Drug-mimicking nanofibrous peptide hydrogel for inhibition of inducible nitric oxide synthase

David G. Leach[1], Jared M. Newton[2,3], Marcus A. Florez[2,3], Tania L. Lopez-Silva[1], Adrianna A. Jones[1], Simon Young[4], Andrew G. Sikora[2], Jeffrey D. Hartgerink*[1]*

[1]Department of Chemistry, Department of Bioengineering, Rice University, Houston, TX 77005.
[2]Department of Otolaryngology-Head and Neck Surgery, Baylor College of Medicine, Houston, TX 77030.
[3]Interdepartmental Program in Translational Biology and Molecular Medicine, Baylor College of Medicine, Houston, TX 77030.
[4]Department of Oral & Maxillofacial Surgery, University of Texas Health Science Center, Houston, TX 77054.

SUPPORTING INFORMATION

Expanded peptide synthesis and purification methods

Peptide synthesis reagents were purchased from EMD Chemicals (Philadelphia, PA). A combination of manual synthesis and an Apex Focus XC (Aapptec) synthesizer was used to synthesize the multi-domain peptides $K_2(SL)_6K_2$, $E_2(SL)_6E_2$, $R_2(SL)_6R_2$ and the precursor to the L-NIL MDP, $K^{Mmt}{}_2(SL)_6K^{Mmt}{}_2$ according to a standard synthetic method previously published.[21] To briefly summarize, each synthesis was performed using low loading Rink Amide MBHA resin (0.32 mmol/g). All amino acid were dissolved in 1:1 DMF:DMSO, and 20% piperidine in 1:1 DMF:DMSO was used to for deprotection reactions. Coupling steps were performed by mixing 4 equivalents amino acid, 4 equivalents of HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate), and 6 equivalents of DiEA (diisoproylethylamine), followed by shaking for 45 minutes. Upon completion of the syntheses the N-termini were acetylated by shaking the resin for 45 min with 5 equivalents of acetic anhydride mixed with 6 equivalents of DiEA in DCM, repeated once.

After acetylation and before cleavage of the peptide from the resin, the following method was used to convert $K^{Mmt}{}_2(SL)_6K^{Mmt}{}_2$ on resin to the L-NIL MDP. Deprotection of the lysine side chains was achieved by removal of the Mmt protection groups with four successive 15 min washes of AcOH/TFE/DCM (1:2:7), washing until the yellow color of released trityl cation was no longer observed. After washing the deprotected resin with DCM, a reaction mixture was added containing 12 equivalents ethyl acetimidate-HCl and 24 equivalents DiEA in DCM, and the mixture was shaken for 3 hours. The reaction was then drained, a second reaction mixture added, and shaken again for 2 hours, followed by drainage and a third fresh reaction mixture added and shaken for 1 hour. This sequence was done to ensure maximum conversion of the lysine side chains to L-NIL functional groups.

Cleavage of peptides achieved by shaking for 3 hours with TFA and protecting scavengers, in a 1:1:1:1:18 ratio of Milli-Q water : triisopropylsilane (TIPS) : anisole : ethane dithiole (EDT) : TFA. Rotary evaporation was used to remove excess TFA, and trituration with diethyl ether yielded crude peptide that was further washed with diethyl ether, dried, and purified by dialysis against Milli-Q water for 5-7 days using 100-500 D MWCO dialysis tubing (Spectra/Por, Spectrum Laboratories Inc., Rancho Dominguez, CA). Dialyzed peptide solutions were adjusted to pH 7.2-7.4, after which they were sterile filtered using 0.2 μm filters and lyophilized to powder for storage and use in experiments. All peptides were analyzed by Autoflex MALDI-TOF MS (Bruker Instruments, Billerica, MA) for confirmation of successful synthesis (**Figure S1**).

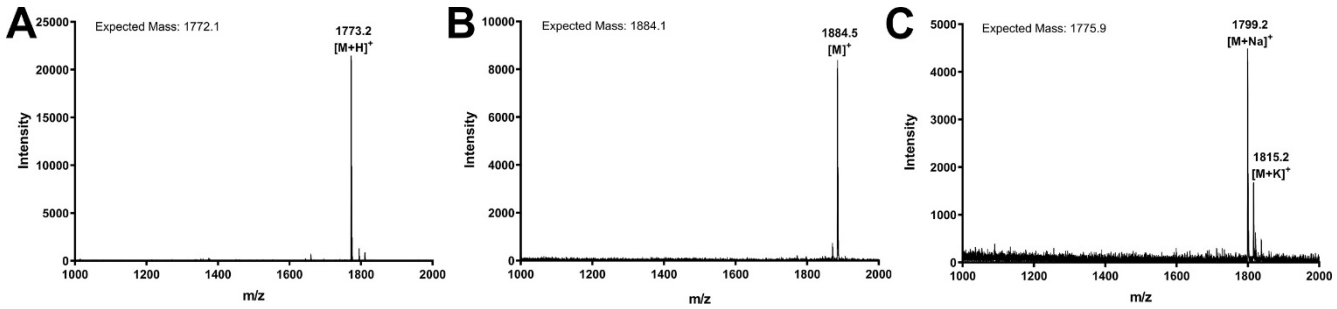


Figure S1. MALDI-TOF mass spectroscopy spectra for synthesized MDPs (A) $K_2(SL)_6K_2$, (B) $R_2(SL)_6R_2$, (C) $E_2(SL)_6E_2$.

Table S1. Antibodies used for immunostaining protocols.

Primary Antibody	*Dilution*	*Source*	*Catalog #*	*Secondary Antibody*
Rabbit anti-Nitrotyrosine	1:200	Millipore-Sigma	06-284	Anti-Rabbit AF® 568
Rabbit IgG isotype control	1:200	Invitrogen	31235	Anti-Rabbit AF® 568
Rabbit anti-αSMA	1:300	Genetex	GTX100034	Anti-Rabbit AF® 568

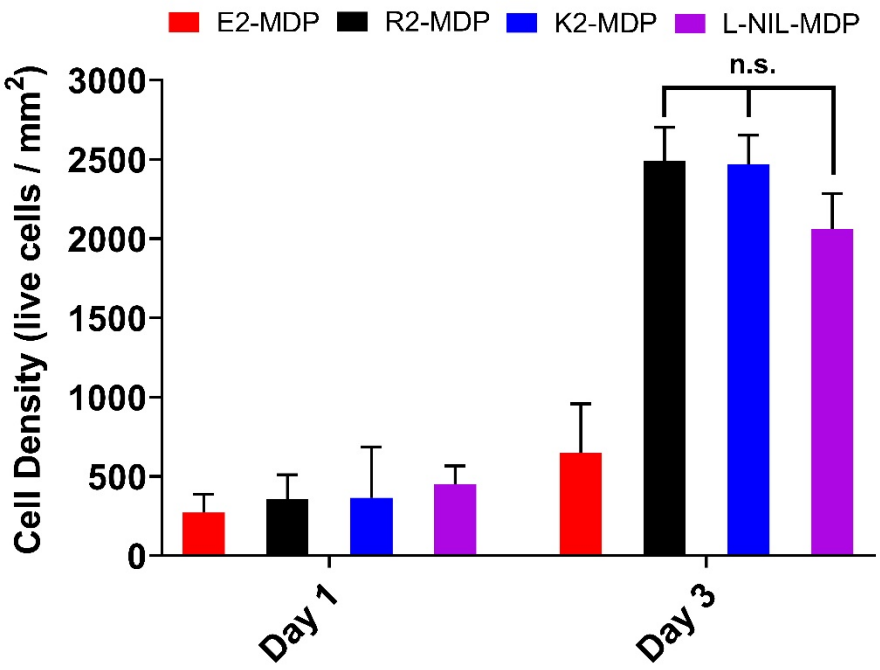


Figure S2. Quantification of live cell density cultured on MDP hydrogel surfaces *in vitro* to evaluate proliferation. RAW 264.7 cells were seeded on top of 70 μL hydrogel pucks under 200 μL of media (changed every two days) and processed under live-dead viability assays at days 1 and 3 (values are mean ± SD with n = 3 images). Cells are observed to not proliferate as readily on the negatively charged E2-MDP compared to the three cationic hydrogels. R2-MDP, K2-MDP, and L-NIL-MDP cell density values at day 3 show no statistically significant differences (n.s. = $p > 0.05$).

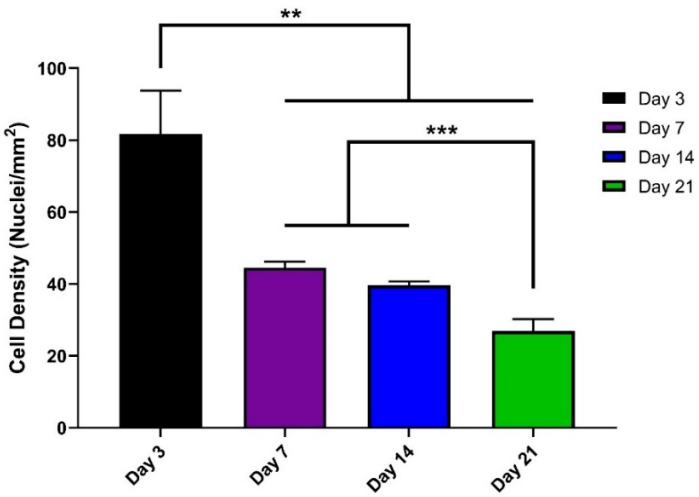


Figure S3. Quantification of cell density *in vivo* in subcutaneous L-NIL-MDP hydrogel implants over time, counting average number of nuclei per mm^2 of tissue section. Cell density is observed to decrease from day 3 to day 21. For each data set values are mean ± SEM with n=3, ** = p-value < 0.05, and *** = p-value < 0.02.

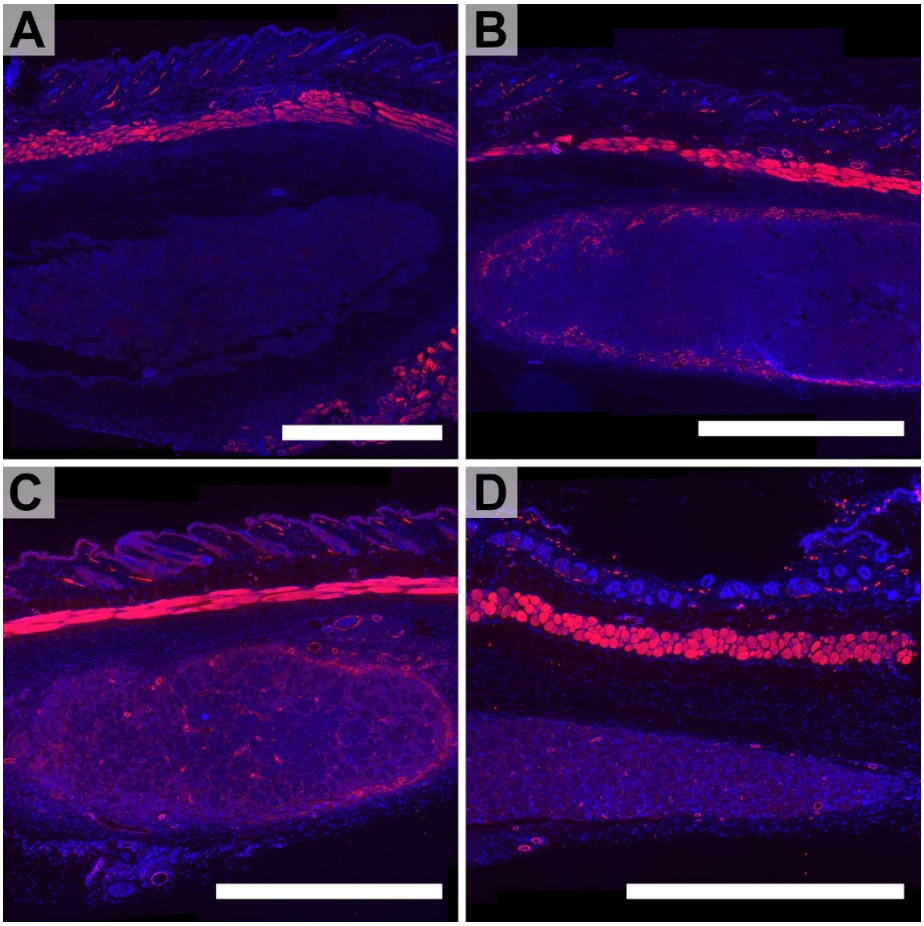
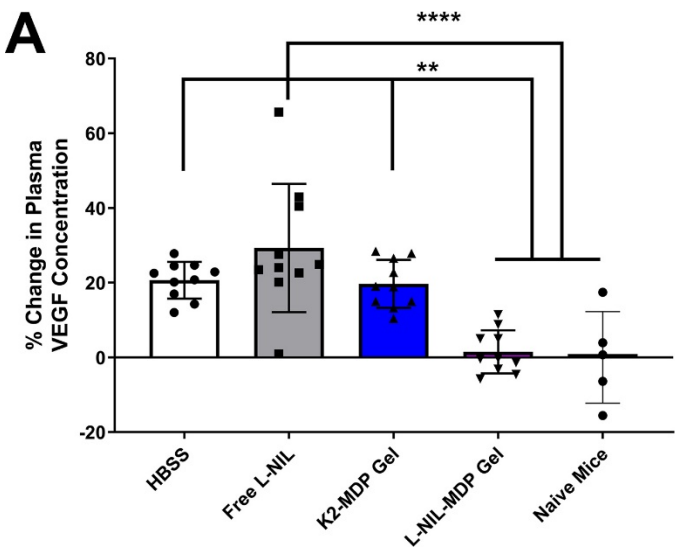


Figure S4. Blood vessel immunostaining of L-NIL-MDP hydrogel implants using anti-αSMA (red) and DAPI (blue) nuclei counterstain, showing some degree of small blood vessel formation in and around the implant. (A) L-NIL-MDP hydrogel 3 days post subcutaneous injection; (B) 7 days post injection; (C) 14 days post injection; (D) 21 days post injection. All scale bars = 1 mm (implant size in general decreases over time due to natural degradation, and thus scale is adjusted to aid visualization).



B

Treatment group	Serum VEGF level (pg/mL)
HBSS	142 ± 7
Free L-NIL	152 ± 11
K2-MDP	143 ± 10
L-NIL-MDP	112 ± 8
Naïve	113 ± 17

Figure S5. Vascular endothelial growth factor (VEGF) serum levels in mice bearing B16-F0 melanoma tumors. (A) Percent change in plasma VEGF concentration from normal levels observed in naïve mice. Data shown are 5 days after 100 μL intratumoral injections of either HBSS buffer control, free L-NIL drug, K2-MDP hydrogel, or L-NIL-MDP hydrogel. In each group n = 10 (except naïve mice n =5), and statistical significance is noted as ** = p value ≤ 0.004, and **** = p value ≤ 0.0002 (values are mean ± SD). Significant VEGF reduction was observed in sera from mice injected with the L-NIL-MDP gel compared to all other tumor-bearing groups, restoring circulating VEGF concentrations to normal levels observed in naïve healthy mice. (B) Raw VEGF serum levels in pg/mL representative of two experiments, with values equal to mean ± SD (n = 5).

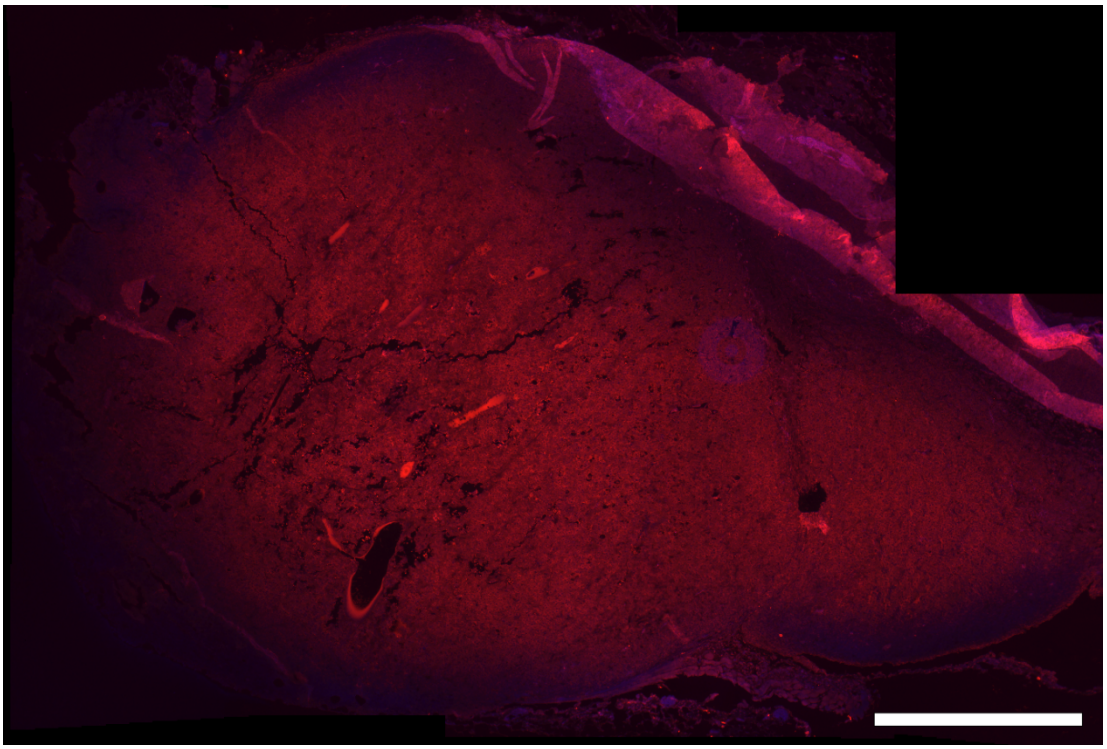


Figure S6. Anti-nitrotyrosine immunostaining of inflamed B16-F0 tumor section used as a positive control for nitrotyrosine quantification studies, using red 568 nm secondary antibody reactive to anti-nitrotyrosine primary and DAPI for nuclei counterstain. Scale bar is 1 mm.

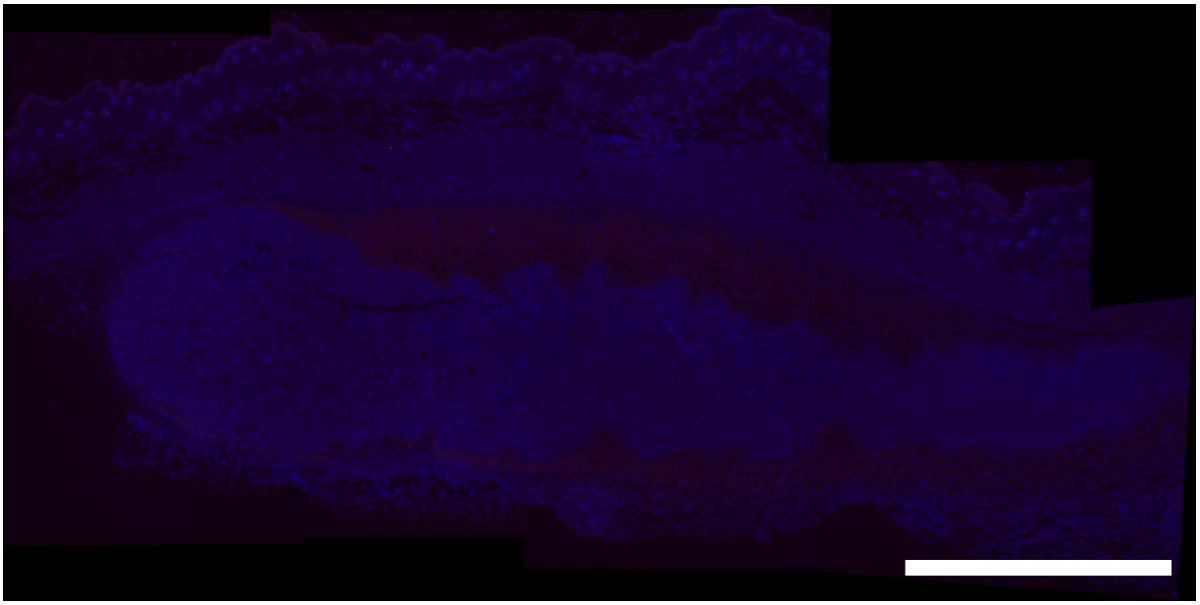


Figure S7. Day 3 L-NIL-MDP isotype control panorama for anti-nitrotyrosine immunostaining study, using rabbit IgG2a isotype control (in place of anti-nitrotyrosine primary), anti-rabbit 568 nm secondary, and DAPI counterstain. Scale bar is 1 mm.

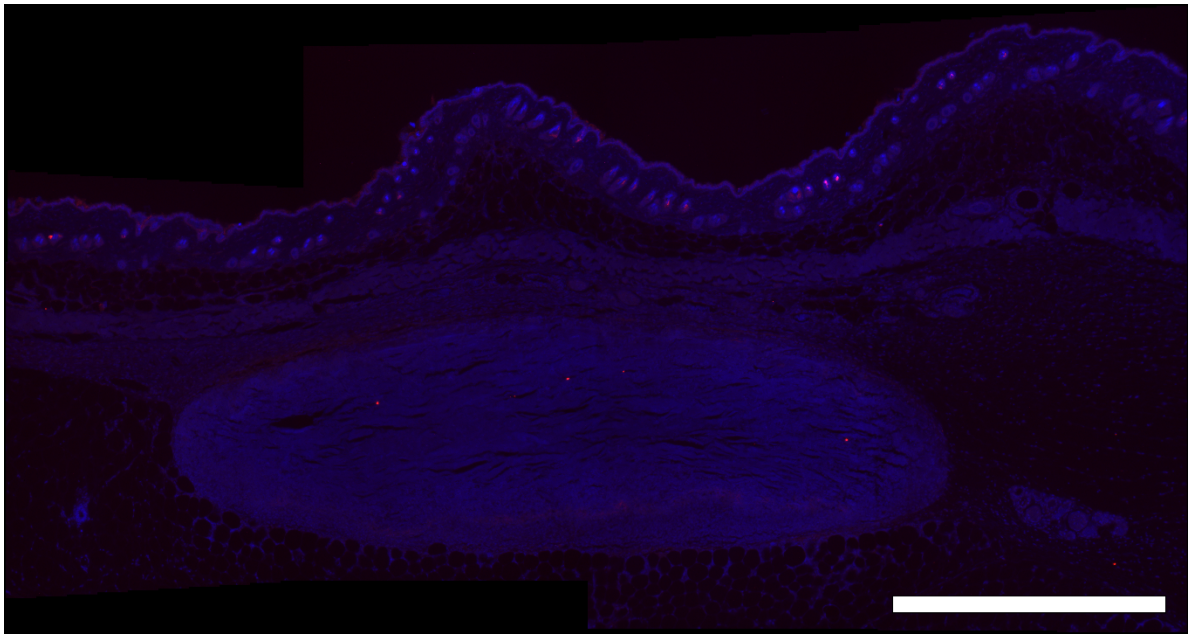


Figure S8. Day 3 K2-MDP isotype control panorama for anti-nitrotyrosine immunostaining study, using rabbit IgG2a isotype control, anti-rabbit 568 nm secondary, and DAPI counterstain. Scale bar is 1 mm.

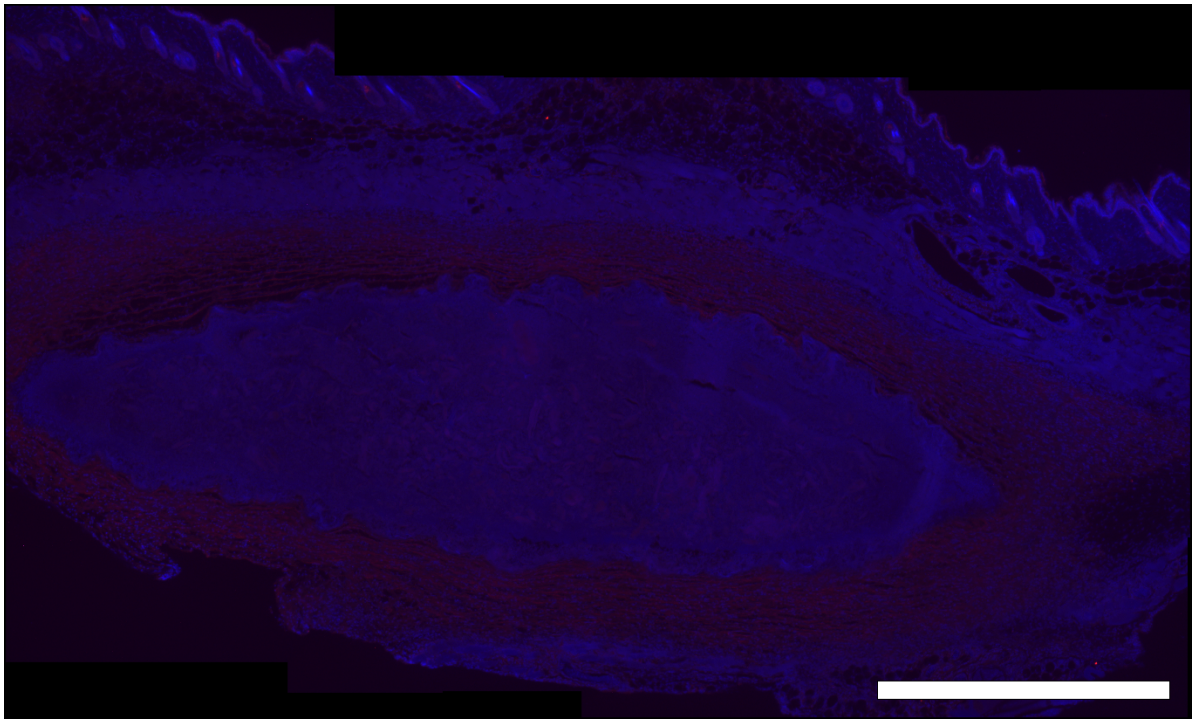
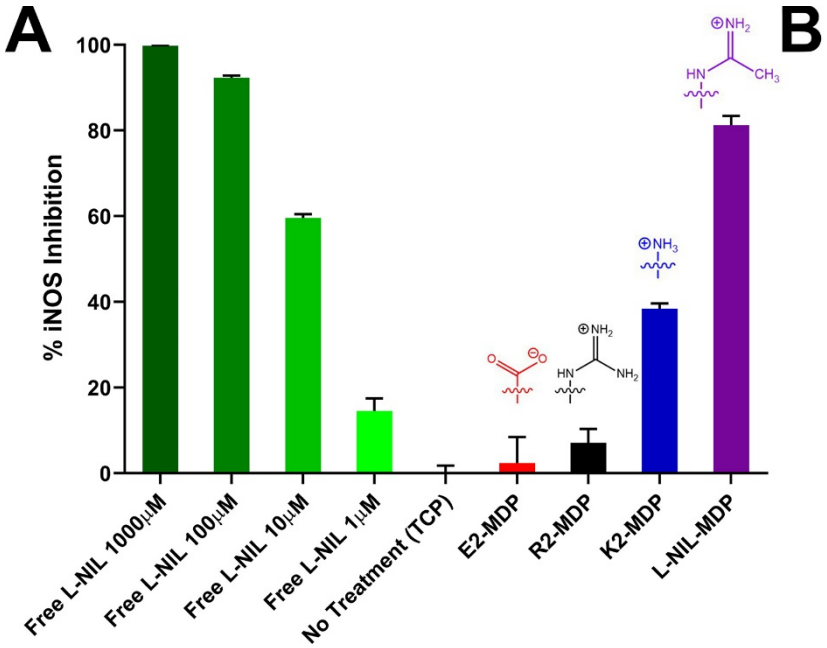


Figure S9. Day 3 R2-MDP isotype control panorama for anti-nitrotyrosine immunostaining study, using rabbit IgG2a isotype control, anti-rabbit 568 nm secondary, and DAPI counterstain. Scale bar is 1 mm.



B

Condition	% Inhibition
1000 μM L-NIL	100 ± 0.1
100 μM L-NIL	92 ± 0.6
10 μM L-NIL	60 ± 0.8
1 μM L-NIL	15 ± 2.9
TCP	0 ± 1.8
E2-MDP gel	2.4 ± 6.1
R2-MDP gel	7.1 ± 3.2
K2-MDP gel	38 ± 1.3
L-NIL-MDP gel	81 ± 2.2

Figure S10. Raw greiss assay results used to evaluate iNOS knockdown efficacy of various materials, with data not normalized for cell viability results obtained at 24 hours. (A) Plot of % iNOS inhibition of RAW 264.7 cells stimulated with Lipopolysaccharide (LPS) and IFN-γ cultured in different surface conditions, showing chemical structures of key surface-exposed functional groups. (B) Quantification of relative inhibition activity; values are from two experiments and are mean ± SD with three replicates tested per condition.