Supporting Information

Nitric Oxide-Scavenging Nanogel for Treating Rheumatoid Arthritis.

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Experimental section

Reagents

Di-tert-butyl dicarbonate, 4-nitro-*O*-phenylenediamine, guanidine hydrochloride, sodium sulfate, palladium on carbon (PD/C), tetrahydrofuran, Celite® 545 AW, acryloyl chloride, trimethylamine, TFA, pyrrolidine, acetonitrile, sodium methoxide, *N,N'*-dimethylformamide, Dowex® 50WX8 resin, acrylamide, *N,N'*-methylenebisacrylamide, APS, TEMED, Lipopolysaccharide (LPS) and thiazolyl blue tetrazoliumbromide (MTT), collagen from chicken sternal cartilage, complete Freund's Adjuvant (CFA) and incomplete Freund's Adjuvant (IFA) were purchased from Sigma Aldrich (St. Louis, MO). Nile blue polyacrylamide was purchased from polysciences (Warrington, PA). Griess reagent kit was purchased from Invitrogen (Carlsbad, CA). DAF-2 DA was purchased from Abcam (Cambridge, MA). ELISA kits for IL-6 and TNF-α were purchased from Koma Biotech (Korea).

Instrumental methods

DLS and zeta potential were analyzed by a Zetasizer Nano S90 system (Malvern Instruments, Worcestershire, U.K.). UV-vis spectra was measured by a UV 2550 spectrophotometer (Shimadzu). NMR was obtaind from Bruker Advance 500 MHz FT-NMR. Fourier Transform infrared (FT-IR) spectroscopy was confirmed by VERTEX70 FT-IR spectrophotometer (Bruker Optics). For atomic force microscopy (AFM), both diluted nanogel samples were dropped into a mica substrate on the heat. Subsequently, samples were dried for 1 day, then AFM images were obtained by VEECO Dimension 3100/Nanoscope V. For transmission electron microscopy (TEM), both diluted nanogel samples for TEM were dropped into a carbon grid and performed negative staining. Then, samples were dried for 1 day and TEM images were obtained by JEM-1011 (JEOL, Tokyo, Japan). Absorbance for Griess assay, ELISA, and MTT assay were measured by a multi-mode microplate reader (SpectraMax® i3, Molecular Devices) and analyzed by SoftMax® Pro 6 software. Confocal laser scanning microscope (CLSM) image was obtained from Olympus FV-1000 and analyzed by OLYMPUS FLUOVIEW

Viewer (ver. 1.7). Histological assay image was obtained by microscopy (Nikon Eclipse 80i, USA). Statistical analysis of data was calculated by GraphPad Prism 7. IVIS imaging was carried out by Davinch in vivo imaging system INV-16M

Synthesis of NOCCL

We synthesized NOCCL using same protocol reported previously by J. Park *et al.*[1] We mixed 4-nitro-o-phenylenediamine and guanidine hydrochloride in ethanol. Di-tert-butyl dicarbonate was added into mixed solution at 35 °C for 40 h. After extraction with ethyl acetate, chromatography was performed. Then, to reduce the nitro group in compound 1, hydrogenation with palladium was performed under a hydrogen atmosphere (40 psi) at 25 °C for 36 h. Subsequently, palladium was removed by using Celite® 545 AW. After filter, acryloyl chloride and triethylamine were added to the filtrate dissolved in anhydrous THF, followed by extraction with ethyl acetate and purification process by chromatography. Finally, to remove the Boc group, trifluoroacetic acid (TFA) was added into reaction mixture at 25 °C for 24 h and pure NOCCL was obtained. H^1-NMR (500 MHz DMSO-d6): δ 9.89 (brs, NH-7), 9.30 (brs, NH-9), 7.20 (d, H-3), 7.19 (d, H-5), 6.84 (dd, H-6), 6.40(m, H-11, H-14), 6.20 (dd, Hb-12, Hb-15), 5.72 (m, Ha-12, Ha-15), 4.98 (brs, NH_2-8, D2O exchangeable)

Preparation of NO-Scv gel and NOX gel

For NO-Scv gel, NOCCL in 100 μL of 1% (v/v) ethanol was diluted with water to make 0.03125% (w/v). Subsequently, we mixed 10 μL of 20% acrylamide and 10 μL of 0.03125 % NOCCL in 1.75mL e-tube. After that, 1μL 10% (w/v) APS and 1μL 5% (w/v) TEMED were added into the solution, with sonication for 2 min. For NOX gel, 10 μL of 20% acrylamide and 10 μL of 0.075% (w/v) *N,N'*-methylene-bisacrylamide were mixed simply. After that, 1 μL 10% (w/v) APS and 1 μL 5% (w/v) TEMED were added into the solution, with sonication for 2 min.

Measurement of NO-scavenging ability of crosslinkers and nanogels

For fresh NO source, we used pyNO solution; pyNO was synthesized according to previous method. Pyrrolidine (1.64 mL, 20.0 mmol) was added to the mixed solution of 15 mL acetonitrile and 5 mL ether. After the addition of pyrrolidine, 3.9 mL of 30 wt % NaOMe solution (20.0 mmol) was added. This solution was then taken into the high-pressure NO reactor, followed by argon purging several times. The reactor was then immediately filled with 90 psi of NO gas and maintained the pressure of NO for 3 days. PyNO was precipitated as white solid during the reaction. After reaction, the product was collected by filteration with washing with cold diethyl ether. The collected product was then keep under vacuum for removal of the solvent. After solvent was dried, pyNO was stored under the -20 °C with sealing. Yield: 2.37 g (15.5 mmol; 77.5 %)

For the measurement of NO-scavenging ability of crosslinkers and nanogels, we prepared 50 μL NO-sample solution containing 5 μg/mL of pyNO solution in distilled water and 0~500 μg/mL of crosslinker solution (NOCCL or NOXCL) or 25 mg/mL of gel solution (NO-Scv or NOX gel). After incubation for 30 min, sample solution was collected for quantification of whole nitrite by Griess assay following the protocol of the manufacturer. In brief, 50 μL of sulfanilamide solution and 50 μL of NED solution were readily added into 100 μL of sample medium and incubated at dark for 10 min. Nitrite solution (0~100 μM) provided by manufacturer was used for standard solution. After incubation, absorbance at 540 nm (azo compound) was measured and the concentration of NO_2^- in medium was calculated by standard curve.

Confirmation of NO-responsiveness by UV-VIS, FT-IR, and NMR

We prepared NO solution using same protocol previous reported.[2] Distilled water (10 mL) in 40 mL vial was purged by nitrogen gas for 30 min, followed by placing the vial into NO apparatus (1.35 atm). The final concentration of saturated NO solution was 1.88 mM at 20 °C. Before NO-responsiveness experiment, NO solution was always prepared freshly.

Benzotriazole formation was observed by UV-vis spectrometry. NO solution (600 ul) containing 0~500 μg/mL of NO concentration was added to NOCCL solution (10mg, 43.2 μmol). Then, we observed 290 nm peak of benzotrialzole in each sample by UV-vis spectrometry.

For the Fourier transform infrared spectroscopy measurement, 500 ul of 100 μM NO solution was added to NOCCL solution (1mg, 4.33 μmol) in 100 μL of 1% ethanol and incubated for 24 h at RT. Subsequently, the sample was perfectly lyophilized and dried, then we prepared IR pellet by using potassium bromide. Finally we carried out Fourier Transform infrared spectroscopy. For the NMR measurement, DMSO was added to the solid pellet, and performed NMR measurement.

Evaluation of cell viability, extracellular NO, and extracellular pro-inflammatory cytokine level

Cytotoxicity, extracellular level of NO, and extracellular pro-inflammatory cytokine level after treatment of NOCCL, NOXCL, NO-Scv gel, and NOX gel were evaluated dose-dependently in murine macrophage cell line (RAW 264.7) and fibroblast cell line (NIH 3T3). In brief, cells were seeded on 6-well plate at a density of 100,000 cells/well under Dulbecco's modified Eagle's medium (DMEM) and incubated for overnight. Medium was replaced by 1 mL of fresh medium containing 5 μg/mL of LPS and 0-50 μg/mL of crosslinkers (NOCCL or NOXCL) or 0~2 mg/mL of nanogels (NO-Scv or NOX gel). After incubation for 24 h, medium was collected for quantification of whole nitrite by Griess assay or cytokine level by ELISA, and viability of residual cells were evaluated by MTT assay.

For Griess assay, medium was collected and centrifuged (3,000 rpm, 10 min) to discard the dead cells or debris in the medium. 100 μL of supernatant was carefully transferred to 96-well plate and evaluated by Griess assay following the protocol of the manufacturer. In brief, 50 μL of sulfanilamide solution and 50 μL of NED solution were readily added into 100 μL of sample medium and incubated at dark for 10 min. 0~100 μM of nitrite provided by manufacturer was used for standard solution. After incubation, absorbance at 540 nm (azo compound) was measured and the concentration of NO_2^- in medium was calculated by standard curve.

In addition, NO-related activation of macrophage was determined by quantification of IL-6 and TNF-α, representative pro-inflammatory cytokines, by ELISA. Briefly, collected medium was centrifuged (3,000 rpm, 10 min) and 100 μL of medium was utilized for detection of cytokines. All the procedures were proceeded according to the manufacturer's protocol. Absorbance at 450 nm was measured and the concentration of cytokines were calculated by standard curve.

Viability of sample-treated cells were evaluated by MTT assay. After discarding medium for Griess assay and ELISA, 1 mL of fresh medium containing 0.5 mg/mL of MTT was added and incubated at dark for 4 h. After incubation, medium was carefully aspirated and violet crystal was fully dissolved with 1 mL of DMSO. 200 μL of each sample was transferred into new 96-well plate. Absorbance at 570 nm (formazan compound) was measured and the relative absorbance of non-treated cells were regarded as 100% viability.

Imaging of intracellular NO by confocal laser scanning microscopy (CLSM)

Intracellular NO level was detected by DAF-2 DA, a cell-permeable NO detection dye. Briefly, RAW 264.7 cells were seeded on the cover glass placed in a 12-well plate at density of 500,000 cells/well and incubated for overnight. Medium was replaced by fresh medium, medium with 5 μg/mL of LPS, LPS with 25 μg/mL of crosslinker (NOCCL or NOXCL), or LPS with 1 mg/mL of gel (NO-Scv or NOX gel), respectively and further incubated for 24 h. After incubation, medium was replaced by fresh medium containing 5 μM of DAF-2 DA and incubated for 40 min. Then, cells were washed with PBS and fresh medium was added, and incubated for additional 20 min to allow de-esterification of the dye. Cells were washed carefully with PBS and fixed with 10% neutrally buffered formalin (NBF) at room temperature at dark condition for 30 min. Cells on the cover glass was mounted in Vectashield anti-fade mounting medium for fluorescence with DAPI (Vector Labs) and observed by CLSM. Nuclei and DAF 2-DA were imaged by DAPI and FITC channel, respectively.

Hemolysis Test

We prepared fresh mouse blood and diluted 10-folds with PBS, followed by centrifugation at 2,000 rpm for 15 min. Samples containing same concentration of NOXCL, NOCCL, Dexa, acrylamide were prepared and added into the centrifuged blood cells. Subsequently, the mixed samples were incubated at 37 °C for 6 h. After incubation, the mixed blood samples were centrifuged at 2,000 rpm for 15 min to make pellets. Except pellets, we transferred supernatants and measured the absorbance of the supernatants at 541 nm, which quantifies the released hemoglobin. PBS and 1x lysis buffer were exploited for negative control (0% hemolysis) and positive control (100% hemolysis), respectively.

In vivo test for NO-scavenging effect of NO-Scv gel in RA and LPS-challenged mice.

We prepared collagen-induced arthritis (CIA) mouse model using DBA/1 mice.[3] For immunization, we injected 100 μl of emulsified solution (1:1) mixing type 2 collagen (2 mg/mL) and complete Freund's Adjuvant (CFA) (1mg/mL) into male DBA/1. After 14 days, we injected again 100 μL of emulsified solution (1:1) mixing type 2 collagen (2 mg/mL) and incomplete Freund's Adjuvant (IFA) (1 mg/mL) into tail, intradermally. After immunization, each sample containing 50 μL of NOX gel or NO-Scv gel (25 mg/ml), 50 μL of Dexa (0.32 mg/ml), and saline was injected into hind paw of different mice. We used a certain range of nanogel concentration that shows maximum effect without toxicity, and consulted reference paper for determining dosage of Dexa[4]. Subsequently, NO level of hind paw tissue and serum were evaluated after 24 h through Griess assay.

We, then evaluated NO level of LPS-challenged mice. Briefly, LPS was administrated i.p. (1 mg/kg), followed by injection of samples in paw of the mice, and NO level of the paw tissue was evaluated after 24 h by Griess assay.

Paw tissues isolated from each mouse were homogenized and used for Griess assay. Tissue fluid was collected and centrifuged (13,000 rpm, 10 min) to remove proteins. 50 μL of fluid was carefully transferred to 96-well plate and evaluated by Griess assay following the protocol of the manufacturer. In brief, 50 μL of sulfanilamide solution and 50 μL of NED solution were readily added into 100 μL

of sample medium and incubated at dark for 10 min. 0~100 μM of nitrite provided by manufacturer was used for standard solution. After incubation, absorbance at 540 nm (azo compound) was measured and the concentration of NO_2^- in medium was calculated by standard curve.

For Griess assay in serum, serum sample was collected from mouse blood and centrifuged (13,000 rpm, 10 min) to remove proteins. 80 μL of fluid was carefully transferred to 96-well plate and evaluated by Griess assay following the protocol of the manufacturer. In brief, 10 μL reductase and 10μL cofactor were added into 80 μL of sample and incubated for 2h. Then 50 μL of sulfanilamide solution and 50 μL of NED solution were readily added into 100 μL of sample and incubated at dark for 10 min. 0~100 μM of nitrite/nitrate provided by manufacturer was used for standard solution. After incubation, absorbance at 540 nm (azo compound) was measured and the concentration of NO_2^- in medium was calculated by standard curve.

In vivo test for therapeutic effect of NO-Scv gel in RA

We prepared collagen-induced arthritis (CIA) mouse model using DBA/1 mice.[3] For immunization, we injected 100 μl of emulsified solution (1:1) mixing type 2 collagen (2 mg/mL) and complete Freund's Adjuvant (CFA) (1mg/mL) into male DBA/1. After 14 days, we injected again 100 μL of emulsified solution (1:1) mixing type 2 collagen (2 mg/mL) and incomplete Freund's Adjuvant (IFA) (1 mg/mL) into tail, intradermally. After immunization, each sample containing 25 mg/ml of gels (NOX gel, NO-Scv gel), 0.32 mg/ml of Dexa, and saline was injected into paw of different mice, intra-articulary. Subsequently, blind test of assessing clinical score was performed according to the previously reported method.[5] After 35 days, we sacrificed mice for CT, ELISA and histological assay. For quantifying paw volume change, area of paw was measured by imageJ software based on day 0.

Preparation of NO-Scv nanogel and NOX nanogel including small molecule dye

NOCCL in 100 μL of 1% (v/v) ethanol was diluted with water to make 0.03125% (w/v). Subsequently,

we mixed 10 μL of 20% acrylamide, 10 μL of 0.03125 % NOCCL and 1 μL of 1% acrylamide-modified Nile blue in 1.75mL e-tube. After that, we added 1μL 10% (w/v) APS and 1μL 5% (w/v) TEMED into the solution, with sonication for 10 min. For NOX gel, 10 μL of 20% acrylamide, 10 μL of 0.075% (w/v) *N,N'*-methylene-bisacrylamide and 1 μL of 1% acrylamide-modified Nile blue was mixed simply. After that, we added 1 μL 10% (w/v) APS and 1 μL 5% (w/v) TEMED into the solution, with sonication for 10 min.

Evaluation of retention time of NO-Scv gel in normal or LPS-challenged paws.

For the preparation of LPS-challenged paws, we injected LPS into paw (5 mg/kg) of normal DBA/1 mice. After 30 min, NO-Scv gel with Nile blue was injected into normal paw or LPS-challenged paw. Subsequently, we monitored the fluorescent of Nile blue (λ_{ex} = 635/λ_{em} =674) through IVIS imaging tool for 7 days.

Evaluation of retention time of NOX gel and small molecule dye in normal paws.

NOX gel or small molecule dye (same concentrations of NOX gel) was injected into each paw of normal DBA/1 mice. Subsequently, we monitored the fluorescent of Nile blue (λ_{ex} = 635/λ_{em} =674) through IVIS imaging tool for 7 days.

Histological assay.

Sacrificed tissues were immersed in 10% neutral buffered formalin, and decalcified with decalcifying solution for 7 days. Then, the decalcified tissues were embedded in paraffin and sliced tissues were stained with H&E, Masson's trichrome and safranin-O. The image was obtained by microscopy (Nikon Eclipse 80i, USA). Quantification of cartilage area was carried out by Safranin-O staining of tissue. Cartilage content of each sample was obtained through normalization by whole area (cartilage and bone marrow) and further normalization by healthy group.

Evaluation of pro-inflammatory cytokines in vivo

Quantification of IL-6 and TNF-α, representative pro-inflammatory cytokines was performed by ELISA. Briefly, sample-treated mouse serums (50 μL) was utilized for detection of cytokines. All the procedures were proceeded according to the manufacturer's protocol. Absorbance at 450 nm was measured, and the concentration of cytokines were calculated by standard curve.

References

1. Park, J.; Pramanick, S.; Park, D.; Yeo, J.; Lee, J.; Lee, H.; Kim, W. J. *Adv Mater.* **2017,** *29,* 17028597.

2. Lee, J.; Park, J.; Lee, H.; Kim, H. I.; Kim, W. J. *J. Mater. Chem. B.* **2014**, *2*, 2616.

3. Courtenay, J. S.; Dallman, M. J.; Dayan, A. D.; Martin, A.; Mosedale, B. *Nature*. **1980**, *283*, 666-668

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5. Ierna, M.; Kerr, A.; Scales, H.; Berge, K.; Griinari, M. *BMC Musculoskeletal Disord.* **2010,** *11*, 136.

Supplementary figures

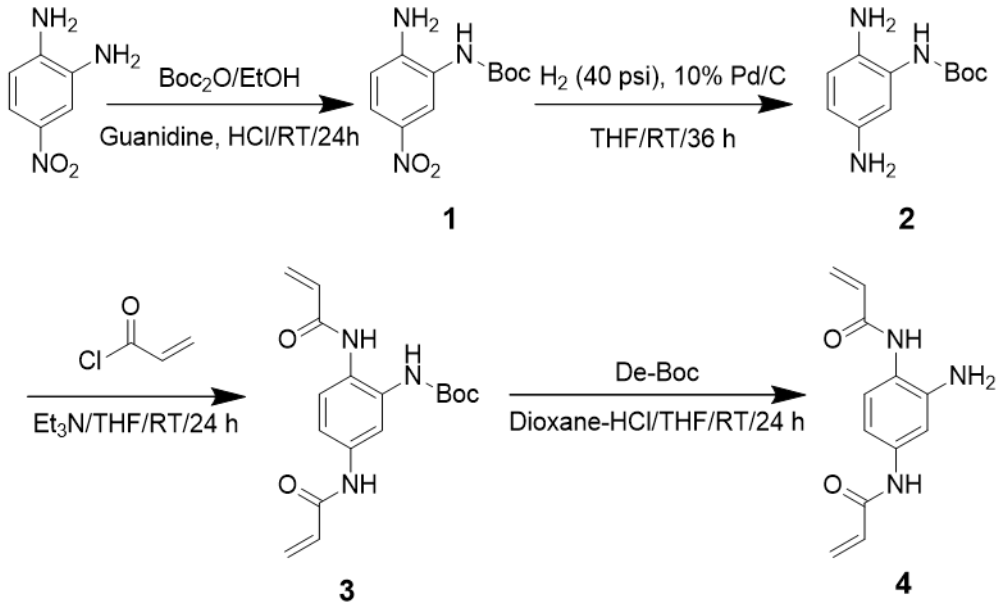

Figure S1. Synthetic scheme of NOCCL.

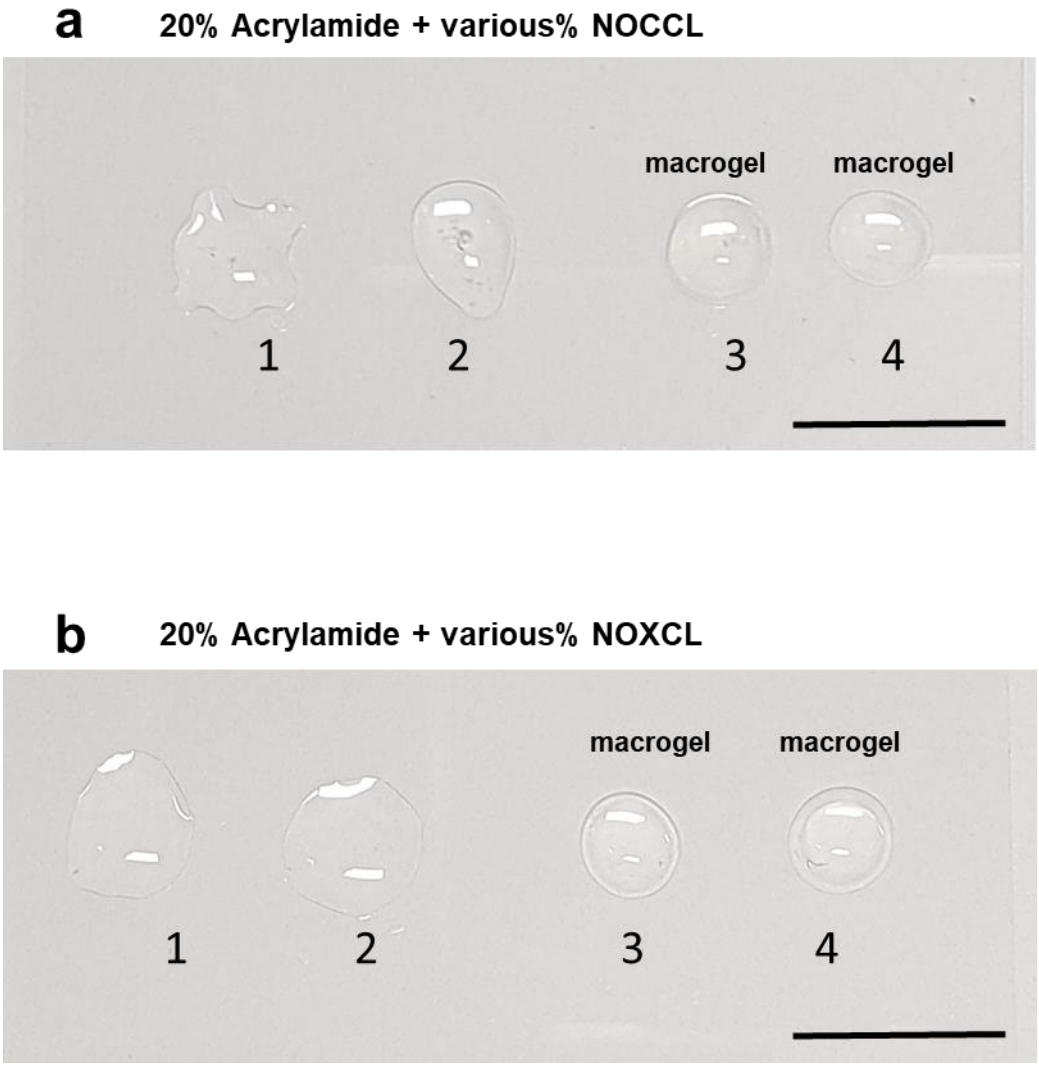

Figure S2. Morphology of gels formed with various concentrations of NOCCL or NOXCL. (a) 20% Acrylamide / NOCCL (**1**: 0.0156 %, **2**: 0.0312%, **3**: 0.0625%, **4**: 0.125%) (b) 20% Acrylamide / NOXCL (**1**: 0.0039 %, **2**: 0.0078%, **3**: 0.0156%, **4**: 0.0312%). (Scale bar = 1 cm).

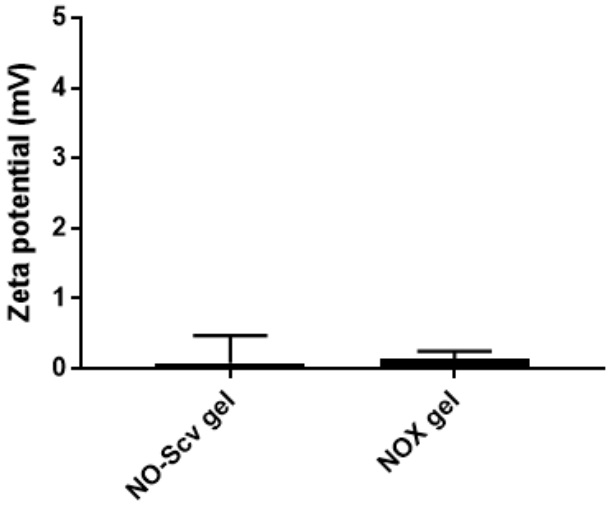

Figure S3. Zeta potential profile of NO-Scv and NOX gel

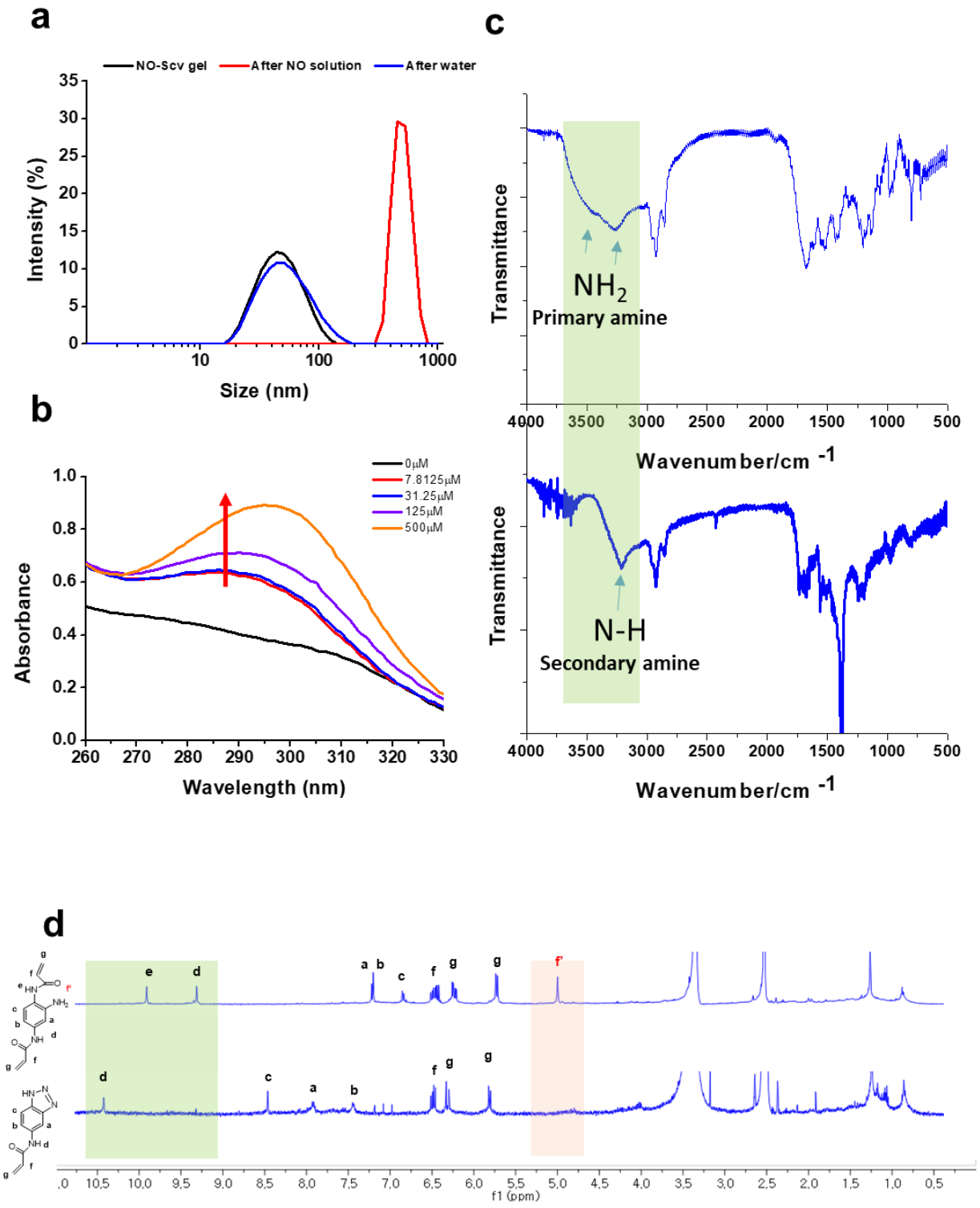

Figure S4. Confirmation of NO-responsive behavior of NO-Scv gel and NOCCL crosslinker. (a) Size distribution of NO-Scv gel after reaction with NO. (b) UV-vis spectroscopy for the confirmation of benzotriazole formation after cleavage of NOCCL by NO. (c) FT-IR measurement for detecting transition of primary amine to secondary amine by cleavage of NOCCL. (d) NMR measurement for

confirmation of NOCCL cleavage by NO.

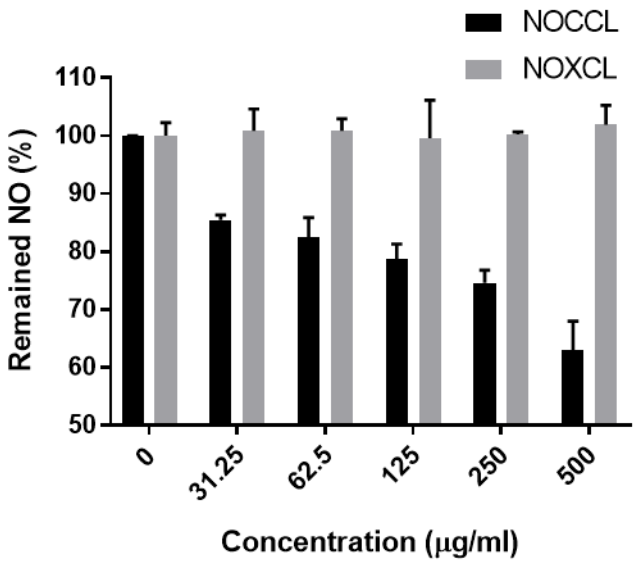

Figure S5. NO-scavenging ability of NOCCL and NOXCL

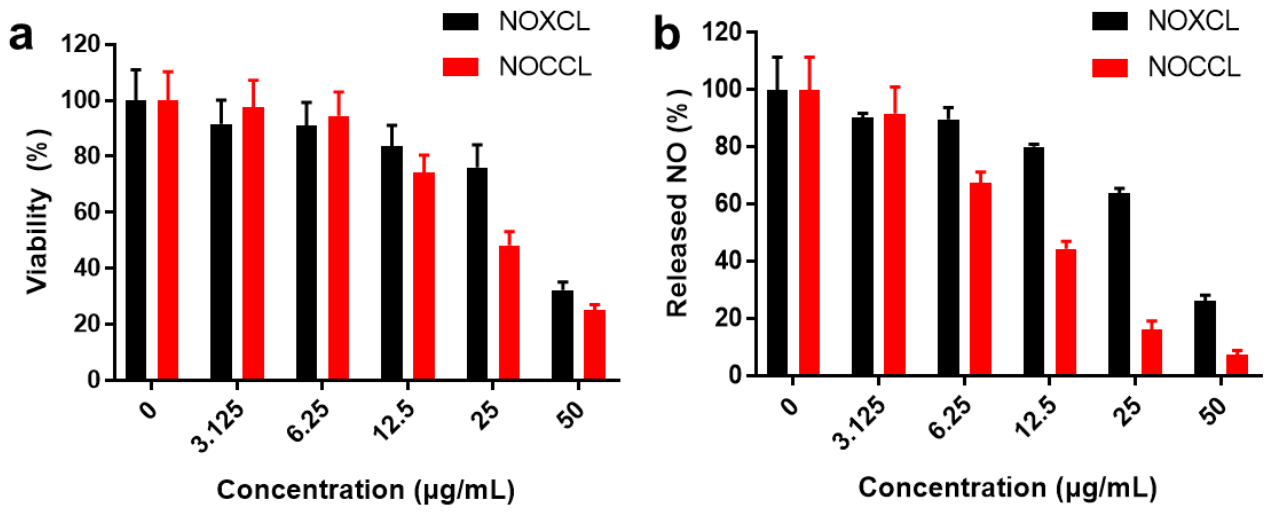

Figure S6. Cytotoxicity and NO-scavenging ability of crosslinkers studied in RAW 264.7 cell line.

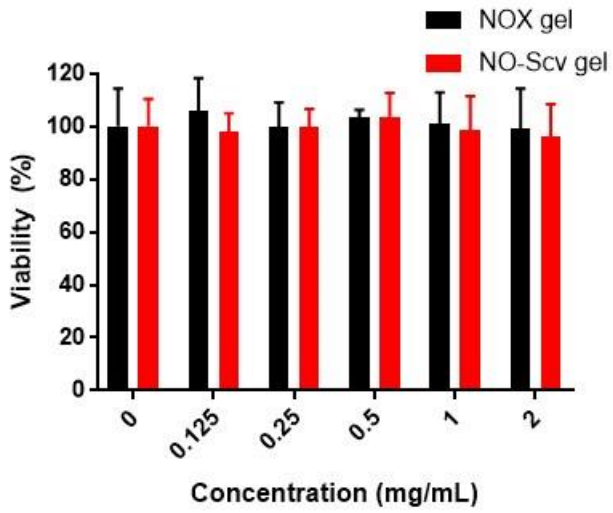

Figure S7. Cytotoxicity of nanogels studied in NIH 3T3 cell line.

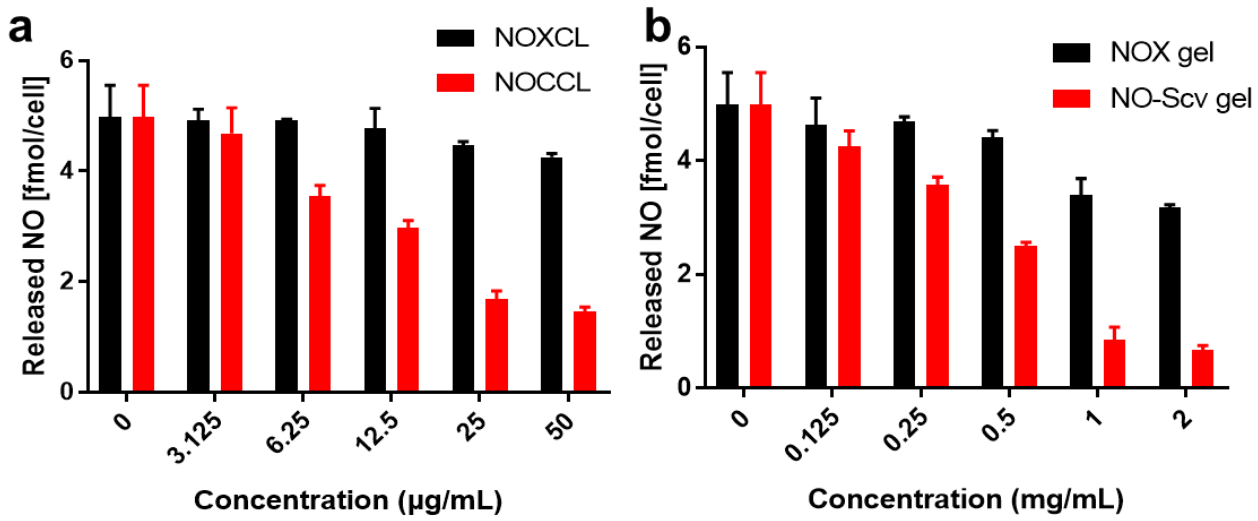

Figure S8. NO concentration released from RAW 264.7 cells after treatment with samples. Amount of released NO was calibrated by cell number.

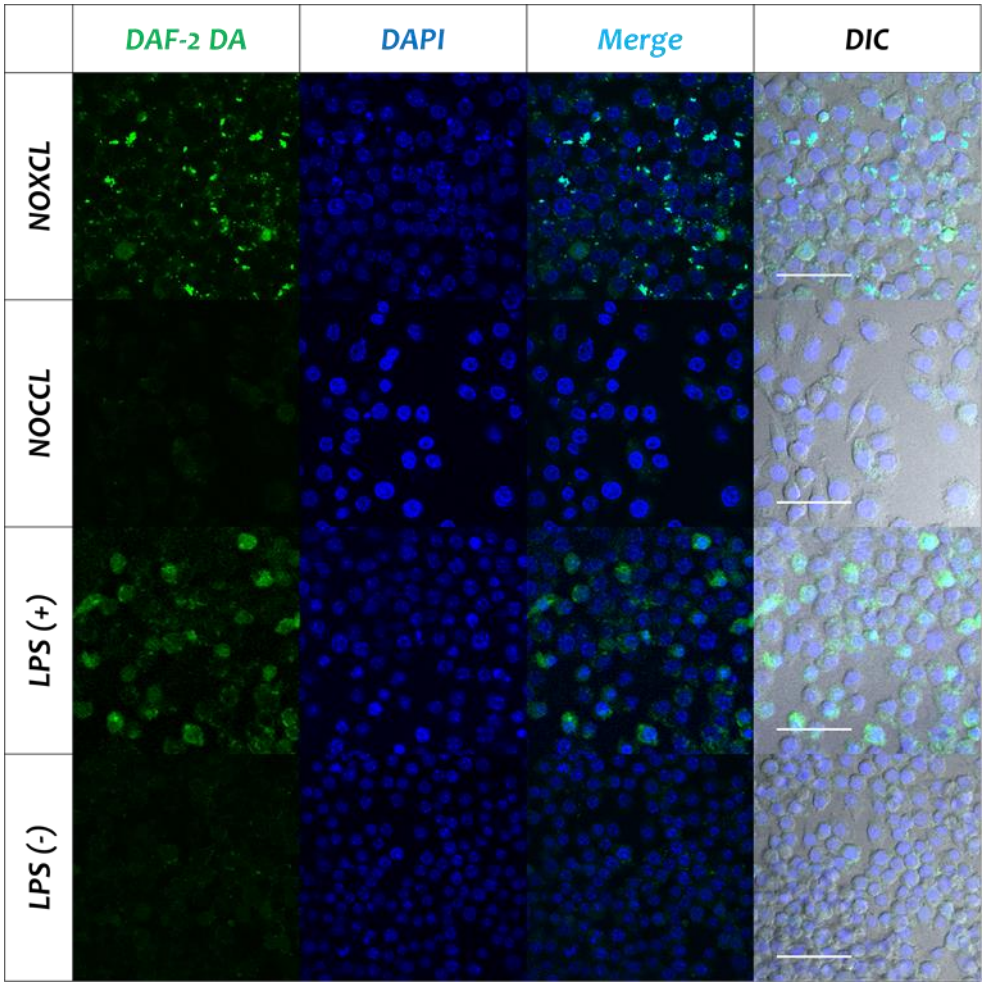

Figure S9. Confocal images of RAW 264.7 cells after treatment with DAF-2 DA and NOCCL or NOXCL. Nuclei were stained with DAPI, and NO was stained with DAF-2 DA (Scale bar = 100 μm).

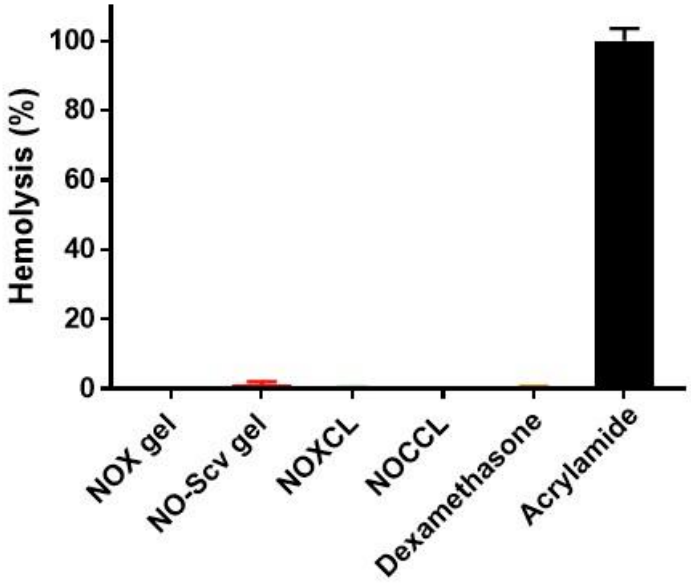

Figure S10. Hemolysis test with nanogels, crosslinkers and dexamethasone.

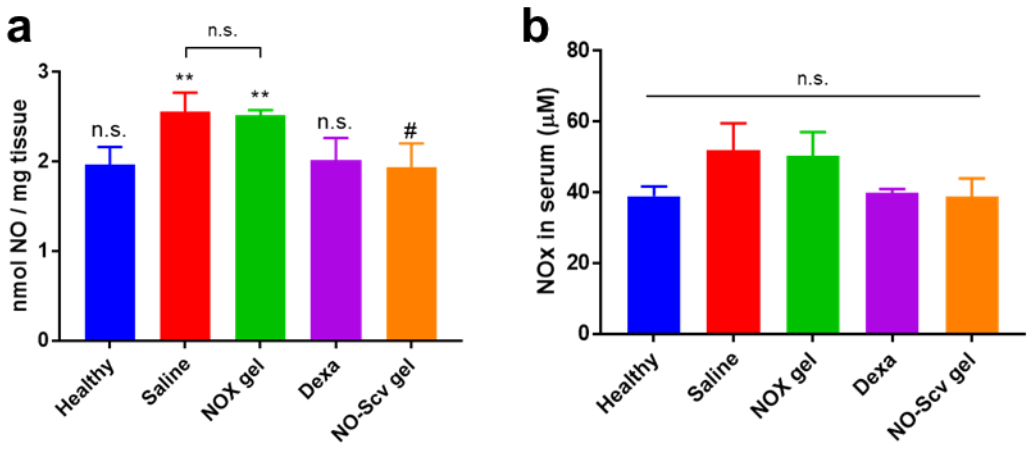

Figure S11. Profile of NO level in Paw tissue (a) and serum (b) of RA mice after treatment with NOX gel, Dexa, and NO-Scv gel. (Mean ± SD, ** $p < 0.01$).

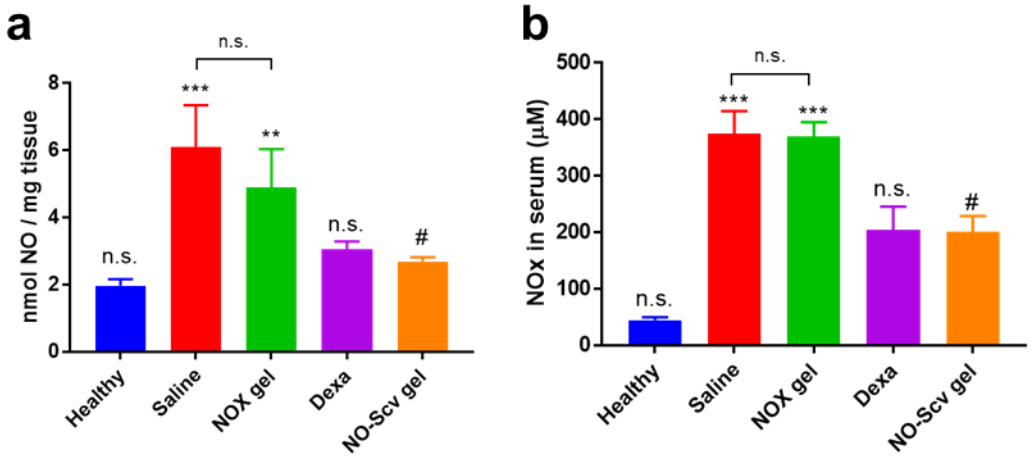

Figure S12. Profile of NO level in Paw tissue (a) and serum (b) of LPS-treated mice after injection of NOX gel, Dexa, and NO-Scv gel. (Mean ± SD, ** $p < 0.01$, *** $p < 0.001$).

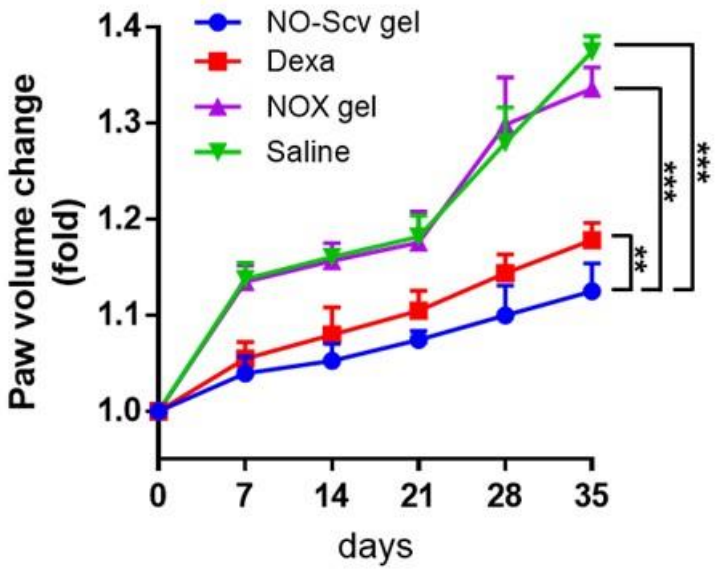

Figure S13. Quantification of paw volume change in RA mouse model. (Mean ± SD, ** $p < 0.01$, *** $p < 0.001$).

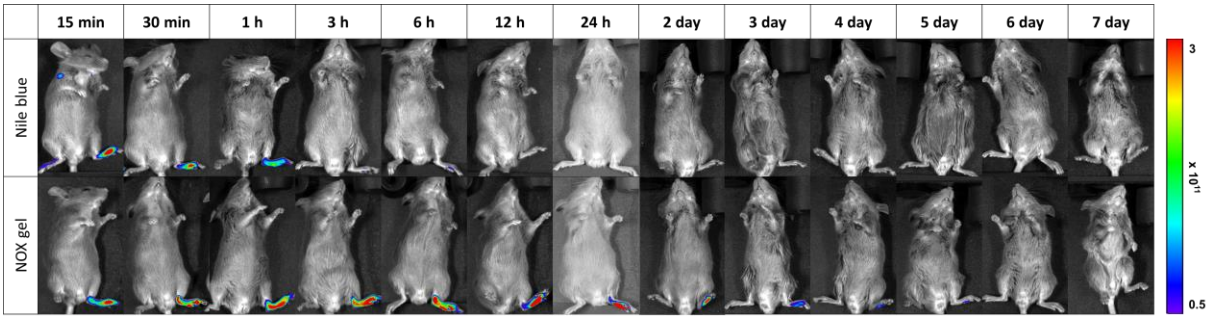

Figure S14a. Image of mice after injection of small molecule dye (Nile blue) or nanogels into paws.

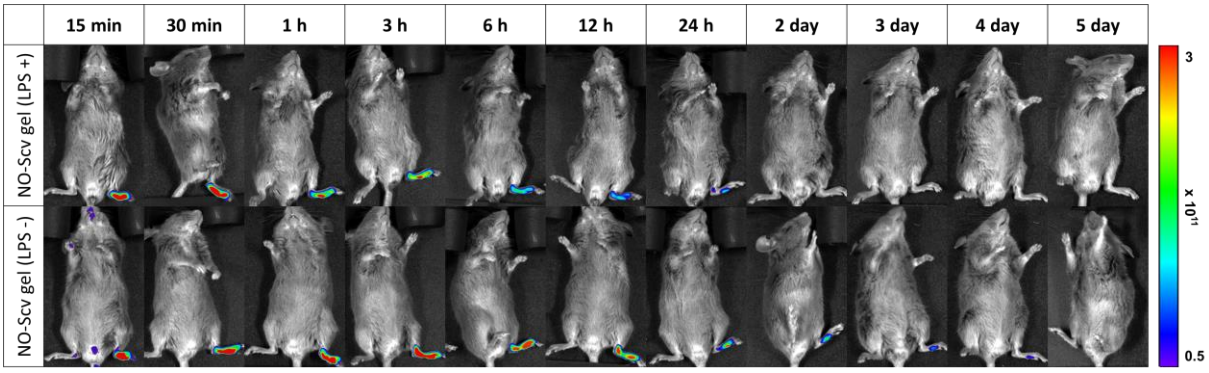

Figure S14b. Image of mice after injection of NO-Scv gel into paw of normal or LPS-treated mouse.

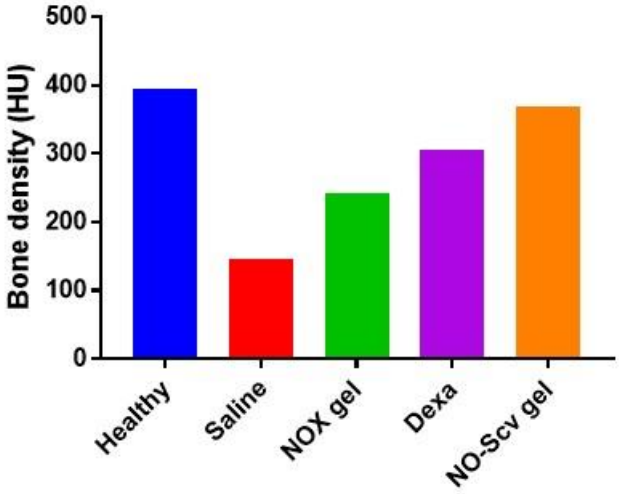

Figure S15. ROI quantification of CT data in RA mouse model.

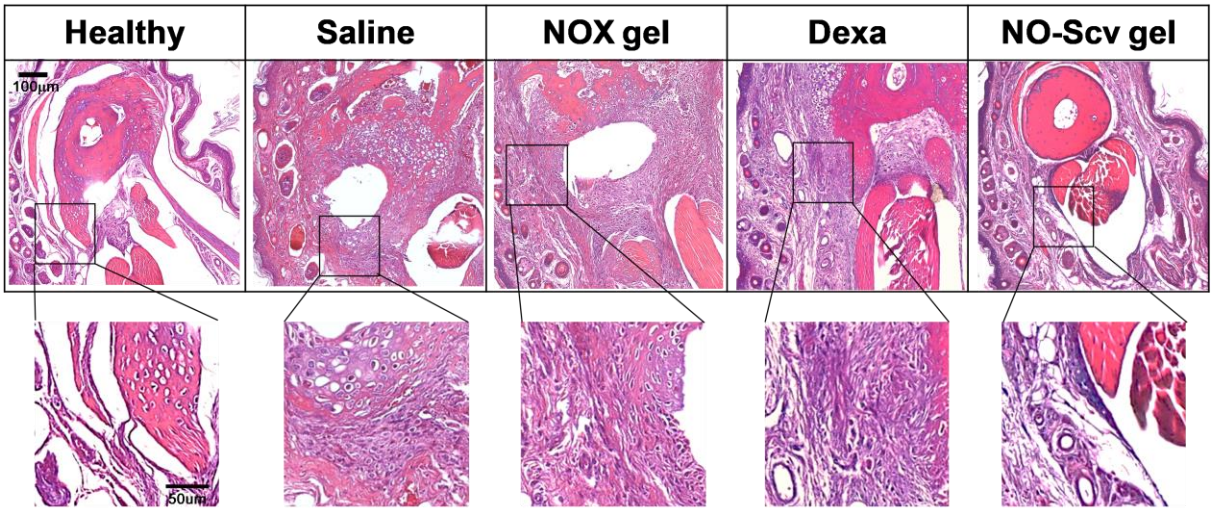

Figure S16. Images of H&E Staining assayed in RA mouse model for detection of synovial fibroblast activation in paw.

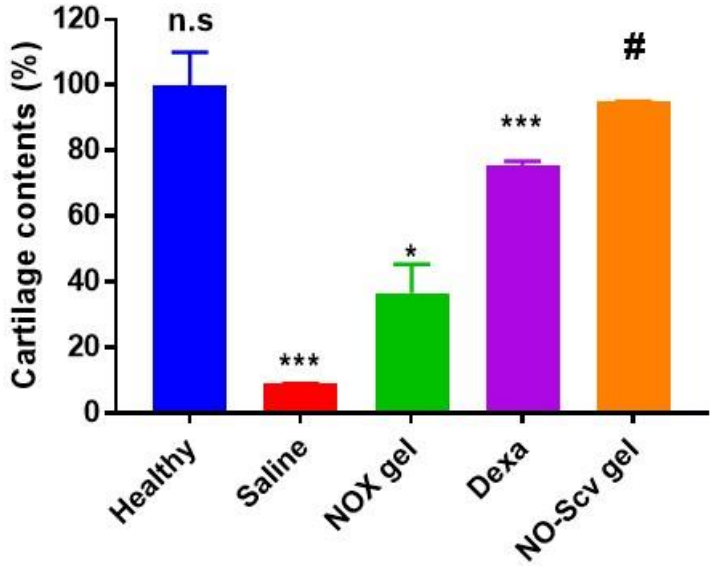

Figure S17. Quantification of safranin-O staining in cartilage (Mean ± SD, * $p < 0.05$, *** $p < 0.001$).

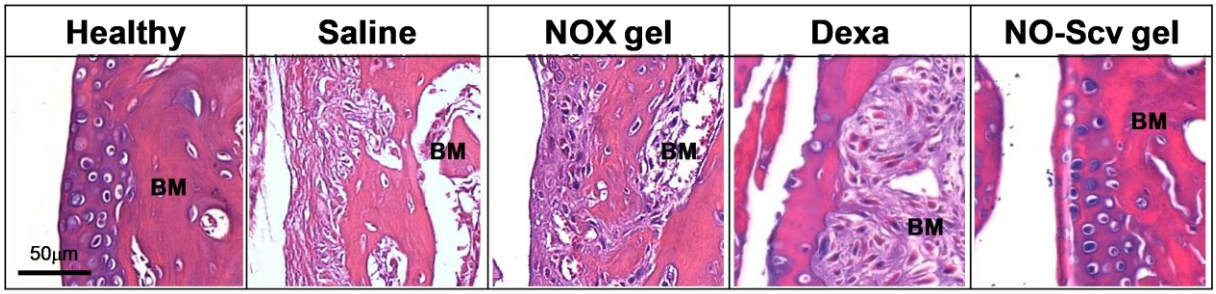

Figure S18. Image of H&E Staining assayed in RA mouse model for detection of chondrocyte in cartilage. BM = bone marrow. (Scale bar = 50 μm).