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Pattern Technologies for Structuring Neuronal Networks on MEAs

John C. Chang and Bruce C. Wheeler

7.1 Introduction

Much progress has been made over the past several centuries regarding the understanding of the human central nervous system. This progress has taken us from knowing the brain as a functionally homogeneous organ to one with interconnected networks of specialized neurons. The initiating study was conducted by Broca who examined a patient with a left-sided posterior frontal lobe lesion which resulted in dysphasia. Broca concluded that the ability to generate coherent speech resided in the dominant posterior frontal lobe and theorized that distinct regions of the brain functioned uniquely. This theory was further developed by Wernicke who speculated that distinct regions of the brain intercommunicated to achieve the observed complex human behavior. Findings from both electrical stimulation and seizure studies support this theory as motor and sensory functions can be localized to specific regions of the brain (Kandel et al., 2000). These experiments provided the foundation for the interconnected network theory, the details of which are being expounded daily by the numerous functional imaging studies reported in the literature (Kandel et al., 2000).

Although the progress in understanding the regional interaction between brain regions has been exceptional, the detailed understanding of the neuronal firing patterns, either in aggregate as in electroencephalography (EEG) or in simultaneous multi-site monitoring as in cortical probes, is desirable. The advantage of understanding the patterns lies in the ability to diagnose diseased states or in cognitive control of exogenous actuators such as robotic arms or input devices to computers (Kandel et al., 2000; Nicolelis, 2001). Diagnosis of seizures is usually confirmed when third-person observation provides suitable symptoms such as absence, tonic or clonic muscular activities, and loss of consciousness, but EEGs can be diagnostic when proper neuronal activities are detected (Kandel et al., 2000). On the other hand, neuronal firing patterns can be harvested to control electronic gadgets as experiments with trained monkeys have shown (Carmena et al., 2003). Thus, knowledge of the patterns of neuronal firing can allow one to decode and predict the state of an organism's neurological function.

With in vivo recording, the successes have come from the easily accessible areas of the brain such as the motor, sensory, and visual cortices. Although it is possible to attain deep brain recording, the difficulty in data interpretation increases dramatically as these structures interact with multiple regions of the brain and are influenced by the behavioral state of the animal (Kandel et al., 2000; Nicolelis and Fanselow, 2002). The multiple factors that influence an intact mammalian nervous system make controlling in vivo networks a monumental task, but properly controlled cell cultures provide an excellent environment for controlling aspects of the experiment that one could not control in the living animal.

Traditionally, cultures of neurons are grown from dissociated cells or slices that preserve the local architecture of the network. In dissociated cultures the innate structure of the brain is destroyed to allow the formation of random new connections. This type of culture grants unprecedented control over the chemical milieu of the culture and access to identified synapses, both of which are difficult to accomplish within the living being (Tao et al., 2000; Ming et al., 2002). To compensate for the loss of network architecture, brain slices have been cultured to maintain the local integrity of the connections, but the tissue architecture is generally lost in the long term (Gaehwiler et al., 1998). Electrical recordings of cultured neurons in both settings with pipette electrodes have demonstrated learning through synaptic potentiation and depression (Cash et al., 1996; Lopez-Garcia et al., 1996; Engert and Bonhoeffer, 1997; Urban and Barrionuevo, 1998); however, extended recordings have been difficult to conduct as the introduction of pipette electrodes can contaminate the culture.

This inability to achieve long-term, multi-site recording has led scientists to pursue recording of cultured neurons on multi-electrode arrays (MEAs). Thomas et al. (1972) first demonstrated the feasibility of recording on MEAs by studying myocardial activity (Thomas et al., 1972), but it was Gross (1979) who demonstrated neuronal recording with MEAs. Since then, numerous neuronal recordings with MEAs have found that dissociated neurons cultured on MEAs retain their dynamic interaction and respond to chemicals in similar fashion as their counterparts in vivo and in vitro (Gross et al., 1997; Jimbo et al., 1998, 1999, 2000; Morefield et al., 2000). Although progress has been made regarding recordings of slice cultures on MEAs, the progress has been limited to the characterization of electrodes and the presence of neuronal activity (Stoppini et al., 1997; Thiebaud et al., 1997; Egert et al., 1998). Although slice cultures may retain the active connections similar to those in vivo, the current MEA technology makes specific site recording difficult in these cultures, as the recording sites are prespecified by the manufacturer. Thus, the ideal situation would be the maintenance of neurological connectivity with placement of the desired control point over the electrodes.

This goal has been made possible by the recent development of surface modification techniques that have been applied to alter the surface properties of both inorganic (silicon oxide and metals) and organic (polymeric) substrates (Weetall, 1976; Moses et al., 1978; Facci and Murray, 1980; Laibinis et al., 1989; Hook et al., 1991; Vargo et al., 1992; Ferguson et al., 1993; Kumar et al., 1994; Lee and

Viehbeck, 1994; Corey et al., 1996; Garbassi et al., 1998). These techniques have successfully modified commonly used insulator and electrode materials such as silicon oxide, silicon nitride, polyimide, polysiloxane, platinum, and gold. The simplest of all techniques is the physisorption technique which can be applied to all surfaces. Next in ease of applicability is silane grafting which can be applied to any surface presenting hydroxyl groups (silicon oxide, silicon nitride, oxidized polyimide, oxidized polysiloxane, and oxidized platinum). Deposited silane presents a uniform functional group that permits specific chemisorption of growth guidance materials. Alternatively, pure noble metal electrodes (platinum and gold) can be modified using functionalized thiol such as 11-mercaptoundecanoic acid. For polymers, however, chemical functionalization (such as hydrolysis) can also produce specific functional groups for chemisorbing macromolecules (Garbassi et al., 1998). These techniques have produced patterned neuronal cultures on substrates without electrodes and are being applied to MEAs (Hickman and Stenger, 1994; Singhvi et al., 1994; Branch et al., 1998; Ma et al., 1998; Ravenscroft et al., 1998; Branch et al., 2000; Chang et al., 2000; James et al., 2000; Chang et al., 2001a,b; Vogt et al., 2003; Nam et al., 2004; Vogt et al., 2004).

The results from patterning neurons in culture have demonstrated that patterned neurons can survive for the long term, establish interconnections, and yield action potentials when grown on MEAs (Kleinfeld et al., 1988; Jimbo et al., 1993; Ma et al., 1998; Branch et al., 2000; Chang et al., 2000; Liu et al., 2000; Prinz and Fromherz, 2000; Lauer et al., 2002). Branch et al. (2000) have demonstrated that neurons cultured in serum-free media can remain patterned for more than four weeks. Patterned neurons have also been shown to interact even at four weeks old through synaptic transmissions (Ma et al., 1998; Chang et al., 2000; Liu et al., 2000). These results demonstrate the feasibility of reconstructing neuronal networks within culture to model in vivo circuitry.

To successfully reconstruct the in vivo circuits, one needs to understand the combination of materials selection, surface functionalization, and polymer delivery techniques that will work with the MEAs employed for culture. The goals of this review are to focus on the materials available to guide neuronal growth, the patterning techniques used to deliver the growth-inducing polymers, and the functionalization methods for insulator and electrode materials encountered in MEAs. As space is limited, the physicochemical properties of the molecules used are not discussed in this review; instead, we focus on the application and the techniques for patterning neurons. A short review of the known properties of patterned networks of neurons and their potential are given to conclude this chapter.

7.2 Materials Selection

In guidance molecule selection, the right combination of foreground (location with cells) and background (location without cells) materials permits the retention of excellent patterns over the duration of the culture. Both foreground and background

materials can be classified into biologically active or inactive materials (Corey and Feldman, 2003). The foreground materials that are biologically inactive include aminated molecules such as poly-lysine, polyethyleneimine (PEI), and aminosilanes which provide an area that is permissive for cellular growth but have not been shown to induce intracellular signaling (Kleinfeld et al., 1988; Hickman and Stenger, 1994; Corey et al., 1996; Rutten et al., 1999; Chang et al., 2000). The active foreground materials include extracellular matrix materials (ECMs) such as laminin, neuronal-cell-adhesive molecules (NCAM), fibronectin, and collagen which induce focal adhesions that may provide intracellular signals (Clark and Brugge, 1995; Schwartz et al., 1995; Ingber, 1997; Banker and Goslin, 1998; Xiao et al., 1999; Kandel et al., 2000). These ECMs can further interact with growth factors such as neurotrophins (NT) or nerve growth factor (NGF) to induce axonal growth (Hari et al., 2004).

For the background, inactive materials include alkylsilanes and polyethyleneglycol which create either a hydrophobic or a protein-resistant surface that repels cells, respectively (Israelachvili, 1997; Sheth and Leckband, 1997; Zhang et al., 1998; Branch et al., 2000; Corey and Feldman, 2003). The active background materials include chondroitin sulfates, myelin-associated glycoprotein, semaphorins, albumin, and serine which range from proteins to amino acids and can resist growth by mechanisms such as cell signaling and protein resistance (Branch et al., 2000; Corey and Feldman, 2003; Jones et al., 2003).

Although numerous guidance molecules exist, not all are useful to the experiments that one has in mind. For example, if one simply desires random growth, one can easily neglect the background molecules. However, if one desires a directed neuronal network, then one will have to select a proper combination of growth-permissive molecule (such as poly-lysine or ECM) plus a growth factor (either NGF or NT) for the foreground and cell-repulsive molecule (such as polyethyleneglycol or chondroitin sulfate) for the background. The complexity of the selection process increases with the directionality within the network.

The complexity of network design using materials selection is demonstrated by the complex intracellular and extracellular signaling that occurs. Reports have shown that substrate adhesivity influences the specificity of the neurites, but that this influence can be molecule and pattern specific (Lochter et al., 1995; Esch et al., 1999). Furthermore, molecules may switch their function based on the intracellular state of the neurons, for example, netrin-1 and semaphorins can alter their attractiveness/repulsiveness based on intracellular concentrations of cyclic nucleotides (Caroni, 1998; Song et al., 1998; Takei et al., 1998; Polleux et al., 2000). As if these complexities were not enough, the developmental history and the chemical gradient further modify the effects of signaling molecules on the growing neurites (Hoepker et al., 1999; Ming et al., 2002). Perhaps the simplest design technique is that proposed by Stenger and Hickman in which speed bumps that slow neurite growth lead to predominant development of dendrites, but even this is technique dependent (Stenger et al., 1998; Vogt et al., 2003, 2004). Thus, directionality design in a neuronal network can be an extremely complicated task (Figure 7.1).

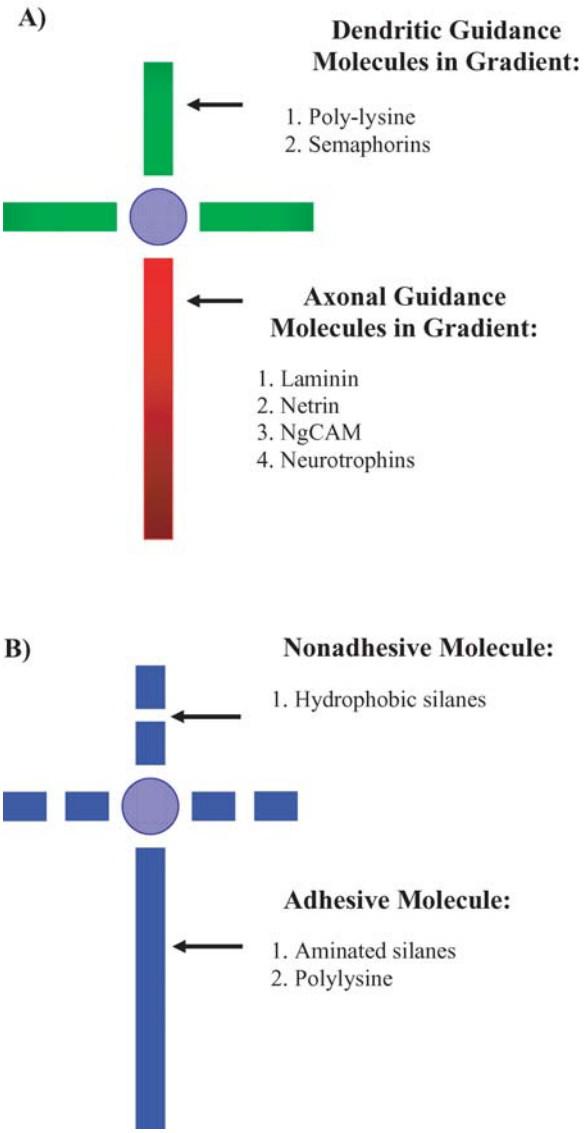

FIGURE 7.1. Proposed directed network designs: two possible designs to achieve directed neuronal networks have been proposed. (A) Signaling technique employs biologically active molecules as the signals to the cells to direct axonal versus dendritic growth. A gradient of these molecules may be incorporated into the design as an additional technique to improve the directionality of the network (Venkateswar et al., 2000). (B) Speed-bump technique employs adhesive and repulsive regions to enhance or retard the growth of the neurites, respectively (Stenger et al., 1998). However, this technique seems to depend on the patterning technique used to create these regions (Vogt et al., 2004).

7.3 Surface Modification Techniques

When culturing neurons on MEAs, the cells encounter a combination of organic and inorganic surfaces that constitute the insulation and the electrodes. The common organic materials used to insulate the MEA are polyimides and polysiloxanes, the structures of which are shown in Figure 7.1 (Gross et al., 1997; Chang et al., 2000). If unmodified, these highly hydrophobic surfaces will not only resist wetting by aqueous solutions but also physisorption and chemisorption of macromolecules conducive for cellular growth (Gross et al., 1997). Thus, it is critical that these surfaces be activated to permit deposition of macromolecules for proper signaling. Alternatively, the electrodes can be insulated using silicon oxides or silicon nitrides (Wise and Weissman, 1971; Shahaf and Marom, 2001). Although these silicon surfaces are more hydrophilic than the organic surfaces, they provide a minimally permissive surface for neuronal survival that is not sufficient for either good cell survival or cell repulsion (a necessary component for maintaining good neuronal patterns in culture). To achieve good patterning and survival on these surfaces, one needs to refunctionalize these surfaces.

Finally, neurons growing on MEA will also encounter electrodes of gold, iridium oxide, platinum, or titanium nitride (Gross et al., 1997; Chang et al., 2000; Weiland and Anderson, 2000; Shahaf and Marom, 2001; Nam et al., 2004). Although metals are permissive for cellular growth, such nonspecific signals to cells may disrupt an experiment designed to study the cellular response to certain extracellular signals (Letourneau, 1975). In addition, electrodes exposed to a proteinaceous solution can accumulate a relatively thick layer of protein that can distort the desired guidance properties of a designed path (Gesteland et al., 1959), and stimulated electrodes can shed platinum into the culture medium (Brummer et al., 1977). Thus, modifying the MEA surface can improve the cell growth, specify desired cell–cell and cell–substrate interactions, and construct specific circuits for neuronal network studies.

The modification techniques can be categorized into three classes, namely, physisorption, functionalization, or grafting. Physisorption simply requires the protein to adhere to the MEA by either the van der Waals or electrostatic forces and involves no chemistry for surface alteration (Lom et al., 1993). Functionalization involves the simple conversion of the original functional group into an alternate functional group that is more amenable for attachment of macromolecules (Garbassi et al., 1998). Grafting, on the other hand, involves attaching a polymeric layer (perhaps more than one molecular layer) that presents a uniform functional group for linking guidance molecules. Under this classification, wet chemical modification of organic surfaces functionalizes the substrates, whereas silane and thiols in general graft the substrates (Garbassi et al., 1998).

7.3.1 Physisorption of Protein

As stated previously, physisorption is the simplest and most generalizable technique for modifying the surfaces of an MEA. Its simplicity and generalizability lie in its independence of specific chemical reaction, because the technique employs either van der Waals or electrostatic forces for binding guidance molecules to the

surface of the MEA (Sadana, 1992; Cheng et al., 1994; Bekos et al., 1995; Taborelli et al., 1995). For this technique to succeed, however, one needs to achieve a clean activated surface for protein or peptide deposition. This can be achieved with sonication for reasonably hydrophilic surfaces (e.g., silicon-oxide and silicon-nitride) or sonication plus gaseous plasma/UV exposure for more hydrophobic surfaces (e.g., polysiloxane and polyimide; Figure 7.2; Chang et al., 2001a,b). Our

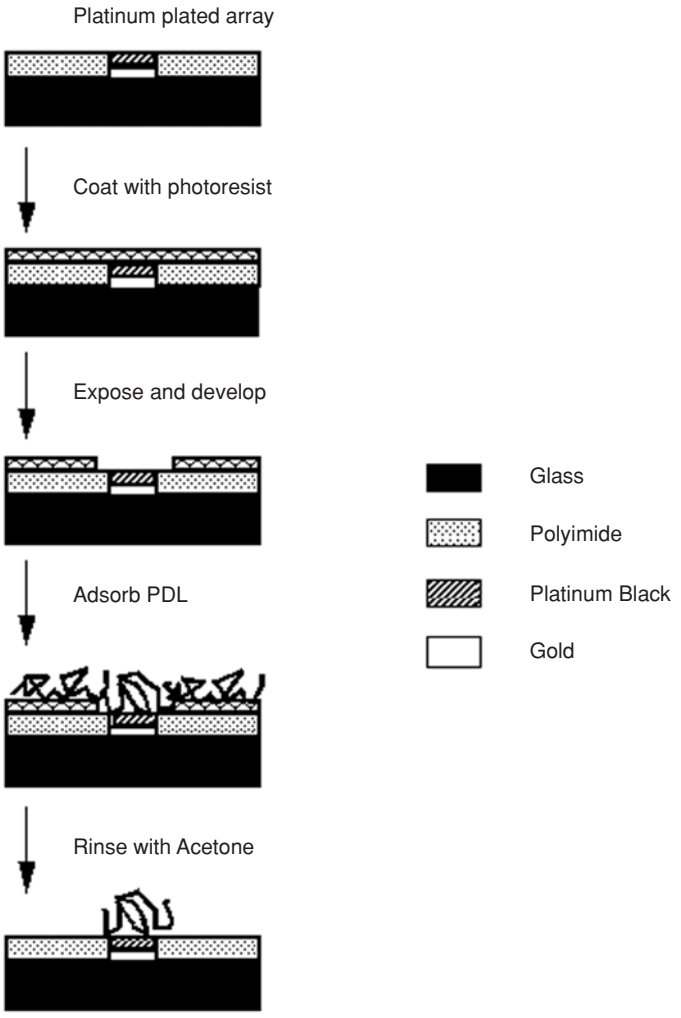

FIGURE 7.2. Lithographic process for patterned adsorption of poly-lysine on MEAs: the process is similar to any lithographic process encountered in a micro-electronic laboratory. The substrate (an MEA in this case) is cleaned via sonication followed by photoresist coating and UV exposure. The developed substrate is then exposed to a protein/macro-molecule solution that deposits the guidance material. Finally, the photoresist is removed via acetone or other solvents that dissolve the photoresist. (Figure reprinted with permission from Chang, et al. 2000; © Kluwer Academic Publishers.)

experience with this technique has been that cells demonstrate robust growth on physisorbed poly-d-lysine surfaces and that patterning can be achieved for short- or long-term depending on the type of background materials used.

Although this technique is simple and generalizable, it does have drawbacks. First, multi-protein deposition in specified patterns is extremely difficult, if not impossible. In order to pattern protein by physisorption, one depends on the successful transfer of pattern to the deposited photoresist using micro-lithography, a process that exposes proteins to denaturants and oxidizing agents, such as acetone, photoresist stripper, and oxygen plasma (Lom et al., 1993; Chang et al., 2000). These harmful agents may destroy the necessary protein conformation that signals the neurons, but the effects may be lessened with dextrose coating (Sorribas et al., 2002). Alternatively, one can ablate the adsorbed protein using a high-energy laser, which may not be easily accessible (Corey et al., 1991). Second, the fidelity of the network structure may depend on the MEA insulation, which may not be the right material for the background. Although it is possible to adsorb a material that inhibits cell growth, long-term patterns have not been achieved with adsorbed background material. In addition, our experience suggests that commonly used silicon nitride does not provide the necessary repulsion that is needed for good, long-term patterns (Chang, 2002), but that oxidized polyimide may provide reasonable background material (Chang et al., 2000). Third, control of the protein conformation is difficult to achieve with physisorption. Although control over the conformation of physisorbed albumin by substrate hydrophicity has been demonstrated (Cheng et al., 1994; Bekos et al., 1995; Taborelli et al., 1995), such a technique may not be applicable to a conformationally sensitive protein which may lose its signaling property with altered structure.

Despite these complexities, we have been quite successful at structuring networks of neurons on MEAs using physisorption and micro-lithography. We have demonstrated long-term patterning of neurons on polyimide, polysiloxane, and silicon nitride MEAs (Figure 7.3A–D; Chang et al., 2000, 2001a,b; Chang, 2002; Chang et al., 2003a). These neurons are electrically active and communicate through synaptic transmissions (Figure 7.3E; Chang et al., 2001a). It is our observation, however, that polyimide in general yields a much better pattern at four weeks than silicon nitride surfaces; we currently do not have sufficient data regarding polysiloxane because these arrays have not survived our culture process on a regular basis. Although silicon nitride can provide good pattern quality at four weeks, this was very sporadic. We speculate that silicon nitride may be less of an ideal surface for patterning because silicon nitride presents an aminated surface which is known to permit cellular growth (Kleinfeld et al., 1988). In order to correct this, one can seek to control the surface groups to regulate culture growth.

7.3.2 Silane Chemistry

Silane modification of substrates is more complicated because of the number (maximum of three) and type of functional groups present on the silicon atom within the silane molecule. The types of functional groups available at the silane terminal are

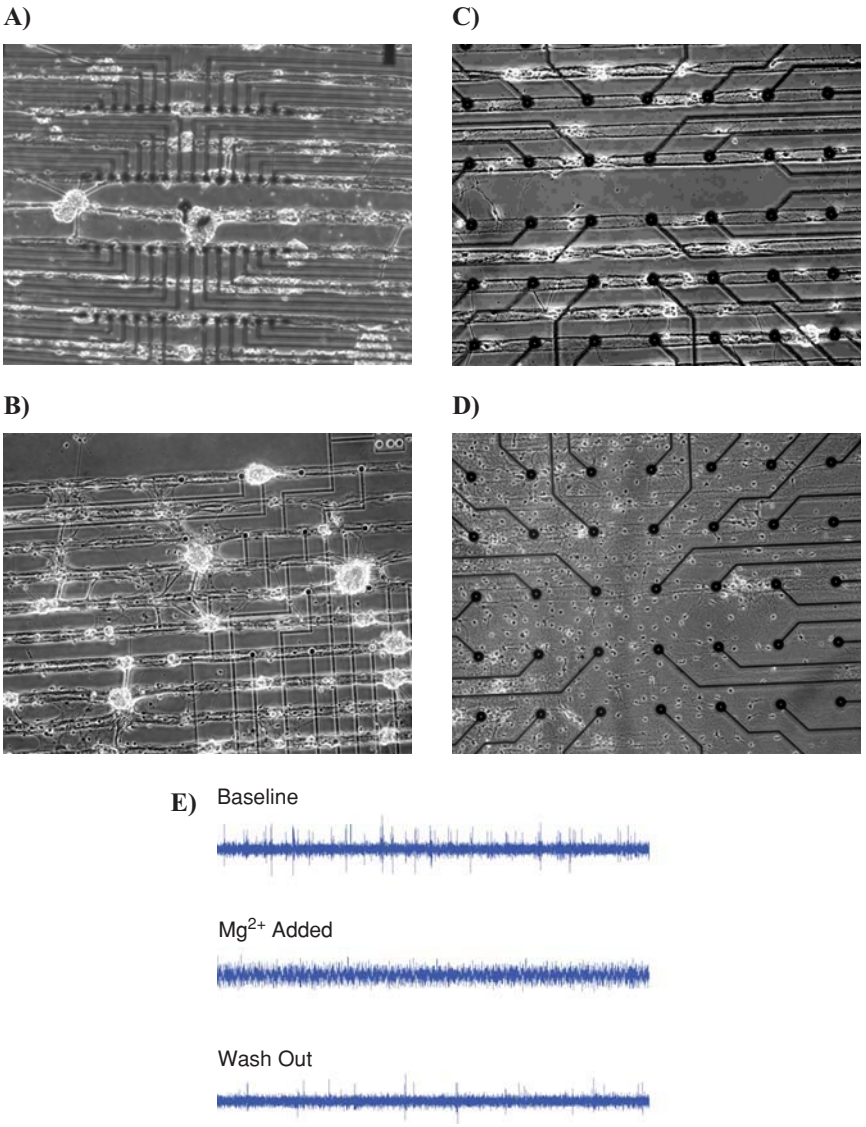

FIGURE 7.3. Neuronal patterning on MEAs with different insulation: all cultures have the same pattern, 40 μm poly-lysine and 60 μm insulation. (A) Eleven days in vitro (DIV) culture on polyimide MEA. (B) Twenty DIV culture on polysiloxane MEA. (C) Thirteen DIV culture on silicon nitride MEA, with good pattern quality. (D) Fourteen DIV culture on silicon nitride MEA, with poor pattern quality. (E) Sample recording of neuronal activity (1.25 sec). Activity can be suppressed with $MgCl_2$, which suggests synaptic communication among the neurons. (A–D are reprinted with permission from Chang, 2002; © 2002 J.C. Chang. E is reprinted with permission from Chang et al. 2001a; © 2001 Elsevier Science.)

methyl (-CH_3, inert), methoxy (-OCH_3, reactive), ethoxy (-OCH_2CH_3, reactive), and chloro (-Cl, highly reactive in the presence of water). As methyl groups are inert with regard to the polymerization, increasing the number of methyl groups at the silicon atom decreases the polymerization potential. With chlorosilanes, the high tendency of the chlorine atom to leave the silicon atom makes this a highly reactive terminal, especially when water is present (Figure 7.4A). Thus, chlorosilane

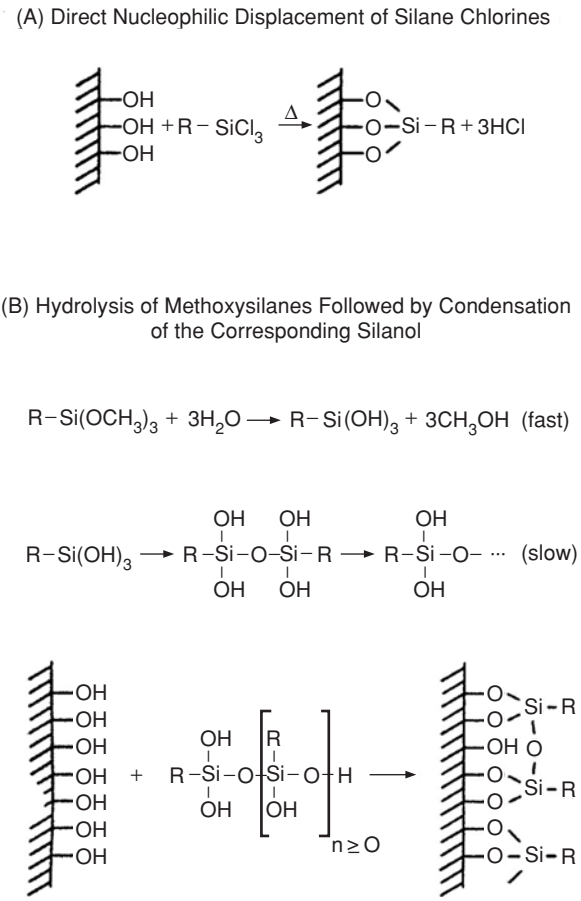

FIGURE 7.4. Silane reaction with hydroxylated surface: (A) chlorosilane reaction with surface-bound hydroxyl groups is a single-step reaction between the chlorine and the hydroxyl groups. Rapid polymerization among the silane molecules can occur with the presence of water. (B) Methoxysilane (and also ethoxysilane) reaction with surface-bound hydroxyl groups requires initial activation with water that converts the methoxysilane to silanols (Si-OH). Silanols then react with surface-bound hydroxyl groups to form a siloxane bond by the condensation reaction (eliminates a water molecule). (Reprinted with permission from Kleinfeld et al., 1988; © 1988 by the Society for Neuroscience.)

generally polymerizes rapidly, sometimes within the solution rather than on the substrate. In contrast to the high reactivity of chlorine, the methoxy and ethoxy groups have a moderate tendency to leave the silicon atom in the presence of water (Figure 7.4B), which makes them ideal candidates for grafting to the hydroxyl presenting substrate. When water is present, the methoxy/ethoxy terminal is converted into a hydroxy terminal that hydrogen bonds with the substrate hydroxy groups and with other silane molecules. When baked, the hydrogen bond is converted into a siloxane bond by the elimination of water molecules. The hydrogen bonds among the silane molecules lead to polymerization, which can be quite extensive. For more details on the silane reaction, one can refer to the book by Pluedemman and the review by Larson (Larson, 1991; Plueddemann, 1991). The readers are also encouraged to contact vendors' technical support to discover more suitable silanes.

In addition to the functional groups present at the silane terminal, the functional groups present at the alkane terminal are equally critical to the success of neuronal patterning. This terminal in combination with the deposited protein or macromolecule determines the type of reaction available for cross-linking to the substrate surface. It typically involves the amine group ($-NH_3$), mercapto group (-SH), carboxyl group (-COOH), or hydroxyl group (-OH), all of which can be linked to the amine group of proteins or macromolecules through a cross-linker (Hermanson, 1996). Although it is possible to radically polymerize this end to proteins using the vinyl group ($-CHCHCH_3$), this type of chemistry is destructive to the conformationally sensitive protein. Thus, the success of patterning depends on the proper selection of the silane, the protein, and the cross-linker. In addition to silane chemistry, the thiol chemistry can also play an important role in structuring neuronal networks in culture.

7.3.3 Thiol Chemistry

Alkanethiols have been shown to interact strongly with metals such as gold, silver, copper, and gallium-arsenide through the formation of the thiolate-metal bond in which sulfur loses its accompanying hydrogen (Dubois and Nuzzo, 1992). This reaction exists naturally when gold is used as the electrode; however, in order to pattern neurons using this technique, one would have to coat the surface of the MEA with a layer of gold, which is the only biocompatible metal named in the list (Nam et al., 2004). Although thiols interact with metals through a single thiolate bond (Figure 7.5), the resulting molecular layer presents a stable uniform layer with minimal defect density for further chemistry and culture (Mrksich et al., 1996; Zhao et al., 1996; Kane et al., 1999). However, the selection of thiols is limited as

$$Au + HS(CH_2)_{10}COOH \xrightarrow{-H} Au{-}S^{-}(CH_2)_{10}COOH$$

FIGURE 7.5. Thiolate formation with gold substrates: thiols react with gold surfaces by the formation of thiolates, which occurs with hydrogen loss by the sulfur atom.

11-mercaptoundecanoic acid is the only commercially available thiol for surface modification. On the other hand, if one's lab has excellent chemical expertise, one can easily synthesize a variety of thiols with different functional groups (Kane et al., 1999).

7.3.4 Inorganic Surface Modification

7.3.4.1 Overview of MEA Materials and Their Reactivity to Silanes

As discussed previously, cells cultured on MEAs can encounter several different inorganic surfaces as either insulation or electrode that may have been modified. Silicon-oxide and silicon-nitride are commonly used as insulation in MEAs and present numerous hydroxyl groups for silane deposition (Corey et al., 1996; Egert et al., 1998; Jung et al., 1998). Platinum, being a noble metal, is generally inert to oxidation, but hydroxyl groups can be created to react with silanes (Moses et al., 1978; Proctor et al., 1985). The details of our experience with these two materials are fully addressed in the following sections, but we confine the discussion on iridium oxide and titanium nitride to the following paragraph because we have limited experience with them.

Although silane linkage to iridium oxide (IrO) and titanium nitride (TiN) has not been demonstrated, silane linkage to these substrates should occur. Reported research has shown that when iridium is oxidized electrochemically to form a thin oxide layer (about 8 molecular layers), the bound oxygen is in the -OH state; however, when the oxide layer becomes thick (about 100 molecular layers), the oxygen is completely bound to the iridium in the form of IrO_2 (Augustynski et al., 1984). Thus, a thin iridium oxide formed from electrochemical activation should be amenable for reaction with silanes (Weiland and Anderson, 2000). However, limiting the activation process to a thin layer may also limit the impedance enhancement gained with a fully activated iridium electrode. This contrasts with TiN electrodes, which permit co-existence of highly reduced electrode impedance and silane modification. Research has shown that sputtered TiN electrode actually consists of titanium nitride, titanium oxynitride (TiN_xO_y), and titanium oxide (TiO_2) at the surface of the electrode (Cyster et al., 2002). When the newly sputtered TiN electrode is exposed to the environment, the surface layer (up to 100 nm) oxidizes to form TiO_2, which can react with silane (Untereker et al., 1977; Nanci et al., 1998). Thus, the columnar structures formed during sputtering improve electrode impedance, whereas oxidation during ambient exposure creates the necessary oxides for silane reaction. However, all this discussion regarding hydroxyl groups on TiN and IrO may be moot as silane deposition may not require the presence of -OH, as demonstrated by silane deposition on gold (Allara et al., 1995).

7.3.4.2 Silicon-Based Insulation Modification by Silane

Although modification of MEAs has considerable similarity to coverslip modification, key differences also exist. The foremost difference is that a MEA is a

composite of materials that behave differently with regard to cleaning agents. Although the silicon-based insulation is stable when exposed to acids such as nitric and sulfuric acid, the electrodes generally are very unstable and will require a gentler cleaning procedure. In addition, the presence of multiple materials also requires one to consider the electrode modification process when designing the insulation modification procedure. Next, the MEA surface can be discontinuous which contrasts with the smooth surface of the coverslips. Although this is a minor point, one should still consider whether the discontinuity would disrupt the integrity of the modification layer, such as a Langmuir–Blodgett layer.

The majority of the goals in modifying MEAs are exactly the same as those for modifying coverslips. With MEA modification, one prefers the cross-linking process to be optimal in the sense that the maximal proportion of desired molecule is delivered to the surface. For example, if one desires to link amine-terminated silanes and lysine on proteins, using glutaraldehyde as a cross-linker, one will cross-link many surface sites even before proteins are linked. Thus, it is desirable to cross-link different functional groups on proteins from those present on the silane. Second, it is preferable that the protein be deposited with specific orientation so that the active sites are exposed to the cells to direct cellular actions. This is especially important when one employs biologically active materials such as laminin or neurotrophins. Third, it is important that the total linkage remain intact for as long as possible so that the neurons retain the pattern for the duration of the experiment. Last, the background should be as free of cells as possible.

In our pursuit of these goals, we have tested a few silanes on coverslips regarding their ability to maintain neuronal patterns. The inspiration for our work includes the reports of Kleinfeld et al. (1988) and Lom et al. (1993). In Kleinfeld's work, a two-step deposition process using micro-lithography was employed to deposit alkylsilane and aminosilane separately to guide the growth of neurons (Figure 7.6A; Kleinfeld et al., 1988). This process first deposits the alkylsilanes on the developed area followed by full-coverslip deposition of the aminosilane. Using the same technology, Lom et al. (1993) tested neuronal preference of substrates and found that neurons preferred ECM proteins, for example, laminin, fibronectin, and collagen IV, over aminosilanes and glass. The least preferred surfaces were alkylsilane or albumin covered (Lom et al., 1993). To improve on pattern quality, Corey et al. (1996) reversed the deposition process (i.e., first patterning the aminosilane and then uniform deposition of the alkylsilane, Figure 7.6B) and obtained excellent neuronal compliance to patterns. In addition, it was found that the pattern geometry also influenced the fidelity of the patterns (Corey et al., 1991, 1996). Although silanes alone are capable of achieving patterned networks of neurons, they do not regulate cellular interactions. To achieve this, one can attach signaling molecules to the silanes.

With protein attachment, however, one desires to maximize the surface concentration of the protein on the substrates. Our initial attempt at delivering proteins to aminosilane modified substrate surface succeeded in patterning neurons (Branch et al., 1998, 2000), but the amount was not optimal because glutaraldehyde

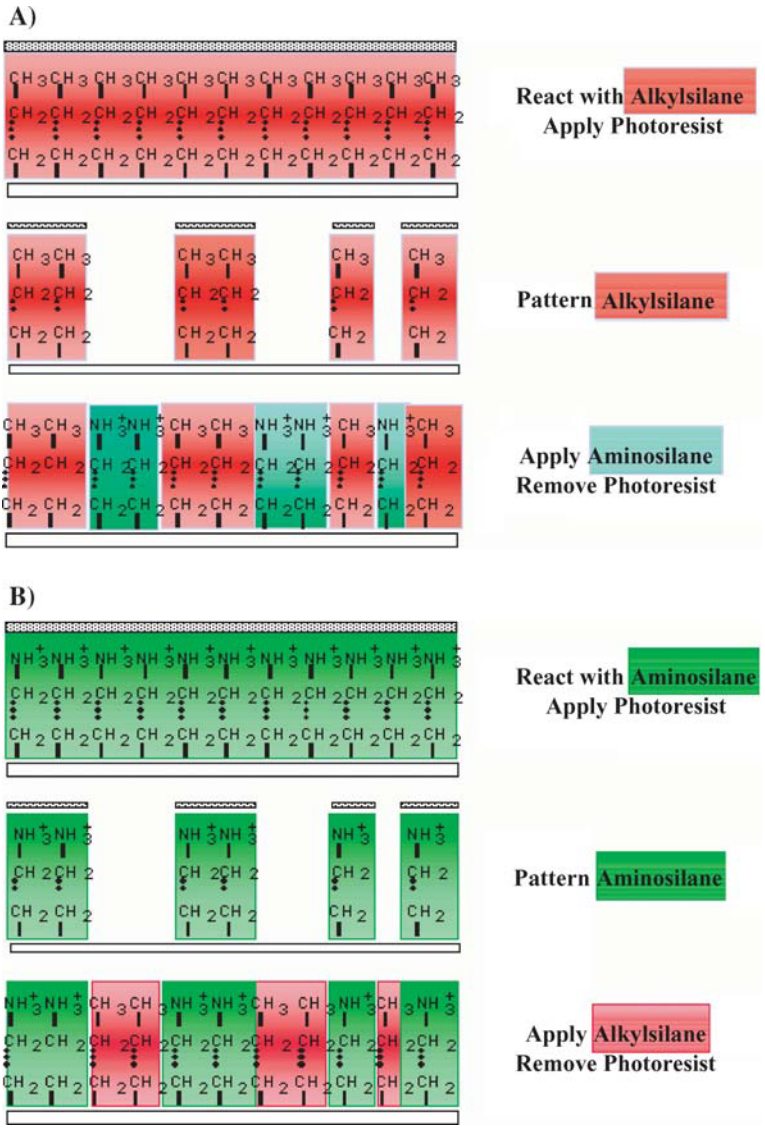

FIGURE 7.6. Silane deposition for neuronal patterning: two types of silane deposition for neuronal patterning have been employed in the literature. (A) The background-first technique was popularized by Kleinfeld et al. (1988) which deposits alkylsilanes first and aminosilanes last. This leads to deposition of aminosilanes throughout the foreground and defects within the background. (B) The selective silane removal technique was popularized by Corey et al. (1996) which deposits aminosilanes first and alkylsilanes last. This technique improves the quality of the patterns because aminosilane is only deposited at the desired location.

cross-linked the amines present on the substrate surface (Figure 7.7A). The linking scheme was improved by using the mercaptosilane to amine linkage that Bhatia et al. (1989) employed to link protein onto glass substrates which markedly improved surface protein density over that of aminosilane to lysine linkage (Figure 7.7B; Bhatia et al., 1989; Branch, 2000).

In addition to optimizing the surface protein density, one also desires longevity in the deposited layers. It is known that silane–glass linkage is sufficiently strong that the film does not break down for nearly four weeks when soaked in 70°C solution (Plueddemann, 1991). This should translate into a silane stability of well over four weeks in 37°C. However, the deposited layer also includes the linkage between the silane and the protein. Our lab had tested the stability of both the silane and the protein layers using patterned neuronal cultures (as an indirect test) and ellipsometry (direct measurement). Branch et al. (2000) found that the neurons retain their patterns, with some breakdown, for well over four weeks. The breakdowns can be attributed to pattern geometry in part (Fromherz and Schaden, 1994; Corey et al., 1996), but also the breakdown of the silane–protein linkage which occurred around the fourth week of soaking (Figure 7.8; Branch et al., 2001). Therefore, the silane–protein linkage should be the target of future improvements as it is currently one of the limiting factors in long-term pattern quality.

With the testing of pattern technology being successful on coverslips, we applied this technology to culturing neurons on MEAs (Figure 7.9; Chang, 2002). In doing so, we had to make some adjustments due to the sensitivity of the electrodes to strongly oxidizing agents. Instead of cleaning the surfaces with pirhana etch or sulfuric acid which can strip the contact pads and the electrodes, we cleaned the arrays by sonicating in acetone and etching in oxygen plasma for ten minutes. Silane deposition on silicon nitride MEAs (purchased from Multi Channel Systems) yielded a silane layer similar to that on coverslips when judged by the static water contact angle. Patterned deposition of poly-d-lysine and polyethyleneglycol resulted in high-fidelity patterns at four weeks; however, pattern breakdown through cell detachment does occur. We speculate that in addition to the silane–protein interface breakdown the adsorbed hydrocarbons on the MEA surface may detach leading to corruption of the neuronal patterns; perhaps a longer oxygen-plasma cleaning will correct this breakdown.

In our work, we have not addressed the protein orientation problem because we consistently used poly-d-lysine as the growth-permitting molecule. However, as we continue to pursue a designed network of neurons, we will inevitably use protein molecules for guidance. Work on inducing axonal growth with laminin has begun in our lab (Corey, 1997), and preliminary work suggests that laminin retains some conformation when linked to the coverslip (Esch et al., 1999). Whether these conformations are sufficient for guidance is a matter that is currently being tested in our lab. If it is the case that laminin is deposited in the wrong orientation, one can alter the amino acid sequences of laminin to aid the proper deposition, a technique that has been applied to orient cytochrome b (Firestone et al., 1996).

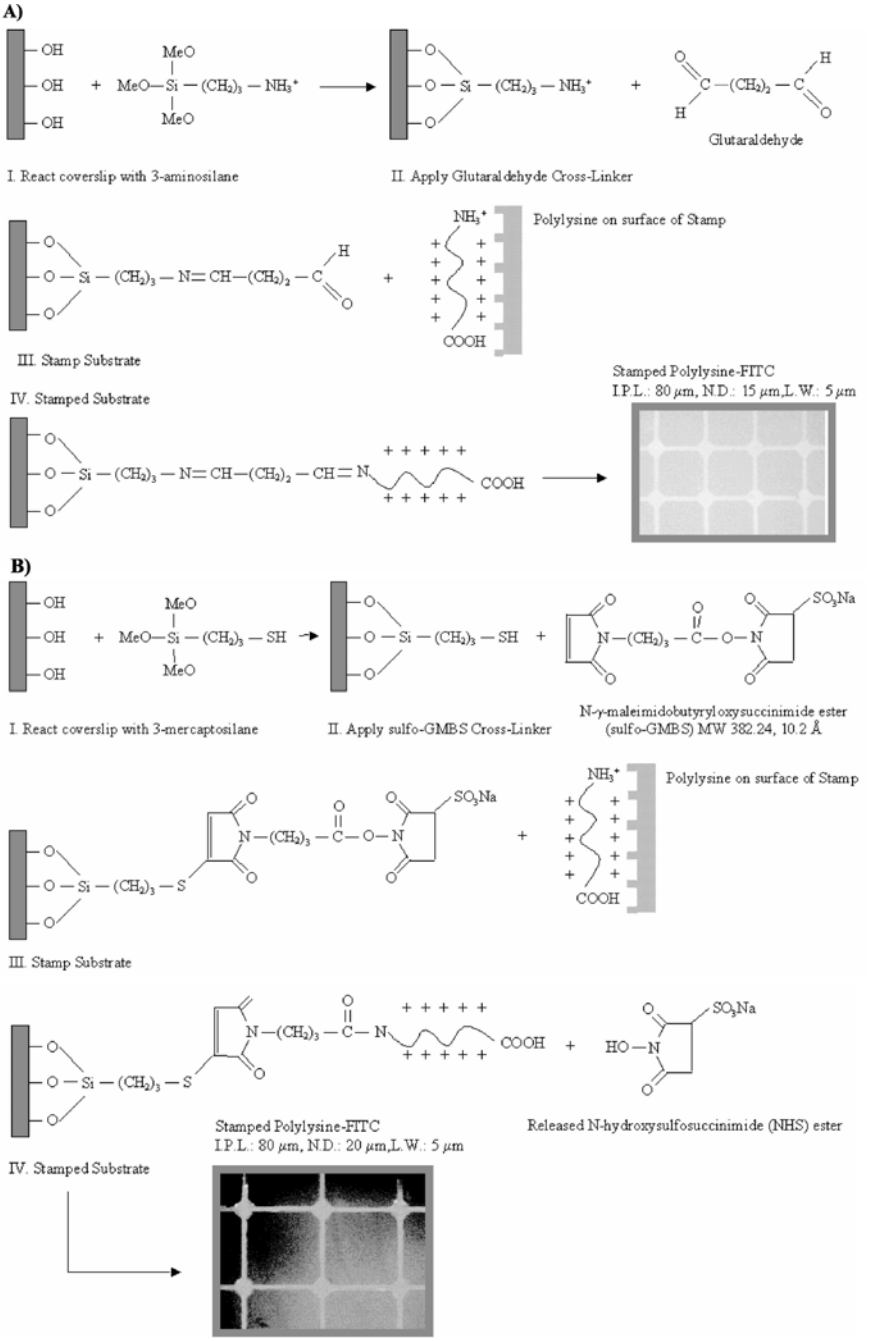

FIGURE 7.7. Reactions of two types of cross-linkers for protein linking to surface bound silanes: (A) aminosilane-coated substrates react with glutaraldehyde to link proteins, but some amounts of the surface amines also cross-link to each other. (B) Mercaptosilane-coated substrate reacts with N-γ-maleimidobutyryloxysulfosuccinimide ester (sulfo-GMBS) to link proteins. Because the functional groups being linked are different, no surface cross-linking is expected.

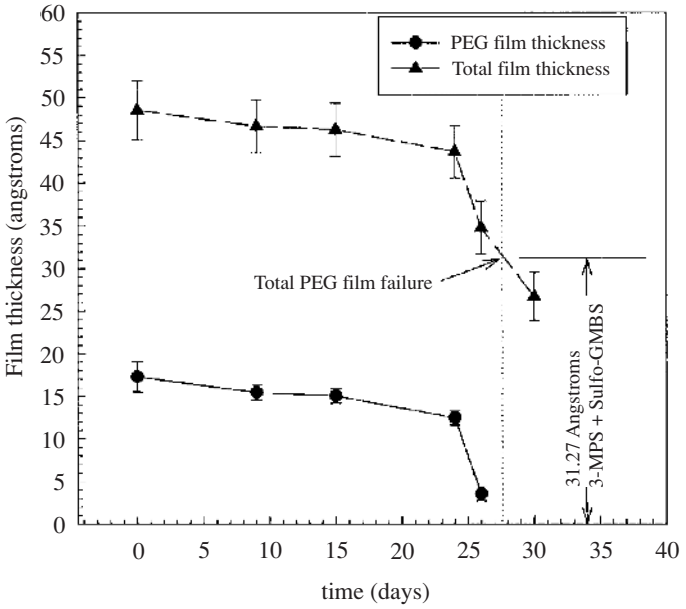

FIGURE 7.8. Degradation of silane–protein linkage in culture condition: the layers tested are the silane and the protein/polyethylene glycol (PEG) layers under culture condition (37°C, PBS, 10% oxygen, and 5% CO_2). The interface between silane and PEG breaks down after three weeks in culture. (Reprinted with permission from Branch et al., 2001; © 2001 Elsevier Science.)

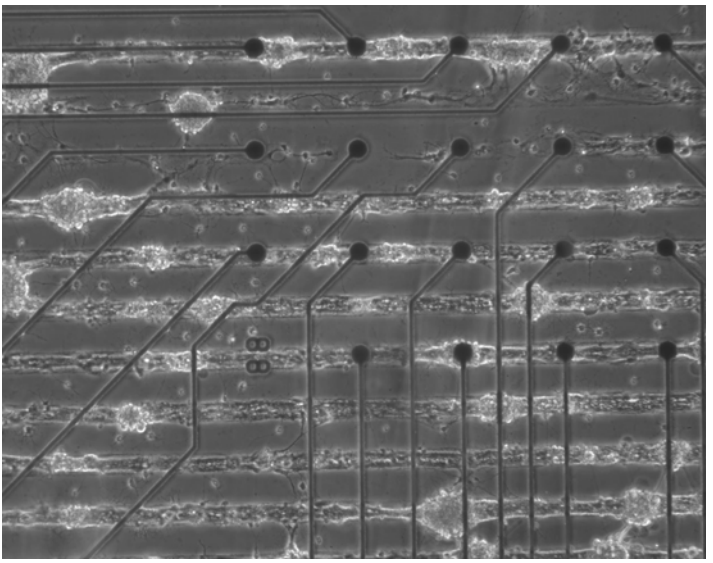

FIGURE 7.9. Patterned culture using silane-coated MEA: the culture is 25 DIV on silicon nitride MEA. The culture remains well patterned in general at three weeks age, but areas of the pattern do suffer breakdown after three weeks.

7.3.4.3 Platinum Modification by Silane

In addition to silicon oxide and silicon nitride, platinum can also be encountered by the neurons on MEAs. Typically, platinum is deposited as platinum black rather than crystalline platinum in order to lower the electrode impedance. For our work, we studied our silane deposition technique on platinum foils to expedite the experiments and applied the technique to platinum black electrodes. The success of the deposition on electrodes was tested by electrode impedance. Successful deposition would lead to an increase in electrode impedance, whereas failed deposition would not cause any change in impedance.

Although platinum is considered a noble metal with resistance to oxidation by oxygen atoms, research has shown that oxygen adsorption results in the formation of hydroxyl groups that are capable of reacting with silanes (Moses et al., 1978). Several techniques have been reported to deposit oxygen onto platinum surfaces. The first and perhaps the simplest is the flame technique in which platinum is heated to incandescence to cause the migration of silicon contaminants to the surface of the platinum (Proctor et al., 1985). This leads to potential silicon oxides on the platinum surface that can react with silanes. Although flaming has been used to activate inert polymer surfaces (Gross, 1979; Gross et al., 1997), it is infeasible to actually heat an MEA to incandescence. Alternatively, electrochemical oxidation of platinum by anodization also places reactive oxygen onto platinum surfaces that can react with silanes (Lenhard and Murray, 1977; Facci and Murray, 1980; Proctor et al., 1985). The final and perhaps the most feasible technique of oxidizing platinum is by oxygen plasma, a frequently used technique for surface cleaning in micro-fabrication. Platinum electrodes have been modified using oxygen plasma to enhance chemical interaction and to produce oxidized species (Chang, 2002; Ward et al., 2002). The species produced are generally PtO which have been shown to react with silanes (Allen et al., 1974; Hammond and Winograd, 1977; Facci and Murray, 1980; Chang, 2002). These data show that oxygen plasma cleaning of MEA results not only in a clean silicon surface for silanization, but also in activated platinum surface for silanization.

In silanizing the platinum surface, we applied our glass silanization protocol and found that silanes can be stably deposited for up to four weeks and resist desorption by electrical stimulation (Chang, 2002). Our data showed that the water contact angle after platinum silanization is similar to that from glass silanization (Chang, 2002). However, this angle was much lower if the platinum had been pretreated with overnight sulfuric acid to remove adsorbed hydrocarbons. This may be attributed to the reaction between the sulfhydryl group of the silane and the unoxidized platinum surfaces. Despite this low contact angle, XPS studies show that silanes are chemically linked regardless of the acid clean status. The silanes remain stably attached at four weeks when immersed in 1X PBS (phosphate buffered saline) at 37°C and exposed to the culture environment (10% oxygen and 5% carbon dioxide). However, there was initial desorption when the substrates were first exposed to the PBS solution. In addition, the silane resisted desorption by applied bipolar pulses lasting for one minute and up to 1.5 V. Thus, we

expect the silanes to remain relatively stable on the electrodes for at least four weeks.

Although the silane may attach to platinum substrates, it is not guaranteed that their presence will make platinum electrodes functional. We compared the impedance of the silane modified and sham-treated platinum electrodes. The results showed an eighteenfold increase in electrode impedance (raising electrode impedance from $<$100 kΩ to $>$1.5 MΩ) for silane-modified electrodes versus a twofold increase in sham-treated electrodes. We speculate that silane polymerization may contribute to this increase and can be decreased by decreasing the number of hydroxyl groups at the silicon atom. Alternatively, one may consider a waterless reaction that usually deposits a monolayer of silanes (Stayton et al., 1992; Firestone et al., 1996; Nelson et al., 2001). Our normal reaction process includes the addition of water to catalyze the conversion of methoxysilanes to silanols, a step that enhances the polymerization. This process can be contrasted with alternative protocols in which the reaction takes place in a waterless environment where the silanes deposited form a relatively thin layer (Stayton et al., 1992; Firestone et al., 1996; Nelson et al., 2001). The disadvantage of the waterless process is the time needed to complete the reaction, usually 12 to 48 hours.

7.3.4.4 Silane Deposition on Titanium Nitride Electrodes

In our work, we applied the silanization process to MEAs with TiN electrodes and grew neurons in patterns for recording purposes (Figure 7.9; Chang, 2002; Chang et al., 2003a). Although we did not directly measure the presence of silanes with XPS, our results were consistent with the findings from platinum electrodes. First, the measured electrode impedance increased to that of the shunt impedance, suggesting that the electrode impedance after modification was much greater than the shunt impedance. Second, the number of active electrodes recorded from patterned cultures on silanized MEA decreased dramatically. Third, even previously silanized MEAs tended to lag unsilanized MEAs in the percentage of electrodes being active. Thus, our data indirectly suggest that TiN can be modified with silane and that silane deposition on TiN electrodes corrupts the impedance.

7.3.4.5 Thiol Deposition on Gold

Although thiols cannot modify glass, they are the most commonly used modifier for noble metal surfaces. This technique can combine the modification of the MEA surface and the electrode into one convenient step if the electrodes were of gold. Nam et al. (2004) demonstrated the feasibility of this process by coating the MEA surface with a thin layer of gold that reacts with thiols (Figure 7.10A,B). It was shown that the 11-mercaptoundecanoic acid modified gold can support neuronal growth in patterns when the poly-d-lysine is cross-linked. This technique did not increase the impedance as much as the silane technique (because recording

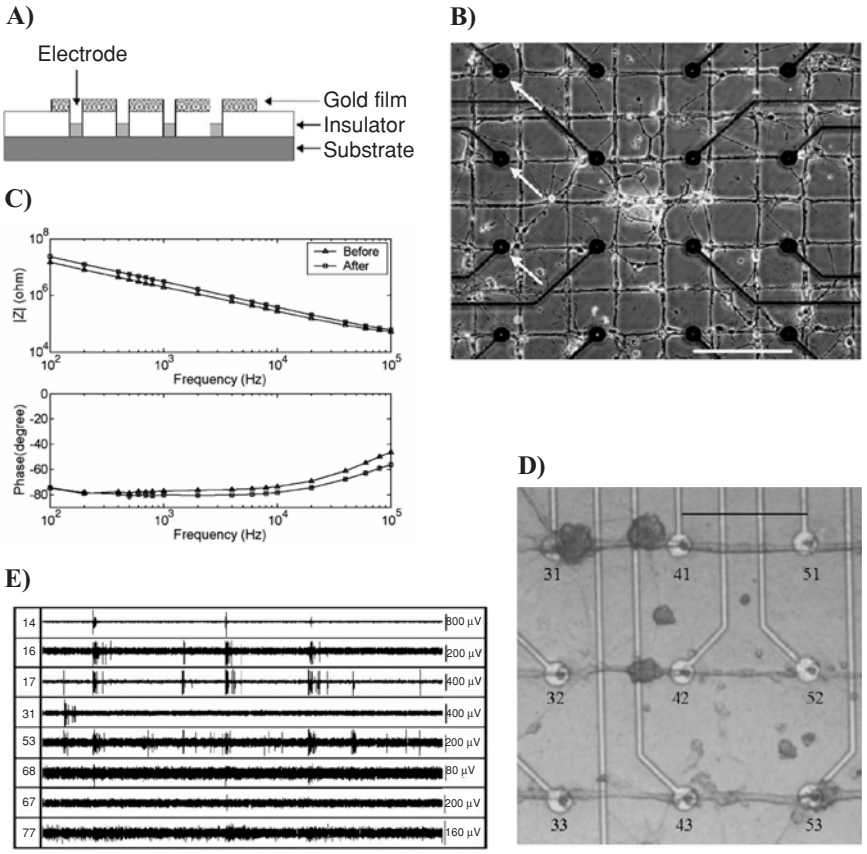

FIGURE 7.10. Patterned culture on thiol-coated MEA: (A) a cross-section view of gold-coated MEA to allow thiol deposition. (B) Twelve DIV culture patterned on thiol-coated MEA. White arrow shows neurites passing by the electrodes. (C) Impedance spectroscopy of electrodes before and after gold coating. The impedance stayed between two to three megohms both before and after the coating. (D) Seventeen DIV culture patterned on thiol-coated MEAs. The electrodes are numbered for reference to the recordings shown in (E). (E) Recordings of neuronal activity from the culture shown in (D). (Reprinted with permission from Nam et al., 2004; © 2004 IEEE.)

is possible, see Section 7.4), most likely due to the presence of defects within the film and to the lack of polymerization between the thiol molecules (Dubois and Nuzzo, 1992). Despite the high impedance of the gold electrodes both before and after thiol modification (Figure 7.10C), the impedance was sufficiently low compared to the shunt impedance that recording was possible, unlike the silane-modified electrodes (Nam et al., 2004). Thus, the combination of gold electrodes and gold-coated MEA surface permits patterned growth and recording of neurons.

7.3.5 Organic Surface Modification

7.3.5.1 Overview of Polymeric MEA Insulation and Its Modification Potential

In addition to silicon-based and platinum substances, the MEAs may incorporate polymeric substances as insulation. Two commonly used polymeric insulators are polyimide and polysiloxane. Polyimide has been used as insulation for nearly 20 years and gives excellent shunt impedance values (Novak and Wheeler, 1986; Boppart et al., 1992; Chang et al., 2000). The procedures for obtaining a stable and biocompatible insulation have also been described in the literature for use in serum-free neuronal cultures (Chang et al., 2000; Nam et al., 2004). In addition to these advantages, polyimide modification procedures by oxygen plasma oxidation and base hydrolysis are well characterized in the literature (Chou et al., 1987; Dunn et al., 1989; Lee and Viehbeck, 1994). As an alternative, one may choose polysiloxane as insulation, a highly hydrophobic material that resists water penetration, because of the ease of modification by oxygen plasma oxidation (Ferguson et al., 1993; Chaudhury, 1995). However, our experience with this insulation in terms of stability has not been as good as polyimide. This insulation tends to detach from the MEA after several uses or during soaks to ensure biocompatibility for cell culture. In contrast, Gross has successfully recorded from polysiloxane coated arrays for nearly a year (Gross, 1994). This may involve a suboptimal interfacial condition between the MEA surface and the polysiloxane that could be improved upon with further experiments.

7.3.5.2 Polyimide Modification

As previously stated, polyimide can be modified by either plasma oxidation or by base hydrolysis. Plasma oxidation relies on the exposure of polyimide to gaseous plasma, usually oxygen when hydroxyls are desired. This process produces several species of oxygen on the polymer surface, such as -OH, -COOH, and -OO (Figure 7.11). Of these, the useful specie is the hydroxyl group when one desires to modify the polyimide with silane (Chou et al., 1987; Vargo et al., 1992). However, if one wishes for direct linkage of protein to the modified polymer, one could choose to use the carboxyl group which reacts with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to link protein onto the polymer surface (Hermanson, 1996). Of these two options, we experimented with linking oxidized polyimide with silane molecules.

In our experience, oxidized polyimide can be modified with silane just as clean coverslips can. The deposited silane layer yields a static water contact angle similar to that deposited on glass coverslip (Table 7.1). After a soaking period of two weeks in PBS under culture environment, the water contact angle decreased slightly (from 56.5 to 50.7), suggesting perhaps film degradation or polymer reorientation. We believe the latter to have stronger influence on static water contact angle because the sulfur-to-nitrogen ratio, which measures the amount of mercaptosilane present on polyimide, was not significantly different between freshly silanized and soaked silanized samples. The silanized polyimide substrates were also used to culture

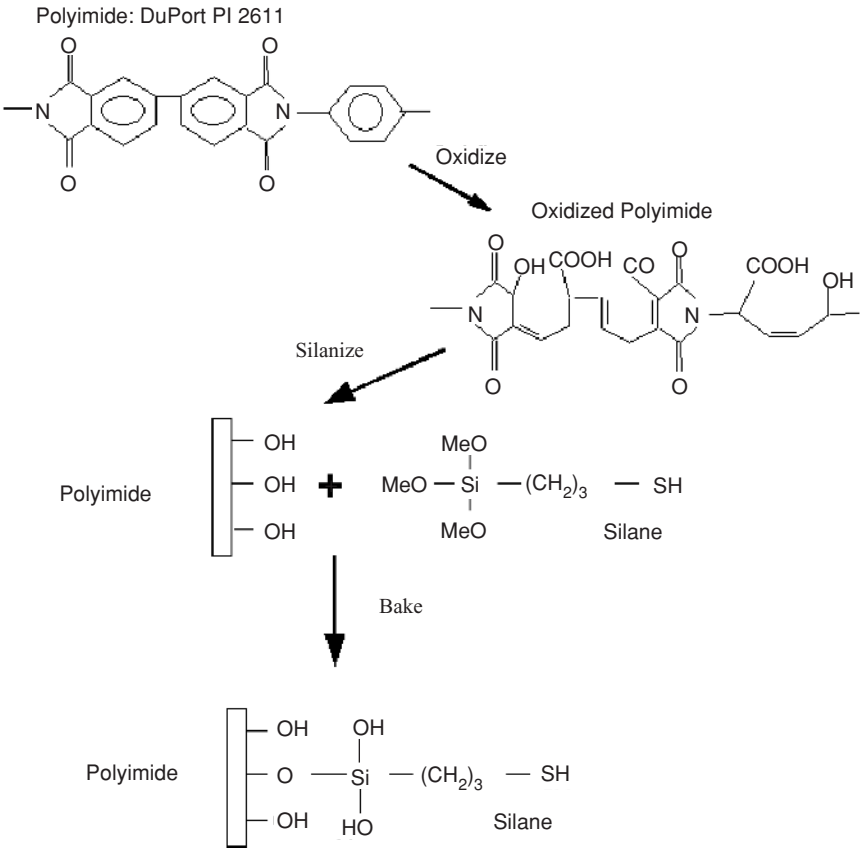

FIGURE 7.11. Polyimide oxidation with gaseous oxygen plasma: oxygen plasma treatment of polyimide produces -COOH, -OH, and -COO·, of which the -OH groups can react with silanes. In addition, the -COOH can react with EDC to cross-link lysines on proteins. (Reprinted with permission from Chang, 2002; © 2002 J.C. Chang.)

patterned neurons, which remained in patterns after two weeks. This was an improvement in pattern quality over unsilanized polyimide substrate that has been stamped with poly-lysine patterns (Figure 7.12A,B).

In addition to oxidizing polyimide with oxygen plasma, one can also generate functional groups with base hydrolysis. Lee and Viehbeck (1994) have shown

TABLE 7.1. Water contact angle of silanized polyimide substrates.

DIV	Contact angle (Mean ± SEM)
0	56.5 ± 2.66
7	48.42 ± 1.12
14	50.67 ± 0.94

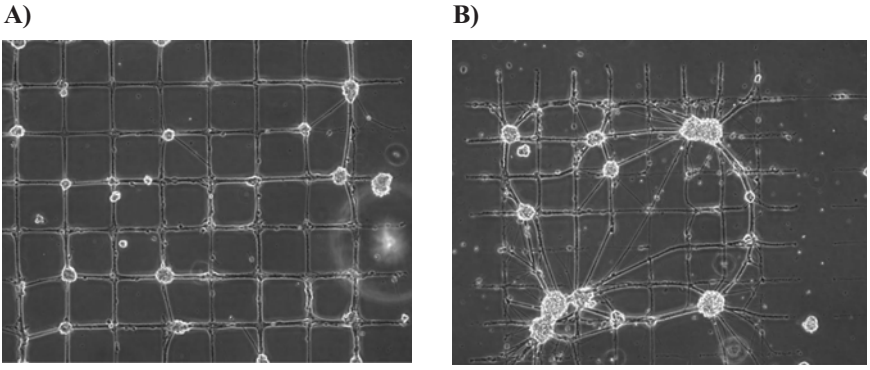

FIGURE 7.12. Comparison of pattern quality on polyimide substrates at eight DIV: (A) silane-linked polyimide yielded high quality patterns at eight DIV. (B) Oxidized polyimide yielded poor quality patterns at eight DIV. ((A) is reprinted with permission from Chang et al., 2003a; © 2003 Elsevier Science Ltd. (B) is reprinted with permission from Chang, 2002; © 2002 J.C. Chang.)

that polyimide exposed to potassium-hydroxide (KOH) produces –COOK and a secondary amine (-NH-). The potassium carboxylate can be exchanged to carboxyl with exposure to an acid solution (Figure 7.13), making the functional group reactive to the previously stated EDC molecule. This is indirectly supported by the water contact angle which becomes very low after exposure to KOH. However, because we desired a simple modification scheme for both the electrode and the insulation, we chose not to pursue this route of modification. Thus, this technique can work in theory, but has not been demonstrated to work in the literature.

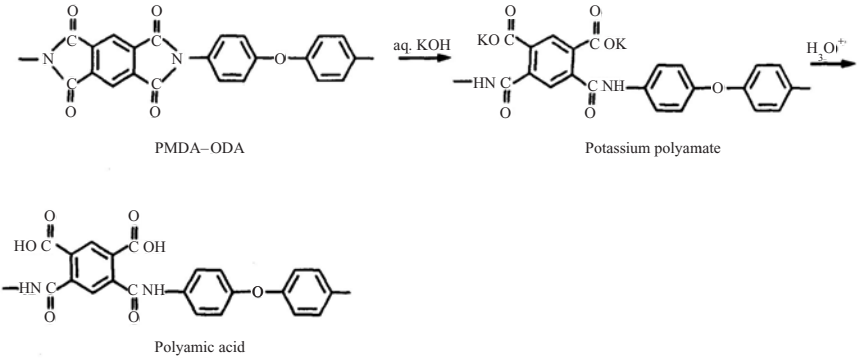

FIGURE 7.13. Chemical modification of polyimide: reaction of polyimide with a base (KOH) yields carboxylate (-COOK) groups that can be converted to carboxyl groups (-COOH) through exchange with an acidic solution. The carboxyl groups can be linked to lysines by using EDC. (Reprinted with permission from Lee and Viehbeck, 1994; © 1994 International Business Machine Corp.)

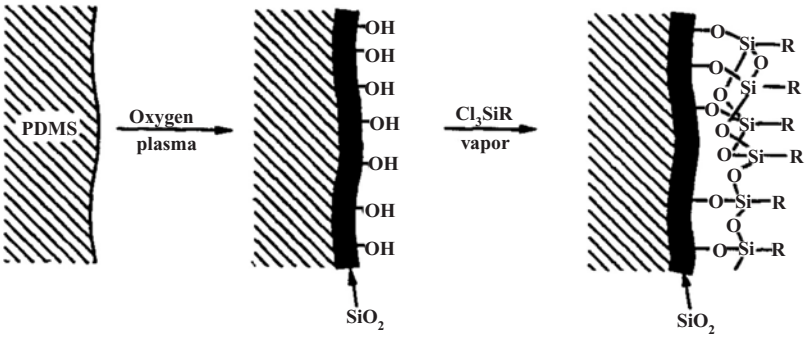

FIGURE 7.14. Oxygen plasma oxidation of poly-dimethyl-siloxane (PDMS): oxygen plasma oxidation of PDMS yields a surface with many hydroxyl groups that can react with silanes, chlorosilanes in this case. (Reprinted with permission from Ferguson et al., 1993; © 1993 American Chemical Society.)

7.3.5.3 Polysiloxane Modification

Another commonly used polymer for MEA insulation is polysiloxane, which is easily modified by oxygen plasma (Gross et al., 1997). The exposure leads to the production of hydroxyl surface groups that permit silane chemisorption (Figure 7.14; Ferguson et al., 1993; Chaudhury, 1995). Published results show that silane films deposited on oxidized polysiloxane are similar to those on silicon oxide surfaces (Ferguson et al., 1993). Although this technique is feasible, we do not use siloxane-insulated MEAs frequently and have not pursued this technique to pattern neurons on MEAs.

7.4 Protein Patterning Techniques

With the surfaces modified, the next step in the patterning of neurons on MEAs is the protein patterning techniques that guide the growth of neurons. These techniques can be classified into two categories, namely, lithography-based and contact-printing-based categories. As the names suggest, lithography-based techniques deliver protein patterns by using micro-lithography, whereas contact-printing-based techniques rely on a molded polymeric structure to transfer protein patterns. Lithographic techniques are simple, especially to the many engineers involved in MEA work. However, this technique requires that the protein be deposited by physisorption and is not compatible with the deposition of multiple proteins. On the other hand, contact-printing uses molded PDMS either as a stamp or as a flow channel to guide the deposition of multiple guidance molecules (Chiu et al., 2000; Jo et al., 2000; Thiebaud et al., 2002). This is a critical issue because patterns with multiple proteins will certainly be used to design directed neuronal networks (Hickman and Stenger, 1994; Wheeler and Brewer, 1994; Stenger et al., 1998; Vogt et al., 2004).

7.4.1 Micro-Lithography

Micro-lithographically based protein patterning has been applied to patterning neurons since the early 1990s and employs micro-lithography to guide protein delivery. The general process is demonstrated in Figure 7.2. The initial step in this class of patterning technique involves the deposition of photoresist which is patterned by exposure to UV light. Depending on which type of photoresist is used (positive or negative), the exposed areas can be foreground or background. After development, the exposed or unexposed areas can adsorb the guidance molecules that remain on the surface during the step to remove the photoresist, assuming a sufficiently gentle process is used to preserve the important protein conformations.

In our experience, this process is the easiest method to achieve at least minimal patterning with excellent survival of the network of neurons because this process does not require complex structures or complex chemistry. The quality of the patterning depends strongly on the quality of the background materials. With polyimide-insulated MEAs, we obtained excellent patterning up to four weeks (Figure 7.3A) for a simple pattern of alternating lines with wide background (60 μm). However, low-fidelity patterning was the norm (18 out of 23 cultures) for silicon-nitride MEAs (Figure 7.3C,D), most likely as a result of the nitrogen within the silicon nitride. Thus, when using micro-lithography plus physisorption, one must consider the type of MEA because the insulation material becomes a critical factor in the quality of the patterns.

Although the background material is crucial, it is possible to present a background different from the insulator; two solutions come to mind. The first is the flood adsorption of background materials, similar to the flood silanization technique that Corey et al. (1996) presented to improve silane patterning. In this method, a background material can be adsorbed to the whole array, which as Corey demonstrated should minimally alter the quality of the foreground pattern. It is possible for one to adsorb high molecular weight PEG or albumin to enhance cell repulsivity of the background (Hoffman, 1996). Alternatively, one could also deposit a hydrophobic silane layer to the nonelectrode areas first followed by micro-lithographic patterning of the foreground. This ought to provide the necessary background while preserving the electrode impedance.

7.4.2 Contact-Printing Techniques

Contact-printing techniques involve either stamps or fluid channels made from poly-dimethyl-siloxane (PDMS), an elastomer that conforms to uneven surfaces, by pouring the monomers into a master mold produced from either silicon or polymeric substrates (Figure 7.15A,B; Branch et al., 1998; Xia and Whitesides, 1998; Corey and Feldman, 2003). When one produces the master from polymeric substrate, reactive-ion etching (RIE) is used to create the grooves within the pattern, producing a valley with right angles. In contrast, silicon masters produced from anisotropic etching have V-shaped valleys. In the direct stamping technique, protein is adsorbed onto the stamp and transferred to the MEA through contact with the

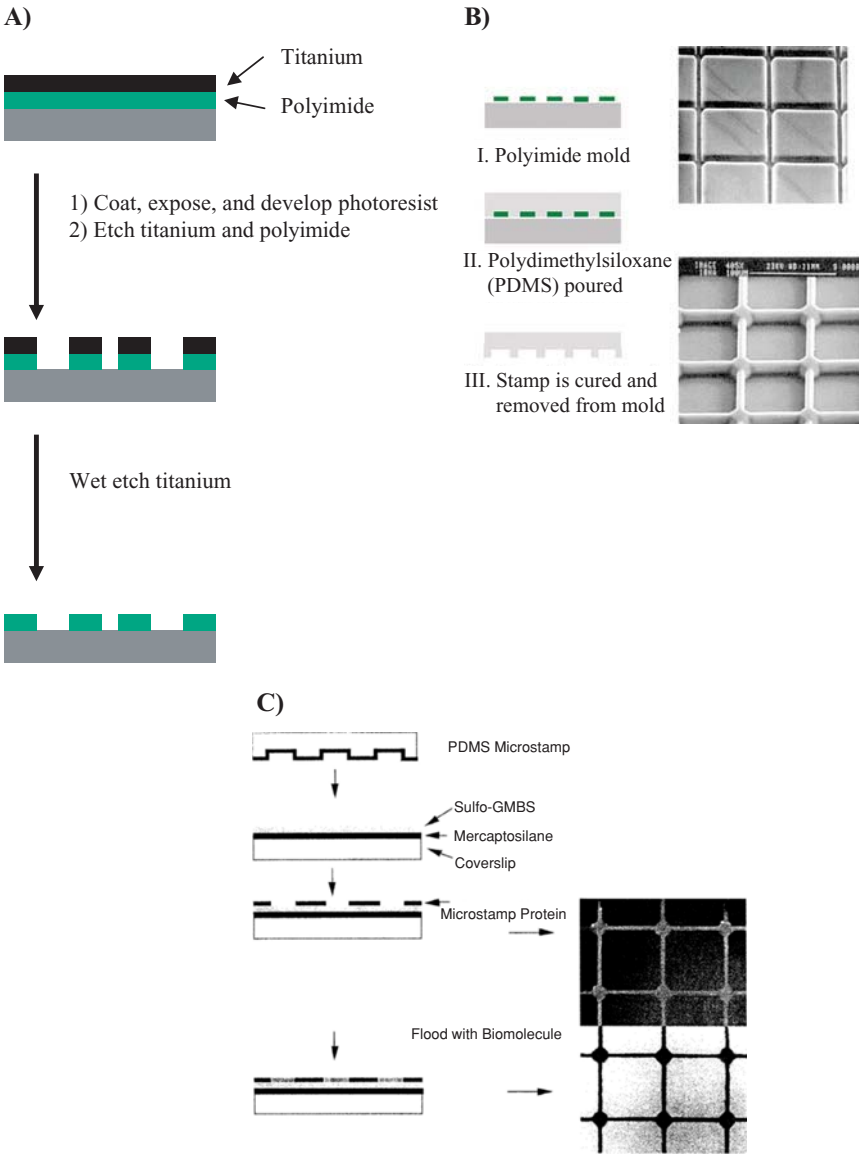

FIGURE 7.15. Fabrication of PDMS micro-stamps: (A) master mold is fabricated on polyimide-coated glass substrate by anisotropic etching of polyimide to create deep relief for stamp fabrication. (B) PDMS stamp is fabricated from the mold by pouring PDMS resin into the mold and curing. (C) Micro-stamping process in which the PDMS stamp is inked with protein and stamped onto activated substrates. The background is flooded with a cell-repulsive material such as PEG. ((C) is reprinted with permission from Branch et al., 2000; © 2000 IEEE.)

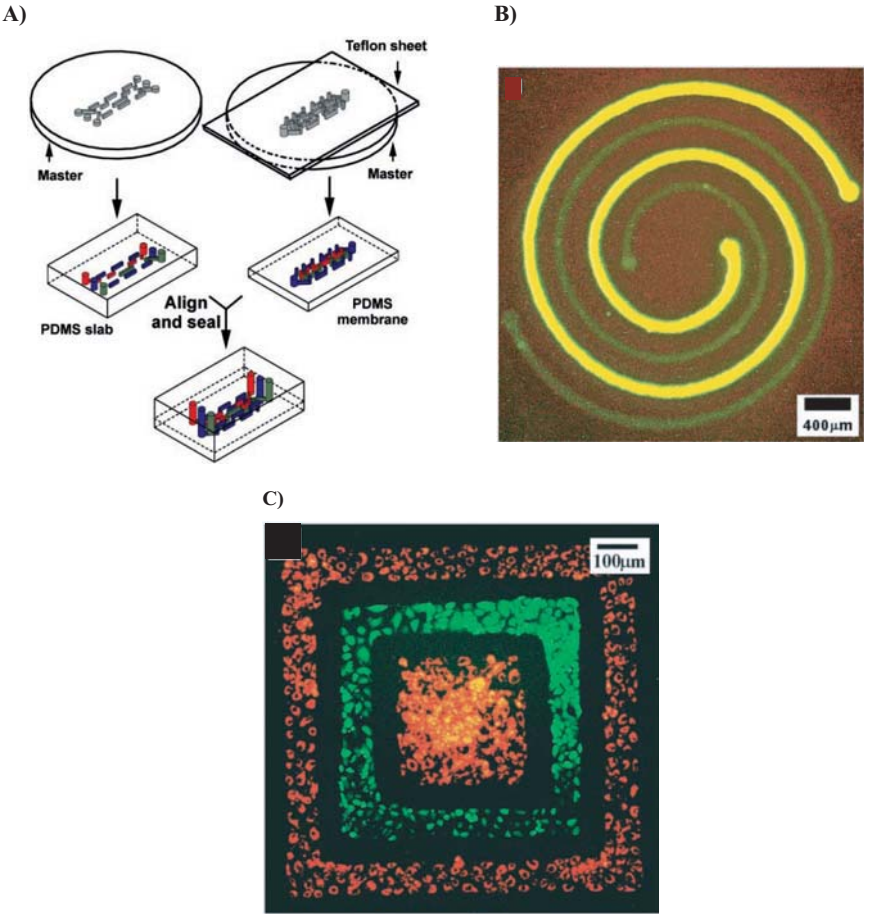

FIGURE 7.16. Micro-fluidic patterning of proteins and cells: (A) the design and assembly of the micro-fluidic channels from two-dimensional sheets of PDMS. The scheme demonstrates a two-layer design. (B) Simultaneous deposition of two proteins both of which are labeled with fluorescein isothiocyanate (FITC). Bovine serum albumin (BSA) is shown in bright green whereas fibrinogen is shown in light green. (C) Simultaneous deposition of two types of cells. Human bladder cancer cells (ECV304 cell line) are shown in green whereas bovine adrenal capillary endothelial cells are shown in red. (Reprinted with permission from Chiu et al, 2000; © 2000 National Academy of Science, U.S.A.)

PDMS stamp (Figure 7.15C). In the fluid-channel technique, micro-channels are designed into the mold to allow in- and out-flow of the protein solution (Figure 7.16A). When the mold contacts the substrates, protein solution is driven into the channels to allow protein adsorption or linking and then washed out with saline. In both techniques, multiple proteins have been delivered to the substrates

to guide the growth of cells, though not necessarily neurons (Branch et al., 1998; Wheeler et al., 1999; Branch, 2000; Chiu et al., 2000).

In our experience with patterning neurons using the micro-stamping technique, several factors influence the reliability of the process and the quality of the protein patterns. The first factor is the importance of a stiff backing for the stamp. As PDMS stamps are quite flexible, the backing provides the stiffness to stabilize the patterns during the transfer process (Corey, 1997; Branch, 2000). The second factor is the aspect-ratio between the height and the width of the patterns. Our experience has been that high aspect-ratios are important in obtaining faithful patterns after transfer (Branch et al., 1998). However, others using stamps produced from silicon masters have shown that shallow ridges can work as well (Bernard et al., 1998). The difference may be the different forces transferred onto the patterns due to the shape of the contact areas. The third is the release layer that improves the detachment of the protein from the stamp. In our experience, more proteins are delivered onto the substrate when a release layer (sodium laurylsulfate, in our lab) is used than when no release layer is used (Chang et al., 2003a). This release layer also allows a stamp to be used for several months, versus needing a new stamp after several uses due to stamp exhaustion (Bernard et al., 2001).

We have used this technique to pattern hippocampal neurons on MEAs and cultured the patterned networks for long term. When the MEAs are silanized or thiolated, we can cross-link the protein and a background material (amine-terminated polyethylene glycol) onto the MEA surface (Branch et al., 2000). The patterns on these arrays can be maintained for up to four weeks, but as stated previously, some patterns may detach from the MEA (Figure 7.9; Chang, 2002; Chang et al., 2003a; Nam et al., 2004). Although we apply a background material, this may not be necessary depending on the pattern geometry because the silane and the thiol both present cell-repulsive surfaces (silane is hydrophobic whereas thiol is negatively charged). However, recordings on silanized arrays have been rare due to dramatically increased electrode impedance, but we have recorded electrical activities from patterned networks on thiolated MEAs (Figure 7.10D,E; Chang, 2002; Nam et al., 2004). We have not done multiple protein patterning on MEAs, but this is not only possible but also an important step in preparing for future experiments.

Although direct stamping is simple, the process can potentially alter the conformation of proteins and is limited to delivering macro-molecules. These deficits can be corrected with the micro-channel technique which delivers either protein or cell solutions directly to the substrate (Chiu et al., 2000; Thiebaud et al., 2002). The tradeoff is that the design complexity of the initial master mold increases dramatically as one must design the in- and out-flow channels and solve the assembly problem in order to create a three-dimensional structure with embedded fluid channels. In addition, the channel surfaces need to be altered so that the fluid flows when pressure is applied, as hydrophobic channels require high pressure to drive aqueous fluid. However, the advantage is that channels can easily deliver multiple proteins to the substrate with one contact versus multiple contacts involved in the direct stamping technique.

Using channel-embedded PDMS structures, several groups have demonstrated the ability to pattern proteins onto substrates to guide cellular growth (Anderson et al., 2000; Chiu et al., 2000; Jo et al., 2000; McDonald et al., 2000). The three-dimensional structures are formed from two-dimensional sheets of PDMS carrying various parts of the final assembly which are assembled after activation by gaseous plasma that makes PDMS sheets extremely reactive towards each other (Anderson et al., 2000; Jo et al., 2000; McDonald et al., 2000). To pattern the substrates, the final assembly is aligned relative to a fiduciary and pressed against the substrate, followed by solution delivery (Chiu et al., 2000; Jo et al., 2000; Thiebaud et al., 2002). The channels guide the delivery of either the cells or protein solution to the designated spots on the substrates (Chiu et al., 2000). Bovine adrenal capillary endothelial and human bladder cancer cells have been patterned and cultured with these channels for approximately one day, and bovine serum albumin and fibrinogen patterning have been demonstrated (Figure 7.16 B,C; Chiu et al., 2000). Similarly, neurons have also been patterned by this technique (Thiebaud et al., 2002). These data strongly suggest that MEA patterning with micro-fluidics should be feasible and should simplify the delivery of multiple protein patterns onto MEAs.

7.5 Effects of Structuring Network: Early Results

These patterning techniques have been applied to the structure networks of neurons on both substrates and MEAs (Ma et al., 1998; Ravenscroft et al., 1998; Chang et al., 2000; James et al., 2000; Liu et al., 2000; Chang et al., 2001a,b; Chang, 2002; Chang et al., 2003a,b). These studies have demonstrated synaptic communication among interconnected cells on both substrates (Ma et al., 1998; Ravenscroft et al., 1998; Liu et al., 2000) and on MEA (Chang et al., 2001). In addition, the patterned networks have been shown to develop robust electrical activity when compared to random networks (Chang et al., 2001a,b, 2003b). When tested on substrates, it was shown that the patterned networks achieved greater glial and synaptic density for the first three weeks of the culture (Chang et al., 2003b; Chang, 2002). However, our results suggest that a random network may catch up in terms of glial and synaptic density after three weeks, which will have to be tested with additional experiments. Thus, the early data suggest that giving neuronal networks a defined structure may influence the developmental process of the network (i.e., neuron and glia interaction) in addition to regulating the interconnectivity between neurons. This finding may have an impact on our understanding mental retardation and genetic diseases that influence the brain as stunted neuron–glia interaction may slow nervous system development and alter information processing.

7.6 Conclusion

Current patterning technology has not only demonstrated feasibility with patterned neuronal culture on MEAs, but also the possibility of altering the developmental

process of the network on the culture. These abilities will help us discover principles of network dynamics and information processing in regard to the functioning of neuronal networks. In addition, the structured development may provide an alternative paradigm for studying neuron–glia interaction in nervous system development. However, the current technology will need improvement in order to reach these important goals. First, stronger linkage between the guidance molecule and the substrate is necessary to prevent degradation of the designed structure. Second, rules of connectivity specification need to be developed, a task that is currently under intense research by several groups but for which practical findings remain elusive.

As we achieve better understanding of internetwork information processing by the central nervous system, we may be able to replace lost neural function regardless of the location of the lesions. As stated in the introduction, robotic arms can be controlled by the firing patterns of prefrontal neurons, implying that we will soon replace lost motor function. However, the challenge of replacing lost dopaminergic neurons (Parkinson's) will be much more formidable as simple cell replacements have induced severe side effects (Freed et al., 2001). Perhaps the solution is to regulate the connectivity of the replacement neurons because the original dopaminergic neurons had very specific connections. Achieving this will make structured network replacement a standard therapy in many central nervous system illnesses as well!

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