Polymer Bulletin 61, 511–520 (2008)
DOI 10.1007/s00289-008-0967-x

Polymer Bulletin

Preparation of biopolymeric micropatterns via non-residual-layered imprinting technique and its application to modify single-celled microelectrodes

Hui-Jung Tang[a], Hsu-Chuang Chen[b] and Yi-Chang Chung[c,d] (✉)

[a] Institute of Biotechnology and Chemical Engineering, I-Shou University, Kaohsiung County 840, Taiwan R.O.C.
[b] Graduate School of Biotechnology, National University of Kaohsiung, Kaohsiung 811, Taiwan R.O.C.
[c] Department of Chemical and Materials Engineering, National University of Kaohsiung, Kaohsiung 811, Taiwan R.O.C.
[d] Center for Micro/Nano Science and Technology, National Cheng Kung University, Tainan 701, Taiwan R.O.C.
E-mail: ycchung@nuk.edu.tw; Fax: 886 7 5919277

Received: 23 January 2008 / Revised version: 5 April 2008 / Accepted: 30 June 2008
Published online: 15 July 2008 –

Summary

A “patch clamp” microelectrode modified on the surface for single cell adhesion and growth was developed using a novel polymer-transfer technique to deposit cell-adhesive polymer layers on the substrate platform. Several hydrophilic polymers–polyvinyl pyrrolidone, chitosan, alginic acid, and gelatin were successfully transferred onto the platform in order to enhance cell adhesion. The process was developed to apply a PDMS stamp pre-adhered with a phosphorylcholine (PC) layer on the protruding areas of the pattern, followed by adsorbing an adhesive polymer to imprint the adhesive layer onto the platform. Plasma activation on the stamp, adsorbed polymers, and/or the substrate was evaluated to provide an accessible method to obtain a nanothick (about 180 nm) polymeric film without any residual layer. XPS characterization on the platform verified that the bioactive polymer was only transferred and coated on the convex surfaces of the pattern. From observing the cell adhesion on the chitosan-modified platform, the number of adhered cells on the chitosan layer was found to be greater than that on the original poly(glycidyl methacrylate) (PGMA) layer, corresponding with the prediction that chitosan can serve as the cell-adhesive layer. The modified patterns were found to confine a single cell on each convex top of the platforms.

Introduction

Single-celled platforms can act as a part of cell-based biosensors, such as planar electrodes for patch clamping, field-effect transistor arrays, microelectrode arrays, electrochemical microsensors and so on [1], for which the patch clamp technique is the main tool for studying ion channels by measuring a voltage change of ionic current in a small patch of cell membrane [2,3]. Patch clamp recording provides an advantage

in facilitating drug screening, and can also directly assay the function of the genes that encode ion channels and transport proteins. However, the method is not currently practical for high throughput because it requires a skilled operator to manually manipulate the pipette onto the cell [4]. Recent efforts to develop micromachined electrode arrays have been successfully fabricated on silicon wafers [5-7], however, there is still little literature about the platform surface modification for cell adhesion.

In order for cell-based biosensors to be effective in their analysis, it is necessary that viable cells are incorporated into the platform that will house them. The deposition should be compatible with high-throughput processes and the cells should be kept alive. As for cell culturing environments, appropriate scaffolds have been developed to regenerate cells and tissues for wound healing, organ reconstruction, and tissue repair. No matter in a human body or in laboratory, the scaffold should be able to induce cells' adhesion, locomotion, and growth. Generally speaking, the scaffold should: be biodegradable, have connective pores, cell-adhesive surfaces and cell-growing stimulants, and so forth. Furthermore, bioactive patterns occurring on micro or nano scale provide an alignment effect for cell spreading and a confined area for cell growth, so the fabrication of micro/nano patterns plays a significant role in developing the platform as a cell scaffold. Inspired by these concepts, a cell-adhesive and biodegradable material has been engineered to coat a platform surrounded by cell-repellent areas to form a micro/nano pattern, and then found to control cells' adhesion and guide their growth [8-10]. The engineered tissue or cell patch was used to fabricate a suitable electrode platform as a scaffold to house a single cell in this investigation.

There are several different methods of patterning active biomaterials, including soft lithography and photolithography to produce cell-specific adhesive areas [8,9]. In practice, bioactive polymer patterns are uneasy to handle in the traditional photolithography techniques. Because developing reagents are aqueous solutions, attempts to etch the hydrophilic patterns usually lead to severe contamination of etchant and probable harm to biomolecules. Also, a polymer carrying some bioactive molecules cannot endure photoirradiation and etching conditions. The micropatterned bioactive polymers used for soft lithography have been published for biomedical applications. Polypeptides were broadly applied using microcontact printing techniques pioneered by Whitesides and coworkers due to a monolayer of proteins being able to adsorb on the PDMS stamp [11]. Polylysine, albumin, laminin and so forth have been printed onto silicon wafers or glass employing the electrostatic interaction between proteins and substrates [12,13]. Some polysaccharides have been derived from extracellular matrices (ECM) or bioactive resources, but there have been very few reports about their use for printing by soft lithography. Hyaluronan (HA) was first reported to be compatible with microcontact printing and molding approaches [14]. The sluggish movement of highly viscous, entangled HA chains during the contact process assisted in conformal contact for complete pattern transfer. To our knowledge, there are no such reports about other bioactive polysaccharides such as chitosan and alginate. Whereas the spin-coated or adsorbed polymer film has continuous coverage over a patterned PDMS stamp, the transferred pattern usually displays ragged edges [15,16].

Hammond's group has developed the "polymer spin-transfer printing" method that is able to spin-coat a polymer thin film directly onto an elastomer stamp at room temperature and to transfer the polymer ink onto the substrate coated with a polyelectrolyte as an electrostatic adhesion promotion polymer [17]. The method has improved the traditional imprinting process with high temperature and pressure during

the formation of the polymer etch barrier. However, the residual layer– the layer remaining in the recessed areas of the etch barrier pattern, made of polyelectrolytes– served as a binder between the polymer etch barrier and the substrate. It is required to remove the residual layer before etching the substrate in maicro/nano fabrication. Removal of residual layers using common Reactive Ion Etch (RIE) techniques always increases the capital cost, environmental contaminants, and the risk of dimensional variation. Polymer inking suggested by Guo et al. was applied to fabricate nano/micro patterns without any residual layer to remove. In Guo's method, the large deviation of linewidth in imprinting caused by the cooling-induced shrinkage and cohesive force of polymers significantly limited the imprinting applications [18]. The comparison between those printing methods for biopolymer micropatterns was listed in Table 1.
In the study, a flexible process was adopted to fabricate a hydrophilic gel pattern without a residual layer on a micromachined electrode for planar patch clamp use. By adhering an amphiphilic surfactant, phosphatidylcholine (PC), on the protruding areas of the stamp pattern, the stamp was then able to selectively adsorb poly(vinyl pyrrolidone) (PVP), alginate, and chitosan, which could then be transferred onto a silicon wafer via the imprinting process. This method does not require high temperature and pressure, as is often the case in imprint lithography. The process was also applied to modify the micromachined electrode. Preliminary cell incubation tests were performed to evaluate the adhesion of single cells for the next step of cell electro-physiological measurement.

Experimental

Enrichment of PC in phospholipids

Extraction of PC-enriched phospholipids has been developed well under liquid chromatography analysis. The commercial Lecithin (TCI, food grade) was precipitated in acetone, extracted by ethanol, and put into a mixed acetonitrile/methanol solvent (4/1 (v/v)) for 30 min of vigorous stir. Those insoluble lipids and most phosphatidylethanolamine were filtered out, giving a PC-enriched, clear-yellow solution. After evaporating the solvent at 40 ^{o}C, the extract was dissolved in 30 ml of ethanol. Zinc chloride (60 wt%) solution was then dropped into the extract solution while stirring to form ivory PC-$ZnCl_2$ complex precipitation. The precipitation was freeze-dried in a vacuum and stored in a refrigerator for use.

Polymer transfer process

The poly(dimethyl siloxane) (PDMS) stamp fabrication process can be taken from other published literature [19]. By lifting to separate the PDMS stamp from the original mold, the stamp with the complementary pattern was ready for printing polymers. As shown in Scheme 1, a clean silicon wafer was coated with 1 mM of octadecyltrichlorosilane (OTS) in toluene solution to obtain a non-sticking surface. The wafer was then spin-coated with 1 wt% of dried PC-$ZnCl_2$ in n-hexane solution for 30 s at 2000 rpm. The stamp was briefly exposed to O_2 plasma for 1 minute using simple plasma equipment (model: AR-100 PC). By impressing the stamp on the PC-coated silicon plate for 3 minutes, the PC-adhered stamp was lifted from the OTS-treated wafer. The PC transfer completeness was evaluated by observing the wafer and PDMS surfaces using an optical microscope. The PDMS stamp was exposed in air

Table 1. Comparison of printing methods for micropatterned biopolymers.

Printing methods for biopolymers	Features	Limits for processing biopolymers	Ref.
Optical lithography	Using a photomask and a photoresist to define a pattern after UV irradiation.	Require using photoresists to define patterns or developing a new photosensitive biopolymer. Equipment cost is high.	9
Micro/nano imprint	Hot-embossing a thermoplastic polymer with a hard mold at above Tg, then cooling to form a replica pattern. UV-curing a mixed oligomer/monomer pressurized by a transparent mold to form a replica pattern.	Using dry etching to remove residual layers. High processing temperature or UV irradiation is adverse to biopolymers.	10
Microcontact printing	Using a rubber stamp adsorbed ink to transfer the thin film patterns on substrates.	Difficult to transfer entangled polymer layers.	11-14
Polymer inking (transfer)	Combine microcontact printing and imprint techniques. Easy to transfer polymer films without residual layer.	By now only for hydrophobic polymers transfer.	18

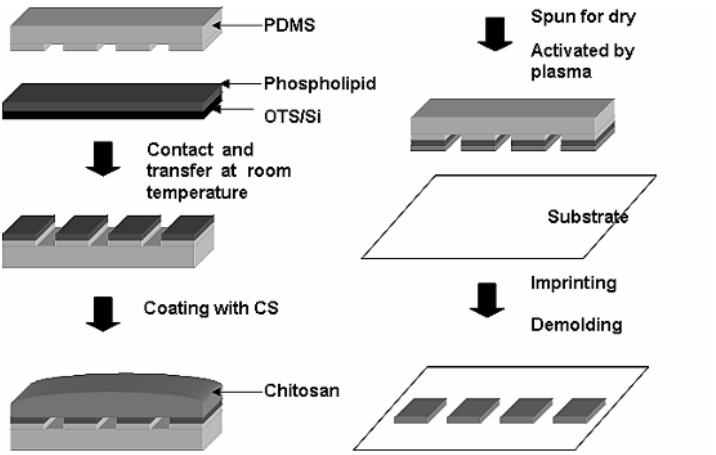
for a given period and then coated with a water-soluble polymer by dipping it into a polymer solution (for example: 1 wt% of chitosan in 1 wt% of aqueous acetic solution;0.5 wt% of poly(vinyl pyrrolidone) in deionized water; 0.1 wt% of alginate in deionized water). After 30 minutes of polymer adsorption, the PDMS stamp was spun to dry. Upon reaching a given hydrostatic pressure temperature on the hot plate for 5 minutes, the hydrophilic polymer was transferred on another silicon wafer (as a substrate) and produced a micropattern without residual layer.

Characterization of surface morphology

A contact anglometer (Magic Drop AT) was used to check the contact angle changes of a no-pattern plate during the process. An optical microscope (Navitar 12X Zoom OM) and a scanning electron microscope (SEM, Hitachi S4100) were directly evaluated for the polymeric thin layers and patterns. The 3D confocal microscope (μSurf, NanoFocus) was used to measure the pattern profile and its thickness.

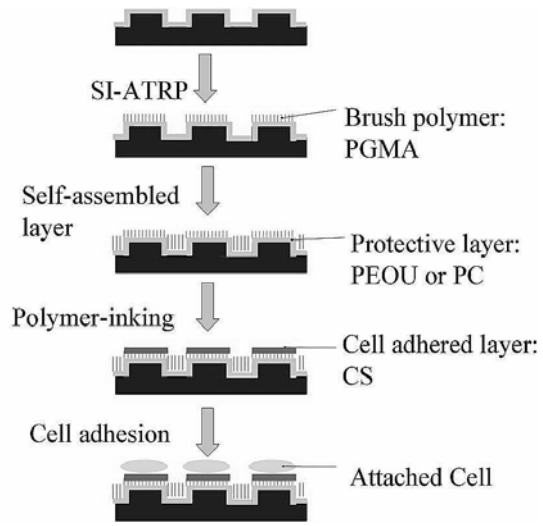
Fabrication of single-cell patterns

The single cell platform for electricity detection was designed and generously donated by Professor C. H. Luo (National Cheng Kung University, Taiwan). Protruding spots ten microns in diameter are distributed across the surface of the silicon chip. Each spot has been etched with a hole that is 2 um in diameter for the next step of electrophysiological measurement. The silicon chip was printed with a flat PDMS stamp pre-adsorbed with chloromethyltrichlorosilane (as an initiator for surface polymerization). Then the chip was immersed into N-(triethoxysilylpropyl)-O-polyethylene oxide urethane (PEOU, MW400~500) to bind the concave portions of the chip for 12 h. After cleaning, the chip was then sealed into a degassed bottle with a multicomponent solution: glycidyl methacrylate (GMA, 4.53 mmol), CuCl (0.453 mmol), $CuBr_2$ (0.091 mmol), and 2,2'-dipyridyl (Bpy, 1.277 mmol)) were mixed in a methanol/water (2/1 (v/v)) solvent. The surface-induced atom transferred radical



Scheme 1. Procedure for surfactant-assisted imprinting technique without residual layers.

polymerization (Si-ATRP) was kept at room temperature for six hours. By cleaning the chip with acetone and methanol/water solvent, the convex spots of the chip were grafted with a thin layer of poly(glycidyl methacrylate) (PGMA). On the PGMA layer, the chitosan was transferred by the imprinting process for cell attachment. The entire process was schematically shown in Scheme 2. X-ray photoelectron spectrometer (XPS, provided by ULVAC-PHI, model: PHI Quantera SXM) was used to characterize the differences in the molecules on the concave and the convex portions of the cell platform by using Mg-K (1253.6 ev) excitation. The surface element analyses were to compare with each one's normalized area, and the chemical-shifting curves were optimized by fitting the single element spectra to estimate the formed bonds.



Scheme 2. Procedure for fabricating the single-celled platform.

Cell adhesion and growing

The cleaned chip was fixed on a culture plate. The sample was covered with L929 osteoblast cell medium, and the system was incubated at 37°C for a given time in a 5 % CO_2 atmosphere. After rinsing the chip with PBS, the adhered cells were fixed with 2.5 % of glutaraldehyde for two hours. After rinsing by PBS three times, the chip was dipped in 1 % osmic acid for one hour. The rinsed chip was then dried with ethanol and the critical point drying process for observing the cell adhesion and growth by an optical microscope. Also the chip was examined via SEM following sputter-coating with an ultrathin gold layer.

Results and Discussion

PDMS was first tested to adsorb PC molecules from OTS-treated wafer directly without O_2 plasma activation. The OTS-pretreated silicon plate assisted in PC transfer from the plate to the convex of the PDMS pattern, causing the water contact angle to change from 120.7^o to 50.5^o. In contrast, in another test with a freshly-cleaned silicon plate (no OTS treatment), the PC layer strongly adhered to the plate, and could no longer be transferred. Optical microscopic observation was carried out to ensure the PC transfer pattern, as Figure 1 shows. Also, the PC-adhered PDMS was tested to print PC molecules onto a clean silicon substrate, giving a pattern suffering from line shrinkage, but still clear (photograph not shown). It is interesting that the PC layer can be transferred below 0 ^{o}C, indicating that the frozen gel state of the PC layer very easily adhered to the PDMS stamp. However, only part of the pattern was transferred in the process. Complete PC adhesion and transfer of the full pattern was thought to enhance polymer transfer; accordingly, the plasma activation on PDMS surfaces was adopted in the following conditions. One minute of plasma irradiation resulted in efficiently hydrophilized surfaces on the PDMS stamp, assisting in homogeneous absorption of PC molecules.

As for the imprinting conditions, imprinting temperature, imprinting pressure, and wetness of polymers were main issues affecting the quality of pattern transfer. When we tried the imprinting process at room temperature and under 1 atm of pressure, the wetness of chitosan was found to dominate the transfer effectiveness. When we decreased the pressure to 0.37 atm, the transferred figures on the pattern shrank and became disconnected, owing to non-conformal contact between the polymer layer and the wafer. After being heated up to 200 ^{o}C, the imprinted chitosan became soft enough to transfer. (While PVP was operated at 140 ^{o}C.) High temperature was held to achieve the glass transition temperature of the polymer during the imprinting process; however, the operating temperature was so high for those polar polymers that it was harmful to plastic and bioactive substrates. Accordingly, low temperature and suitable pressure in this inking process were considered preferable. Because of the high molecular weight property of chitosan, the polymer entanglement tends to form a thick film across the micropattern, leading to ineffective chitosan transfer from PDMS to the silicon substrate. By absorbing a sufficient amount of water to soften the polymer chains for conformal contact between PDMS and the substrate, chitosan was able to transfer at the room temperature. The similar situation can be found in PVP, alginate and gelatin transfer. Alternatively, chitosan adsorbed on the protruding part of the PDMS stamp was activated by oxygen plasma before contact with the substrate. The result showed a completely transferred pattern for the chitosan polymer having

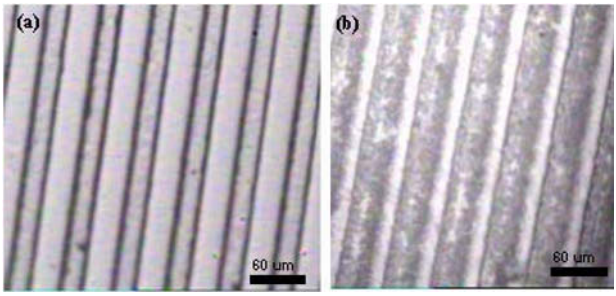


Figure 1. Microscopic photographs of patterns after PC adhesion from OTS-treated wafer to PDMS stamp. (a) PDMS mold; (b) OTS-treated wafer.

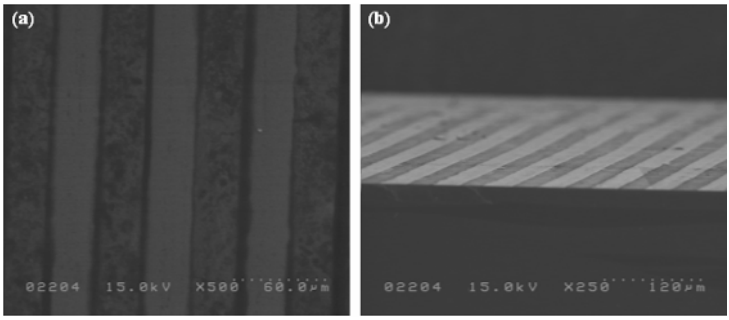


Figure 2. SEM photographs of the transferred chitosan ruled pattern. (a) Top view. (b) Tilted view.

high molecular weight and hard polymer chains. In Guo's publication, a high temperature was applied to soften the given polymers, leading to the dimension shrinkage on the transferred pattern after cooling [18]. We chose gentle conditions so that imprinted polymers at ambient temperature under O_2 plasma treatment or not could produce well-transferred micropatterns without apparent figure shrinkage and residual layers, as shown in Figure 2.

The transferred pattern having no residual layer on the substrate displayed the uniform thickness (about 180 nm), shown in Figure 3. The thickness was able to be controlled by adsorption with various concentrations of chitosan solutions and the immersion more hydrophilic than the concave ones. Hydrophilic polymers– PVP, alginate, or chitosan– in a diluted solution were also able to adsorb on the convex patterns. That is why no residual layer was found. The imprinting process was then carried out under a slightly normal pressure to force the polymers to adhere to the silicon wafer at ambient temperature.

The C1s peaks at 285 eV and at 286.4 eV may be assigned to the hydrocarbon (C-C-C) bond and the ether group (C-O-C) by the deconvolution of C1s peaks, as shown in Figure 4 (a). The peak at 287.2 eV based on carbon with nitrogen group (C-N) coming from transferred chitosan (convex layer) or PGMA/PC (concave layer) [19]. The peak near 289.3 eV based on residual amide groups of chitosan due to partial deacylation from chitin, which is obviously found in the spectrum of convex layer and weighted as 4 % of total carbon species (gray area in Figure 4(a)). However, only about 1.9 % of amide groups in total carbon species were found on the concave layers. Moreover, the

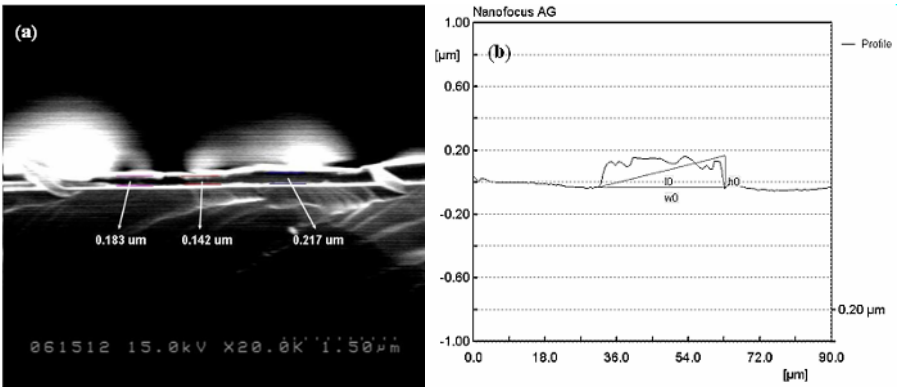


Figure 3. Thickness evaluation of the transferred convex pattern. (a) SEM photograph from a cross-section view of the pattern; (b) measurement of the Z-directional depth profile by a 3D confocal microscope.

N1s analysis on the pattern surfaces showed that a sharp N1s peak at 400 eV based on amine groups in chitosan was found on the convex surfaces, while a vague N1s peak appeared on the spectrum of concave surfaces, suggesting the chitosan-rich layers were transferred. XPS measurement demonstrated the polymer transfer process was able to transfer chitosan onto the convex surfaces of the cell platforms.

The application of water-soluble polymer transfer was significant to the fabrication of single-celled platform for patch clamp use. As shown in Figure 5 (b), the chitosan layer can be transferred onto the convex platform, while the concave area was treated with biocompatible PEG molecules. In cell attachment measurement, the platform displayed the surface property that only single cells could be adhered, as shown in Figure 5 (b), (c) and (d). Cells were found to selectively adhere onto the chitosan-transferred platforms, other than on the PGMA-grafted surfaces. The selectivity was about 2:1. Furthermore, the concave substrate surfaces coated with PEOU still adsorbed many cells, showing the concave patterns favored by cells and that the morphology of the surface broadly affects cell adhesion and migration, similar to the conclusions of previous literature [20]. A single cell adhered onto the platform was found to grow and extend its pseudopods to cross the platform. When cells were

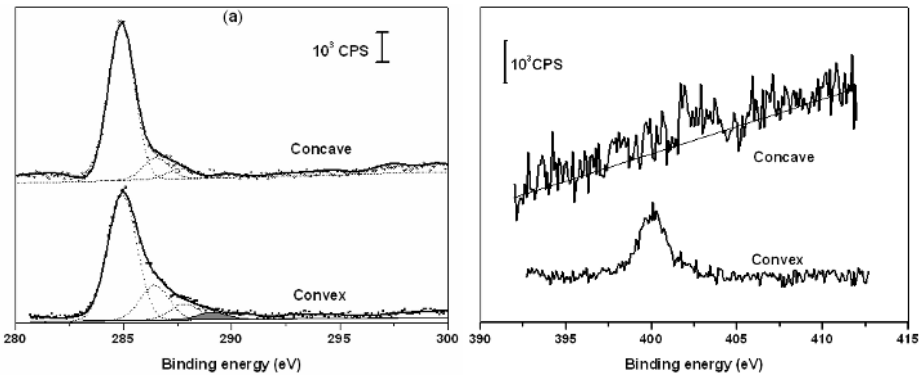


Figure 4. XPS spectra of the patterned platform. (a) C1s and (b) N1s analyses for the coating on the concave and the convex portions. Inset show the P2p spectrum of the concave portion.

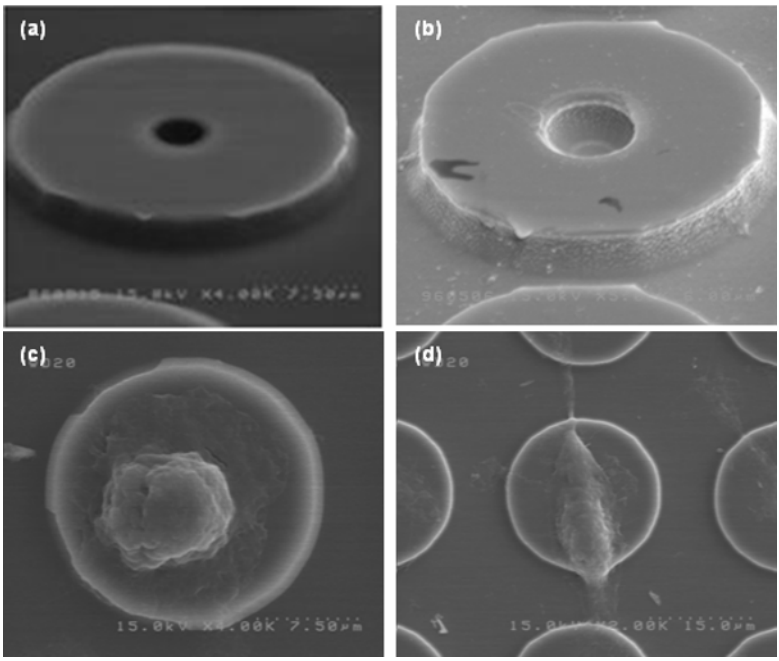


Figure 5. (a): Microphotograph of original platform. (b): Microphotograph of the Chitosan-transferred platform where a thin film is formed on the protruding surfaces. (b) and (c): Microphotographs of cell adhesion on the platform. Cells were aggregated on the concave plane, while a single cell tended to adhere on the convex platform coated with chitosan after three hours of incubation. The single cell was able to grow and to spread the pseudopod after one day of incubation, as shown in (d).

applied to incubate with different shapes of platforms, only a single cell attached on a platform maintaining round shape in any shape of platform (data not shown).
As the polymer transfer technique can be used to easily fabricate residue-free microstructured areas over a whole square-centimeter pattern, it is likely to extend and apply this process to larger areas using standard commercial processing equipment and adjustment of the factors in small-scale fabrication. The process entails the formation of bioactive polymer microstructures on a range of surfaces, including silicon wafers, metal oxides, glass, and flexible polymer substrates. The technique can also be used to fabricate multi-tiered microstructures, especially for multilayered scaffold construction.

Conclusions

The bioactive polymers– PVP, alginate, gelatin and chitosan– were successfully transferred onto the wafer without a residual layer by using imprinting techniques. The PDMS stamp pre-adhered with PC on the protruding areas of the pattern and followed by adsorbing gels was able to imprint the chitosan layer onto the wafer. Plasma activation on the stamp, adsorbed chitosan, and/or the substrate was able to provide an accessible method to obtain a nanothick polymer pattern. Preliminary cell adhesion and growth tests were performed to find that most cells attached on the

electrode platform are single, which would be helpful to further electrophysiological detection.

Acknowledgements. The authors thank the Ministry of Economic Affairs of R.O.C. for financial support under Contract No. 95-EC-17-A-07-S1-0018. The Precision Instrumentation Development Center of the NSC at National Cheng Kung University is also appreciated for assistance with SEM operations. Prof. Guo SM and Chang SC (Department of Biomedical Engineering, I-Shou University) are appreciated for helping cell incubation tests.

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