Supplementary Materials

1. Methods

1.1 *In vitro* inhibition effect of micelles against the inflammatory cells of Raw264.7

To investigate the inhibition effect of micelles on cell growth we stimulated Raw264.7 macrophages with lipopolysaccharide (LPS, 1 μg/mL) using the HUVEC cell line as control. After incubation overnight, both Raw264.7 and HUVEC cells were treated with different concentration of PMs, MTX-PMs, R-MTX-PMs, NIM-PMs, R-NIM-PMs, M/N-PMs and R-M/N-PMs in which the amount of NIM in the NIM formulations was 5-times higher than that of MTX in the MTX formulations. This was consistent with the amount used *in vivo* treatments. After 24 h incubation, cell viability was measured by the MTT assay. Briefly, the culture media in each well were removed and the cells were washed with PBS. 20 μL of MTT solution (5 mg/mL) was added and incubated for another 4 h. The medium was removed, and the formazan crystals were dissolved by adding 100 μL of DMSO. The cells were incubated for additional 15 min at 37 °C with gentle shaking. Absorbance at 490 nm was measured using a Varioskan Flash Microplate Reader (Thermo, USA). The cell viability (%) was calculated using the following formula: Cell viability (%) = (A_{sample} - A_{blank}) / ($A_{negative}$ - A_{blank}) × 100%. Empty wells were used as a negative control and wells containing cell culture medium alone as a blank control.

1.2 Stability study of micelles

We used PBS containing 20% fetal bovine serum to estimate the stability of micelles in blood-mimicking conditions. This experiment was similar to the *in vitro* release study. In brief, 2 mL of the freshly prepared MTX-PMs, NIM-PMs, R-MTX-PMs, R-NIM-PMs were mixed with the same volume of the special medium and then placed in dialysis bags (n=3) with a molecular weight cut-off of 3.5 kDa (Millipore).

The sealed dialysis bags were suspended in 100 mL PBS (pH = 7.4) and the whole device was placed in a water bath at 37 °C with a stirring speed of 50 rpm for 24 h. At the pre-designed time points of 0.5, 1, 2, 4, 6, 8, 12, 24, 2.0 mL of the solution from the PBS medium was taken out and replaced with fresh PBS. The sample was placed under optical microscope to observe the morphological changes. Every sample was brought up to 5 mL with methanol and filtered through a 0.22-μm membrane (Millipore). The concentration of MTX or NIM in various micelles was determined by the HPLC method as described in the Methods section to calculate the release percentage.

1.3 Tissue distribution of MTX-PMs, R-M/N-PMs in a rat model of collagen-induced arthritis

Both the arthritic rats and the healthy rats were used to analyze the *in vivo* distribution of micelles with MTX as the model drug. A single intravenous injection of MTX-PMs or R-MTX-PMs was administered to rats at a dose of 12 mg MTX/kg body weight. At 0.25, 1, 4 and 6 h after injection, rats were sacrificed, blood was collected *via* the eye sockets, and plasma, various organs of heart, liver, spleen, lung, kidney as well as entire hind limbs were quickly harvested (n=5). The samples were prepared as described [1] for the detection of MTX by HPLC as mentioned in section of Characterization of Micelles.

2. Results

2.1 Viability of inflammatory cells exposed to micelles

To evaluate the *in vitro* inhibition effect of micelles on the inflammatory cells, we took the lipopolysaccharide-stimulated Raw264.7 and HUVEC as the model cells. MTT assay was used to determine the cell viability, in which MTX-PMs, R-MTX-PMs, NIM-PMs, and R-NIM-PMs were loaded with a single drug, whereas the M/N-PMs and R-M/N-PMs carried the combination of both drugs.

According to the literature, the best ratio of NIM and MTX is 5:1 in the *in vivo*

experiment [2]. Therefore, in the MTT assay, we used the MTX concentrations of 0.05, 0.5, 5, 10, 20 μg/mL and the corresponding NIM concentrations of 0.25, 2.5, 25, 50, 100 μg/mL. In the combined group the total concentration was 0.3, 3, 30, 60, 120 μg/mL, respectively. As shown in **Figure S1**, among all the formulations, PMs showed no inhibitory effect on both Raw264.7 and HUVEC cells while treatment with others resulted in cell-growth inhibition in a concentration-dependent manner.

For Raw264.7 cells, after separate treatments with 20 μg/mL of MTX-PMs and R-MTX-PMs, the cell viability was 35.76 ± 5.11% and 27.36 ± 4.12%, respectively; and after treatments with 100 μg/mL NIM-PMs and R-NIM-PMs, the cell viability was 30.25 ± 4.27% and 18.21 ± 0.85%, respectively. The treatments of M/N-PMs and R-M/N-PMs led to a cell viability of 32.26 ± 2.80% and 17.13 ± 0.74%, respectively. These results indicated that RGD significantly enhanced growth-inhibition effect of micelle formulation on the inflammatory cells.

The viability of HUVEC cells showed a similar changing trend as observed in the Raw264.7 cells. After treatments with MTX-PMs and R-MTX-PMs (20 μg/mL), the cell viability was 58.49 ± 2.96% and 50.38 ± 3.47%, respectively and the cell viability after treatment with NIM-PMs and R-NIM-PMs (100 μg/mL) was 46.25 ± 5.54% and 27.62 ± 3.25%, respectively. Finally, the cell viabilities following treatment with M/N-PMs and R-M/N-PMs (120 μg/mL) were 43.76 ± 3.50% and 32.70 ± 2.59%, respectively.

These results indicated that the combined treatment showed a better inhibitory effect on cell proliferation than the single formulations.

2.2 Stability analysis

During the 24-h incubation of micelles with the blood-mimicking medium no aggregation or precipitation was observed. As shown in **Figure S2,** the accumulated release profile was similar to the result from the *in vitro* release profile indicating that the serum had no effect on the release of MTX or NIM from micelle formulations.

Thus, the micelles prepared in this study were stable in the blood-mimicking conditions.

2.3 Selective biodistribution *in vivo*

As shown in **Figure S4 (A-H)**, the concentration of MTX in blood showed no difference between MTX-PMs and R-MTX-PMs; both in healthy and arthritic rats, the major organs for micelles of MTX-PMs and R-MTX-PMs to reach were liver and spleen at all time points. However, in arthritic rats, both MTX-PMs and R-MTX-PMs accumulated selectively in joints, in which R-MTX-PMs showed greater targeting ability to joints that MTX-PMs did. Over time, the MTX in blood decreased, while that in arthritic joints increased gradually, which was consistent with the results from the real-time fluorescence imaging analysis.

References

[1] Lin Y, Li Y, Wang X, Gong T, Zhang L, Sun X. Targeted drug delivery to renal proximal tubule epithelial cells mediated by 2-glucosamine. J Control Release. 2013, 167 (2013): 148-156.

[2] Al-Abd AM, Inglis JJ, Nofal SM, Khalifa AE, Williams RO, El-Eraky WI, et al. Nimesulide improves the disease modifying anti-rheumatic profile of methotrexate in mice with collagen-induced arthritis. Eur J pharmacol. 2010, 644(1-3): 245-50.

Figures

Figure S1

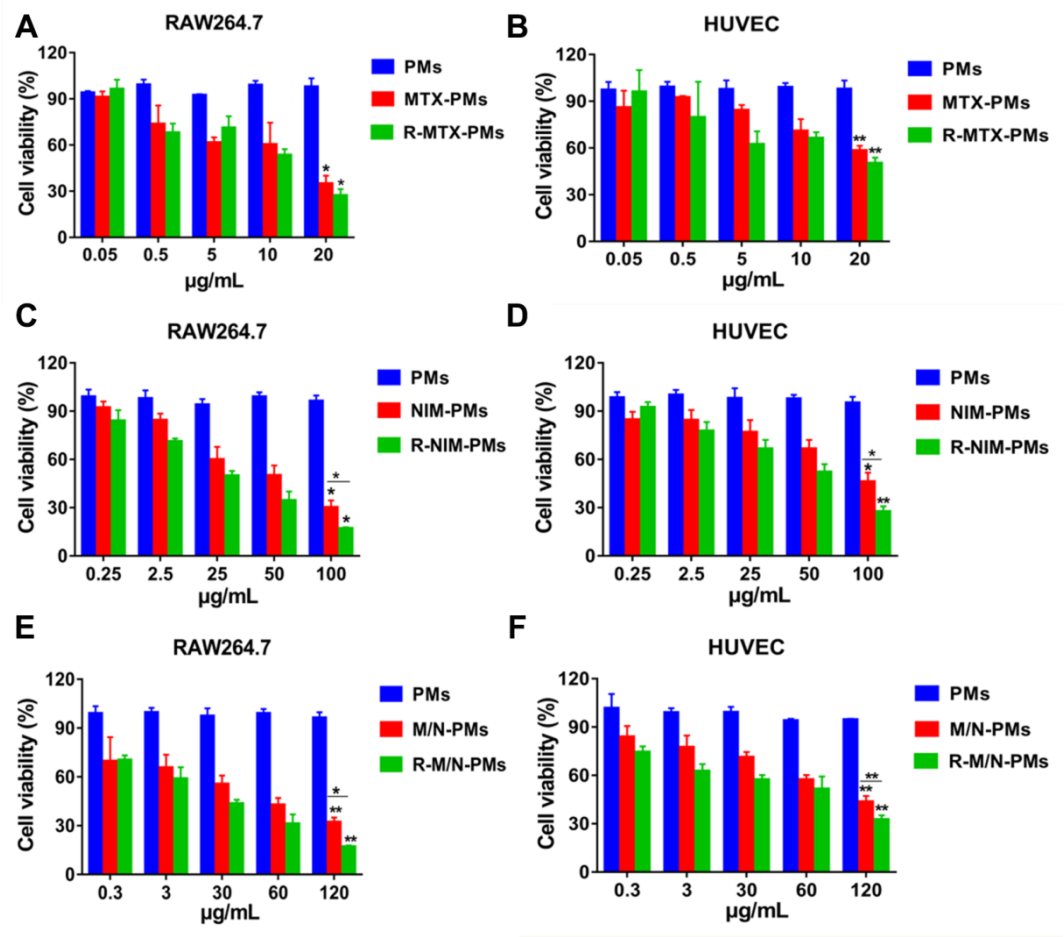

Figure S1. Cell viability of LPS-stimulated RAW264.7 (A, C, E) and HUVEC cells (B, D, F) incubated with various micelle formulations. Both Raw264.7 and HUVEC cells were cultured overnight with complete media containing 1 μg/mL LPS and incubated with various formulations at different concentration for 24 h. MTT assay was conducted to determine the cell viability. Results are presented as means ± SD (n=3). Symbols *P* represent statistical significance with $^{*}P < 0.05$ and $^{**}P < 0.01$.

Figure S2

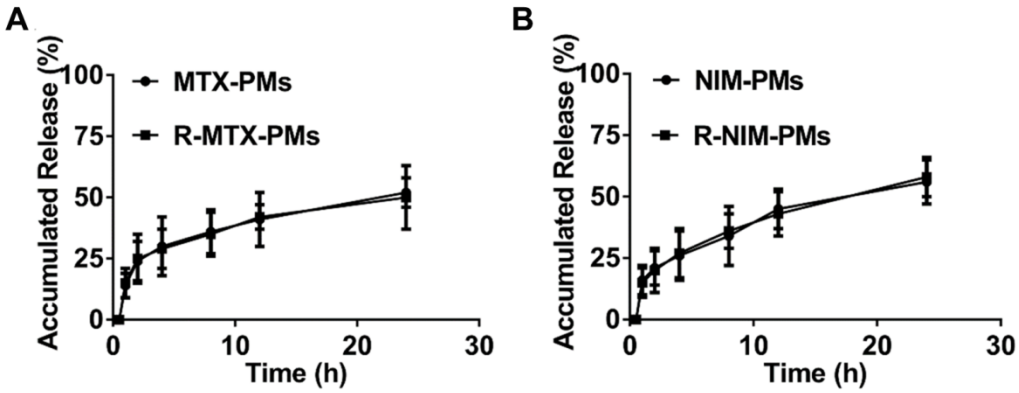

Figure S2. Stability analysis of micelles in the blood-mimicking conditions. Freshly prepared MTX-PMs, R-MTX-PMs, NIM-PMs, R-NIM-PMs were mixed with the same volume of the PBS medium containing 20% fetal bovine serum. Samples was collected at different time points to detect MTX or NIM by HPLC for determining the accumulated release. Results are presented as mean ± SD (n = 3).

Figure S3

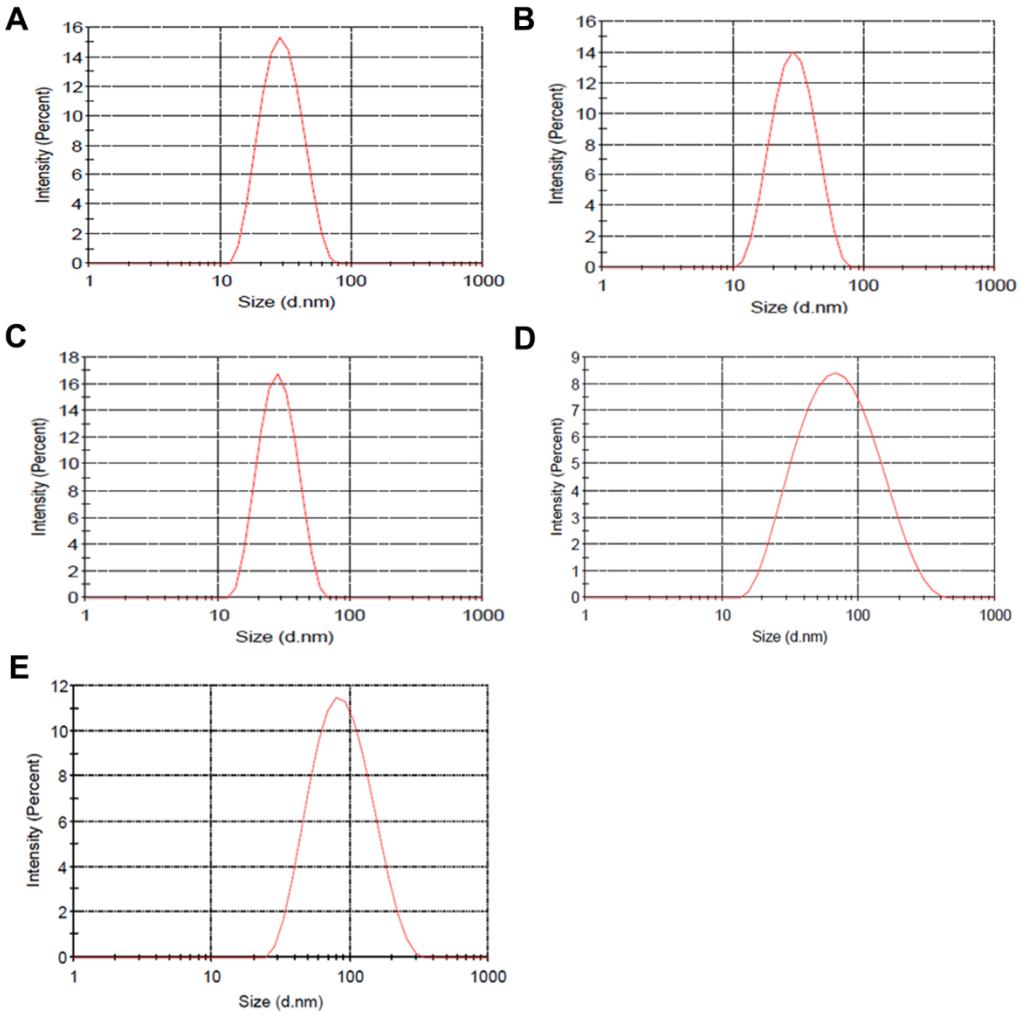

Figure S3. Particle size distribution of PMs (A), MTX-PMs (B), NIM-PMs (C), R-MTX-PMs (D) and R-NIM-PMs (E) measured by the dynamic light scattering method using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, U.K.).

Figure S4

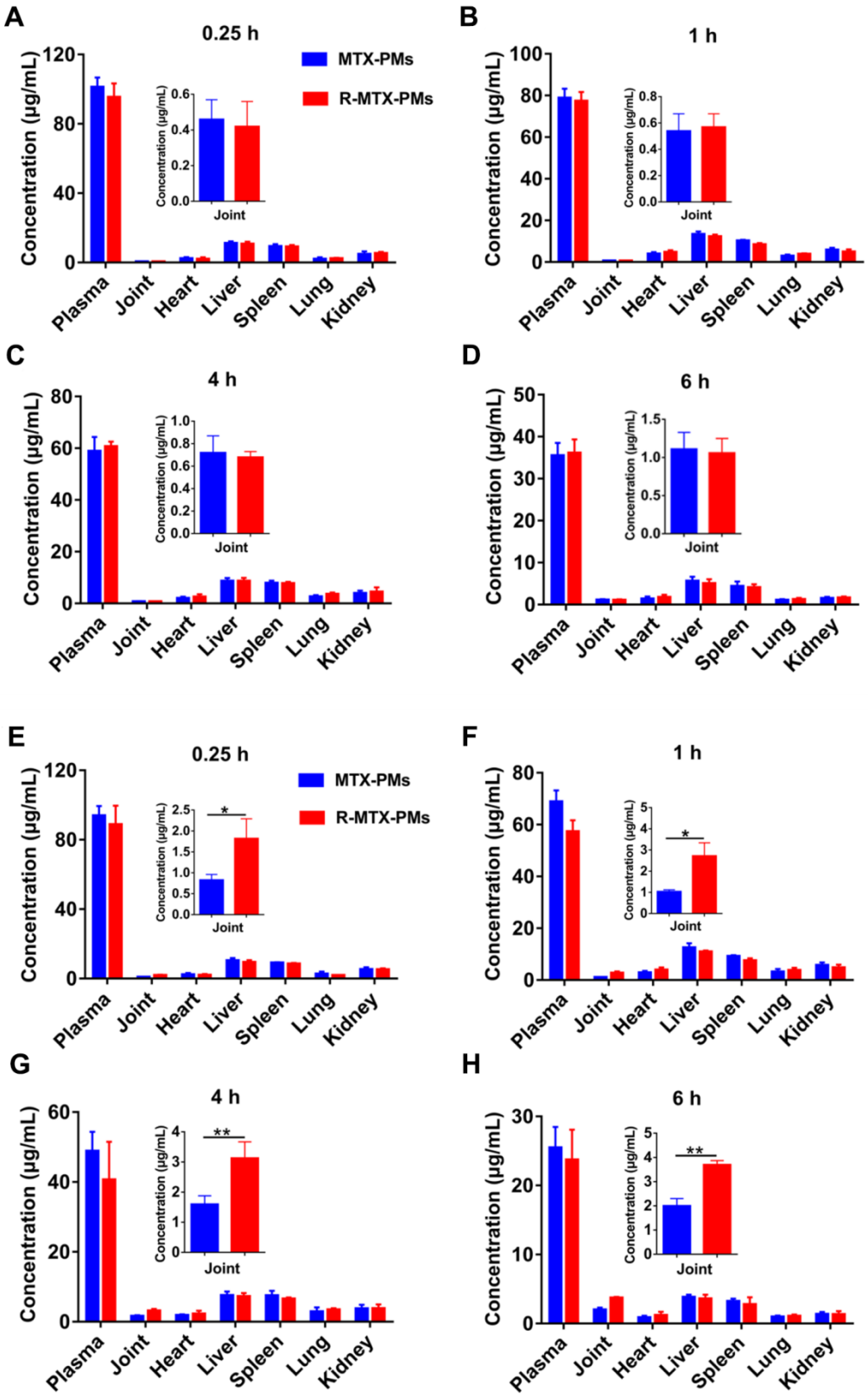
A
0.25 h
MTX-PMs
R-MTX-PMs
Concentration (μg/mL)
Joint
Plasma
Joint
Heart
Liver
Spleen
Lung
Kidney
B
1 h
C
4 h
D
6 h
E
0.25 h
F
1 h
G
4 h
H
6 h
*
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Figure S4. *In vivo* biodistribution of MTX-PMs and R-MTX-PMs in healthy rats (A-D) and arthritic rats (E-H). Rats were injected with either preparation *via* the tail vein and sacrificed at various times points of 0.25 h, 1 h, 4 h and 6 h. Samples of plasma, heart, liver, spleen, lung, kidney, and joints were immediately collected and analyzed by HPLC. Data were presented as mean ± SD (n = 5, *P < 0.05)