FABRICATING IN VITRO NANOMATERIAL SCAFFOLDS THROUGH INTEGRATED CIRCUIT COMPATIBLE MICROFABRICATION TO MODULATE MAMMALIAN CELLULAR BEHAVIORS

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4.1. INTRODUCTION

The past several decades have brought about an ever-increasing requirement for nanomaterial-based scaffolds that could be used to: a) explore cellular behaviors by mimicking in vivo extracellular matrix conditions, or b) modulate cellular behaviors by manipulating them on material interfaces. This chapter describes the development of easy-to-handle, high-throughput methods – a combination of common integrated circuit (IC)-compatible manufacturing procedures with features of mass production capacity – to create in vitro nanomaterial scaffolds and platforms to modulate cellular behavior and growth micropatterning for a variety of mammalian cells. Microfabrication has already been integrated into techniques of photolithography, reactive-ion etching, metal nanoparticle-assisted etching, and chemically-based surface modification. These approaches enable us to sufficiently design and create artificial scaffolds with small-scale features to probe cell-substrate interactions.

In the natural environment, cell-to-cell or cell-to-milieu interactions are crucial for the formation of organs and tissues in vivo. Exploring the interactions between materials and mammalian cells is a meaningful and interesting subject in various research communities including tissue engineering, regenerative medicine, and biosensors [1]. The ability to manipulate mammalian cell-material surface interactions has offered us a myriad of profound knowledge into biology-related research including mechanotaxis, cell viability, and stem cell differentiation [2,3]. Cell-material surface interactions are largely and intimately determined by the interplay of adhesive molecules at the cell-material interface. These adhesive molecules affect various cellular functions, such as division, proliferation, cell migration, differentiation, and structural protein distribution [4-6]. Various molecular mechanisms influencing the way that cells discern and react to their surrounding milieus have been explored, including ligand-integrin interactions [7-10], surface hydrophobicity or hydrophilicity, [11-13] and topography [14-21]. Because biomedical engineering places great significance on cell adhesion, the development of biomimetic materials to enable cell attachment and sustain desirable architecture has been widely investigated [22,23]. Notably, it has been shown that the actin cytoskeleton is a critical and dynamic structural component of the mammalian cell milieu that responds to changes in surface modification and, in doing so, alters characteristics of adhesion. Substrate-related modifications (often stimulations), for instance, can enhance actin polymerization and affect focal adhesion complexes [24]. Various collaborations between biology and engineering researchers have sought to develop biocompatible scaffolds to modulate cellular responses in vitro. As a result, controlling cellular environments using artificial scaffolds has become
an area of devoted, albeit difficult, research that carefully and thoughtfully integrates the regulation of both scaffold function and cell development. Most research in this field makes use of plastic cell culture dishes to perform experiments, but such surfaces are morphologically dissimilar to the natural extracellular matrix. Recent advances in the development of three-dimensional environments that can provide a more natively relevant surrounding support scaffold for cells have spawned crucial advances in understanding various physiological and pathological conditions [25-29]. In other literature, the differences between \textit{in vitro} and \textit{in vivo} outcomes have been extensively discussed [30].

Several approaches have been used to create nanomaterial-based scaffolds for \textit{in vitro} experiments. Research exploring cell-material surface interactions at the nanometric scale can be catalogued into two groups, the examination of cellular responses to i) adhesion molecules on various substrates (chemically based approach) [31,32], or to ii) surface stiffness [33-37] and topographic features (physically based approach) [38-41]. With regard to chemical approaches, various chemicals or proteins micropatterns can be used to efficiently modulate cellular function. For example, cell adhesion and neurite outgrowth can be modified by chemically patterned surfaces [42-44]. Further, self-assembled monolayers of alkanethiols have been shown to influence the action potential of neurons [8]. Physically-based approaches, which use micro-/nano-topographically altered surfaces, can influence cytoskeletal remodeling, cellular morphology, and cellular spreading [45,46]. The effects of topographic features, including the effects of a variety of micro-/nano-scale geometries on cellular behaviors, have been investigated. Such investigations include experimental review of microposts [47-50], nanorings [51,52], microgrooves [53], microperiodic structure [54,55], micropillars [56,57], nanopillars [58], and nanofibers [59]. In a notable example of both physical and chemical influences working together to affect cellular response, substrate topography has been shown to work in concert with chemical signaling to regulate cell behaviors [53]. The topographical dimension of adhesive molecules, such as collagen fibers, laminin, and fibronectin exists in the nanometric scale in nature; hence, to mimic \textit{in vivo} conditions, it is necessary to examine the physiological and physical effects of nanoscalar surface modifications, especially with regard to the manner by which they affect chemical contact sites for the regulation of cell activity. At present, our understanding of the physical and chemical properties that, together, affect interactions between the cell and the substrate surface and modulate specific cell behavior remains immature. This chapter, which discusses nanomaterials including oxidized silicon nanosponges and functionalized chitosan membranes coated with different monolayers of functional groups, may provide a suitable preliminary platform to further investigate cellular behaviors using biocompatible and biomimetic conditions that resemble \textit{in vivo} milieux. The development of suitable artificial structures \textit{in vitro} is
crucial in several medical fields including nerve regeneration, small-scale biomedical devices, and nanofabrication methodology.

Another challenging issue in biomedical engineering is determining how to array cells with a desired pattern on artificial scaffolds. The specific arrangement of cells is crucial to understand cell-to-scaffold interaction, but it is also important as a means of mimicking \textit{in vivo} conditions. For example, native neural cell cultures are composed of a random arrangement of neural cells, a situation that is hard for researchers to emulate because neural cells prefer to aggregate into non-uniform groups. Using microfabrication, scientists can control neural cell adhesion and growth, which allows for experiments that better illuminate genuine neurobiological mysteries [42,60-63]. Current promising progress in microfabrication has allowed for cellular patterning in desired regions. The common microfabrication techniques for creating precise, solid surface micropatterns include nanoimprint lithography [64], microcontact printing [65-68] and microfluidic-based processes [69]. Microcontact printing technology, developed by Dr. Whitesides’ research group (Harvard University), has become the most common approach for transferring protein patterns onto cultural substrates [70]. However, disadvantages of this method, namely surface sticking and an unstable yield rate, need to be overcome before it is suitable for mass production. Also, because the elastomeric stamp in this process is flexible, deformation frequently occurs when pressing the stamp onto a solid surface.

Studies have examined a variety of surface property changes that can be leveraged to specifically arrange cells on desired regions [11,71]. The capacity for mammalian cell micropatterning is a preliminary requirement for the development of artificial stents, suitable biosensors, and implantable artificial tissues. For this reason, the demand for nanomaterial-based \textit{in vitro} scaffolds that can micropattern mammalian cells has been increasing, especially in the fields of regenerative medicine and tissue engineering. The core of this chapter, which is based on a selection of the past five-years’ published research, includes an examination of the following nanomaterial-based devices: 1) artificial scaffolds to be used as \textit{in vitro} platforms for studying cellular biology while mimicking the natural environment of the extracellular matrix (\textit{e.g.}, silicon wafer and glass materials); and, 2) implantable materials (\textit{e.g.}, chitosan membranes) to assist regenerative medicine, including organ transplant and nerve conduit procedures.
4.2. OXIDIZED SILICON NANOSPONGES

To investigate cellular behaviors in biomimetically and physiologically-relevant situations \textit{in vivo}, oxidized silicon nanosponges have been fabricated using inorganic, oxidized silicon wafers, a commonly used material in IC manufacturing processes. It is worth noting that IC manufacturing industries using silicon-based platforms have the following characteristics: 1) robustness; 2) ease-of-use; and, 3) expandability to mass production. Therefore, silicon-based platforms have considerable potential for various applications in the field of biomedical engineering. Furthermore, oxidized silicon substrate can be modified with various functional group monolayers to detect cell responses, including organization of the cytoskeleton, biochemical changes, and cell adhesion. Moreover, because photolithographically-based microfabrication can be used to create specifically micropatterned regions and hydrophobic nanosponges on silicon surfaces, micropatterning of mammalian cells can be achieved to facilitate the insightful examination of cellular morphology. Sections 4.2 and 4.3 largely describe Yang's studies examining mammalian cell response and patterning on oxidized silicon nanosponges [72,73].

4.2.1. Surface modification of silicon substrates

Nanosponges have been fabricated on monocrystalline silicon surfaces \textit{via} Ag-nanoparticle-assisted etching (Figure 1a) [74,75]. In these experiments, a silicon wafer was immersed into 0.01 M silver nitrate solution for approximately 5 min. Following formation of a metallic catalyst layer, the substrate was soaked in etchant containing hydrogen fluoride (HF, 49 % wt) and hydrogen peroxide (H$_2$O$_2$, 30 % wt) at a mixture ratio of 3 : 1 (v / v) for 3 min. The procedure for fabricating nanosponges on a silicon surface was formerly developed by Peng \textit{et al.} [75]. In order to enhance biocompatibility, the silicon surfaces were fabricated to create a 20 nm-thick silicon dioxide layer. The wafer surfaces were hydroxylated by O$_2$ plasma treatment for 10 min \textit{via} vapor deposition of silanol groups in a vacuum chamber (Figure 1b). Following this, a silanol-hydroxyl reaction was used to create a self-assembled molecular layer on the surface [76]. In total, six types of surfaces containing various chemical modifications and nano-topography were created, including pristine oxidized silicon surfaces, perfluorodecyltrichlorosilane (FDTS)-grafted oxidized silicon surfaces, (aminopropyl)trimethoxysilane (APTMS)-grafted oxidized silicon surfaces, pristine oxidized silicon nanosponge surfaces, FDTS-grafted oxidized silicon nanosponge surfaces, and APTMS-grafted oxidized silicon nanosponge surfaces.
Figure 1. Fabrication of modified silicon substrates. (a) Ag nanoparticle-assisted etching was used to fabricate silicon nanosponges. Through vapor deposition process, different chemical functional groups were self-assembled on silicon nanostructures. (b) The scheme of the self-assembled functional groups. Surface chemical modification was created by \( \text{O}_2 \) plasma treatment for surface hydroxylation. Next, the reaction between hydroxyl and silanol reacted. Finally, the monolayer was self-assembled on the interface [72].
4.2.2. Substrate surface characteristics

The hydrophobicity of the aforementioned surfaces was measured using a water contact angle meter. The results indicate that the water contact angles were $29^\circ \pm 2^\circ$, $110^\circ \pm 3^\circ$, $41^\circ \pm 2^\circ$, $1^\circ \pm 1^\circ$, $148^\circ \pm 4^\circ$ and $2^\circ \pm 1^\circ$ for pristine oxidized silicon substrate, FDTS-grafted oxidized silicon substrate, APTMS-grafted oxidized silicon substrate, pristine oxidized silicon nanosponge, FDTS-grafted oxidized silicon nanosponge, and APTMS-grafted oxidized silicon nanosponge, respectively (Figure 2). Note, FDTS functional groups can increase substrate surface contact angle on more hydrophobically coated surfaces. While, the nano-topography of surfaces coated with functional groups may enhance hydrophobicity, silicon surfaces grafted with APTMS functional groups were still hydrophilic and displayed a contact angle of less than $90^\circ$.

![Figure 2. Contact angles and SEM images of modified substrates. (a) Pristine oxidized silicon substrate; (b) FDTS-grafted oxidized silicon substrate; (c) APTMS-grafted oxidized silicon substrate; (d) Pristine oxidized silicon nanosponge; (e) FDTS-grafted oxidized silicon nanosponge; (f) APTMS-grafted oxidized silicon nanosponge [72].](image)

4.2.3. Cellular morphology on oxidized silicon surfaces

Chinese hamster ovary (CHO) cells were selected to study the interaction between cells and the aforementioned substrates. Figure 3 displays morphological images of CHO cells following 2 h of culture. The CHO cells grown on oxidized silicon surfaces appeared analogous to those grown on culture plates (Figure 3a). Figure 3d displays CHO cells stretched out and showing a rounded-up shape on pristine oxidized silicon nanosponges. Figures 3b and 3e show CHO cells cultured on FDTS-grafted substrates but, in these images, nanospikes are no longer observed and the cells have a smaller rounded-up shape. On the APTMS-grafted substrates, CHO cells were known to spread up to 100 $\mu$m in size (Figure 3c, 3f). This morphology is caused by the $\text{NH}_2^+$ functional group of APTMS [77]. In comparison, CHO cells were seeded on
pristine oxidized silicon nanosponges and pristine oxidized silicon substrate for durations of 30, 60, 120, and 240 min. Figure 4 shows that, despite the fact that CHO cells showed flat morphology on the pristine oxidized silicon substrates, the cells appeared to be rounded-up on the pristine oxidized silicon nanosponges, and differentiated numerous nanospikes visibly emanate from the somas attached to the nanoposts of the oxidized silicon nanosponges. Nanosponges were thus seen to offer physical support for cells to adhere to the oxidized silicon surfaces.

Figure 3. SEM images of CHO cells seeded on functionalized surfaces after 2 hours of culture. (a) Pristine oxidized silicon substrate; (b) FDTS-grafted oxidized silicon substrate; (c) APTMS-grafted oxidized silicon substrate; (d) Pristine oxidized silicon nanosponge; (e) FDTS-grafted oxidized silicon nanosponge; (f) APTMS-grafted oxidized silicon nanosponge [72].
Figure 4. SEM images of the contact morphology of cells seeded on pristine oxidized silicon substrate and oxidized silicon nanosponge after different culturing durations (a-30: after 30 min of culture on pristine oxidized silicon substrate, b-30: after 30 min of culture on oxidized silicon nanosponge). Cells cultured on pristine oxidized silicon substrate for 30, 60, 120 and 240 min, respectively [72].

4.2.4. Cytoskeleton remodeling on different surfaces

Cytoskeletal orientation and focal adhesion formation are products of the interactions between cell shape and the extracellular matrix [78]. Cytoskeletal organization of CHO cells was examined to determine the influence of nanosponges via staining with rhodamine-conjugated phalloidin (Figure 5). Note, the CHO cells seeded on pristine oxidized silicon nanosponges showed
filamentous actin clustered in a punctuate manner, which could represent the adhesive dots of cell-nanoposts. These results indicate that actin polymerization is restricted along a specific direction when CHO cells encounter nanoscale adhesive dots on nanosponges.

Figure 5. Confocal microscope images of the nucleus and cytoskeleton of CHO cells seeded on silicon surfaces. (a) Pristine oxidized silicon substrate; (b) FDTD-grafted oxidized silicon substrate; (c) APTMS-grafted oxidized silicon substrate; (d) Pristine oxidized silicon nanosponge; (e) FDTD-grafted oxidized silicon nanosponge; (f) APTMS-grafted oxidized silicon nanosponge [72].
4.2.5. Cell attachment assays

Cell attachment assays have been carried out by re-culturing CHO cells for different time durations. Greater cell adhesion was observed in cultures using nanosponges compared to pristine oxidized silicon substrates, FDTS-, and APTMS-grafted substrates (Figure 6). These outcomes suggest that nano-topography is a determining factor in cell immobilization.

![Figure 6](image)

**Figure 6.** Cell attachment after 30, 60, 120 and 240 min of culture on silicon substrates with various surface modification. The error bars represent the standard deviations of cell number. The *p* values were compared with the pristine oxidized silicon substrates [72].

4.3. MICROPATTERNED SILICON SUBSTRATES

AZ4620 photoresist was micropatterned onto a monocrystalline silicon wafer surface to act as the protective layer during photolithography. The micropatterned silicon wafers were selectively etched using Ag nanoparticle-assisted etching. Subsequently, the surface was chemically modified as previously mentioned. Finally, photoresist was removed with acetone, and the silicon substrate with micropatterned features was prepared for further cell behavior study (Figure 7).
Figure 7. Schematic of the integrated circuit (IC)-based microfabrication for fabricating micropatterned silicon substrates. The designed patterns were fabricated through photolithography and Ag nanoparticle assisted etching. And then, the chemical surface modification was created via vapor deposition. Finally, mammalian cells were seeded on a micropatterned silicon surface [73].

4.3.1. Cell response to micropatterned silicon substrates

Because CHO cells could cross over the nanosponge gap between two flat silicon stages, the distance between two flat silicon stages was adjusted (Figure 8a, 8b). The maximum distance between two flat silicon stages that CHO cells could cross was approximately 40 µm (Figure 8c, 8d). For this reason, the desired distance of nanosponge gap for two flat silicon stages was set to 80 µm to prevent cells from crossing. The results indicate that HIG-82 fibroblasts and CHO cells prefer to attach onto a flat oxidized silicon surface rather than onto FDTS-grafted oxidized silicon nanosponges. With increasing cell culture time, cells gradually migrated from FDTS grafted oxidized silicon nanosponges. Further, Figure 9 shows that when HIG-82 fibroblasts and CHO cells were arranged on pristine oxidized silicon stages, HIG-82 fibroblasts were prone to connect together but CHO cells were not. CHO cells re-arranged on pristine oxidized silicon stages but the cell-to-cell connection was not discovered.
Figure 8. SEM images of CHO cells crossed over two silicon stages; (a) top view, (b) cross-section view. (c) Schematic of CHO cells crosses over two silicon stages. (d) The distance between two silicon stages with nanosponge gap that CHO cell would cross over versus the suspended percentage. The largest distance between two silicon stages was 40 µm for CHO cell to cross over. The error bars represent the standard deviations of the suspended percentage [73].

Figure 9. Staining images of F actin (red) and nucleus (blue). CHO cells and HIG-82 fibroblasts were at different culture durations on micropatterned silicon substrates [73].
4.3.2. Cell fusion analysis

Membrane fusion, including endocytosis and intracellular transport, is an integrative biological procedure [79]. In this research, micropatterned silicon substrate with a hydrophilic-hydrophobic boundary (created using pristine oxidized silicon surfaces and FDTS-grafted oxidized silicon nanosponges) was used to examine whether the cell-to-cell connections of HIG-82 fibroblasts were due to membrane fusion. To verify whether membrane fusion occurred when HIG-82 fibroblasts connected together, NBD C6-HPC was used to mark the phospholipid membrane. The results indicate that few fibroblasts fused together when cultured on micropatterned oxidized silicon substrates after 3 days of culture (Figure 10a). Further, when the width of flat regions was reduced to 5 µm, more HIG-82 fibroblasts were likely to fuse together; indicating that cell density may play a critical role in membrane fusion, since HIG-82 fibroblasts were not prone to stay on FDTS-grafted nanosponges. On the other hand, single CHO cells placed on 5 µm width flat regions re-arranged quite quickly, but did not fuse together (Figure 10b).

![Cell membrane staining. The picture area is about 590 µm by 440 µm; the total cell number in one picture was approximately 300 via calculating the nucleus in a staining picture. (a) Staining pictures of nucleus (blue), F actin (red) and membranes (green). (b) Fusion ratios of CHO cells and HIG-82 fibroblasts after 3 days of culture. The error bars represent the standard deviations [73].](image)
4.4. FUNCTIONALIZED CHITOSAN MEMBRANES

Chitosan-based biomaterials (chitin-based biopolymers) derived from nature, have been approved by the US Food and Drug Administration (US FDA). Typically, chitosan is used in a myriad of biotechnologically-related applications for implantable materials, including neural regeneration, and drug carriers [80-84]. Additionally, chitosan is a biodegradable polymer that is a suitable carrier of implantable materials for regenerative medicine [85,86]. Despite technological advances, it is still challenging to modify physically-based factors (e.g., topography, geometry, and stiffness) on chitosan membranes. Recent advances in the development of chitosan membranes (a naturally derived biomaterial or “soft” material) prepared via microfabrication may help overcome existing restrictions and achieve several objectives in the field of tissue engineering. Chitosan-based platforms possess the following unique advantages: 1) they can be used to easily control cell function, and 2) they are suitable for tissue regeneration, due to mechanical properties, biological properties, and biomimetic characteristics. Furthermore, nanostructures on chitosan surfaces can provide uniform but non-periodic nanostructures that more closely mimic physiologically relevant microenvironments. Note, micro/nanoscaled features can be integrated onto chitosan membranes via multiple IC-based manufacturing processes. These modified chitosan membranes may be used to study cellular behaviors and micropatterning of mammalian cells. Paragraphs four and five describe Shuai’s studies, which display mammalian cell response and patterning on chitosan membranes with surface modification [87,88].

4.4.1. Surface modification of chitosan membranes

The scheme of microfabrication for chitosan membranes with flat and nanostructured surfaces is displayed in Figure 11. Chitosan solution contains 1 % (w/v) chitosan powder (190–310 kDa) in 1 % v/v acetic acid. Flat chitosan membranes were prepared by soaking diced silicon wafers in chitosan solution. After casting chitosan solution onto flat or nanostructured silicon wafers, the chitosan membranes were generated by drying for 6 h in an oven at 60 °C. Finally, diced treated silicon wafers were immersed in 0.1 M NaOH so that newly cast chitosan substrate could be peeled from the silicon wafer material. Functionalized chitosan membranes were modified through molecular vapor deposition on flat chitosan surfaces [72,73], and four types of chitosan membranes were created, including flat chitosan membranes, O₂-plasma-treated chitosan membranes, FDTS-grafted chitosan membranes and nanostructured chitosan membranes, to regulate cellular behaviors.
Figure 11. (A) Fabrication of chitosan membranes. The flat chitosan membranes were created via soaking chitosan solution onto a silicon mold, and then flat chitosan membranes were peeled off of the silicon mold in 0.1 M NaOH solution. (B) Fabrication of nanostructured chitosan membranes. The nanostructured chitosan membranes were created by soaking chitosan solution onto silicon nanosponges. After solvent evaporation, chitosan thin film processed a nanostructured surface that duplicated the silicon nanosponge [88].

4.4.2. Surface characteristics of chitosan membranes

Various chitosan membrane morphologies are shown in Figure 12. As evidenced by measurements of water contact angle for each of the chitosan surfaces examined, those chitosan surfaces treated with O₂ plasma demonstrated more hydrophilicity because there were more hydroxyl groups on their surface. FDTS-grafted surfaces produced a rise in measurable contact angle due to the presence of more fluorine groups. Note, the hydrophilicity of chitosan membranes was also enhanced by nano-topographically scaled features on the chitosan membranes. To closely examine surface differences, flat and nanostructured chitosan surfaces were examined via atomic force microscopy (AFM). Due to size restrictions of the AFM cantilever probe, accurate measurement of nanostructured chitosan surface was difficult, but differences between flat and nanostructured chitosan surfaces (relatively rough surface) could be distinguished.
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4.4.3. Cellular morphology on modified chitosan surfaces

To examine cellular response to surface modification, NIH/3T3 fibroblasts were cultured on various chemically/physically modified chitosan membranes. Figure 13 displays phase contrast and staining image results from these experiments, which indicate the following: 1) cell proliferation was inhibited when NIH/3T3 fibroblasts were seeded onto nanostructured (nanotextured) chitosan surfaces, 2) NIH/3T3 fibroblasts cultures grew to a larger projected cell area on O$_2$ plasma-treated surfaces than on FDTS-grafted and nanostructured surfaces, and 3) NIH/3T3 fibroblasts spread and proliferated on O$_2$-plasma-treated chitosan surfaces. Our study suggests that, because the distance between chitosan-based nanostructures (about 80–100 nm) was consistent with the distance between two integrin clusters of cells, cell spreading was limited. Therefore, the extension of actin fibers was restricted as well.

**Figure 12.** Surface morphologies of physically/chemically modified chitosan membranes (a) SEM images and contact angle examination of chitosan surfaces with different surface modifications including flat, O$_2$-plasma-treated, FDTS-grafted and nanostructured. (b) Atomic force microscopy images of flat and nanostructured chitosan membranes [88].
To probe the interactions between integrin α1β1 in NIH/3T3 fibroblasts and chitosan-based nanostructures, rhodamine fibronectin was immobilized onto chitosan-based nanostructures to specifically bond with integrin α1β1 on cell membranes. Figure 14 shows that fibroblasts adhered and spread out on the nanostructured chitosan membranes after 1 h of culturing due to the fibronectin coating, which promoted both cell spreading and attachment. Results indicate that integrin clusters truly connected to chitosan-based
nanostructures. Furthermore, we observed more NIH/3T3 fibroblast integrin $\alpha_1\beta_1$ expressed in cultures grown on the flat chitosan membranes than in cell cultures grown on nanostructured chitosan membranes (Figure 15).

**Figure 14.** Staining images of nucleus (blue) and integrin $\alpha_1\beta_1$ (green) of NIH-3T3 fibroblasts seeded on nanostructured chitosan membranes with rhodamine fibronectin (red) treatment after 1 h (a and b) and 24 h (c and d) of culture. The results display that fibroblasts spread out on the nanostructures after 1 h of cell culture due to fibronectin coating, an extracellular matrix protein, support cell attachment and spreading out. These figures suggest that integrins truly attached onto single chitosan-based nanostructures and several integrins could gather onto single nanostructures. (e) Scheme of integrin attached onto chitosan-based nanostructures (nanosponges) with rhodamine fibronectin coating [88].
Figure 15. (a) Staining images of integrin of NIH-3T3 fibroblasts on flat and nanostructured chitosan surfaces after 48 h of culture. (b) Staining intensity of expressed integrin α1β1 of single fibroblast per its adhesive area on flat and nanostructured chitosan membranes. Data are mean ± standard deviation (N = 5, n = 10) [88].

4.5. SINGLE-CELL CHITOSAN MICROARRAY

Micropatterning single cells at specific locations would be beneficial for probing cellular responses to drugs. A single-cell chitosan microarray with mixed flat and nanostructured surfaces was cast from a silicon mold that was fabricated through microfabrications including photolithography and Ag nanoparticle-assisted etching. First, photoresist was removed from the silicon mold, and then chitosan solution was cast onto the mold. This chitosan membrane was dried in an oven at 60 °C for approximately 6 h. The single-cell chitosan microarray was then peeled off of the silicon mold after soaking in 1 M NaOH. HeLa cells (cancer cells) were subsequently cultured on the surface of these microarrays for drug screening experiments (Figure 16). HeLa cells were treated with a chemical compound, cytochalasin D, which inhibited actin polymerization by disturbing actin microfilaments and causing cell apoptosis. Finally, in order to determine the percentage of apoptotic cells per total cells, annexin V-FITC was applied to label cells (Figure 17). The results show that approximately 59 % of the HeLa cells underwent apoptosis after 1 h with 10 mM cytochalasin D treatment, compared to merely 4 % apoptotic when HeLa cells were treated with cytochalasin D-free medium. In a final experiment, cytochalasin D was cleared away from cell cultures via washing with phosphate-buffered saline (PBS). Subsequently, these cells were incubated in cytochalasin D-free medium again for 1 h and for 24 h to examine actin microfilament morphology. The results show that, after just 1 h, recovered cells were still apoptotic. Nevertheless, the apoptosis percentage was reduced to approximately 30 % because the recovery duration was increased. This finding suggests that cells may re-enter the cell cycle and may have been able to reconstruct their filamentous actins.
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Figure 16. The fabrication of single-cell chitosan microarray. Micropatterned silicon mold was fabricated via photolithography and chemical etching. And then the single-cell patterns on chitosan membrane were transferred from silicon mold via solution casting. After peeling from mold with NaOH solution, single-cell chitosan microarray was prepared for cell culture [88].

Figure 17. Drug screening applications on single-cell chitosan microarray. (a) Staining images of HeLa cells on microsquared chitosan microarrays. Color representation: F-actin (green), G-actin (red) and nucleus (blue). (b) Apoptotic cell percentage of HeLa cells cultured on a single-cell chitosan microarray and pristine chitosan membranes after 1 h of incubation with cytochalasin D treatment. Then, cytochalasin D was cleared away for next 1 h and 24 h incubation (recovery period). In the control group, HeLa cells were seeded in the medium without cytochalasin D treatment. Data are mean ± standard deviation. (n = 20, N = 6[six samples]). (c) Staining images combined phase contrast images. HeLa cells were labeled with DAPI and Annexin V-FITC [88].
4.6. NANOROUGH GLASS SURFACES

Because the mechanosensitivity of embryonic stem cells (ESCs) to the physiological milieu remains unclear, some researchers have recently shown interest in regulating cell-nanoscale surface interactions to further explore stem cell response on material interfaces [89-91]. Among these pieces of research, Chen et al. developed an easy-to-handle microfabrication method for precisely controlling and micropatterning the nanoroughness of glass surfaces. The glass material, a common material used in cell culture, possesses the following advantages: 1) chemical ligands can be easily modified on its surface to promote cell affinity, 2) glass material is transparent, so it is beneficial for immediate observation via optical microscope, and 3) topographical modifications are easily fabricated on silica-based glass wafer surfaces via IC-compatible microfabrication. This study indicates that nanoroughness on glass surfaces could be useful for examining resultant regulatory signaling for different human embryonic stem cell (hESC) behaviors. Section 4.6 describes Chen's studies regarding the microfabrication of nanorous glass surfaces and their application for probing stem cell responses [92,93].

4.6.1. Microfabrication method for creating local nanoroughness

Specifically nanoroughed regions can be created on a glass wafer via IC-compatible microfabrication, including photolithography and reactive-ion etching (RIE) techniques. In Chen's studies, the photoresist layer was micropatterned onto each glass surface using photolithography. Then, the non-photoresist-protected regions with variously shaped designs were bombarded with RIE over different durations to generate randomly nanoscaled surfaces [94]. Note, the reactive ion species generated via SF₆ and C₄F₈ gases striking the unreactive glass substrate resulted in some defects, causing the glass surface to be reactive toward the etchant species. Finally, photoresist was removed by solvents, and the nanorous glass substrate was prepared for further stem culture purposes (Figure 18).

![Fabrication of nanorous glass surface](image)

**Figure 18.** Fabrication of nanorous glass surface. The photoresist layer was micropatterned on a glass wafer surface via photolithography. Then, the glass wafer was fabricated via RIE process to generate the nano-scale surface. After the RIE process, photoresist was removed (stripped) with solvents. The nanorous glass substrate was prepared for further stem culture purposes [93].
4.6.2. Surface characterization of nanorough glass surfaces

Glass surfaces were processed with RIE at different durations in order to generate various levels of surface nanoroughness. Figure 19 shows statistical and scanning electron microscope (SEM) images displaying the different levels of nanoroughness produced by variations in glass substrate etching time. The roughness of the unprocessed glass surfaces, measured via atomic force microscope (AFM), was about 1 nm (the root-mean-square roughness, Rq). After the glass wafers were fabricated via RIE, the Rq ranged from 1–150 nm, which corresponded to different treatment durations. Furthermore, by precisely designing masks for photolithography, variously shaped nanorough islands could be achieved.

![Figure 19](image)

**Figure 19.** (a) The glass surfaces were processed with RIE at different durations in order to generate various levels of surface nanoroughness. (b) SEM images of micropatterned glass surfaces, including nanorough characters, square, circle, and triangle islands. (c) SEM images of glass surfaces with (middle and bottom) and without (top) treatments of RIE processes, with their rms nanoroughness indicated [93].

4.6.3. Cellular responses of hESCs on nanorough surfaces

Nanorough glass surfaces with different extents of nanoroughness were used as a growth substrate to probe functional responses of hESCs. First, the glass surfaces were coated with vitronectin to enhance self-renewal of hESCs. In terms of observation via scanning electron microscopy, hESCs showed highly branched, spreading morphology on a smooth glass surface (Rq = 1 nm) after 24 h of culture. However, hESCs showed short cytoplasmic extensions and less spreading on a nanorough glass surface (Rq = 150 nm). Furthermore, hESCs
displayed selectivity of cell immobilization between different extents of glass substrate nanoroughness. The results show that hESCs preferred to selectively adhere and aggregate onto the available smooth islands (Rq = 1 nm) rather than onto the nanorough regions (Rq = 70 nm) after 24 h of culture. Further, hESCs kept their stemness and showed positive expression of Oct3/4+ during the selective adhesion process (Figure 20a). Note, Oct3/4− hESCs, which expressed spontaneous differentiation, did not show any adhesion preference and preferred to randomly immobilize on the micropatterned glass surfaces (Figure 20b). With increased surface roughness, Oct3/4+ hESCs adhered less to surfaces regardless of treatment with Y27632, which is typically applied to promote survival of hESCs (Figure 20c). Figure 20d shows that hESCs were prone to self-renewal on the non RIE-treated glass surfaces after 7 days of culture.

![Figure 20](image.png)

**Figure 20.** (a) Phase-contrast images show that Oct3/4+ hESCs displayed selectivity of cell immobilization between different degrees of nanoroughness after 24 h of culture. (b) Phase-contrast images shows that Oct3/4− hESCs randomly attached on a micropatterned nanorough glass surface. (c) Adhesion percentage of hESCs on different roughnedd surfaces after 24 h of culture. (d) Rate of Oct3/4+ hESCs on the different level of nanoroughness glass surfaces after 7 days of culture [93].

### 4.6.4. Coculture system on nanorough glass surfaces

To further explore whether other cell types displayed responses similar to those of hESCs regarding glass surface roughness, NIH/3T3 fibroblasts were seeded onto the nanorough glass surface for examination. The results show that NIH/3T3 fibroblasts preferred to attach to the micropatterned nanorough islands with different geometries (Figure 21a, 21b). The micropatterned
nanorough glass surfaces could be used to control the adhesion location of NIH/3T3 fibroblasts and the adhesion selection of NIH/3T3 fibroblasts was contrary to that of hESCs. Intriguingly, these differences in cell preference, i.e., the desire to attach onto flat or nanorough surfaces, offered a strategy to part different cell types for coculture systems. The results show that hESCs and NIH/3T3 fibroblasts selectively attach onto flat and nanorough regions after 48 h of culture (Figure 21c). Statistical analysis indicates high cell separation efficiency when using micropatterned nanorough glass surfaces to separate hESCs and NIH/3T3 fibroblasts (Figure 21d).

Figure 21. (a) Phase-contrast images of clusters of NIH/3T3 fibroblasts adhering onto nanorough islands (Rq = 70 nm) with various patterns after 24 h of culture. (b) Cell adhesion percentage of NIH/3T3 fibroblasts on the flat glass surface (Rq = 1 nm) and the nanorough glass islands (Rq = 70 nm). (c) Merged optical microscopic image of coculture Oct3/4+ hESCs and NIH/3T3 fibroblasts spatially separating on a micropatterned nanorough glass surface after 48 h of culture. Cells were stained for nucleus (DAPI, blue) and Oct3/4+ (red). (d) Percentages of NIH/3T3 fibroblasts and hESCs located on the flat (Rq = 1 nm) and nanorough (Rq = 70 nm) regions of the micropatterned nanorough glass surfaces after 48 h of culture [93].
4.7. CONCLUSION

This chapter describes three types of new nanomaterial-based, in vitro cell culture platforms for modifying surfaces through IC-based microfabrication techniques, all of which display mass production potential. First, oxidized silicon nanosponges can be easily fabricated with various micro-/nano-surface patterns and have been shown to provide an easy-to-use, high-throughput in vitro platform for obtaining comprehensive insight into cell morphogenesis and exploring cellular responses in biomimicking environments. Secondly, chitosan material, a US FDA / US Environmental Protection Agency (US EPA)-approved biomaterial, demonstrates a wide range of potential applications as an implantable material for organ transplants and wound dressing. More importantly here, we found that single-cell chitosan microarrays, which can be cast from silicon molds, are useful for generating micro-/nano-structures on soft material surfaces that can subsequently be used for drug screening applications. Furthermore, chitosan membranes can also be functionalized through chemical modification to probe cellular behaviors. Thirdly, nanorough glass surfaces can be fabricated via microfabrication strategies to provide an efficient regulatory signal over different hESC behaviors as well as provide a selectively separating coculture system. We believe that silicon wafers, chitosan, and glass nanomaterial-based platforms are beneficial for creating inexpensive but stable in vitro cell culture devices for biomedical engineering and regenerative medicine.

REFERENCES