Supporting Information

Nanotopography facilitates *in vivo* transdermal delivery of high molecular weight therapeutics through an integrin-dependent mechanism

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SUPPORTING INFORMATION: Experimental procedures; histogram summarizing depth of microneedle penetration; and pre and post device application images of rat skin. This material is available free of charge via the Internet at http://pubs.acs.org

Materials and Methods

Film and device fabrication

Nanostructured films are made, as previously described, by nanoimprint lithography whereby a thermoplastic is heated above its glass transition temperature and pressed into a silicon mold (*38*). Molds are fabricated by electron beam lithography, allowing for precise design of submicron structures. This method has allowed the fabrication of thin films with varying nanotopographies. Films used in the in vivo studies were the highest performer in prior drug delivery studies and are made from polyether ether ketone (PEEK) with nanocolumns of three different lengths and widths: 1um length - 360-370nm width; 400nm length - 158-170nm width; and 200nm length - 80-115nm width. For the in vitro studies, a simple topography composed of PEEK or polypropylene nanocolumns measuring 300 nm high and 200 nm wide was used. These films were used for *in vitro* studies because they were similarly high performing as the *in vivo* topography and offered a simple baseline design from which to iterate future studies to isolate nanostructure parameters.

The *in vivo* devices are made of an impermeable backing, a reservoir for a drug solution, a rate-controlling membrane, and silicon microneedle array (MNA) with or without a nanostructured film. The MNA is made up of 200 μm long, 90 μm wide microneedles with a pitch of 400 μm and a density of about 750 needles per square cm. The MNA is 25 mm by 25 mm and the entire device is 45 mm by 45 mm including the adhesive zone to affix the device to the skin. Using heat, the nanostructured thin films are vacuum sealed onto the MNA.

In vivo drug delivery

The nanostructured surfaces were integrated into a 25 mm by 25 mm transdermal microneedle patch for *in vivo* drug delivery studies in a rabbit model. 11 New Zealand White healthy rabbits

weighing approximately 4 kg were used. Each animal was closely clipped to remove the fur in the left flank region. The study consisted of 3 groups: (1) the subcutaneous injection group receiving 2.5 mg/animal (n=2), (2) the nanotopography-wrapped microneedles group receiving 1.875 mg/animal (n=5), and (3) the smooth microneedles group receiving 1.875 mg/animal (n=4). The *in vivo* test facility used for this animal study is registered with the United States Department of Agriculture to conduct research in laboratory animals and is AAALAC accredited. The study was reviewed and approved by the Facility's Institutional Animal Care and Use Committee (IACUC). Blood samples were drawn from an ear vein to measure the etanercept concentration in the blood serum at 0, 0.5, 2, 6, 24, and 48 hours. All animals were euthanized following collection of the last blood sample. Etanercept was assayed in duplicate samples from each serum collection using Human sTNF-receptor ELISA kit (R&D Systems, Minneapolis, MN) which has a minimum detectable dose of 0.6 pg/mL.

For the rat studies, 12 Sprague Dawley rats weighing approximately 450 g were used. Each animal was closely clipped and Nair hair removal product was applied to remove fur in the central back region. The study consisted of three groups: (1) the nanotopography-wrapped microneedles group receiving 12.5 mg/animal (n=4), (2) the smooth microneedles group receiving 12.5 mg/animal (n=4), and (3) reservoir patch group receiving 12.5 mg/animal (n=4).). The *in vivo* test facility used for this animal study is registered with the United States Department of Agriculture to conduct research in laboratory animals and is AAALAC accredited. The study was reviewed and approved by the Facility's Institutional Animal Care and Use Committee (IACUC). Blood samples were drawn from the tail vein to measure the etanercept concentration in the blood serum at 0, 8, 24, 36, 48, and 72 hours. All animals were euthanized following collection of the last blood sample. Etanercept was assayed in duplicate

samples from each serum collection using Human sTNF-receptor ELISA kit (R&D Systems, Minneapolis, MN) which has a minimum detectable dose of 0.6 pg/mL.

Primary human keratinocyte cultures

Primary human keratinocytes were derived from healthy neonatal foreskin. Epidermis was dissociated from dermis with dispase, and keratinocytes were grown to confluency in human epidermal media without antibiotics (CellnTec Media CnT-PR) (Zen-Bio, Raleigh, NC). Upon reaching confluency, the cells were dissociated and seeded on transwell inserts to differentiate them into multilayered cultures. Cells were air-lifted on day 3 of transwell culture, and media was exchanged daily into the basolateral chamber (CellnTec media CnT PR-D) (Zen-Bio, Raleigh, NC).

Stratified keratinocyte immunofluorescence staining

Day 8 primary human keratinocytes were used for all immunofluorescence studies. Cells were exposed to no device, flat control film, or nanostructured film on the apical surface (air interface) for 24 hours. After 24 hours, the devices were removed, and cells were fixed for 10 minutes in 100% methanol at -20 degrees Celsius and then for 20 minutes in 4% paraformaldehyde at room temperature. After fixation, the membranes were paraffin embedded, sectioned, and placed onto glass slides. For staining, the slides were de-paraffinized, blocked with 4% bovine serum albumin (BSA) for 1 hour, and then incubated with primary antibody diluted in 4% BSA for 12 hours at 4 degrees Celsius. The following primary antibodies were used: mouse anti-claudin-1 (Invitrogen 374900, Carlsbad, CA) (1:200); rabbit anti-claudin-4 (Abcam ab53156, Cambridge, England) (1:200). Following incubation with primary antibody, the sections were washed with 1x phosphate buffered saline (PBS) and then incubated with secondary antibody diluted in 4% BSA for 1 hour at room temperature. The following secondary antibodies were used: rabbit

Alexa 568 (1:500) and mouse Alexa 488 (1:500). Following incubation with secondary antibody, the sections were washed and protected with a cover slip.

For reversal studies, the device was removed after 24 hour exposure, the cells were fed new media, and fixation and staining were performed after another 24 hours in the same manner as outlined above.

Caco-2 cell culture

Caco-2 cells from the human intestinal line (American Type Culture Collection (ATCC), Manassas, VA) were cultured in MEM with Earle's BSS supplemented with 20% FBS, 1% penicillin-streptomycin, and 1% sodium pyruvate, in an atmosphere containing 5% CO_2 and 90% relative humidity at 37 degrees Celsius. The cells were subcultured at 90% confluency by trypsinization with 0.05% trypsin-EDTA.

Caco-2 immunofluorescence and TEM

Caco-2 cells were grown to confluency on Transwell inserts (BD Falcon, Franklin Lakes, NJ) until tight junctions formed as measured by TEER (World Precision Instruments, Sarasota, FL). No film, a flat film, or a nanostructured film were placed in contact with the cell monolayer. After two hours in contact with the films, immunofluorescence samples were fixed with 3.7% paraformaldehyde (PFA) for 15 minutes at room temperature. Samples were blocked and permeabilized with a solution of 1% BSA with 0.1% Triton-x in a phosphate buffer solution (PBS) for one hour at room temperature. The samples were incubated with a primary antibody: mouse anti-claudin-1 (Santa Cruz Biotechnology XX7, Santa Cruz, CA) (1:200), rabbit anti-claudin-4 (Abcam ab53156, Cambridge, England) (1:200), mouse anti-occludin (33-1500 Invitrogen, Carlsbad, CA) (1:100), rabbit anti-pFAK (Life Technologies 44-6246, Carlsbad, CA) (1:100), FITC conjugated mouse anti-vinculin (Sigma F7053, St. Louis, MO) (1:100), rabbit

anti-pMLC (Cell Signaling Technologies 3671S, Danvers, MA) (1:50), rabbit anti-integrin beta1 (Abcam AB52971, Cambridge, England) (1:100), or rabbit anti-ZO-1 (Invitrogen 40220, Carlsbad, CA) (1:100) at 37 degrees Celsius for 1 hour. After washing with PBS, a secondary antibody solution goat anti-rabbit Alexa 488 (Life Technologies A11008, Carlsbad, CA) (1:100) or goat anti-mouse Alexa 488 (Life Technologies A11029, Carlsbad, CA) (1:100), and rhodamine phalloidin (Life Technologies R415, Carlsbad, CA) (1:40) was added for 1 hour at 37 degrees Celsius. The samples were then mounted with vectashield HardSet mounting medium with DAPI (Fisher Scientific, Waltham, MA) for imaging with a Nikon FN-1 Microscope (Melville, NY).

Samples for TEM were fixed in 2.5% glutaraldehyde (EM grade) in PBS solution for 1 hour at room temperature. Samples were dehydrated with 25%, 50%, 70%, 95%, then 100% ethanol solutions. For resin infiltration, samples were incubated with 1:1 solution of 100% ethanol and Epon resin for one hour, then incubated, covered, overnight in pure Epon resin. To embed samples, samples were placed in fresh epon resin and put in an oven at 60^0 C for two days. Samples were imaged on a H-7500 TEM (Hitachi, Los Angeles, CA).

TEER and drug delivery studies

Caco-2 cells were grown to confluency on transwell inserts (BD Falcon) until tight junctions formed as measured by TEER (World Precision Instruments, Sarasota, FL). Cells were incubated with 2 mg/mL RGDS peptide, mouse anti-integrin beta1 (Millipore MAB2253, Temecula, CA) (1:25) and anti-integrin alphaV (Millipore MAB2021Z, Temecula, CA) (1:5), or 330 μM MLCK inhibitor peptide 18 (aka PIK) (Millipore 475981, Temecula, CA) for one hour at 37 degrees Celsius. No film, a flat film, or a nanostructured film were then placed in contact with the cell monolayer and FITC-BSA (Sigma, St. Louis, MO) was added to the apical chamber to a final

concentration of 0.1 mg/mL. The basolateral chamber was sampled at time points and the amount of FITC-BSA transported was measured on a UV-vis spectrophotometer or the TEER was measured. After the final time point, MLCK treated samples were then fixed and stained as above for microscopy.

Statistical analysis

Data are reported as mean values +/- standard deviation. Multiple comparisons were analyzed with a one-way ANOVA test followed by the Holm t-test. P-values of less than 0.05 were considered statistically significant

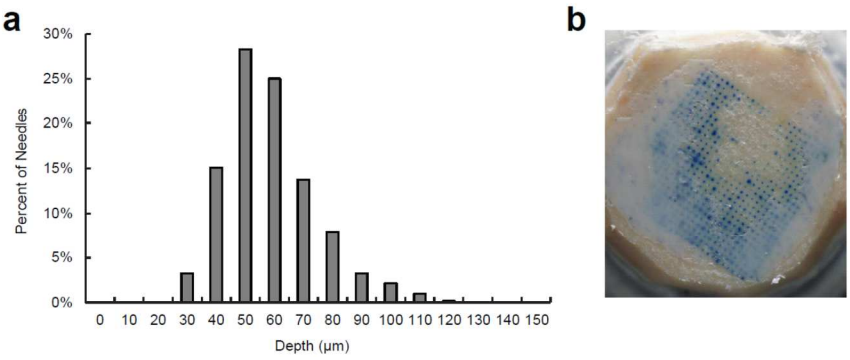

Figure S1. Microneedle penetration in rat skin. (a) The depth of MNA penetration ranged from 30 to 120 microns. Average depth of penetration in rat skin was 58 microns. (b) Depth of penetration of the MNAs was assessed in rats by soaking the skin samples in methylene blue following application of the MNA. The skin was then cryosectioned, and the depth of dye penetration was measured for each point in the array.

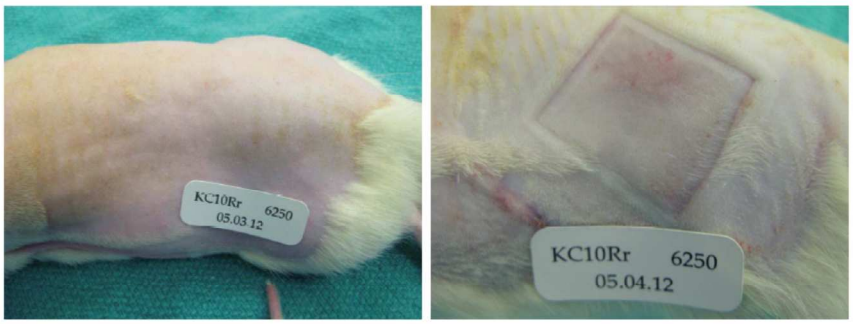

Figure S2. Transdermal device application does not produce erythema or irritation of skin. Pre and post-application pictures of transdermal device on rats. There was no evident erythema or irritation of the exposed skin following application of the devices.