Nanotomicroscale Porous Silicon as a Cell Interface for Bone-Tissue Engineering**

By Wei Sun, J. Edward Puzas, Tzong-Jen Sheu, Xi Liu, and Philippe M. Fauchet*

An ideal material for orthopedic tissue engineering should be biocompatible, biodegradable, osteoconductive, osteoinductive, mechanically stable, and widely available.\[1-4\] Porous silicon (PSi), a silicon-based material, fulfills these criteria. It is biocompatible\[5,6\] and biodegradable,\[7\] and supports hydroxyapatite (HA) nucleation.\[6\] The micro-/nanoarchitecture of PSi may regulate cell behavior. The surface chemistry of PSi is flexible so that the interfacial properties between this material and living cells can be tailored easily by chemical modifications. Here, we report that PSi can support and promote primary osteoblast growth, protein-matrix synthesis, and mineralization. We also show that the osteoconductivity of PSi and other cellular responses can be controlled by altering the micro-/nanoarchitecture of the porous interface. With this material, we are closer to a functional biomaterial with both osteoconductivity and drug-delivery functions.

Recently, tissue-engineering strategies using engineered biomaterials that support and promote bone-tissue growth have been proposed for reconstructive surgeries. The goal of a tissue-engineering approach is to repair and regenerate damaged human tissue with biomaterial-based devices. The approach requires functional cells derived from the target tissue, a matrix supporting those cells, bioactive molecules regulating cellular behavior, and the integration of this composite in the damaged tissue.\[1\] Si, a semiconductor material, has the potential to achieve all the properties required for a tissue-engineering strategy. The physical and chemical properties of Si are widely known because of its wide use in the microelectronics industry. Moreover, sophisticated microfabrication techniques allow precise structures to be formed on Si substrates, some of which have been proposed for medical care.\[8-10\] The recent discovery of the biocompatibility, biodegradability, and bioactivity of PSi\[5-8,14\] has opened the door for implantable applications of this Si-based material. After implantation of Si-based bioactive glass into rabbit bone, the elevation in Si concentration was only found at the implant site and not in other organs, and the implanted Si was efficiently excreted by urine.\[17\] Furthermore, the large surface-to-volume ratio\[18\] and the chemical flexibility\[19\] of PSi makes it attractive for immobilizing bioactive molecules for drug-delivery purposes.\[20-22\] These findings suggest that PSi can be a candidate for orthopaedic tissue engineering.

Our investigations on the osteoconductivity of PSi were carried out using nanoscale (<15 nm, NanPSi), mesoscale (ca. 50 nm, MesPSi), and macroscale (ca. 1 μm, MacPSi) pores in vitro. The PSi samples were produced by electrochemical etching of p-type Si wafers in HF-based electrolytes. The various pore configurations were achieved by changing the Si substrate, the electrolyte content, or the current density (see Supporting Information for detailed experimental information). As shown in Figure 1, MacPSi had pores with openings close to 1 μm; MesPSi had pores with pore openings around 50 nm; and NanPSi had a spongy porous structure with pore sizes under 15 nm. Unlike polished Si wafers, which do not degrade in cell media, PSi can be degraded in such a solution. Preliminary experiments showed that freshly-etched MesPSi degraded faster than MacPSi in cell media (see Supporting Information). The observation indicates that PSi, rather than Si, has potential in vivo degradation, and that MacPSi may be the most favorable candidate for bone-tissue engineering in terms of both biodegradability and stability. To protect PSi from gradual oxidation and degradation, a chemical oxidation in hydrogen peroxide was carried out after etching to form a thin oxide layer on the surface.\[16\] Primary rat calvaria cells (osteoblasts) or rat osteosarcoma cells (ROS 17/2.8) were seeded onto PSi substrates for from 1 h to 5 weeks and the substrates and cells were assayed both qualitatively and quantitatively. Standard cell culture in 24-well polystyrene culture plates was used as a control.

The adhesion of osteoblasts to PSi surfaces was quantified by direct counting of the attached cells. The viability of the attached cells was determined by an adenosine triphosphate (ATP)-based cell-viability assay. In adhesion studies (0.5–4 h), PSi chips bound slightly fewer osteoblasts than the tissue-culture plate, but the difference was not statistically sig-
cells adhered to substrates, they tended to spread out on the microenvironment to foster osteogenetic cells growth. That MacPSi provides osteoblasts with the most favorable geometry of the substrates influences cell behavior. Considering attachment, viability, and gene expression, we conclude that it promotes osteoblast growth better than the other forms of PSI.

To verify whether the biological functions of the osteoblasts grown on PSI were affected by the substrate, real-time reverse transcriptase polymerase chain reaction (RT-PCR) was employed to quantify three characteristic biomarkers of bone formation: alkaline phosphatase (AP), osteocalcin (OC) and type I collagen (Col1). The ROS 17/2.8 cell line was used in these studies because we found it easy to extract RNA from this cell line. After culturing these cells on PSI for 7 days, the three genes were detected on all three types of PSI (Fig. 2c). MacPSi maintained the transcription of all these biomarkers at a high level, comparable to the control surface. So MacPSi is not toxic to osteoblasts and allow their survival on MacPSi or that the cells have higher metabolic activities at these time points than those grown on the other surfaces. NanPSi than the control. This could be attributed to the fact that fewer cells were attached to these samples initially. Higher viability was found on MacPSi than on control-tissue plates at days 5 and 7, suggesting that either more osteoblasts survived on MacPSi or that the cells have higher metabolic activities at these time points than those grown on the other surfaces. MacPSi and NanPSi surfaces (Fig. 3a), but remained more separated and rounded on the MesPSi substrates. Within 3–5 days of culture, these adhered cells migrated, proliferated, and clustered to form mineralizing nodules (Fig. 3b), a feature common in the process of bone formation. Upon maturation, the osteoblasts secreted an extracellular matrix (ECM) that could support further mineralization. Coll1, which constitutes approximately 95% of this protein matrix in bones, was detected on all PSI samples from immunofluorescence after 1 week of culture (Fig. 3c). OC, a major noncollagenous bone-matrix protein and late marker of osteoblast maturation, was also present on all the samples after 2 weeks of culture (Fig. 3d). High resolution SEM images demonstrated the presence of a fibrous mesh around cultured osteoblasts with the banding characteristics of Col1 (Fig. 3e and f). These observations confirmed that PSI supports the functionalization of osteoblasts.

A semiquantitative investigation on the mineralization of cultured osteoblasts on PSI samples further supported this finding. After 7 days of culture, calcified ECM layers were detected on MacPSi but not on the other two types of PSI. After 2 weeks, calcified layers were found on all PSI substrates. Using a dual fluorescence labeling method with propidium iodide (PI) and calcein, we were able simultaneously to visualize the cells and the mineralized matrix (Fig. 3g). The upper part of the image shows a fully calcified ECM layer with few cells remaining. In the lower part of the image, mineralization did not take place and more individual cells were detected. Figure 3h is an SEM image of an ECM layer deposited by the osteoblasts on MacPSi, and Figure 3i shows a cross section of the wafer with penetration of the mineralized matrix into the pores. The corresponding energy dispersive X-ray (EDX) spectrum of this layer is shown in Figure 3j. The atomic ratio of the major elements was obtained by quantifying the spectra. The Ca to P ratio in the matrix on MacPSi was 1.72, suggesting the formation of an apatite-like material. This ratio is in the range (1.65–1.77) found in human bone minerals. The Ca content was lower with NanPSi and the lowest with MesPSi. The finding is consistent with a low OC transcriptional level in the cells. The osteoblasts cultured on MacPSi seem to differentiate and mature faster than on the other substrates.

As MacPSi enhanced the osteoblast viability (Fig. 2b) and mineralization (described above) and maintained the expression of the biomarkers of bone formation (Fig. 2c), we conclude that it promotes osteoblast growth better than the other forms of PSI. The micrometer pore and the abundant flat Si surface present around the pores on MacPSi anchor the cells firmly while providing them with enough space to spread. This topography activates a cascade of intracellular signaling path-

Figure 1. Images of the surface morphology of the three forms of PSI. a) MacPSi: straight pores with openings close to 1 µm (scanning electron microscopy (SEM)), b) MesPSi: branching pores with pore openings of ca. 50 nm (SEM), c) NanPSi: spongy porous structure with pore sizes less than 15 nm (atomic force microscopy (AFM)).
ways and thus guides the cells to proliferate and fulfill their function efficiently. In contrast, nanoscale pores on NanPSi, though they may mimic protein-binding sites, may not anchor the cells firmly and provide the same mechanical signals to regulate cell behavior. The dense sub-micrometer pores and the very limited flat surface of MesPSi appear to hinder the spread of the bound cells and inhibit further growth. Thus, by tuning the local geometry of implant material, it is possible to control the mineralization and integration of the implant into a host.

In summary, we have demonstrated that PSi displays promising osteoconductivity. Different architectures of PSi induced different cellular responses of osteoblasts in terms of adhesion, metabolic activity, protein synthesis, and mineralization. MacPSi performed better than MesPSi and NanPSi in supporting osteoblast growth and sustaining their function. Considering its higher rate of mineralization, its potential biodegradability, and its potential drug-delivery function, MacPSi is a compelling biomaterial for bone-tissue engineering. The ability of all three forms of PSi to support cell growth makes them good candidates for tissue engineering in general. The large surface-to-volume ratio and flexible surface chemistry also make them appealing for microscale medical devices with both drug-delivery and scaffolding functions. The knowledge obtained in this study on the interaction between living cells and a semiconductor material will be the foundation for further development of electronic and optoelectronic biointerfaced devices. One possible future application would be to use electric fields or light to control the PSi-based device in order to attract host cells and adjust their behavior as well as to release embedded drugs that promote tissue regeneration.

Received: February 15, 2006
Revised: August 16, 2006
Published online: March 6, 2007

Figure 2. The adhesion, metabolic activity and biomarkers of osteoblasts on PSi (n = 3 and error bars represent standard errors). a) Direct count of osteoblasts stained by propidium iodide (PI) on PSi and a control surface after incubation times of 0.5, 1, 2, and 4 h. b) Cell-viability assay of osteoblasts cultured on PSi and a control after 4, 120, and 168 h (luminescence after incubation times of 0.5, 1, 2, and 4 h. b) Cell-viability assay of osteoblasts stained by propidium iodide (PI) on PSi and a control surface for 7 days (all the data are normalized to β-actin level).
Figure 3. Cell, extracellular matrix (ECM), and the mineralization on PSi. a) Osteoblasts adhere and spread out after 18 h of incubation (stained by PI dye). b) Cultured osteoblasts cluster to form nodules after 5 days (stained by PI dye). c) Immunofluorescence of Col1 after 1 week of culture (Rhodamine fluorescence). d) Immunofluorescence of OC after 2 weeks of culture (Rhodamine fluorescence). e) SEM image of an osteoblast surrounded by fibrous mesh after one week of culture. f) Fibrils with banding characteristics of Col1 after one week of culture. g) Ca-rich protein matrix with green fluorescence (stained by calcine) and osteoblasts with red fluorescence (stained by PI dye) at the mineralization front of the ECM laid down by osteoblasts grown on MesPSi for four weeks. h) SEM image of a protein layer on MacPSi (top view). i) SEM image of a protein matrix within pores (cross section). j) EDX spectrum of a protein layer (the brightest part) shown in (h). The table shows the atomic percentage of the major elements detected (Si is from the substrate and Au is introduced by sputtering for visualization purposes).