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Femtosecond laser-patterned nanopore arrays for surface-mediated peptide treatment

Angela L. Zachman, MS[a], Lucas H. Hofmeister, MS[a], Lino Costa, PhD[b], Timothy C. Boire, BS[a], Yu-Shik Hwang, PhD[c], William H. Hofmeister, PhD[b], Hak-Joon Sung, PhD[a,*]

[a]*Department of Biomedical Engineering, Vanderbilt University, Nashville, TN, USA*
[b]*Center for Laser Applications, University of Tennessee Space Institute, Tullahoma, TN, USA*
[c]*Department of Maxillofacial Biomedical Engineering, Kyung Hee University, 1 Hoegidong, Dongdaemun-gu, Seoul, South Korea*

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Abstract

The major goal of this study was to create easy-to-use, reusable substrates capable of storing any peptides or bioactive molecules for a desired period of time until cells uptake them without the need for bioactive molecule or peptide-specific techniques. Nanopore arrays of uniform size and distribution were machined into fused silica substrates using femtosecond laser ablation and loaded with peptides by simple adsorption. The nanopore substrates were validated by examining the effect of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) loaded nanopores on macrophage phagocytosis and intracellular production of reactive oxygen species (ROS) with and without the pro-inflammatory lipopolysaccharide (LPS). Our results demonstrated that nanopores were generated in a uniform array fashion. Ac-SDKP peptides were stably stored in nanopores and internalized by macrophages. Significant reductions in ROS production and phagocytosis in macrophages were observed over control substrates, even in combination with LPS stimulation, indicating that loading Ac-SDKP peptides in pores significantly improved the anti-inflammatory effects.

From the Clinical Editor: This team of scientists intended to create easy-to-use, reusable substrates for storing peptides or bioactive molecules for a desired period of time before cellular uptake occurs, and without the need for bioactive molecule or peptide-specific techniques. They demonstrate the successful generation of nanopores in a uniform array that stably stores Ac-SDKP peptides in the nanopores. When peptides were internalized by macrophages, significant reductions in ROS production and phagocytosis were observed, indicating improved anti-inflammatory effects.

Key words: Nanopore; Ac-SDKP; Cellular uptake; Anti-inflammation

Surface-mediated or reverse-transfection is a well-established method to deliver genetic information to cells more effectively and efficiently by either simple adsorption or covalent tethering of nucleotide vectors to a culture substrate prior to cell seeding.[1] Covalent attachment is the most common surface-mediated protein delivery method but availability to cells, long term stability, and bioactivity are questionable with this approach.[1-4] Non-covalent approaches to peptide delivery generally result in burst release of peptide from the surface and are difficult to tailor for longer-term studies.[5-7] We hypothesized that loading peptides into nanopore arrays would increase the efficacy of peptide treatment similar to surface-mediated transfection for long-term treatment of cells *in vitro*. The goal was to create easy-to-use, reusable substrates capable of storing any peptides or

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*Corresponding author: Department of Biomedical Engineering, Vanderbilt University, Nashville, TN 37235, USA.

E-mail addresses: angela.l.zachman@vanderbilt.edu (A.L. Zachman), lucas.h.hofmeister@vanderbilt.edu (L.H. Hofmeister), lcosta@utsi.edu (L. Costa), timothy.c.boire@vanderbilt.edu (T.C. Boire), yshwang@khu.ac.kr (Y.-S. Hwang), hof@utsi.edu (W.H. Hofmeister), hak-joon.sung@vanderbilt.edu (H.-J. Sung).

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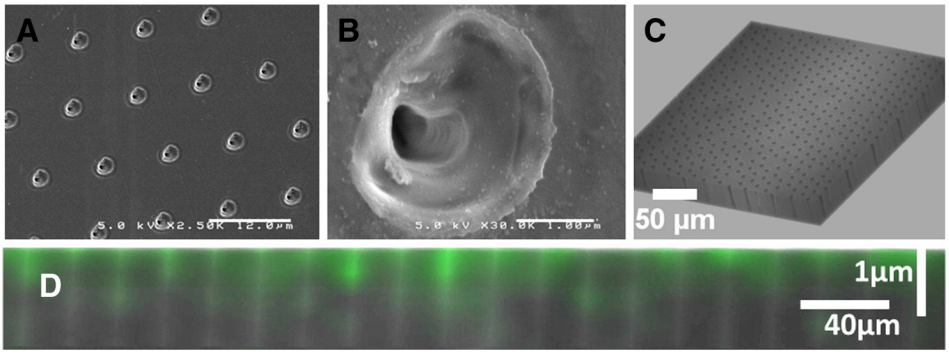

Figure 1. (**A**) SEM of 1 million nanopore array showing uniformity of pore size, shape, and distribution. (**B**) SEM of a single nanopore showing pore morphology. (**C**) Z-stack confocal micrograph of empty nanopore array. (**D**) Z section of FITC-SDKP loaded nanopore array after two days of incubation in culture media.

bioactive molecules for an extended period of time until cells uptake them without the need for pretreatment or peptide-specific techniques.

The effect of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) loaded nanopores on macrophage phagocytosis and intracellular production of reactive oxygen species (ROS) was examined with and without the pro-inflammatory endotoxin lipopolysaccharide (LPS). Macrophages produce ROS in response to inflammatory stimuli (e.g., LPS treatment) to destroy foreign materials and then phagocytose the debris to prevent damage to other cells.[8,9] The tetrapeptide, Ac-SDKP, found in wound fluid, attenuates inflammatory and fibrotic responses by decreasing infiltration and adhesion of macrophages as well as the production of inflammatory cytokines.[10,11]

Methods

Nanopore arrays of uniform size and distribution were machined into fused silica substrates using femtosecond laser ablation with a single 160 fs pulse.[12] For in vitro experiments, arrays of 200 × 200 20 µm-spaced nanopores were arranged in a square lattice. Nanopore substrates were characterized by scanning electron microscopy (SEM). Peptides were loaded into the nanopores immediately following laser machining. Briefly, a 50 µL drop of 6 mg/mL Ac-SDKP in ultrapure water was deposited on the pattern or on a flat surface for peptide without pore controls, dried under vacuum overnight, and washed with sterile water prior to use. Confocal microscopy was used to confirm the presence of the fluorescently-labeled peptide FITC-SDKP after 48 hour incubation in culture media.

For *in vitro* studies, RAW 264.7 cells (ATCC, Manassas, VA) were cultured as suggested by the supplier. Cells were seeded onto each substrate at a density of 6.3×10^3 cells.cm^{-2}. Cells were allowed to attach for 15 hours, then the media were replaced with normal or LPS (1 mg.mL^{-1}, Sigma-Aldrich, St. Louis, MO), or FITC- SDKP (0.5, 0.25, or 0.125 mg.mL^{-1}) containing media. Endpoint analysis was carried out 72 hours after treatment.

Phagocytosis was measured using a Vybrant® Phagocytosis Assay Kit (V-6694, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Intracellular superoxide was stained with 5 ug.mL^{-1} dihydroethidium (DHE) and counterstained with 5 ug.mL^{-1} Hoechst. Fluorescence intensities of *Escherichia coli* particles phagocytized and intracellular superoxide were quantified using a plate reader (Tecan Group, Ltd Männendorf, Switzerland). Results were represented on plots as average ± standard deviation of 45 technical replicates per sample (n = 2 for nanopore samples loaded with Ac-SDKP). Statistical analyses were performed by using one-way ANOVA tests followed by one-tailed *t* tests for equal variances. For all tests, significance was designated as $P < 0.05$.

Results

SEM images showed that nanopore substrates were highly uniform in space, distribution, and pore size. Nanopores had roughly-elliptical openings with average major and minor axes of 2.95 and 2.56 µm (Figure 1, *A* and *B*). Below the surface the pore diameter reduces to around 540 nm. Confocal microscopy of empty nanopores indicated that nanopores could be as much as 40 µm deep (Figure 1, *C*). Confocal microscopy of FITC-SDKP loaded nanopores showed that nanopore substrates remained loaded with FITC-SDKP after 48 hours of incubation in culture media (Figure 1, *D*, Figure 2, *H*). Confocal microscopy also confirmed that nanopore fluorescence is not due to auto-fluorescence of the pore structure. FITC-labeled SDKP peptides were visible inside macrophages after 4 day culture on FITC-SDKP loaded nanopores, whereas peptide delivered in solution was only internalized at the highest concentration (Figure 2).

Ac-SDKP release significantly reduced intracellular superoxide production and phagocytosis over control substrates, even in the case of LPS stimulation (Figure 3). When loaded in pores,