *E. coli* colony patterns for a chip used (a) the first-time (b) the fifth-time. The SAM patterned chips with 20 μm (diameter) spots and 1000 μm spacing were used.

3.4 Conclusions

For on-chip bacterial cultures, three different growth methods were developed. For the LB liquid method, the chips with cell patterns were covered with LB media and incubated at typical culture conditions. The result showed that colony patterns were achieved but the cell detachment problem during culturing limited the application of this method. For the top agarose method, a diluted growth agarose solution was added on top of a chip that was placed on an agar plate. After gelation, the agar plate was incubated. This method also suffered from the same problem as the liquid LB method before the solidification of the liquid gel. To overcome this disadvantage, the N/C membrane transfer method was employed. The cell patterns on a chip were transferred to a N/C membrane, which was then cultured on an agar plate. Since no liquid media were used in this procedure, the cell detachment problem was eliminated. The effect of inter-spot spacing on colony patterns was investigated. The result demonstrates that the spacing does not have substantial influence. This growth method could be applied to both SAM patterned chips and gold patterned chips. However, transfer of cell patterns was not complete, especially for small-sized adhesion spots. Future work should concentrate on enhancing the transfer efficiency between chips and membranes. For all of these culture methods, effective removal of non-adherent cells was vital. The same protocol for exhaustive washing was used.

3.5 References

1. Koh, W.-G.; Revzin, A.; Simonian, A.; Reeves, T.; Pishko, M., *Biomedical Microdevices* **2003**, 5, 11-19.

2. Lan, S.; Veiseh, M.; Zhang, M., *Biosensors and Bioelectronics* **2005**, 20, 1697-1708.

3. Kaya, T.; Nagamine, K.; Oyamatsu, D.; Shiku, H.; Nishizawa, M.; Matsue, T., *Lab on a Chip* **2003**, 3, 313-317.

4. Kaya, T.; Nagamine, K.; Matsui, N.; Yasukawa, T.; Shiku, H.; Matsue, T., *Chem. Comm.* **2004**, (2), 248-249.

5. Inoue, I.; Wakamoto, Y.; Moriguchi, H.; Okano, K.; Yasuda, K., *Lab on a Chip* **2001**, 1, 50-55.

6. Umehara, S.; Wakamoto, Y.; Inoue, I.; Yasuda, K., *Biochemical and Biophysical Research Communications* **2003**, 305, 534-540.

7. Yang, N., Cell Arrays on Chemically Modified Chip Surfaces, University of Alberta, Edmonton, **2004**.

8. Sambrook, J.; Fritsch, E. F.; Maniatis, T., *Molecular Cloning*. ed.; Cold Spring Harbor Laboratory Press: **1989**; pp 18.60-18.61.

Chapter 4 Animal Cell Patterning

4.1 Introduction

Compared to bacterial cells, animal cells have more complicated structures and functions. Transfection technologies, used to introduce foreign DNA into a host animal cell, have been commonly used in biological research. Genomic analyses of animal cells provide information about gene expression in complex biological systems. The increasing interest in studying animal cells also comes from medical science, because the normal and pathogenic development in animals is closer to human than bacteria. Therefore, drug screening tests on animal cells may yield more reliable results for drug candidates. High-density arrays of animal cells can be employed to perform these assays in a high-throughput way and thus hold promise in biochemical and biomedical applications. A series of techniques are available for cell patterning, including photolithography[1], microcontact printing[2], and membrane-based patterning[3], as described in Chapter 1.

In this chapter, the SAM patterning method (with PLL treatment), which was presented in Chapter 2, was used to fabricate microchips. Hela and Jurkat cells were used for the deposition tests on the patterns. The cell patterns on various-sized spots were investigated and compared between these two kinds of cells.

4.2 Experimental

4.2.1 Chip Fabrication

Microchips were fabricated using the SAM patterning method as described in Chapter 2. Device D, with the smallest spacing in the cell array photomask, was used for fabrication. (The layout of the photomask is illustrated in Figures 2.3 and 2.4.) Briefly, gold coated chips were immersed into cold piranha solution (3:1, 98% H_2SO_4/30% H_2O_2) overnight to completely remove the contaminant on the surfaces, followed by flushing thoroughly with autoclaved DI H_2O (deionized H_2O from an Ultra-pure water system, Millipore, France) and drying with nitrogen gas. These chips were then rinsed with absolute ethanol and immersed into 1 mM ethanolic thiol-EG-OMe (1-mercaptoundec-11-yl-triethylene glycol methylether, synthesized according to the

method described by Ni Yang[4]) solution overnight. The chips were then exposed to 254 nm UV light through a photomask for 3 hours and dipped into 1 mM ethanolic thiol-OH (6-mercapto-1-hexanol, Aldrich) solution for 1 hour. Upon removal, the chips were flushed with absolute ethanol and blown dry. Before use, the chips were treated with 0.1 mg/mL PLL (poly-L-lysine, MW 88,000, Sigma) for 5 minutes, then flushed with DI H_2O and blown dry with nitrogen.

4.2.2 Animal Cell Culture Preparation[5]

Hela cells, one of the human cervical carcinoma cell lines, were maintained in a humidified incubator supplemented with 5% CO_2 at 37 °C. DMEM (Dulbecco's Modified Eagle's Medium, Gibco, Invitrogen Cooperation) added with 10% FBS (fetal bovine serum, Gibco) and 50 μg/mL of gentamicin (Gibco) was used as culture media. Normally, cells were cultivated in a culture dish (100 mm diameter × 15 mm depth, Fisher Scientific) which contained about 15 mL media. After two days, cell growth was monitored by a microscope. When cells were saturated on the surface of the culture dish, they were ready to be harvested. Cell harvest was done by using 0.05%/0.53 mM trypsin/EDTA (Gibco) to float the cells, following a 5 minute incubation at 37 °C, and they were then pelleted by centrifugation (1000 rpm, 5 minutes). Harvested cells were briefly washed with PBS (phosphate buffered saline, Gibco) twice. For the deposition test on microchips, the concentration of cell solutions was diluted to about 1×10^6 cells/mL with PBS.

Jurkat cells, derived from human T cell leukemia, were also used for the deposition test. RPMI 1640 (Roswell Park Memorial Institute Medium, Gibco) with the same supplements was used for cell incubation. The preparation procedure is the same as that for Hela cells, except that only centrifugation was performed to harvest Jurkat cells. The final concentration of cell solutions used was about 1×10^6 cells/mL.

4.2.3 Cell Deposition Test

Chips were disinfected by treatment with 70% ethanol for 5 minutes prior to use. These chips were rinsed with autoclaved DI H_2O and placed on the animal cell culture dishes (Fisher Scientific). Prepared cell solution was poured into the dishes and incubated for 1 hour at room temperature. The chips were gently washed with PBS twice to remove

non-adherent cells and supplied with completely fresh media. After incubating at 37 °C overnight, the chips were investigated using a reflecting light microscope (BH-2 Olympus, Japan).

4.2.4 Chip Regeneration

Used chips were disinfected with 70% ethanol and then immersed into cold piranha solution overnight, followed by flushing with a large quantity of DI H_2O. With this cleaning process, these chips could be recoated with SAMs and reused at least five times.

4.3 Results and Discussions

4.3.1 Hela Cell Patterning

The SAM patterning method created the cytophilic (thiol-OH SAM with PLL) and cytophobic (thiol-EG-OMe SAM) domains on gold surfaces. For patterning cells, the SAM patterned chips were incubated in a Hela cell solution (about 1×10^6 cells/mL) overnight. On device D, there are seven regions with variously sized and shaped adhesion spots: 20 μm-circle, 10 μm-circle, 6 μm-circle, 3 × 6 μm-rectangle, 3 × 4 μm-rectangle, 2 × 6 μm-rectangle, and 2 × 4 μm-rectangle. Cell patterns on each adhesive region were investigated using a microscope.

The Hela cell pattern on the 20 μm-circle adhesive region is shown in Figure 4.1. In order to locate the spots on microchips, a grid with a line spacing of 80 μm was used as a reference because the center-to-center spot spacing was 80 μm. This image clearly indicates that most cells clustered around the adhesion spots but extended beyond where the adhesion material was located. The small amount of cells not on the adhesion spots might also be those cells that grew during the overnight incubation. The spatial distribution of cells was investigated by counting the number of cells on each spot. Two hundred spots were randomly selected for this study. The percentage of the spots containing a certain number of cells is illustrated in Figure 4.2. The results show that the majority of adhesion spots accommodated 2 to 4 cells/spot and each of these quantities had a similar probability. The maximum number of cells one spot contained was observed to be 7 cells/spot in our experimental conditions. The possibility for a 20 μm spot to adsorb only one cell, however, was lower than 10% in this experiment. In order to

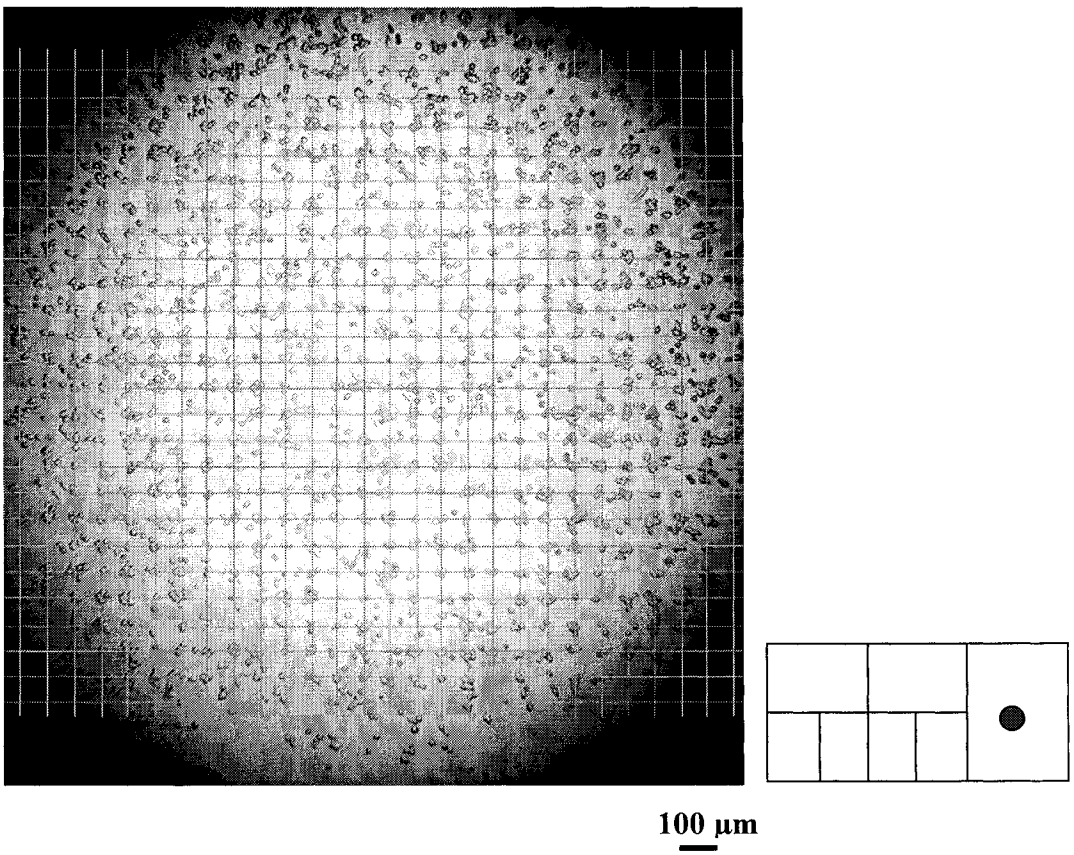


Figure 4.1 Image of the Hela cell pattern on the 20 μm-circle adhesive region on a microchip fabricated using the SAM patterning method. Adhesion spots were located at the intersections of the grid lines. The line spacing is 80 μm. The spot on the insert indicates the location on the chip where this picture was taken.

achieve a single cell array on the 20 μm spots, a much lower cell concentration would be needed, or a shorter incubation time. Based on counting the total number of cells on the 200 spots, the average was 3.3 cells/spot. Under microscope, we observed that the Hela cell floating in a suspension had a circular-shaped with a diameter of about 20 μm. Since a 20 μm spot could adsorb more than one Hela cell, the cells might stick to only a portion of the adhesion surface, stack on each other, or take advantage of their ability to change shape to fit in the region of the spot.

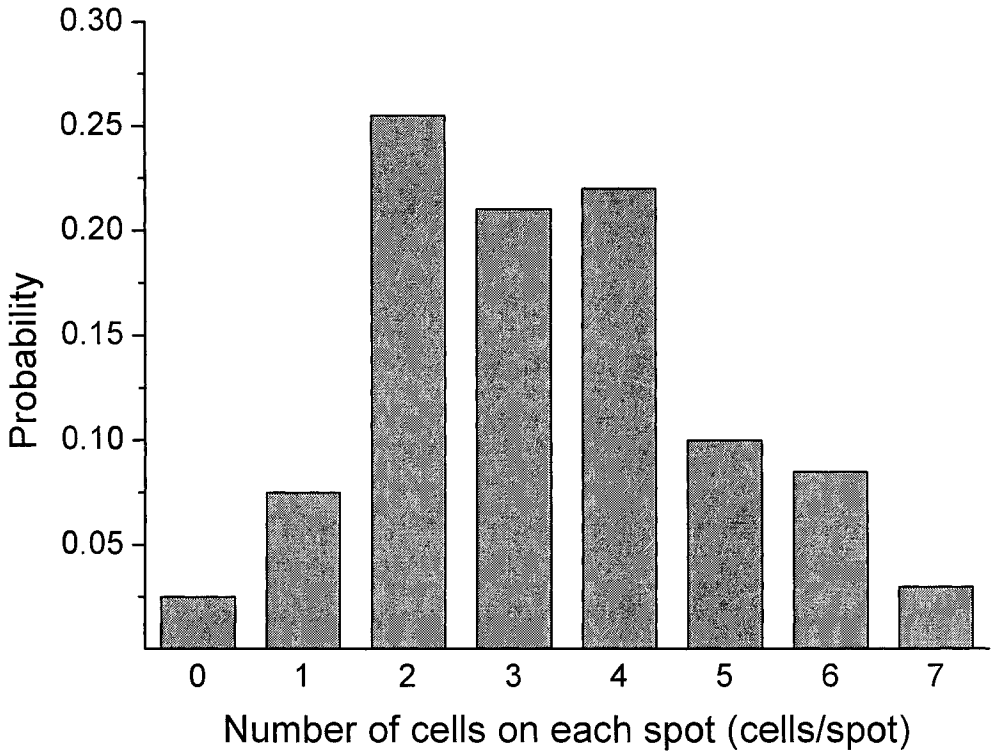
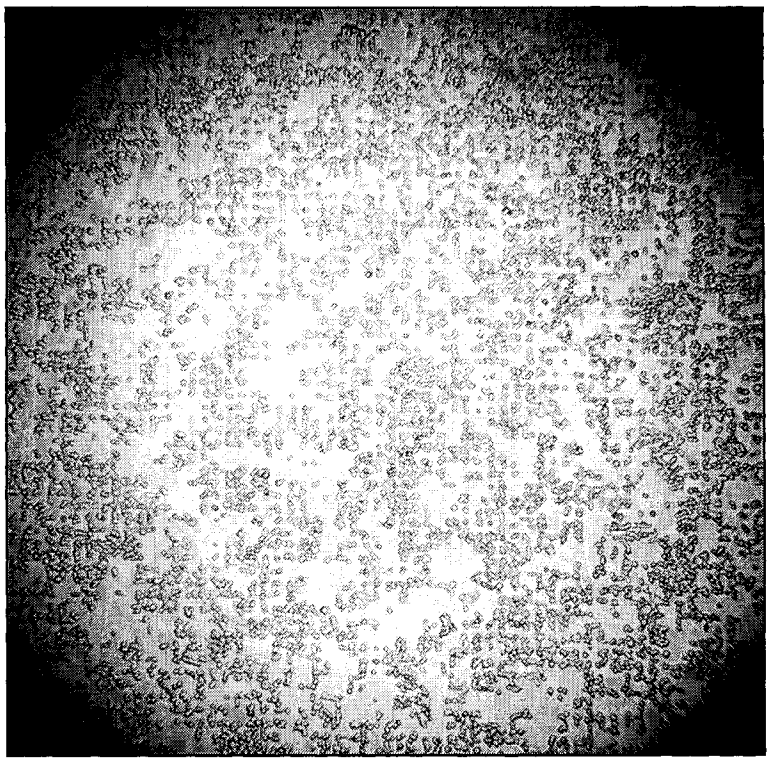
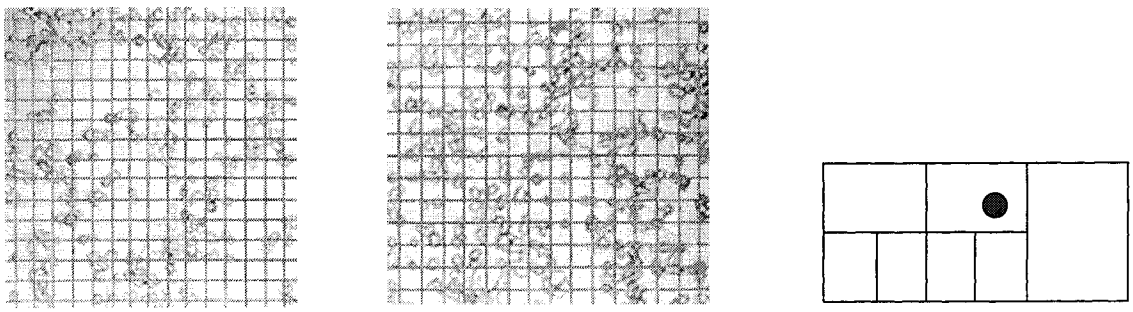


Figure 4.2 Plot of Hela cell distribution on the 20 μm-circle region on a chip fabricated using the SAM patterning method. The concentration of the cell solution used for incubation was about 1×10^6 cells/mL.

Figure 4.3 shows the patterned Hela cells on the 10 μm-circle adhesive region. Grids were drawn on two enlarged pictures for clarity. Adhesion spots are located at the intersections of the grids, with a center-to-center spacing of 40 μm. From the image, we see that although a large portion of cells coated the patterned array, many adhesion spots were empty. An increase in the cell concentration from 1×10^6 cells/mL may eliminate some empty sites. The same statistical studies as mentioned above were performed on the 10 μm spots. The results demonstrate that about 40% of the adhesion spots had no cells. A single cell per spot occurred with the highest probability (about 45%), but the possibility of two or more cells were also relatively high, at about 16%. The average number was 0.8 cells/spot, while for the 20 μm spots, the number was 3.3 cells/spot. This is consistent with our expectation that a larger spot area should accommodate more cells.





(a) (b)

Figure 4.3 Image of the Hela cell pattern on the 10 μm-circle adhesive region on a microchip fabricated using the SAM patterning method. The insert (a) contains two enlarged pictures of the cell pattern. Adhesion spots were located at the intersections of the grid lines. The line spacing is 40 μm. The spot on the insert (b) indicates the location on the chip where the picture was taken.

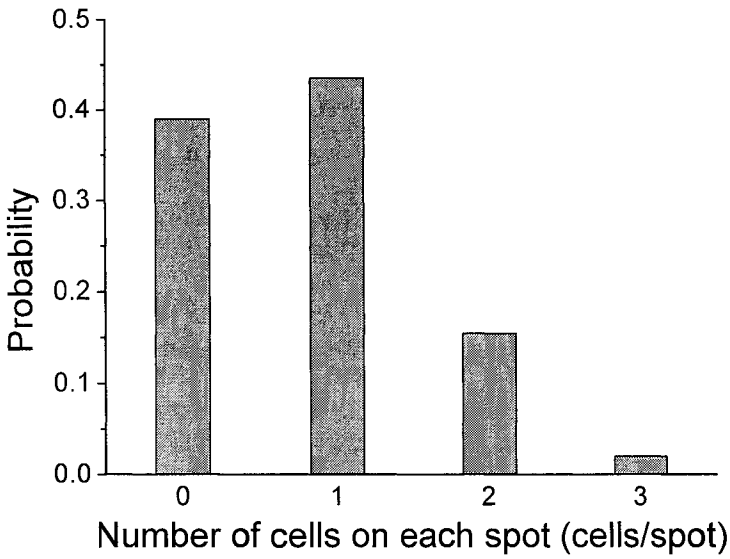


Figure 4.4 Plot of Hela cell distribution on the 10 μm-circle region on a chip fabricated using the SAM patterning method. The concentration of the cell solution used for incubation was about 1 × 10^6 cells/mL.

The ratio of average cells per spot for 20 to 10 μm was 4.1:1, consistent with the surface area ratio (4:1). The results also show that decreasing the spot size can be used effectively to enhance the probability of obtaining a single cell per spot.

For the 6 μm-circle adhesive region, no pattern was observed after incubating with a cell solution, as shown in Figure 4.5. The results were the same for other regions with even smaller dimensions of adhesion spots: 3 × 6 μm-rectangle, 3 × 4 μm-rectangle, 2 × 6 μm-rectangle, and 2 × 4 μm-rectangle (data not shown). These spots are too small for Hela cells to fit in, but they can stretch up to 100 μm in length when adhering to a surface. Since the adhesion spots were only separated by 20 μm center to center, Hela cells were capable of bridging between several adjacent spots[6] (shown in Figure 4.6), which would lead to an apparently random distribution of cells. This phenomenon became more evident as the spot size and the spacing decreased. In addition, the overnight incubation might cause more cells to attach to the areas outside the adhesion spots.

Both SAMs and PLL play important roles in patterning cells on microchips fabricated using the SAM patterning method. Figure 4.7 shows the Hela cell pattern on the 20 μm-circle region, with a defect caused by the absence of a SAM due to a lack of gold coating. The contrast between the much more random distribution of cells in the defect area with the obvious pattern of cells on the array demonstrates that SAMs are crucial to the cell patterning. The influence of PLL was investigated by comparing cell

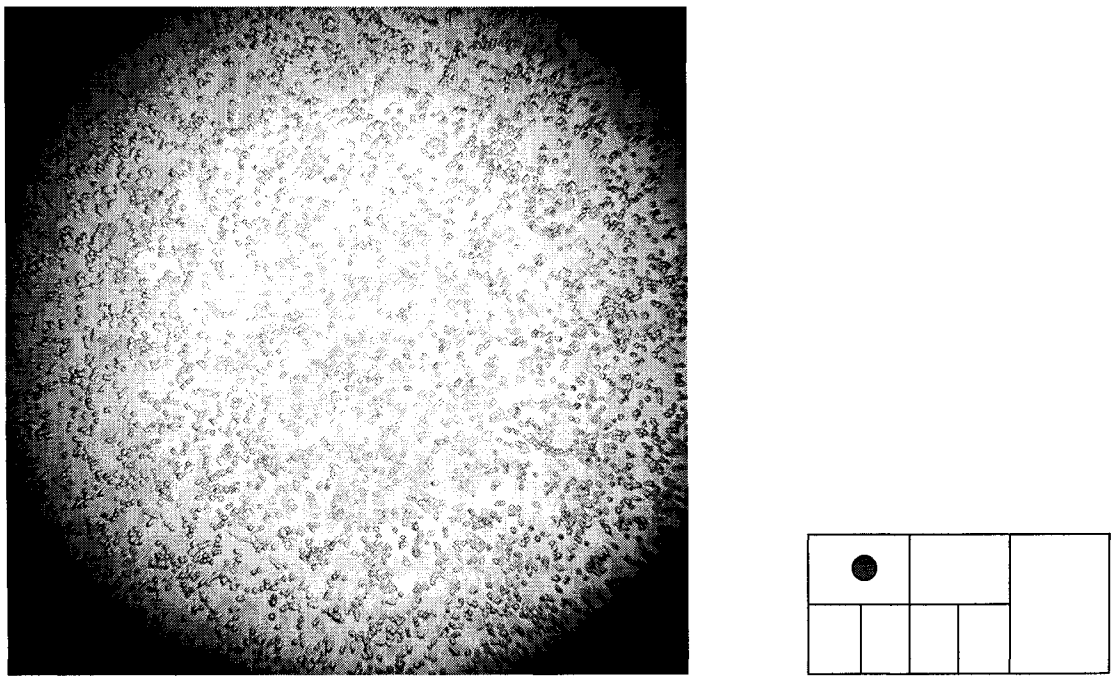


Figure 4.5 Image of Hela cells on the 6 μm-circle adhesive region on a microchip fabricated using the SAM patterning method. The spacing between adhesion spots is 20 μm. The spot on the insert indicates the location on the chip where this picture was taken.

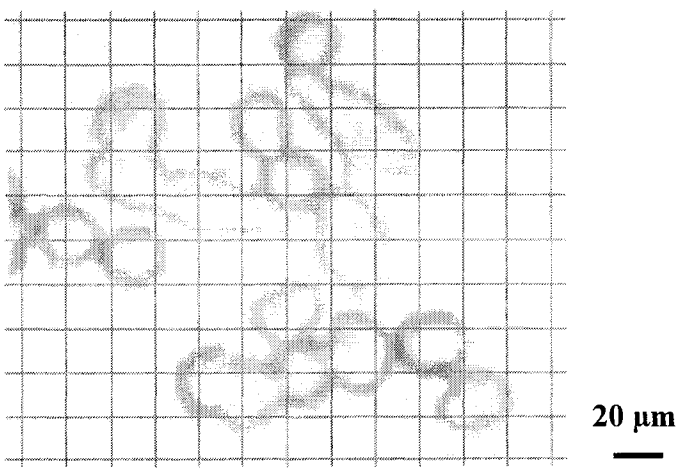


Figure 4.6 Enlarged image of Hela cells on the 6 μm-circle adhesive region on a microchip fabricated using the SAM patterning method. Adhesion spots were located at the intersections of the grid lines. The line spacing is 80 μm. The spacing between adhesion spots is 20 μm.

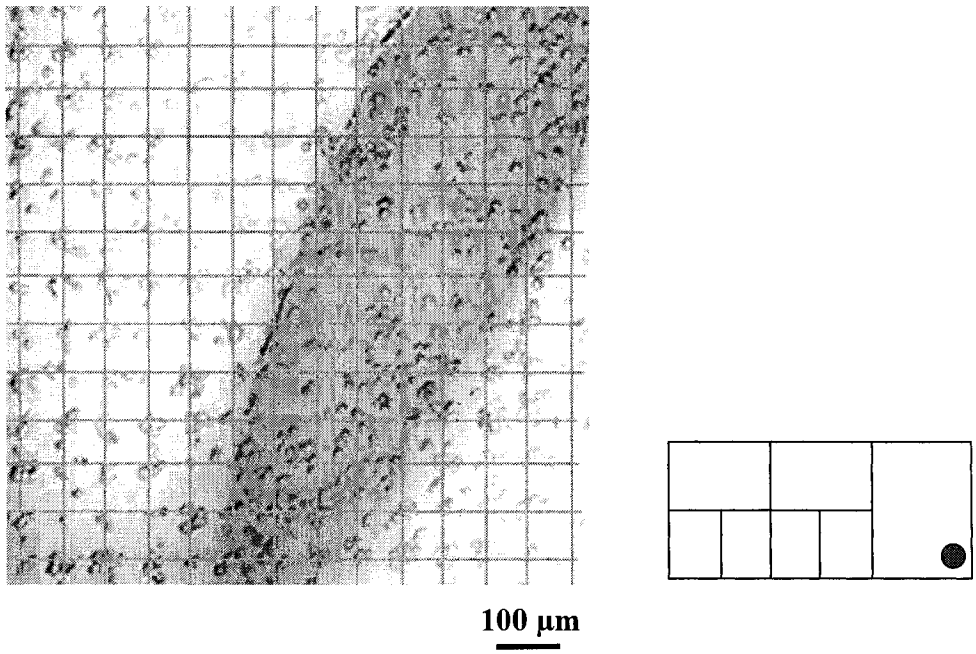


Figure 4.7 Image of the Hela cell pattern on the 20 μm-circle adhesive region on a microchip fabricated using the SAM patterning method. A defect on the right was caused by a lack of gold coating. Adhesion spots were located at the intersections of the grid lines. The line spacing is 80 μm. The spot on the insert indicates the location on the chip where this picture was taken.

patterns on two microchips, for which one was treated with 0.1 mg/mL PLL for 5 minutes while the other was not. The resulting patterns are shown in Figure 4.8. A clear pattern was observed for the PLL-treated chip, but no pattern resulted on the chip without PLL treatment. In cell growth, PLL is commonly used to increase the hydrophilicity of hydrophobic surfaces. The random distribution of cells on the chip without PLL treatment may indicate that the SAM of thiol-OH alone is not strong enough to preferentially adsorb cells.

Different inter-spot spacings may also affect the cell patterns. In this study, we only tested device D, with the smallest spacing in the photomask. When using smaller spots, the larger spacing may be better for Hela cell attachment, as the chance of cross contamination caused by the simultaneous attachment of one cell to several adjacent spots will be diminished.

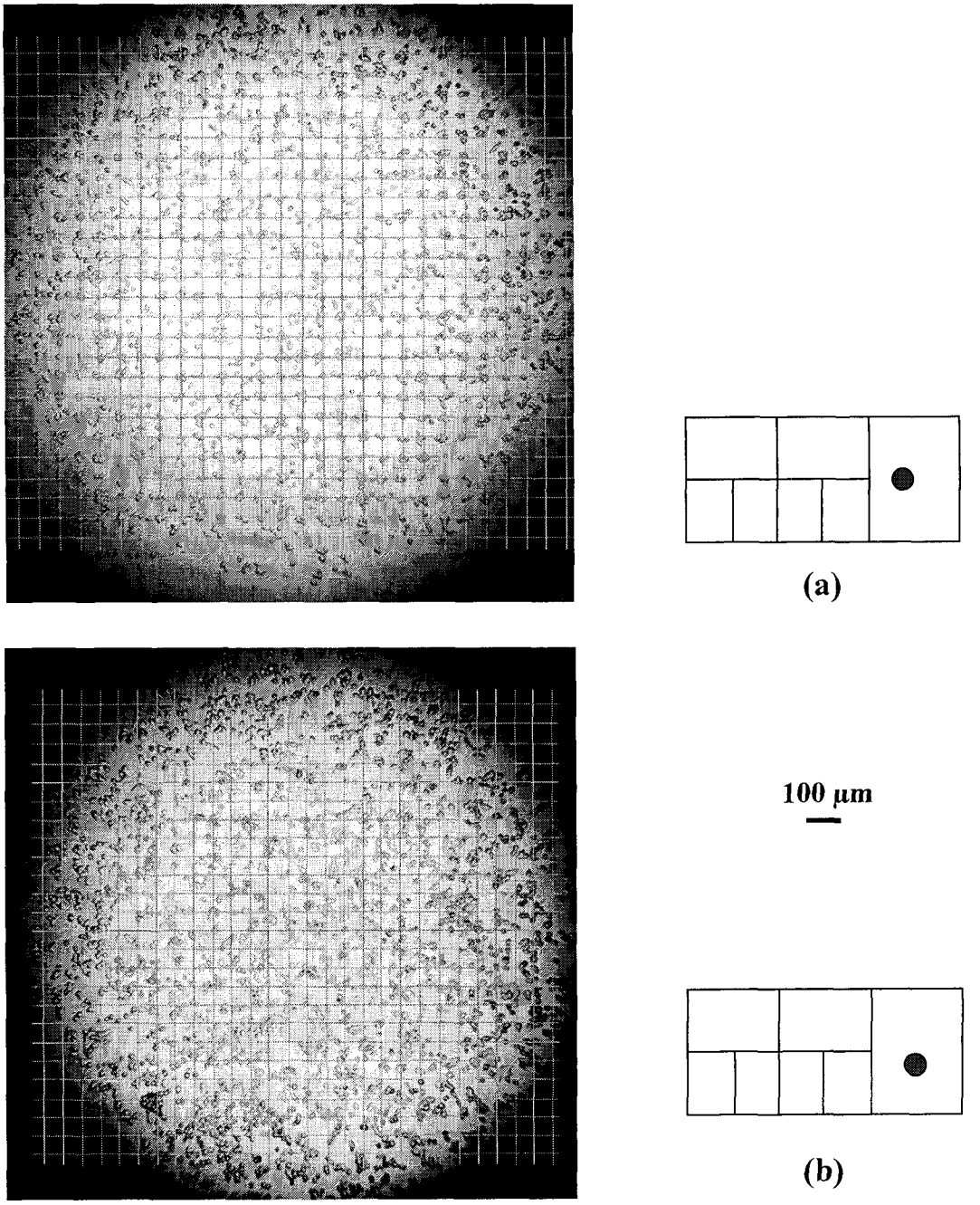


Figure 4.8 Images of Hela cells on the 20 μm-circle region on microchips (a) with PLL treatment (b) without PLL treatment. The chips were fabricated by the SAM patterning method. The grid lines with a spacing of 80 μm on the images were used as a reference for locating adhesion spots. The spot on each insert indicates the location on the chip where the corresponding picture was taken.

4.3.2 Jurkat Cell Patterning

The cell deposition test was also conducted with Jurkat cells by incubating the SAM patterned chips in a Jurkat cell solution (about 1×10^6 cells/mL) overnight. Unfortunately, under the microscope, no pattern was observed even on the 20 μm adhesion spot array (data not shown).

The Jurkat cell is a suspension-cultured cell, which is non-adherent to a surface. The Hela cell, a kind of weakly-adherent cell, is able to attach to a substrate. The different patterning results from these two cells may suggest that the PLL treated thiol-OH SAM can adsorb adherent and weakly-adherent cells, but is not able to capture non-adherent cells. Since thiol-OH encourages the adherence of proteins, treating the patterned surface with adhesion proteins, such as fibronectin[7] and collagen[8], may be a good way to enhance the binding affinity of non-adherent cells to the surface.

4.4 Conclusions

In this chapter, preliminary deposition tests were performed with Hela and Jurkat cells on microchips fabricated using the SAM patterning method (with PLL treatment). The resulting patterns of Hela cells indicate that they could be arranged in arrays on the 20 μm-circle and the 10 μm-circle adhesive regions. Both SAMs and PLL are demonstrated to be important in the cell array formation for Hela cells. However, on the regions with smaller-sized adhesion spots, cells became randomly distributed, which was probably due to the fact that one cell could bridge several adjacent spots. Cell distribution was studied on both 20 and 10 μm spots. The statistical studies show that in our experimental conditions, most 20 μm adhesion spots and a relative high percentage of 10 μm spots contained multiple cells. In order to achieve single cell per spot, further studies are needed to investigate the influence of the concentration of cell solution and determine the optimal conditions for cell deposition. Patterning of Jurkat cells did not show any clearly-defined patterns. The results demonstrate that the PLL treated thiol-OH SAM is not good at adsorbing non-adherent cells. The incorporation of adhesion proteins may promote the preferential attachment of these cells to the patterned surface.

4.5 References

1. Copper, E.; Wiggs, R.; Hutt, D. A.; Parker, L.; Leggett, G. J.; Parker, T. L., *J. Mater. Chem.* **1997,** 7, 435-441.

2. Lee, Z.-W.; Lee, K.-B.; Hong, J.-H.; Kim, J.-H.; Choi, I.; Choi, I., *Chem. Lett.* **2005,** 34, 648-649.

3. Ostuni, E.; Kane, R.; Chen, C. S.; Ingber, D. E.; Whitesides, G. M., *Langmuir* **2000,** 16, 7811-7819.

4. Yang, N., Cell Arrays on Chemically Modified Chip Surfaces, University of Alberta, Edmonton, 2004.

5. Freshney, R. I., *Culture of Animal Cells - A Manual of Basic Technique*, Alan R. Liss, Inc., New York, 1983.

6. Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E., *Science* **1997,** 276, 1425-1428.

7. Hyun, J.; Ma, H.; Zhang, Z.; Beebe, T. P. Jr.; Chilkoti, A., *Adv. Mater.* **2003,** 15, 576-579.

8. Rohr, S.; Fluckiger-Labrada, R.; Kucera, J., *Eur. J. Physiol.* **2003,** 446, 125-132.

Chapter 5 Conclusions and Future Work

The objective of this thesis is to explore a practical, convenient method for patterning cells on a solid substrate with a high-density grid pattern, and a suitable on-chip incubation approach to develop colony arrays. The results of our work demonstrate that this cell array can be successfully obtained using the two methods we developed. The challenge associated with growing cells on chip has been addressed by various methods. We found that the nitrocellulose (N/C) membrane transfer method is suitable for on-chip cell capture with subsequent culturing. This chapter summarizes the results and discussion presented in the preceding chapters, and advances suggestions that may lead to an improvement of the current experiments.

In Chapter 2, we presented the two fabrication methods, which we refer to as the gold patterning method and the SAM patterning method for creating high-density cell arrays. The gold patterning method was developed by our former group member, Ni Yang, and was described in detail in her thesis "Cell arrays on chemically modified chip surfaces." Briefly, a SAM pattern was generated by coating of cell-repellent thiol-EG-OMe (1-mercaptoundec-11-yl-triethylene glycol methylether) on a patterned gold surface, which was fabricated using DRIE and photoresist lift-off. An adhesion coating of PEI (polyethyleneimine) was then deposited in open regions. Using this method, the surface pattern can be detected under a microscope. After bacterial deposition, clear cell patterns were obtained. The relationship between the average numbers of cells per spot with bacterial concentration can be modeled by the Langmuir isotherm, as described in Ni's thesis. The SAM patterning method was newly developed in our lab. In this method, adhesion spots were defined by exchanging photooxidized thiol-EG-OMe SAM on a glass surface with thiol-OH SAM. Surface characterization using infrared reflectance absorbance spectroscopy (IRRAS) indicated that this method provided good surface chemistries for patterning bacteria. Compared to the gold patterning method, the fabrication process is simpler and faster, but adhesion spots can not be detected.

The results of cell patterning show that the SAM patterning method does not work with spot areas smaller than 3x 6 rectangle even after treatment with PEI, which can

enhance the adhesion ability of thiol-OH coated spots. However, we can not increase the PEI treatment time to enhance adhesion, since too long a time turns the hydrophobic surface of thiol-EG-OMe to hydrophilic one. Because small-sized spots are better for achieving a single cell array, future work will involve the investigation and application of new materials that have stronger interaction with *E. coli*.

Chapter 3 investigated different methods for on-chip culturing of *E. coli*. For the LB liquid method, chips were covered with a few mL of LB media and incubated using typical culture conditions. As a result, colony patterns were achieved. But the cell detachment problem caused cross contamination during culturing, which limited the application of this method. For the top agarose method, a diluted agarose growth solution was added on top of a chip that was placed on an agar plate. After gelation, the agar plate was incubated. This method suffered from the same problem as the liquid LB method in the stage before the solidification of the liquid gel. To solve this problem, the N/C membrane transfer method was developed, in which the cell patterns on a chip were transferred to a N/C membrane and incubated on an agar plate. Since no liquid media were used in this procedure, the cell detachment problem was eliminated. This growth method is suitable for on-chip *E. coli* incubation and could be applied to both SAM patterned chips and gold patterned chips. However, transfer efficiency was not very good, especially for small-sized adhesion spots. Future work should concentrate on enhancing the transfer efficiency between chips and membranes. A straightforward way is to increase the chip-membrane contact time. Because bacteria do not have enough oxygen and nutrients during the transfer step, a long contact time may lead to the death of substantial portion of cells. Therefore, a change to this transfer step is needed. One possible method is to apply an electrical field to accelerate the transfer rate and thus increase the transfer efficiency.

In Chapter 4, deposition tests were performed with two kinds of animal cells, Hela and Jurkat cells on microchips fabricated by the SAM patterning method. The resulting patterns of Hela cells indicate that they could be arranged in arrays on the 20 μm-circle and the 10 μm-circle adhesive regions. Both SAMs and PLL are demonstrated to be essential in the cell array formation. However, on the regions with smaller-sized adhesion spots, cells became randomly distributed, probably due to cells bridging several adjacent

spots. Cell distribution was studied on both 20 and 10 μm spots. The statistical studies show that in our experimental conditions, most 20 μm adhesion spots and a relative high percentage of 10 μm spots contained multiple cells. In order to achieve single cell per spot, further studies are needed to investigate the influence of the concentration of cell solution and determine the optimal conditions for cell deposition. Patterning of Jurkat cells, one kind of suspension-cultured cells did not show any clearly-defined patterns. The results demonstrate that the PLL treated thiol-OH SAM is not good at adsorbing non-adherent cells. This leads to a suggestion that the incorporation of adhesion proteins, such as fibronectin and collagen may promote the preferential attachment of these cells to the patterned surfaces.