Electronic Supplementary Information

Superfast and controllable microfluidic inking of anti-inflammatory melanin-like nanoparticles inspired by Cephalopod

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Experimental Section

Materials

Dopamine hydrochloride, melanin from *Sepia officinalis*, Hank's Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), and Versene solution were purchased from Life Technologies Gibco®, USA. CellTiter-Glo® assay was purchased from Promega Corporation, USA. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), L-Glutathione reduced (GSH), 2', 7'-Dichlorofluorescin diacetate (DCFH-DA), peroxidase from horseradish (HRP) enzyme and hydrogen peroxide solution ($H_2O_2 \geq 35\%$ v/v) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). 10× Phosphate Buffered Saline (PBS), non-essential amino acids (NEAA), L-glutamine 200 mM, penicillin (100 IU mL^{-1}), streptomycin (100 mg mL^{-1}) and trypsin (2.5%) were purchased from HyClone™, GE Healthcare Lifesciences (Logan, UT, USA). Triton X-100 was purchased from Merck Millipore (Darmstadt, Germany). Fluorescein isothiocyanate (FITC) isomer I was purchased from TCI (Tokyo, Japan). CellMask™ DeepRed and trypsin-ethylenediamine tetraacetic acid (EDTA) were purchased from Invitrogen, USA. Recombinant murine interferon-gamma (IFN-γ), recombinant murine interleukin (IL)-4, and recombinant murine IL-13 were purchased from PeproTech® (Stockholm, Sweden). Lipopolysaccharide (LPS), APC anti-mouse CD206 (MMR) antibody and PE anti-mouse CD80 antibody were purchased from BioLegend® (San Diego, CA, USA). Human plasma were purchased from Finnish Red Cross (Veripalvelu) from anonymous donors. All materials were use as received.

Fabrication of co-flow microfluidic device

Device for MN production: The co-flow microfluidic device was assembled from the borosilicate glass cylindrical capillaries and the glass slides. The inner capillary was tapered to the diameter of 120 μm using a micropipette puller (P-97, Sutter Instrument, USA), and

inserted inside the 1st outer capillary with an inner diameter of 1.12 mm (Fig. 1a). The end of the 1st outer capillary was aligned to the 2nd outer capillary. Three blunt needles, in which represent the inner, 1st and 2nd outer flow, were connected to the microfluidic device. The polyethylene tubes were then linked to those needles to allow the independent injection of the inner and outer fluids controlled by pumps (PHD 2000, Harvard Apparatus, USA). A transparent epoxy resin (5 min® Epoxi, Devcon, USA) was used to seal the microfluidic device where required.

Synthesis and characterization of melanin nanoparticles (MNs) by microfluidics

MNs were synthesized using the co-flow microfluidic device described above. The inner fluid containing dopamine hydrochloride dissolved in Milli-Q water (200 mg mL^{-1}) was pumped into the inner tapered glass capillary at a flow rate of 1 mL h^{-1}, while the 1st outer fluid containing 0.1 M of NaOH solution was parallelly pumped simultaneously at a flow rate of 20 mL h^{-1}. MNs were immediately formed by self-polymerization reaction and the reaction was stopped by the 2nd outer fluid (2.5 mL h^{-1}), 1 M of HCl solution, at different distances (3, 4.5 and 12 cm; between the tip of the tapered capillary and the end of the 1st outer cylindrical capillary). The obtained particles were then collected, centrifuged at 16110g for 10 min, and washed twice with Milli-Q water before further use. For the synthesis of FITC labelled MNs, FITC was added into the inner fluid at a concentration of 0.2 mg mL^{-1}, and the rest of the procedure was as the same as the method mentioned above.

The particle size, polydispersity index (PDI) and zeta-potential were measured using dynamic light scattering (DLS) on a Malvern Zetasizer Nano-ZS (UK). The particle stability was studied by adding 100 μg MNs in 1 mL of 50% human plasma, and incubated at 37 °C for 2 h. The z-average size and PDI were measured every 30 min. The morphology of the different nanoparticles was evaluated by transmission electron microscope (TEM) (Tecnai F12, FEI Company, USA). The chemical composition was confirmed by FTIR on a Bruker Vertex 70

spectrometer. The samples were mixed with KBr (FTIR grade, Sigma-Aldrich, USA) and pressed into pellets. The pellets consisted of 1 mg of sample and 200 mg of KBr. The FTIR spectra were recorded in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} using OPUS 8.1 software. The UV-Vis absorbance spectra were acquired on a UV-1600PC spectrophotometer (VWR, USA).

Synthesis of MNs by bulk method and kinetics study

The MNs (low dopamine concentration) were synthesized by a bulk method, as reported elsewhere.[2] Dopamine hydrochloride (1.5 mg mL^{-1}) was introduced into the basic NaOH solution (0.01 M) with vigorous stirring at room temperature, to allow the self-polymerization of the MNs. After each time point, 100 µL of the reaction solution was sampled and diluted with 900 µL of MilliQ-water for DLS measurement. The DLS measurement setting was manually optimized, according to previously published literature.[3] Specifically, the laser position was fixed at 4.65 mm to avoid static scattering from the cell wall. The sub-measurement duration was set to 3 s with 50 repetitions for each measurement cycle, and the measurement was repeated 3 times for each sample. Only the data which passed the DLS internal quality test were plotted. After 24 h, the reaction was quenched by the addition of 1 M HCl, and MNs were collected by centrifugation at 16110g for 10 min. The particles were washed again with Milli-Q water twice and characterized by TEM.

The MNs (high dopamine concentration) were synthesized by mixing the inner fluid (dopamine hydrochloride solution at 200 mg mL^{-1}) and the 1st outer fluid (0.1 M of NaOH solution) in a beaker with vigorous stirring. After a few seconds, 1 M of HCl solution was added to quench the reaction and the solution was centrifuged at 16110g for 10 min. The resulted particles were further washed twice by Milli-Q water characterized by DLS and TEM.

H_2O_2 scavenging

The decomposition of H_2O_2 (10 μM) was determined using Amplex® UltraRed reagent. Firstly, The MN solutions at different concentrations (10, 20 and 50 μg mL^{-1}) were individually mixed with 10 μM of the H_2O_2 solution for 1 h. The HBSS buffer (pH 7.4) containing either H_2O_2 solution or working solution (0.1 mmol Amplex® UltraRed and 1 U mL^{-1} HRP enzyme in HBSS buffer) were used as positive and negative controls, respectively. The samples were centrifuged at 16110g for 5 min, and the supernatant (50 μL) was taken and incubated with a working solution (50 μL) for 30 min. The fluorescence intensity of the mixture after the reaction was measured using a microplate reader at the excitation/emission wavelength of 565/590 nm. The samples were tested in triplicates.

Cell culture

RAW 264.7 murine macrophage cells (passage numbers 4-10) were cultured in DMEM with 4.5 g L^{-1} glucose, supplemented with 10% FBS, 1% L-glutamine, 1% NEAA, penicillin (100 IU mL^{-1}) and streptomycin (100 mg mL^{-1}). The cells were cultured in the 5% CO_2-incubator at 37 ℃, and 95% relative humidity. The culture media were changed every other day. Prior to each test, the cells were passaged and incubated for 5 min using 0.25% (v/v) trypsin EDTA/PBS.

THP-1 human monocytic leukemia cells (passage numbers 14-16) were cultured in RPMI 1640, supplemented with 10% FBS, 1% L-glutamine, 1% NEAA, penicillin (100 IU mL^{-1}) and streptomycin (100 mg mL^{-1}). The cell suspension was maintained in the 5% CO_2-incubator at 37 ℃, and 95% relative humidity. The culture media were changed every 2-3 days.

Cytotoxicity studies

The in vitro cytotoxicity was performed using a CellTiter-Glo® luminescent cell viability assay (Promega Corp., WI, USA). The cells were seeded overnight on a 96-well plate at density of 1×10^4 cells per well in cell culture medium. MN from 50 to 500 μg mL^{-1} were tested. Triton X-100 solution (1% v/v in cell culture medium), and cell culture medium without any tested

materials were used as negative and positive controls, respectively. All samples were tested in four replicates.

Nanoparticle cellular uptake study

For confocal imaging of nanoparticle cellular uptake, RAW 264.7 cells were seeded in Lab-Tek® chambered borosilicate coverglass (8-chamber, ThermoFisher Scientific, USA) at a density of 1.5×10^4 cells per well in cell culture medium. Then FITC labelled MN (50 μg mL^{-1}) added to the cells, and incubated for 1 h. The cells without any particle incubation were used as control. After incubation, the particles were gently removed and the cells were washed with PBS for 3 times. The cells were then incubated with CellMask™ DeepRed for 5 min at room temperature, followed by washing with PBS for 3 times. Finally, the cells were imaged by a Leica TCS SP5 II HCS-A confocal microscope (Leica Microsystems, Germany). The images acquired were processed by Fiji 1.51 software.

For flow cytometry analysis of cellular uptake, RAW 264.7 cells were seeded at the density of 75,000 cells per well in a 24 well plate in cell culture medium. Then, cells were incubated with FITC labelled MN (50 μg mL^{-1}). After incubation, the particles were removed, and the cells were washed twice with PBS before detachment by trypsin. The detached cells were centrifuged at 317g, washed again with PBS, and incubated with trypan blue for 15 min to quench the fluorescence on the cell surface. After trypan blue incubation, the cells were washed by PBS, and analyzed on a BD LSR-II Cell Analyzer flow cytometer (USA).

Imaging of intracellular ROS

For confocal imaging of the intracellular ROS, RAW 264.7 cells were seeded in Lab-Tek® chambered borosilicate coverglass at a cell density of 1.5×10^4 cells per well in cell culture medium. After attachment, the cells were stimulated using LPS (100 ng mL^{-1} in PBS) and IFN-γ (2 ng mL^{-1} in 0.1% BSA) prepared in cell culture medium. After 24-h stimulation, the cells were treated with the new fresh stimulants at the same concentrations containing samples

MNs at 20 μg mL^{-1} for 5 h, followed by DCFH-DA staining (40 μM in dissolved in PBS containing 0.1% DMSO). Finally, the cells were washed and stained by CellMask™ DeepRed, before imaging by a Leica TCS SP5 II HCS-A confocal microscope. The images acquired were processed by Fiji 1.51 software.

Quantification of intracellular ROS scavenging capability of MNs

For a quantitative analysis of ROS scavenging capability of MNs, RAW 264.7 cells were seeded in a 96-well plate at a density of 1.5×10^4 cells per well in cell culture medium. After overnight attachment, the cells were incubated with MNs or L-Glutathione reduced (GSH) at 5, 20, 100 μg mL^{-1} for 1 h. Then the particles were removed, and the cells were washed by PBS once, followed by the addition of DCFH-DA (10 μM in dissolved in PBS containing 0.025% of DMSO). After 45 min incubation, the DCFH-DA was removed and cells were challenged by 100 μM of H_2O_2. After 45 min or 90 min, the fluorescence of the samples were measured by Varioskan™ LUX multimode microplate reader (λ_{ex} 485 nm, λ_{em} 535 nm). The cells without H_2O_2 challenge were used as negative controls. All samples were tested in three replicates.

***In vitro* anti-inflammatory therapeutic efficacy evaluation**

RAW 264.7 cells were cultured in 24-well plate in cell culture medium at the density of 50,000 cells per well. The cells were incubated overnight and stimulated by 100 ng mL^{-1} of LPS and 2 ng mL^{-1} of IFN-γ prepared in cell culture medium for 1 day. MNs (20 μg mL^{-1}) were added into the cells in the present of 20 ng mL^{-1} LPS and 0.4 ng mL^{-1} IFN-γ during the treatment for 2 days. The cells, in which stimulated by LPS and IFN-γ at the same concentrations according to the treated groups, were used as the M1 phenotype control, and the cells, in which stimulated by anti-inflammatory cytokine IL-4 (20 ng mL^{-1}) and IL-13 (20 ng mL^{-1}), was used as M2 phenotype control. After 3 days, the culture supernatants were collected and frozen at −20 ℃ for enzyme linked immunosorbent assay (ELISA) analysis, and

the expression of CD80 and CD206 on the cell surfaces was detected by immunostaining with the CD80 and CD206 antibodies. The cells were washed with PBS twice and detached by Versene (200 μL per well) for 15 min at 37 ℃.Then, 200 μL of PBS-EDTA was added into each well, and the cells were transferred to each glass tube. The cells were centrifuged at 317g for 5 min and washed with PBS twice, followed by immunostaining the cell pellets with APC anti-CD80 and PE anti-CD206 at the concentration of 1 μg mL^{-1} in PBS at 4 ^{o}C for 30 min. After that, the cells were washed again with PBS twice, and subsequently analyzed by flow cytometry. In each group, cells without antibody staining were used as the negative control. The fold of change of MFI in each sample (in triplicates) was calculated as [MFI of antibody-stained sample/MFI of non-stained negative control]. All flow cytometry data were processed by FlowJo software.

The culture supernatants were frozen at -20 ℃ until analysis with pre-coated ELISA kits (PeproTech®, Stockholm, Sweden) according to the manufacturer's protocol. Secreted tumor necrosis factor-alpha (TNF-α) and IL-10 were used as M1 and M2 markers, respectively.

Statistical analysis

The experiments were performed in at least triplicates and the values are represented as mean ± standard deviation. The data were analyzed by one-way ANOVA, followed by Tukey's post-test on OriginPro 2018. The levels of significant differences were set at probabilities of *P < 0.05, **P < 0.01, and ***P < 0.001.

Supporting Data

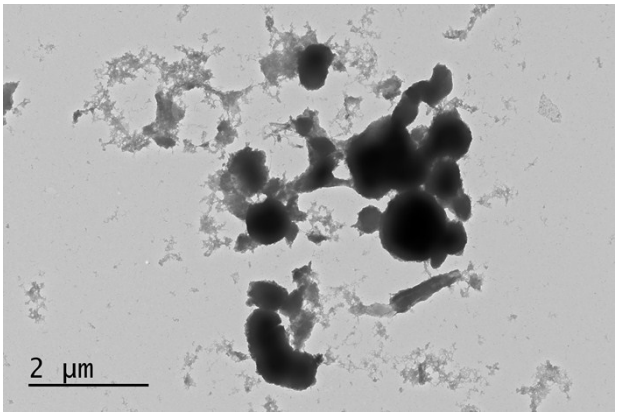

Fig. S1. The TEM image of MNs prepared by bulk method at 200 mg mL^{-1} dopamine after 20 h reaction.

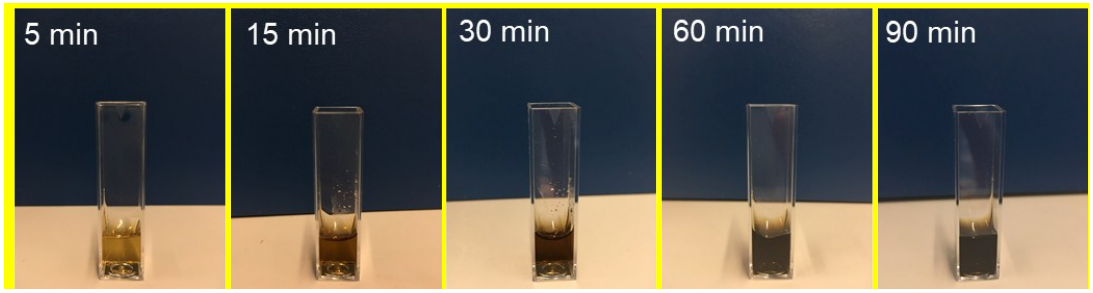

Fig. S2. The representative images of reaction solutions during MN bulk synthesis at 1.5 mg mL^{-1} dopamine from 5 min to 90 min.

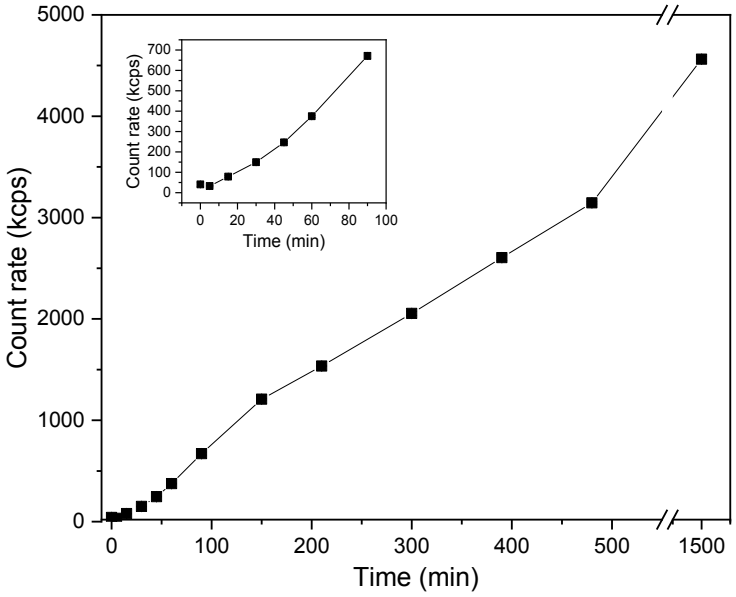

Fig. S3. The DLS derived count rate variations during MN bulk synthesis at 1.5 mg mL^{-1} dopamine. The inset figure showed the enlarged curve from 0 min to 90 min.

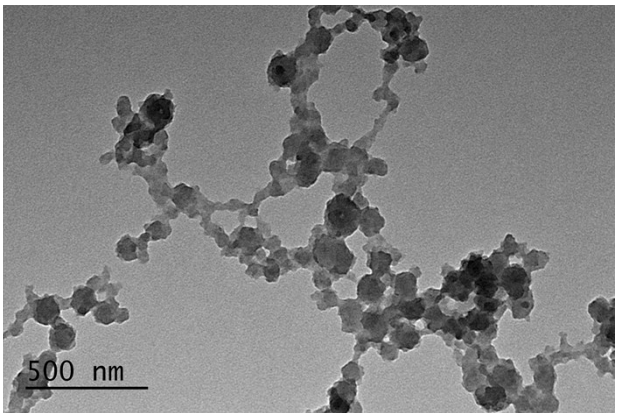

Fig. S4. The TEM image of MNs prepared by bulk method at 1.5 mg mL^{-1} dopamine after 20 h reaction.

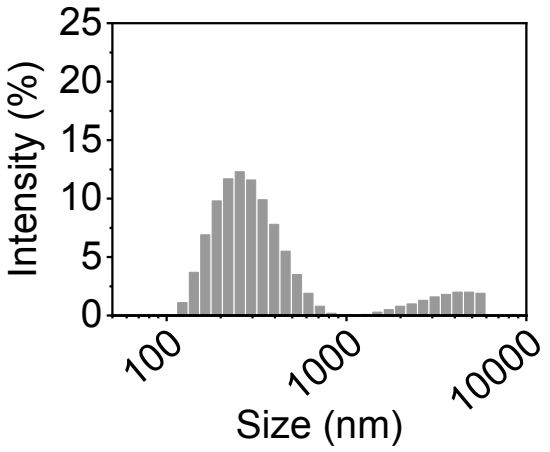

Fig. S5. The DLS distribution of MNs from *Sepia* reconstituted in water.

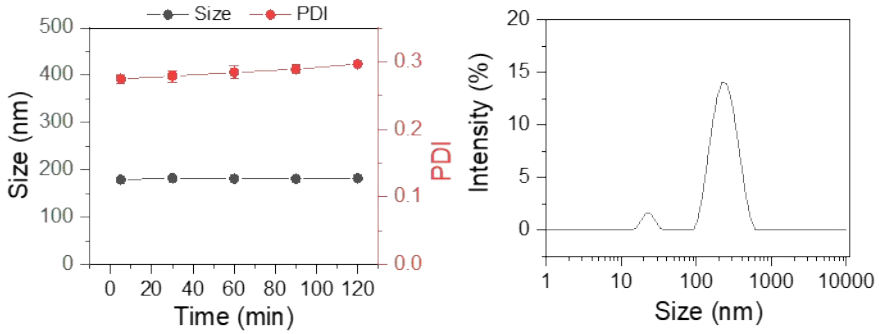

Fig. S6. Left: Z-average size and PDI of MNs prepared by microfluidic method 50% of plasma (middle). Right: Size distribution curve based on intensity after 2 h stability study.

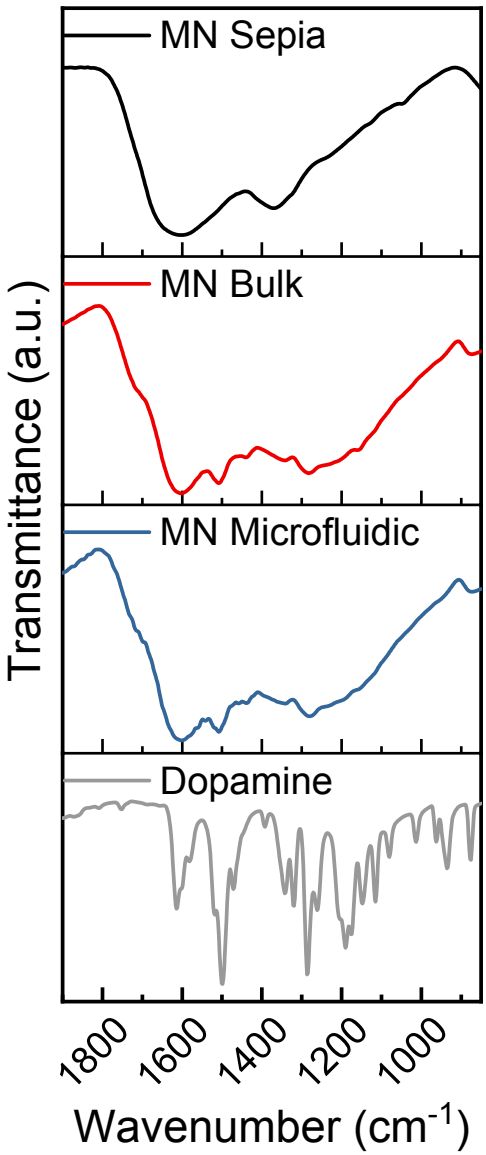

Fig. S7. The zoomed-in FTIR spectra of MNs from *Sepia* (black), MNs synthesized by bulk method (red), MNs synthesized by microfluidic method (blue) and dopamine (grey).

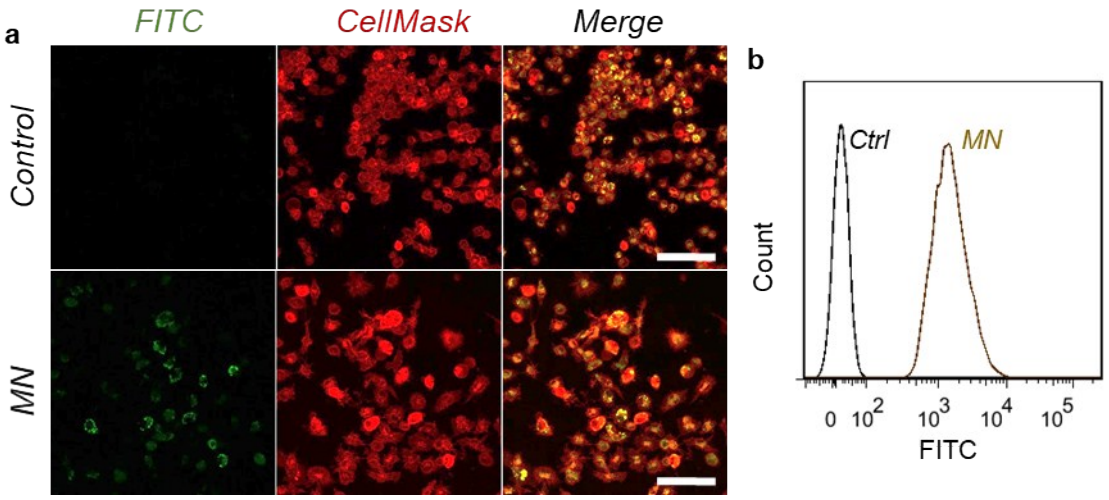

Fig. S8. The uptake of MNs synthesized by microfluidic method on RAW 264.7 cells. (a) The confocal images of RAW 264.7 cells treated without particles (control), and with FITC-labelled MNs. The cell membranes were stained by CellMask™ Deep Red. Scale bar = 100 μm. (b) The flow cytometry results of RAW 264.7 cells treated with FITC-labelled MNs.

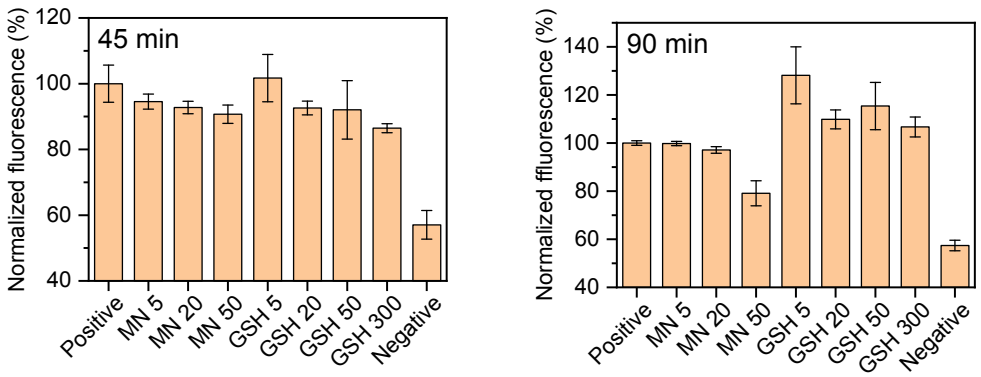

Fig. S9. The ROS level of RAW 264.7 cells after 100 μM H_2O_2 challenge at, with or without the preloading of GSH and MNs at different concentrations (5, 20, 50 and 300 μg mL^{-1}). The results were normalized to the positive control group, which was challenged by H_2O_2 without any scavenger. The negative control group represents cells cultured at normal condition without H_2O_2 challenge. Data are presented as the mean ± s.d. (n = 3).

Table S1. Zeta-potential of all MNs in Milli-Q water.

Sample	Zeta-potential (mV)
Natural MN from *Sepia*	–29.3 ± 2.3
Synthetic MN by microfluidic method	–29.4 ± 0.5
Synthetic MN by bulk method	–28.5 ± 2.0

References

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