Femtosecond Laser-Patterned Nanopore Arrays for Spatio-Temporal Control of Bioactive Molecule Release

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Statement of Purpose: This study sought to fabricate novel patterned cell culture substrate surfaces to release biologically active molecules in a spatio-temporally controlled manner for *in vitro* testing. Nanoscale pore arrays of uniform size and distribution were machined into fused silica substrates using femtosecond laser ablation. This study characterized the nanopores using optical methods and validated the selective loading and release of an anti-inflammatory peptide, Ac-SDKP, into and out of the pores, respectively. The effect of controlled Ac-SDKP release on macrophage responses, including reactive oxygen species (ROS) production, phagocytosis, apoptosis, and tumor necrosis factor (TNF)-α production, were then examined using the Ac-SDKP-loaded nanoscale pore arrays as a cell culture substrate in comparison to unloaded nanoscale pore arrays and glass substrates with and without pro-inflammatory endotoxin lipopolysaccharide (LPS).

Methods: Arrays of 200×200 regularly spaced nanoscale pores arranged in a square lattice were fabricated on the surface of 22×22 mm^2, 500 μm thick UV grade double side polish fused silica by locally ablating the surface of the fused silica with a single 160 fs laser pulse tightly focused by a dry microscope objective (Nikon CF Plan Achromat 79173) with a numerical aperture of 0.85.[1] Immediately after laser machining, a 6 mg/mL solution of Ac-SDKP in nanopure pater was deposited on the nanopore array and dried under vacuum. In order to remove excess peptides, the substrates were washed with nanopure water and dried under ultrapure nitrogen. For release kinetic studies, A 1 million-pore substrate was loaded with Ac-SDKP as described above. The substrate was incubated with 100 uL ultrapure water which was replaced daily for two weeks. Ac-SDKP concentration in the releasate was measured by LC-MS/MS through a waters HSS T3 1.8um pore size 1 by 100 mm C18 column (Waters Synapt-G2). Murine macrophages (Raw 264.7) were seeded on the substrates at a density of one cell per pore and cultured for 24 hours before administering test conditions. For non-pore substrates, cells were seeded with the same amount of Ac-SDKP (6 mg/mL). Cells were stimulated with 1ng/mL LPS for 72 hours. Phagocytosis was then measured using a Vybrant® Phagocytosis assay kit (Life Technologies); and stained with dihydroethidium (1μg / mL DHE) to measure ROS production. TNF-α production was measured by ELISA (PeproTech Rocky Hill, NJ). Cells were stained with Hoechst (Sigma) and imaged. The readout values from the cell assays were normalized to the cell number counted from images of the corresponding test conditions. Cell apoptosis was identified by plasma membrane blebbing and abnormal Hoechest staining (e.g., nuclear fragmentation and condensation) in images. The number of these apoptotic cells was calculated to a percentage out of the total cell number seen in each image field.

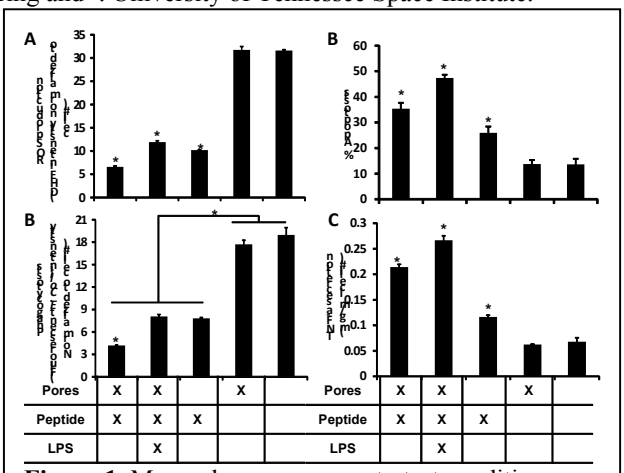

Figure 1. Macrophage responses to test conditions. A) ROS production measured by DHE. B) Phagocytosis measured by fluorescent E-Coli particle uptake. C) Apoptosis measured by D) TNF-α production measured by ELISA. *P<0.05 vs. the other groups or the lined comparison group

Results: Fluorescently labeled Ac-SDKP was successfully loaded into nanopore substrates and verified by confocal imaging. Sustained release of the peptide was confirmed for a period of 7 days of incubation. *In vitro* studies showed that Ac-SDKP release significantly reduced ROS production and phagocytosis over control substrates, even in in the case of LPS stimulation (Fig 1a,b). Cell adhesion, apoptosis and TNF-a production increased significantly in the presence of Ac-SDKP peptides (Fig 1c,d). When loaded in pores, the Ac-SDKP effects were improved significantly, compared to the treatment without pores. These findings are aligned with the previous studies, including ours,[2] that LPS[3] or cell adhesion[2] induces apoptosis in macrophages through the autocrine production of TNF-alpha.

Conclusions: This study is the first to demonstrate that femtosecond laser-machined nanopore arrays are an effective method of achieving sustained effects of functional molecules for *in vitro* cell studies. Inflammatory activities were reduced in Ac-SDKP loaded nanopores compared to the other conditions. In the presence of Ac-SDKP in pores, cell adhesion and spreading increased with overproduction of TNF-a, leading to apoptosis in macrophages. These results suggest a potential mechanism to study in the future. The successful application of nanopore arrays will enable chemokine gradients and spatially uniform release of functional molecules in *vivo*.

References: 1. White YV. Optics Express 2008; 16:1411-14420. 2. Zachman AL. Acta Biomaterialia 2012 [PMID:23128157]. 3. Xaus J. Blood 2000;95:3823-31.

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