

Micro- and Nanopatterning for Bacteria- and Virus-Based Biosensing Applications

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Abstract

Current technologies capable of rapidly and accurately detecting the presence of infectious diseases and toxic compounds in the human body and the environment are inadequate and new, novel techniques are required to ensure the safety of the general population. To develop these technologies, researchers must broaden their scope of interest and investigate scientific areas that have yet to be fully explored. Lithography is a common name given to technologies designed to print materials onto smooth surfaces. More specifically, micropatterning encompasses the selective binding of materials to surfaces in organized microscale arrays. The selective micropatterning of bacteria and viruses is currently an exciting area of research in the field of biomedical engineering and can potentially offer attractive qualities to biosensing applications in terms of increased sensing accuracy and reliability. This chapter focuses on briefly introducing the reader to the fundamentals of bacterial and viral surface interactions and describing several different micropatterning techniques and their advantages and disadvantages in the field of biosensing. The application of these techniques in healthcare and environmental settings is also discussed.

1. Introduction

There is a great need for the development of techniques that can rapidly screen for the presence of infectious diseases or toxic compounds in the human body and the environment (Suh et al. 2004). Viral outbreaks, such as severe acute respiratory syndrome (SARS) and avian flu, have created a rising awareness about toxic and infectious agents in our surroundings. Antimicrobial agents have been developed to help treat patients suffering from exposure to harmful bacteria and viruses; however, bacteria, fungi, and viruses have all begun to develop resistance to the antimicrobial agents directed against them (Moellering 1995). Microbial resourcefulness and resilience have never been more evident than in their ability to develop resistance to chemotherapeutic agents (Wood and Moellering 2003). Bacteria accomplish this through the modification of their DNA by chromosomal mutation and then by acquiring resistance genes via conjugation, transformation, and even transduction (Wood and Moellering 2003). An example of this is the demonstration of transferable fluoroquinolone resistance genes in *Klebsiella*

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pneumoniae shown by Tran (2002). Additionally, the emergence of past viruses that were previously eradicated through the delivery of vaccines reaffirms that faster and more reliable techniques to identify viruses and bacteria must be found. This can be seen in the devastating re-emergence of wild poliovirus in Namibia (Roberts 2006). This recent outbreak has resulted in many deaths and has the potential to become a very serious threat. This example also illustrates the increasing need for bacterial and viral detection on a global basis. With the recent advances in global transportation the ability of a lethal microbial infection to spread across the world is greater now than ever before.

Traditional laboratory diagnoses of viral and bacterial infections typically involve the use of direct fluorescent antibody assays, viral load testing and cell culturing (Whiley and Sloots 2005). Techniques such as Enzyme-Linked ImmunoSorbent Assay (ELISA) use two antibodies, one linked to an enzyme and the other specifically bound to an antigen, to identify virus presence (Ivanov and Dragunsky 2005). The enzyme acts as an amplifier, converting a substrate to a chemogenic or fluorescent signal that is easily detected by a spectrophotometer (Ivanov and Dragunsky 2005). This technique may be highly sensitive, however, it requires highly trained technicians and high maintenance equipment, which make it unfeasible in many underdeveloped regions of the world (Respass, Rayfield and Dondero 2001). Another method of cell culturing bacteria involves placing a sample in a plated environment and waiting for colonies of bacteria to form and grow. This technique is simple but too time consuming to be considered valuable.

Other viruses, such as HIV, can be detected through the use of viral load testing (Rich et al. 1999). Viral load testing detects cell-free plasma viral RNA through amplification techniques, such as polymerase chain reaction (PCR). Diagnostic techniques such as these provide quick results; however, they are generally not sensitive enough to provide reliable and consistent results (van et al. 2001).

Viruses and bacteria can also be detected through cell culturing methods. This involves obtaining a sample of interest and filtering it with a membrane capable of removing the microbes of interest. The microbes are subsequently detached from the membrane and transferred into a highly concentrated solution. Following this, a host cell is entrenched in the solution, and if there are microbes present, they will infect the host cell and initiate conformational changes that will be easily visible by light microscopy. These methods provide robust and sensitive results but are labor-intensive, time consuming and depend on optimal sample transport for virus isolation (Doller et al. 1992).

To minimize these potential difficulties, miniaturization of cell-based bioassays is currently under active study for the detection of toxic compounds in the environment (Dunn and Feygin 2000). Specifically, bacteria cells are useful in sensing applications because their analytic specificity is easily modified by genetic engineering and their dynamic structure as compared to mammalian cells (Rainina et al. 1996). Recent miniaturization of such assays has included the patterned collection of cells in microsystems, where cellular adhesion can be easily controlled through physical and chemical characteristics such as hydrophobicity, hydrophilicity and surface charge (Koh et al. 2003).

The application of micro and nanopatterning to the problems outlined above has the potential to eliminate the difficulties mentioned and generate newer, easier, cheaper and more reliable bacterial and viral detection methods. Micropatterning can be described as the selective binding of materials to surfaces in organized arrays at the micro and nanoscale. Currently used to pattern cells and proteins, micropatterning incorporates microfabrication techniques with materials science and surface chemistry to explore the interactions between cells that require surface attachment for proper function (Falconnet et al. 2006).

Micropatterning bacteria and viruses have the potential to offer three significant advantages not present in current detection technologies. First, screening sample volumes can

be greatly minimized with the application of micro and nanotechnology. This technology has the potential to significantly reduce the required sample volume for detection testing, therefore making sample collection easier. Secondly, micro and nanopatterning may also be used to increase screening throughput. This is possible because of the potential to test for many different bacteria in a very small volume of sample. Many different bacteria and viruses could be patterned on a single surface, thereby increasing the number of simultaneous detections. Finally, micro and nanopatterning may also offer higher sensitivity than current technologies. This may be attributed to the ability to sense and track bacteria at much smaller scales.

The goal of this review is to briefly describe the fundamentals of bacterial and viral surface interactions, to discuss the emergence of micro and nanotechnologies used to pattern bacteria and viruses as well as give examples of specific applications. Furthermore, we will discuss challenges that exist in the development of this technology.

2. Fundamentals of Bacterial and Viral Surface Interactions

To understand the fabrication of micropatterned surfaces for bacteria, one must understand the mechanism of bacterial adhesion. When a bacterium initially comes in contact with a substrate surface it binds reversibly through non-specific interactions (Bonin, Rontani and Bordenave 2001). This leads to eventual irreversible binding through the formation of a protein-ligand interaction (Razatos et al. 1998). Therefore, the adhesion of bacterium, and most other cell types, is dependent on the formation of a protein layer on the surface of the substrate. As such, prevention of this protein layer can be applied to create biocompatible materials that resist bacterial adhesion. Such materials include polyethylene glycol (PEG) polymers, self-assembling monolayers (SAMs) terminated by a PEG group, polysaccharides and phospholipids (Kingshott 1999). Lipids can also be involved in the initiation of bacterial adhesion, such as on the surface of hydrogel contact lenses (Franklin 1993); however, inhibiting protein formation is considered the primary objective when designing biocompatible materials (Kingshott 1999).

Viral infection of cells is initiated by the attachment of the virus to cell receptors present on the cell membrane (English and Hammer 2005). Therefore, by inhibiting receptor-mediated attachment, viral infections can potentially be reduced (English and Hammer 2005). The attachment is mediated by viral attachment proteins (VAPs) which are unique to each individual type of virus (Wickham et al. 1990). Several virus types have their VAPs and respective receptors identified. For example, Human Immunodeficiency VAP is a 120-kD glycoprotein that binds to a CD4 receptor present on the surface of T-cells (Wickham et al. 1990). Although many virus types bind through receptor-mediated binding, the nature of this binding varies quite significantly. This can be in terms of number of VAPs per virus and receptor types as well as binding affinity (Wickham et al. 1990). Although receptor-mediated linkages are considered the most common form of viral attachment to cells, there are obviously different methods with which to place viruses onto surfaces. Cheung et al. (2003) report that the fabrication of nanoscale virus arrays can be completed through the placement of a chemoselective linker to the surface of the virus, which enables the virus to attach to a patterned template that is manufactured using scanning probe nanolithography. In another study, Suh et al. (2006) tested the binding affinities of M13 viruses to surfaces using P3 and P9 antibodies. Fig. 32.1 shows scanning electron microscope (SEM) images of the M13 virus on a silicon wafer. Studies such as these may potentially be used to create virus arrays useful for the creation of miniaturized electronic devices or biosensors (Mao 2003).

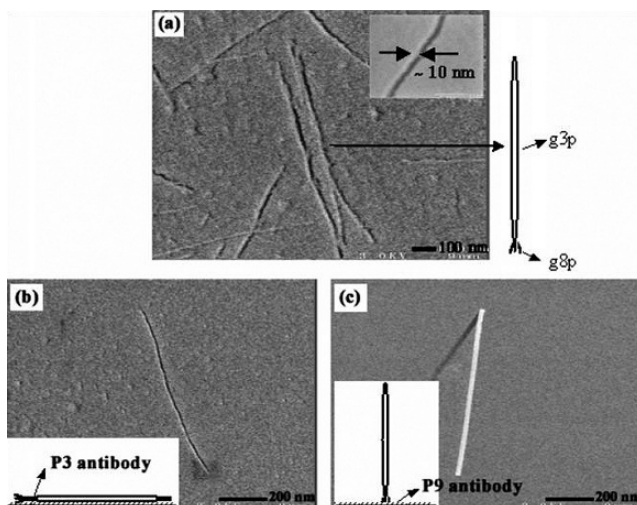


Figure 32.1. (a) SEM image showing the M13 viruses that were cast from a solution onto a silicon wafer. The width of the virus was increased from ~ 6 nm to ~ 10 nm because of the gold coating (inset). (b-c) SEM images showing the transition of virus morphology on the P3 and P9 antibody treated silicon wafer, respectively (Reproduced with permission from Nano Letters 2006, 6, 1198–1201. Copyright 2006 American Chemical Society).

3. Technologies for Patterning

3.1. Overview

There have been multiple techniques utilized in the development of bacterial and viral micropatterning. These techniques have been successfully adapted from other fields of research, ranging from microelectronic fabrication to miniaturization of patterning of mammalian cells (Falconnet et al. 2006). Photolithography, soft lithography and scanning probe lithography are described below and examples of relevant research are also included.

3.2. Photolithography

Historically, photolithography has been the most commonly used technique in micropatterning (Xia and Whitesides 1998). This technique was first implemented in the integrated circuit fabrication industry and has been adopted into the biomedical field (Xia and Whitesides 1998). Photolithography involves the placement of the precursor solution onto the substrate of choice. Although there are several variations to photolithography, the main characteristics are the use of a photoresist mask with the pattern of interest and illumination of the precursor solution under UV light.

Koh et al. (2003) report that substrate surface can be modified with the use of a polymer. They suggest the first step in photolithography is to make a precursor solution consisting of the polymer to be photopolymerized and a UV light photo-initiator. The precursor solution is then spin coated onto the surface of the substrate in order to ensure that the substrate is covered with a thin, uniform coat. Correspondingly, faster spin coat speeds result in thinner wells, which reduce the overall volume of the well (Koh et al. 2003). Photopolymerization is then performed under UV light, where only the exposed regions of the precursor solution experience photopolymerization. The areas covered by the mask can then be washed away, leaving the mask's projection patterned on the surface.

Disadvantages to photolithography include high costs associated with the equipment necessary to perform this technique, the required access to a clean room and the method's ineffectiveness at patterning non-planar surfaces (Xia and Whitesides 1998).

3.3. Micromolding (Soft Lithography)

Soft lithography is a technique that uses elastomeric molds to transfer patterns to the surface of the substrate (Whitesides et al. 2001). Soft lithography incorporates many different techniques, including replica molding (Ng et al. 2002), micro contact printing (μ CP) (Takayama et al. 1999), microtransfer molding (Zhao, Xia and Whitesides 1996) and capillary force lithography (Suh et al. 2004). In most of the aforementioned techniques a stamp is created by molding an elastomer around a replica of the desired pattern, thus creating a negative replica. Most of the research occurring in soft lithographic techniques focuses on the use of PDMS as the elastomer (Ismagilov et al. 2001; Whitesides et al. 2001; McDonald and Whitesides 2002; Ng et al. 2002). PDMS has many of the desired characteristics of the elastomer, including being biocompatible and permeable to gases. In addition, it can be used for cell culture (Xia and Whitesides 1998). Softer materials such as PDMS offer exciting alternatives to photolithography due to their decreased costs, chemical versatility and potential biodegradability (Ismagilov et al. 2001; Whitesides et al. 2001).

3.3.1. Replica Molding

Replica molding (illustrated in Fig. 32.2a) is a very useful technique for replicating a patterned three-dimensional (3-D) solid surface into a reusable, elastomeric form. This process begins with the formation of a structure referred to as a *master* (Whitesides et al. 2001). The master is the original mold that was created through photolithography (as previously described). This master is initially cast in an elastomer, such as PDMS, and allowed to cure. This is accomplished by simply pouring the elastomer over the master mold. Once cured, the elastomer is peeled off of the master and the resulting "replica" can then be used in microcontact printing as a stamp. Elastomers are used as the replica material due to their soft structure (Whitesides et al. 2001). The nature of the master is typically rigid, and therefore the soft nature of the elastomer helps facilitate separation of the two. The master may also be exposed to $\text{CF}_3(\text{CF}_2)_6(\text{CH}_2)_2\text{SiCl}_3$ overnight to help ease the separation of the master and the elastomer replica (Whitesides et al. 2001).

3.3.2. Microcontact Printing

Microcontact printing (illustrated in Fig. 32.2b) is used in conjunction with replica molding to create micropatterned wells that can be used for bacteria and virus patterning (Whitesides et al. 2001). The stamp produced in replica molding is coated with an alkanthiol and brought into contact with a thin coat of gold that sits on top of a silicon substrate (Whitesides et al. 2001). When the stamp is removed the alkanethiol leaves a self-assembled monolayer on the surface of the gold. The monolayer self assembles due to the tendency of sulfur atoms in the thiol chain to coordinate the gold surface and expose the terminal groups of the alkanethiol. Sulfur will react similarly with silver; however, gold is only used in bacterial and viral applications because silver is cytotoxic (Whitesides et al. 2001). The stamp is then removed and the SAM is left on the surface of the silicon in the shape that is printed on the surface of the stamp. The concentration of the alkanthiol used can affect the quality of the SAM and the resolution of the printed features. Microcontact printing is advantageous in situations where it is desirable to transfer large patterns or series of patterns onto a substrate in one step (Whitesides et al. 2001).

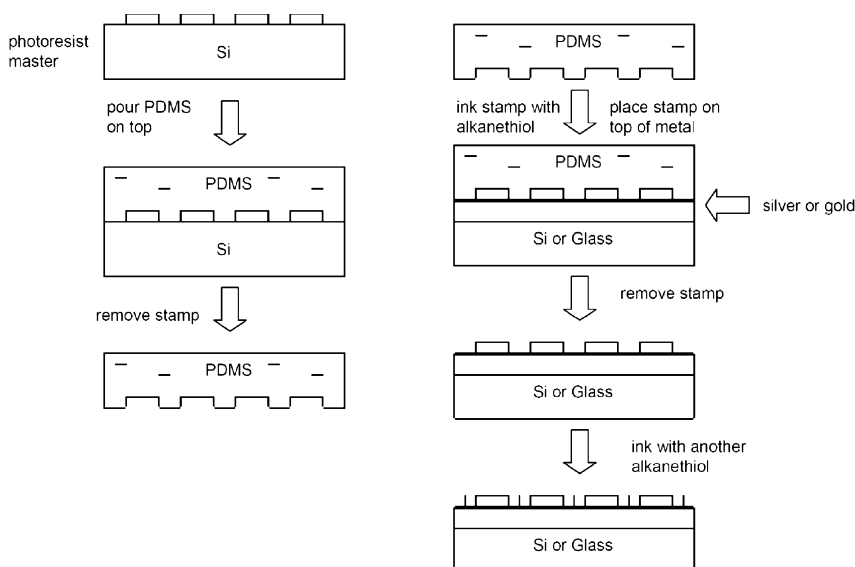


Figure 32.2. A schematic outline of micropatterning by preparation of PDMS stamp using replica molding (a), followed by pattern transfer by microcontact printing (b).

St. John et al. (1998) used microcontact printing to stamp an antibody grating pattern on a silicon surface in a way as to permit the binding of antigen to the antibody, thus creating a biosensor. The performance of the sensor was tested by encapsulating *E.coli* cells on the antibody stamped lines and measuring the intensity of a first-order diffraction beam resulting from the attachment. They found that the diffraction intensity increased with cell density on the surface of the antibody.

Through microcontact printing Morhard et al. were able to use a similar approach in their report of the attachment of covalently coupled antibodies to the surface of a gold substrate. These antibodies were able to selectively bind *E.coli* at well-defined adsorption sites. An incident laser beam was then used to measure light intensity changes in the diffraction pattern of the surface of the substrate, thus verifying the presence of the bacteria.

3.3.3. Microtransfer Molding

In microtransfer molding a patterned PDMS mold is used as a replica of the pattern of interest (Xia and Whitesides 1998). A drop of polymer solution is placed onto the PDMS mold and covered with a substrate. After heating or irradiating, the mold is peeled off of the liquid precursor, leaving a patterned microstructure present on the surface of the substrate. The most advantageous use of microtransfer molding is when molding on a non-planar surface (Xia and Whitesides 1998). This is particularly useful when constructing 3-D microstructures using a layer-by-layer methodology (Xia and Whitesides 1998). Another advantage of microtransfer molding is its ability to fabricate microstructures over large surface areas in a relatively short period of time (Xia and Whitesides 1998).

3.3.4. Capillary Force Lithography

Capillary force lithography is another soft lithographic technique that involves the placement of a PDMS stamp onto the surface of a spin-coated polymer film (Suh, Kim and Lee 2001). Depending on the capillary forces and the surface interactions of the polymer

with the stamp, a variety of different polymeric shapes and structures could be formed. The placement of the stamp is followed by a short period of time in which the stamp and the substrate must remain undisturbed. During this time the polymer forms a negative replica of the stamp by spontaneously moving into the stamp's void space via capillary action. If the thickness of the film is small compared to the height of the stamp then the polymer moves to the edges of the stamp rather than fill the void space, where it localizes (Suh, Kim and Lee 2001).

Suh et al. (2004) demonstrated that bacterial cells could be micropatterned using host-parasite and virus-antibody interactions. In this procedure, virus-antibody interactions were introduced to enhance selectivity. It was determined that the adhesion of bacteria was significantly reduced on the surface of PEG when compared to that of silicon or glass (Suh et al. 2004). However, it was also determined that non-specific adhesion became evident when the concentration of bacteria was increased (Suh et al. 2004). This led to a reduction in specificity, which is very undesirable. Fig. 32.3 illustrates the procedure used for creating bacteria microarrays using capillary lithography.

3.4. Scanning Probe Lithography

Scanning probe lithography (SPL) encompasses a wide range of techniques that all share some similar qualities. Each involves the nanoscale surface modification of a substrate using

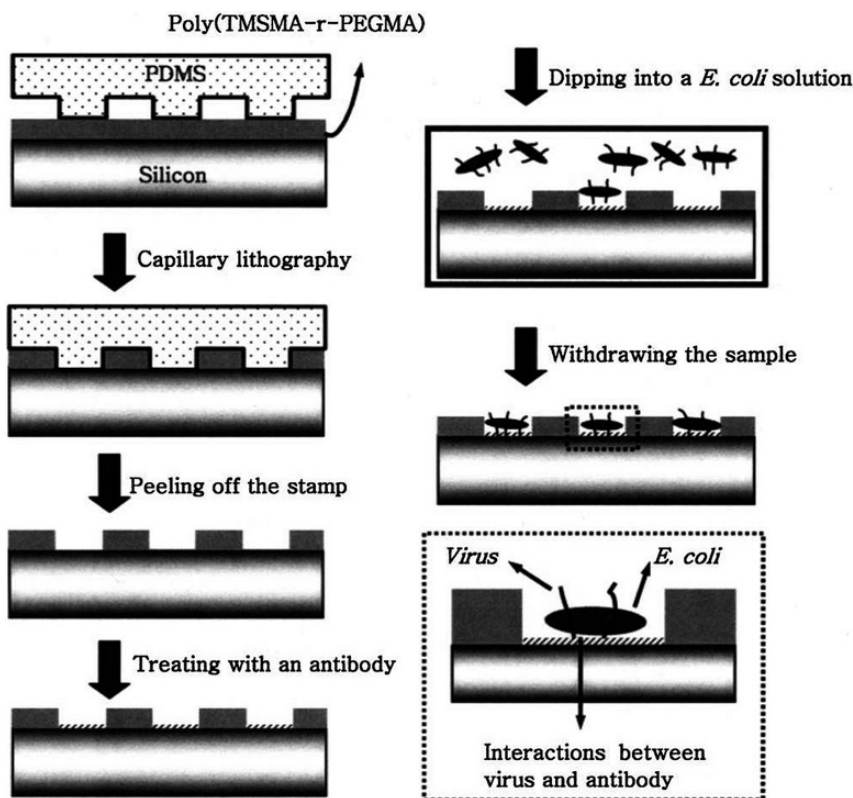


Figure 32.3. Experimental procedure of bacterial array fabrication using capillary lithography (Reprinted from Suh et al. 2004 with kind permission of Springer Science and Business Media).

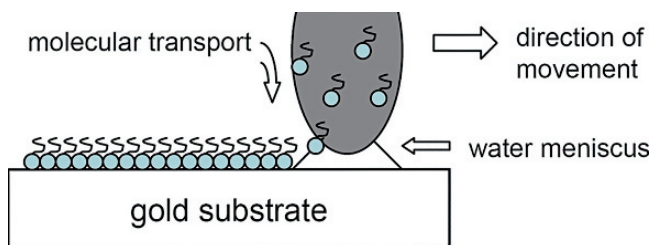


Figure 32.4. Schematic illustration of Dip-Pen nanolithography.

an atomic force microscope (AFM). The four most commonly used SPL techniques are dip-pen lithography, nanoshaving, nanowearing and nanografting.

As shown in Fig. 32.4, dip-pen nanolithography uses an AFM, operated in contact mode, to deposit thiolated molecules onto the surface of a gold substrate. The tip of the microscope is extremely thin and coated with a thiol, such as 1-octadecanethiol (ODT). When the tip of the microscope is covered with ODT and brought into contact with the surface of the gold, a water meniscus forms between the two. The size of the meniscus is controllable by the relative humidity present on the surface of the gold. The size of the meniscus affects the contact area between the tip and the gold, and correspondingly the resolution of the image. From the development of this novel technique, other forms of nanolithography have begun to emerge. These include nanoshaving, nanowearing and nanografting. These techniques permit scientists to create patterns of thiol groups in the gold substrates.

Nanoshaving uses an AFM to exert very high pressure onto the surface of molecules that have adsorbed to the surface of a substrate. This high pressure creates a high shear force on the attached molecules, which results in their immediate displacement. It is important to note that the load applied by the microscope must be higher than the displacement threshold of the attached molecules. Nanoshaving is commonly used because of its ability to fabricate holes and trenches in a single scan. Nanowearing is quite similar to nanoshaving, the key difference being that a lower force is applied to the molecules over an extended period of time. This allows groups to be gradually removed, a major advantage when working near the edge of a substrate and around sites that have been damaged or defected. Nanografting is another nanoscale technique that uses AFM technology. AFM tips remove molecules from the substrate surface by lightly brushing against their terminal chains. In the case of thiol groups attached to a gold substrate, if the surface of the AFM is immersed in a different thiol, it can be attached to the gold in the place of the removed thiol group.

All of the aforementioned techniques can be applied to the micro and nanopatterning of bacteria, viruses and proteins onto surfaces. These applications offer exciting new advances in the biomedical and biosensing fields, and shall be discussed in the following section.

4. Biosensing Applications and Examples

4.1. Overview

Biosensors can be adapted to many different applications, from sensing the presence of a toxin in the atmosphere to sensing an infectious disease in the body. The ability to selectively place bacteria and viruses on patterned surfaces presents tremendous opportunities in many different biomedical and biosensing applications. In conjunction with this, the ability to micro and nanopattern proteins capable of binding bacteria and viruses is equally important. Bacterial

adhesion to substrate surfaces is typically accomplished by culturing solutions in LB broth and then applying them directly to the micropatterned substrate. The substrates must then be incubated in order to allow the bacteria time to adhere to the surface. Subsequent to the incubation period, the substrates are rinsed with fresh LB media and stained for imaging of the cells (Koh et al. 2003). Fig. 32.5 shows several different images of the process of bacterial patterning on a silicon substrate. Viral attachment is completed in much the same manner; however, recent work has been done using nanolithographic techniques and with chemoselective linkers to fabricate similar viral assemblies (Cheung et al. 2003). These methods can then be used to investigate how the interaction of intervirions affects such things as assembly morphology and kinetics.

Biosensors typically consist of a biologically specific recognition system, a signal emitted from this system when the target binds and a physical or electrochemical transducer to selectively and quantitatively convert this binding reaction into a machine-readable output signal (Hall 2002). The recent development of biosensor technology has reduced the interface between the biological recognition system and the transducer to the nanometer scale (Hall 2002). An example of this is the generation of a pH change at the surface of an electrode in response to

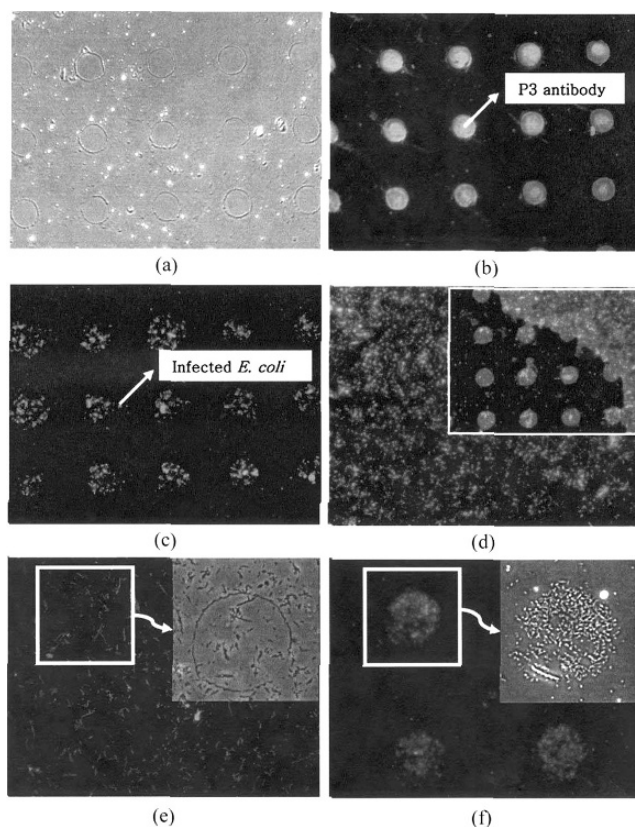


Figure 32.5. (a) An optical micrograph for the initial microstructure of 50 μ m circles; (b) a fluorescent micrograph for (a) stained with FITC-labeled secondary antibody; (c) an optical micrograph for aggregated arrays of *E. coli* onto the same pattern; (d) a fluorescent image of *E. coli* adsorption onto a bare silicon substrate without a predefined pattern (control) the inset shows the boundary between the patterned and non-patterned regions; (e) a fluorescent image of bacteria patterning without the aid of virus (inset: the corresponding optical image for the box region); and (f) a fluorescent image for the pattern stained with a different dye (inset: the corresponding optical image for the box region) (Reprinted from Suh et al. 2004 with kind permission of Springer Science and Business Media).

the release of CO₂ by urease (Cullum and Vo-Dinh 2000). This change can then be transduced by any range of highly sensitive electrochemical or fluorescent methods (Hall 2002). Dill et al. (1999) applied this principle for the detection of *Salmonella* in poultry using biotin–fluorescein-conjugated anti-*Salmonella* antibodies with an anti-fluorescein urease conjugate attached to the immunocomplex.

4.2. Healthcare Applications

Biosensors have a wide range of applications in healthcare. Miniaturized point-of-care tests that can be operated by consumers are projected to emerge in coming years over centralized laboratories with large-scale tests. An example of this is the development of glucose biosensors for patients with diabetes. Glucose biosensors have been generally found to exhibit high affinity, high sensitivity and fast response for the detection of glucose in the blood stream.

The adhesion of bacteria to mammalian cells is a common cause of infection within the human body. The ability to understand and inhibit this adhesion has the potential to be a valuable tool in the development of anti-bacterial therapeutics. There are several methods to evaluate the activity of potential inhibitors of bacterial adhesion, including hemagglutination inhibition assays and solid-phase binding (ELISA-type) assays (Qian et al. 2002). Hemagglutination inhibition assays are based on the conglomeration of erythrocytes in the presence of lectins and other proteins that are capable of the recognition of ligands on the surface of the cell (Goldhar 1995). The degree of complexity of the carbohydrates presented on the surface of erythrocytes often hinders agglutination; therefore, results from hemagglutination inhibition assays are not always reproducible (Qian et al. 2002). Solid-phase binding assays generally involve ligand or receptor non-covalent adsorption to a surface. Ligand or receptor density is difficult to control, however, and bovine serum albumin (BSA) is used to block the uncoated surface sites and therefore reduce nonspecific adsorption. Even when the ligands are attached covalently, ligand density can vary, and blocking may be necessary.

In response to this, the use of SAMs on gold substrates is structurally well defined and offers great control over ligand density and environment (Ulman 1996). Previous results demonstrate that SAMs prepared from alkanethiols terminated with oligo(ethylene glycol) groups effectively resist the nonspecific adsorption of proteins and the nonspecific adhesion of mammalian cells (Prime and Whitesides 1991; Qian et al. 2002). Therefore, SAMs with alkanethiols terminated in oligo(ethylene glycol) groups and alkanethiols terminated with the ligand of interest provide an excellent model surface with which to study bio-interfacial problems (Qian et al. 2002). This was demonstrated by Qian et al. through the development of arrays of SAMs for the study of bacterial adhesion (2002). Specifically, the authors studied the adhesion of uropathogenic *E.coli*, which when bound to mammalian cells in the bladder and/or urinary tract, is a leading cause of urinary tract infections (Barnett and Stephens 1997). They suggest that SAMs consisting of alkanethiols terminated in oligo(ethylene glycol) groups provide biospecific surfaces that promote the adhesion of uropathogenic *E.coli* through interactions between multiple pili present on the surface of the bacteria and multiple copies of mannosides present on the SAM surface (Qian et al. 2002). This work helps to provide methods with which to study the effects of bacterial adhesion, which can then be applied to the development of new and novel therapeutics to prevent bacterial attachment and help eliminate bacterial based infections.

Bacterial infections may also be caused by the formation of a biofilm within the human body. Biofilms form on the surfaces of materials when bacteria first adsorb to a material's surface and then excrete a slimy, glue-like substance (commonly called an exopolysaccharide matrix) that anchors them in place (Costerton, Stewart and Greenberg 1999). Due to their

inherent resistance to antimicrobial agents, the development of engineered biofilms could potentially be a valuable tool for understanding their mechanisms of formation and the development of techniques to treat the infections they cause (Stewart and Costerton 2001). Bos et al. (2000) began research in this area when they used photolithography and the formation of biofilms on teeth and voice prosthesis to illustrate that biofilm preferential growth did not exist between hydrophilic and hydrophobic surfaces. Cowan et al. (2001) continued this research with the development of poly-L-lysine (PLL) surfaces to enhance bacterial adhesion to surfaces of interest. Previously, the attachment of OmpR (oroitidine-5'-monophosphate) has been used to promote bacterial attachment to a surface (Vidal et al. 1998); however, laboratory strains of *E.coli* lack the required *ompR* gene, and therefore are non-adherent. Cowan et al.'s (2001) technique demonstrated that bacteria could be patterned onto surfaces, and artificial biofilms could be engineered. This technology could be potentially applied to fields where biofilm formation is preferred, such as in the reduction of biochemical oxygen demand in the treatment of wastewater (Nicolella, van Loosdrecht and Heijnen 2000).

4.3. Detection of Toxins in the Environment

As mentioned previously, there is an increasing need for the ability to detect toxic substances in the environment. The increased specificity offered by techniques such as SPL may make it possible to detect very small amounts of toxins in the air. The sources of these toxins could range from bioterrorists to extended growth of mold in a home. In either case, the fast, reliable detection of the toxin is required to maintain safety.

SAMs on gold substrates offer some unique characteristics that make them excellent for the application of biosensing. Firstly, SAM can be miniaturized on gold through the use of microcontact printing (Whitesides et al. 2001). Secondly, the high density and ordered nature of long-chain alkanethiols closely resembles the structure present in lipid bilayer membranes (Chaki 2001). This feature has the potential to provide novel substrates for biological systems (Chaki 2001). Furthermore, the compatibility of SAMs with metal substrates make electrochemical measurements simple and enable easy applications for biosensors requiring measurements of current or potential (Chaki 2001). Finally, the ability of the SAM to remain stable after interface with immobilizing molecules for biological sensing integrated with an electrochemical transducer make SAMs tremendous options for biosensor fabrication and development (Chaki 2001).

Conversely, the ability of bacteria to selectively attach to micropatterned substrates has also been studied for micro and nanosensing applications. Bacterial cells have been found to be relatively robust when compared to their mammalian counterparts, a characteristic that is important in biosensing applications. The increased density of selective attachment of bacterial cells could result in an increased amplification of signal transduction. This property is of significant interest in the field of biosensor development.

There has also been research into the development of methods in which spores can be micropatterned onto the surface of a glass substrate (Park et al. 2004). The increased stability of spores, in comparison with other conventional cells used in micropatterning, as well as their ease of manipulation could be of great benefit in the development of cell-based sensors and detectors (Park et al. 2004). Specifically, microcontact printing has been used to micropattern wells of *Bacillus thuringiensis* (BT) spores that displayed enhanced green fluorescent protein (EGFP) (Park et al. 2004). In the presence of nutrients such as sugars and amino acids the spores germinated into vegetative cells, thus suggesting that any microbial cell could be micropatterned by spatially addressing its spores onto a substrate and then allowing them to germinate. Such cells would include bacteria, yeasts and filamentous fungi, which are all spore-forming microbial cells.

4.4. Real Devices and Challenges

The examples given above show there has been a significant increase in the amount of research being done in the field of micropatterning of bacteria and viruses. The methods currently employed in the detection of viruses are considered unreliable, inconsistent, expensive and unfeasible in regions of the world where simplicity and cost effectiveness are mandatory. Research currently being completed is a direct result of the inefficiencies present in other approaches used, and initially the technologies previously described show great promise in bacterial and viral detection.

Traditional photolithographic techniques continue to be unavailable to regions of the world that do not have access to clean-room laboratories and large, expensive facilities. However, there are significant steps being taken toward approaches that focus on the use of photoresist masks and UV illumination without the use of conventional substrates and precursor solutions. These advances eliminate the need for clean rooms and suggest that further work may be completed to ease the use of photolithography in areas where it has previously been inaccessible.

Soft lithography has characteristics that are unique in microfabrication, and that enable the techniques it encompasses to be carried out conveniently, rapidly and relatively inexpensively (Whitesides et al. 2001). Procedures involving larger features (on the scale of $>1\mu\text{m}$) can also be conducted in an unprotected laboratory environment, thereby enhancing their usefulness in locations where normal microfabrication settings are available or where equipment costs must be minimized (Xia and Whitesides 1998). Generally, the aforementioned techniques are also based on concepts that do not require specialized technicians to perform them—another advantage over current technologies. Due to these advantages, soft lithographic approaches offer significant potential in the micro and nanopatterning applications.

Dip-pen lithography currently boasts a significant increase in resolution, when compared to other lithographic techniques; however, more development is necessary to make it feasible in areas of the world where skilled technicians and expensive laboratory equipment are not readily available. The techniques that offer this higher resolution at the nanoscale can be expected to be concentrated on in future research for the continued miniaturization of techniques and methods of patterning bacteria and viruses.

5. Conclusions and Future Perspectives

The development of the micropatterning techniques outlined above continues to be a major topic of interest in the field of biomedical engineering. The development of micropatterning methods has tremendous implications in the growth of biosensors and biomedical applications. These applications include the development of new and novel antibiotics as well as biomaterials capable of inhibiting bacterial adhesion, which makes them useful for human implantation. The understanding of biofilm formation may also make it possible to inhibit bacterial adhesion, therefore enhancing the capabilities of such biomaterials. The continued miniaturization of lithographic patterning techniques has the potential to enhance patterning resolution, thus creating more bacterial and viral specificity and effectively enhancing the capabilities of prospective biosensors. Correspondingly, continued miniaturization will also help the fight against bacteria that have recently been found to be resistant to traditionally prescribed antibiotics. These developments will be coupled with the goal of reducing the capital costs associated with the equipment necessary to perform these methods and the requirement for skilled technicians to operate this equipment. These aspects could potentially allow these techniques to be used on a worldwide platform, ranging from inside North American laboratories to the far-reaching portions of under-developed societies. This is a crucial aspect of the detection of bacteria and viruses because the spread of these potentially dangerous microbes is far easier today than

even ten years ago. The probability of a foreign toxic agent reaching a developed country has become much greater with the advancement of transportation, and this technology is a vital component in the fight against such an attack. If history has taught us anything, it should be that bacterial and viral outbreaks can occur at any place and at any time, and it is in everyone's best interest to be ready for them.

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