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Compatibility of Primary Hepatocytes with Oxidized Nanoporous Silicon**

By Vicki Chin, Boyce E. Collins, Michael J. Sailor, and Sangeeta N. Bhatia*

Silicon has begun to receive increasing attention for use in biomedical applications. In particular crystalline silicon has been utilized as a textured surface to guide cell alignment,^[1,2] to encapsulate cells for implantation,^[3] and as an electroactive substrate to stimulate excitable cells.^[4,5] Several properties of silicon have led to its use in these diverse applications: 1) well-described silane chemistries for immobilization of adhesive ligands, 2) wet and dry micromachining capability to form three-dimensional structures on biologically relevant length scales, and 3) semiconductor properties that allow incorporation of microelectronic elements. In comparison, porous silicon, a nanocrystalline material generated by etching of crystalline silicon in hydrofluoric acid, has been less extensively utilized for biomedical applications. Its open pore structure and large surface area, combined with unique properties such as photo and electroluminescence have provided a platform for sensors for non-biological species (e.g., solvents, gases, and explosives) as well as biological species (deoxyribonucleic acid (DNA), proteins).^[6–8] Indeed, the range of tunable pore sizes (5 to 1200 nm) in porous silicon spans a range of sizes important in biology; a small DNA fragment is on the order of a few tens of nm, proteins are generally in the 100 nm range, and bacteria and cells can be a few micrometers in diameter.^[9]

Some researchers have begun to explore the use of porous silicon as a biodegradable material for the slow release of drugs or essential trace elements to cells or as an in vivo diagnostic.^[10–12] Promising findings by Canham et al. have shown

hydroxyapatite nucleation on porous silicon in vitro, suggesting that porous silicon, in contrast to crystalline silicon, could be a bioactive surface.^[13,14] Nonetheless, porous silicon has not been extensively characterized as a material for implantation or the formation of hybrid (biological/non-biological) devices in vitro.^[15] Studies on the compatibility of this material with mammalian tissues have been limited to immortalized cell lines that are known to be relatively robust. Furthermore, immortalized cell lines such as those investigated by Bayliss et al. proliferate in vitro, therefore, potential cytotoxic effects of nanoporous silicon may not be readily discerned in “bulk” metabolic assays of viability.^[16,17]

The aim of this study was to characterize the attachment, viability, and function of primary rat hepatocytes, a notoriously difficult cell to grow in culture. Attachment and viability were assessed by fluorescent microscopy of cells probed with vital dyes on nanoporous silicon, in contrast to bulk metabolism assays that have been previously used. To investigate the long-term effects of the porous silicon material on cell viability and function, we also examined the maintenance of liver-specific functions over two weeks of continuous culture.

The attachment and spreading of primary hepatocytes on nanoporous silicon were compared across a variety of culture conditions. The porous Si samples were prepared in an electrochemical etch as described previously.^[18] The porosity of the porous Si substrates was approximately 70 %, with an average pore size of 2–5 nm.^[19] The samples were then subjected to oxidizing conditions which generate a thin (approx. 5 nm) oxide layer. Initially, the cells are exposed to a silicon oxide surface rather than pure silicon; however, Fourier transform infrared spectroscopy (FTIR) showed that the oxidized porous silicon also contains the Si–Si crystalline vibrational mode, indicating that nanocrystalline Si is still present (data not shown). The oxidized surface is therefore similar to the surface of bioactive glass, which has been shown to be compatible with osteoblasts.^[20,21] Although this renders the silicon surface less electroactive, electrical control of the surface may be retained through electron tunneling through the thin oxide layer.

Figures 1A–C present representative micrographs of primary hepatocytes that have been labeled with fluorescein diacetate 24 h after they were seeded onto porous Si substrates in the absence of adhesive serum proteins, in 10 %-serum containing media, and in the presence of both 10 %-serum containing media and collagen I coating. Cells appear to preferentially adhere and spread on porous silicon in the presence of type I collagen. In the absence of collagen but in the presence of serum and associated adhesion molecules (e.g., vitronectin, fibronectin, etc.), some cells attach and spread well, but to a lesser degree than the collagen adsorbed case. In the absence of any non-endogenous cell adhesion molecules, cell attachment was sparse and spreading was minimal. Figure 1D shows the percent of surface area covered by cells as determined by image analysis. There was significantly more coverage on the collagen treated porous silicon ($32 \pm 3.6\%$) than on either the serum treated or untreated porous silicon

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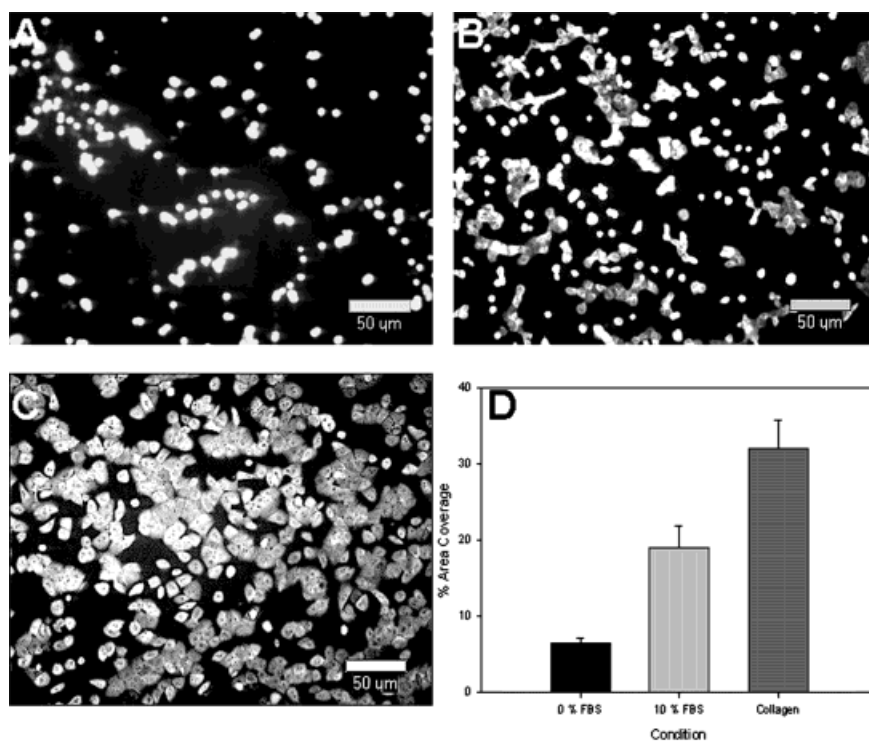


Fig. 1. Representative optical micrographs of primary rat hepatocytes stained with FDA on porous silicon substrates. A) Hepatocytes on untreated porous silicon. B) Hepatocytes on porous silicon pre-treated with 10 % (v/v) FBS. C) Hepatocytes on porous silicon pre-treated with a solution containing collagen I. D) Summary graph showing the fraction of substrate area with adherent cells as a function of substrate treatment.

($19 \pm 3\%$ and $6 \pm 0.7\%$ respectively). The serum-treated sample also displays significantly more cellular coverage than the untreated sample.

Notably, one of the challenges in studying the mechanism of cell-substrate interaction on biomaterials is discerning the relative role of chemical interactions with cell surface receptors and physical topography of the surface. Typically, topographical features have been difficult to characterize and control in the absence of associated chemical modifications. Using nanoporous silicon, the physical and chemical properties of a substrate may be decoupled in this system. Through atomic force microscopy (AFM) imaging, the topology of the substrate surface has been characterized and shown to be similar irrespective of the chemical modification used. AFM images shown in Figures 2A–C reveal that the nanotopology of the porous silicon is relatively unchanged with the addition of cell matrix and adhesion molecules, implicating surface chemistry as the dominant variable. The similarity of the images suggests that differences in cell attachment were the result primarily of the chemical modification rather than the nanotopology of the surface.

The long-term effects of continuous hepatocyte culture in the presence of porous silicon as compared to crystalline silicon and tissue culture polystyrene are presented in Figure 3. Figure 3A shows the percent of adherent cells that are viable on porous silicon for the first 5 days after isolation. Cells in all conditions remained viable at comparable levels, suggesting that cell viability on nanoporous silicon approached that found on a comparative biocompatible standard, tissue culture polystyrene.

Figures 3B and C indicate rates of albumin and urea production of the cultures over two weeks. The synthesis of albumin is a widely accepted marker of hepatocyte synthetic function, and the secretion of urea is an indicator of an intact nitrogen metabolism pathway. The daily production of both albumin and urea in the porous silicon cultures are comparable to cells cultured on polystyrene as well as crystalline silicon over the entire culture period. These data suggest that there are no gross, long-term (~ weeks) cytotoxic effects of nanoporous silicon on primary hepatocytes despite the harsh electrochemical reaction conditions and high concentrations of HF used in the preparation of porous Si. The production of silane from porous silicon degrading in an isotonic solution, observed in Canham^[13] also does not seem to affect hepatocyte function.

In summary, porous silicon is a nanoporous semiconductor substrate that couples many of the beneficial features of crystalline silicon with unique properties such as its capability to act as a biosensor and the tunability of pore sizes over biologically relevant length scales. We have shown using both a long-term biochemical study and direct fluorescent imaging of live cells that primary cells attach, spread, and function on this material.

These results demonstrate the feasibility of integrating existing porous silicon biosensor technology with functional cells, which may enable real-time, non-invasive monitoring of cellular function and metabolism and aid in the formation of complex BioMEMS (biological micro-electro-mechanical systems). Furthermore, tuning of pore size provides a tool to examine the effects of nano- to micro-scale topography on cell behavior independent of surface chemistry.^[1,22,23]

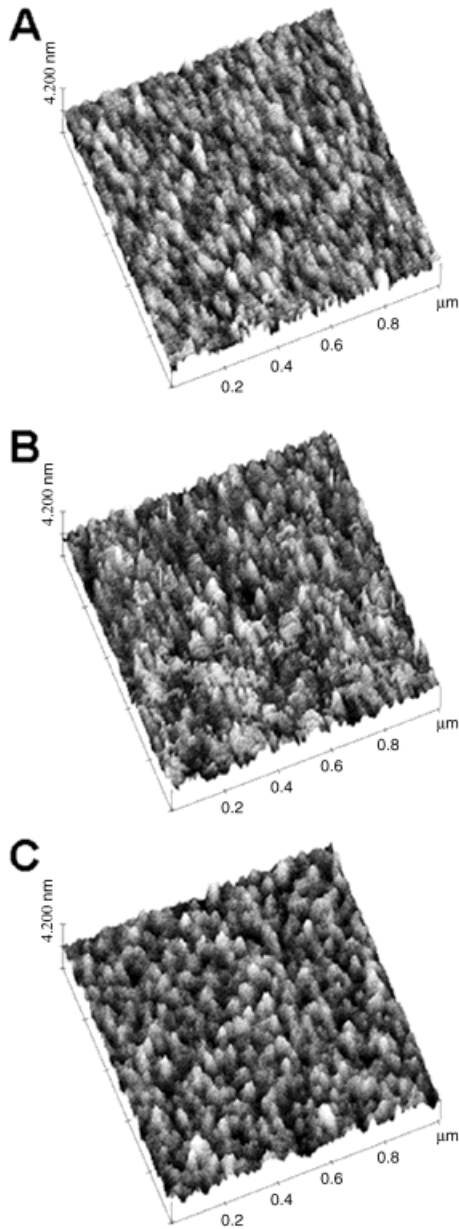


Fig. 2. AFM images of the three types of porous Si substrate preparations used in this study. All porous silicon samples were prepared under identical etching parameters and then modified with the chemistry of interest. The images were obtained after chemical modification, and represent the appearance of the samples immediately prior to hepatocyte seeding. A) Untreated porous silicon. B) Porous silicon pre-treated with 10 % (v/v) FBS. C) Porous silicon pre-treated with a solution containing collagen I. Area statistical rms roughness values for (A), (B), and (C) are 0.229, 0.303, and 0.282 nm, respectively.

Experimental

Hepatocyte Isolation and Culture: Hepatocytes were isolated from 2–3 month old adult female Lewis rats (Charles River Laboratories, Wilmington, MA) weighing 180–200 g, by a modified procedure of Seglen [24]. Detailed procedures for isolation and purification of hepatocytes were previously described by Dunn et al. [25]. Briefly, 200–300 million cells were isolated with viability between 85 % and 95 %, as judged by trypan blue exclusion. Nonparenchymal cells, as judged by their size (<10 μm in diameter) and morphology (non-polygonal or stellate), were less than 1 %. Culture medium was Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10 % fetal bovine

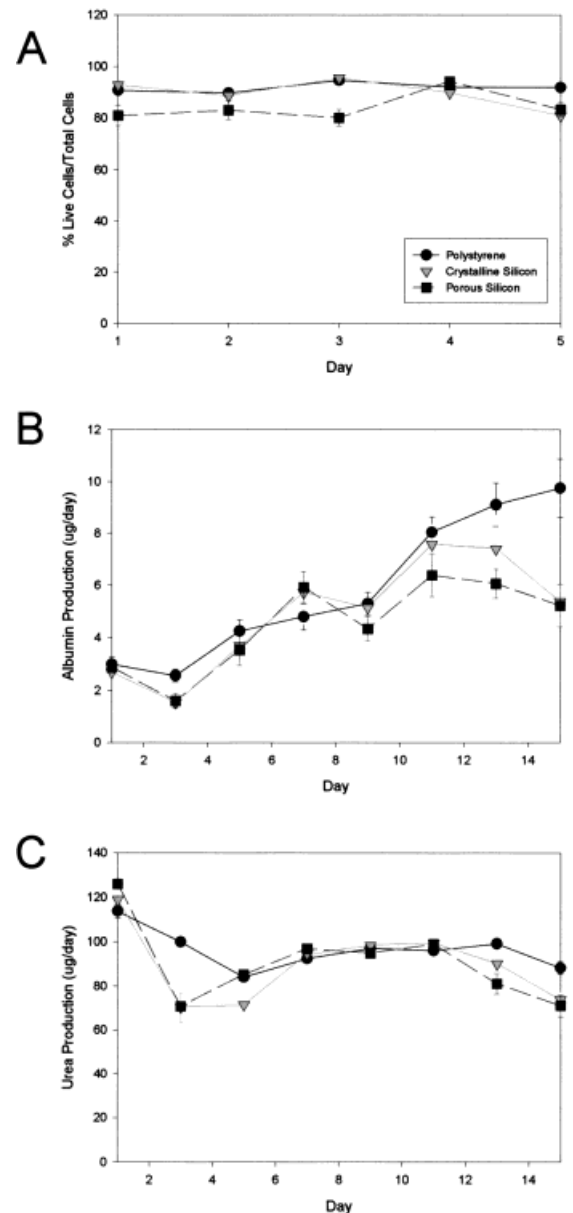


Fig. 3. Viability and function of cells on porous silicon. A) Graph indicating the percentage of live staining cells of the total cell number as a function of time. B) Total albumin secretion level per day over a two week period for the three conditions examined. C) Total urea production per day over a two week period for the three conditions examined.

serum (FBS, Sigma, St. Louis, MO), 0.5 U/mL insulin, 7 ng/mL glucagon, 20 ng/mL epidermal growth factor, 7.5 μg/mL hydrocortisone, 200 U/mL penicillin, and 200 μg/mL streptomycin.

Cell Culture on Crystalline and Porous Silicon: Hepatocytes were cultured on silicon chips prepared in the following manner: Porous Si samples were prepared by anodically etching p-type silicon (5–10 Ω/cm resistivity, (100) orientation, B-doped, supplied by Silicon Quest Inc.) in a 1:1 v/v mixture of ethanol and aqueous hydrofluoric acid (49 %) at 5 mA/cm² for 2 min in the absence of light. A mesh Pt counter electrode was used to ensure a homogenous electric field. Each sample was then rinsed thoroughly with ethanol and dried under a nitrogen stream.

Crystalline Si samples were rinsed with ethanol and dried under nitrogen stream. Both types of Si samples were exposed to ozone for 15 min using an ozone generator (Ozomax, Quebec, Canada) with a flow rate of 8 g/h of O₃ to provide a stable, oxidized, silanol-terminated surface.

Prior to cell seeding, Si samples were cleaned by exposure to oxygen plasma on a Technics 500 II Asher at a base vacuum of 80 mtorr and O₂ pressure of 120 mtorr at a power of 100 W for 4 min. Samples were then sterilized by immersion in 70 % ethanol for 1 h. After sterilization, samples were rinsed 4× in sterile water. Collagen I was adsorbed to the surface by incubation with 0.25 mg/mL collagen I (Vitrogen, Cohesion Technologies, Palo Alto, CA) for 1.5 h at 37 °C. As a control, collagen I was also adsorbed to polystyrene cell culture wells (Falcon). After incubation, Si samples were washed with sterile water and placed in the cell culture well pre-treated with 0.1 % bovine serum albumin (BSA) to prevent cell adhesion on non-Si surfaces. Hepatocytes were seeded in culture media and allowed to attach overnight.

Functional Analysis of Hepatocytes on Silicon Substrates: After overnight attachment, the samples were washed once with media. The substrates were overlaid with a layer of collagen gel to stabilize cell function [26]. Specifically, a 10:1 Vitrogen/10× DMEM solution was applied and allowed to gel for 1 h at 37 °C. After gelation, culture media was added. Media was collected and changed every other day for twelve days. Samples were stored at –80 °C for subsequent analysis for urea and albumin content. Urea synthesis was assayed using a commercially available kit (Sigma Chemical Co., kit No. 535-A) Albumin content was measured by enzyme-linked immunosorbent assays (ELISA) as described previously [27]. Rat albumin and anti-rat albumin antibodies were purchased from ICN/Cappel Laboratories (Cochranville, PA, USA).

Atomic Force Microscopy: AFM was performed on chemically modified surfaces identical to those used in cell culture to demonstrate the presence of attached collagen. AFM images were obtained under ambient conditions using a Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments, Santa Barbara, CA) operating in tapping mode using a 2–5 nm diameter tip. Roughness measurements were determined as area statistical root mean square (rms) roughness, the standard deviation of the z-data, using an algorithm provided in the instrument software.

Microscopy: Specimens were observed and recorded using a Nikon Diaphot microscope equipped with a SPOT digital camera (SPOT Diagnostic Equipment, Software Version 2.2, Sterling Heights, MI), and MetaMorph Image Analysis System (Universal Imaging, Westchester, PA) for digital image acquisition. Viability of hepatocytes was determined using the vital dyes fluorescein diacetate (FDA, Sigma) and propidium iodide (PI, Sigma). Immediately prior to imaging, cells were labeled with 2 mL of a solution containing 5 µg/mL PI and 5 ng/mL FDA. Hepatocytes on silicon were inverted onto supports prior to labeling to facilitate microscopy. The labeled hepatocytes were immediately viewed by fluorescence microscopy using excitation wavelength/emission wavelength settings of: 492/517 and 541/565 nm for FDA and PI, respectively. Viability was then quantified using the Metamorph Image Analysis system with 10–15 fields per condition per day.

Statistics and Data Analysis: Experiments were repeated two to three times with duplicate or triplicate culture plates for each condition. One representative experiment is presented where the same trends were seen in multiple trials. Error bars represent standard error of the mean. Statistical significance was determined using one-way ANOVA (analysis of variance) on Systat 9 (SPSS Science, Chicago, IL) with Bonferroni post-test analysis with $p < 0.05$.

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Microporous Films Prepared by Spin-Coating Stable Colloidal Suspensions of Zeolites**

By Svetlana Mintova* and Thomas Bein*

Micro- and mesoporous materials exhibit a particularly rich diversity of structures and applications.^[1] Recently, molecular sieves with particle sizes in the nanometer range (10–700 nm) have been targeted for novel technologies including reactive membranes, optical coatings, dielectric layers, and selective chemical sensors.^[2–7] A number of papers are dedicated to the direct growth of these microporous layers on different substrates including silicon, ceramics, porous supports, and metals directly from hydrothermal precursor gels or solutions.^[8–10] Another approach widely used for the preparation of ultrathin films involves the attachment of nanosized zeolite seed layers on various substrates prior to hydrothermal synthesis of a secondary layer.^[11–15] Usually the microporous materials used for preparation of the seed layers are synthesized in colloidal form from clear aluminosilicate or aluminophosphate solutions, resulting in a mean particle size in the range of 50–600 nm.^[16–19] Using this method, continuous zeolite films have been deposited on supports of different nature, shape, and size. Very often, however, even supports compatible with the

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