# A novel indomethacin / methotrexate / MMP-9 siRNA *in situ* hydrogel with dual effects of anti-inflammatory and reversing cartilage disruption for the synergistical treatment of rheumatoid arthritis

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## 1. Synthesis and characterization of disulfide-crosslinked polyethyleneimine (PEI-SS)

The thiolated PEI was synthesized in two steps (Fig. S1) following a modified method as described in the literature[1]. The pH of the 1.2k PEI solution (2 g) was adjusted to 7.2 with 0.5 N hydrochloric acid, and the resultant solution was lyophilized. The lyophilized product was dissolved in methanol (30 ml) and purged with nitrogen for 20 minutes. A 5-fold molar excess or a 10-fold molar excess of propylene sulfide compared to PEI was slowly added dropwise, and stirred at 60°C for 24h under a nitrogen atmosphere. The reaction solution was evaporated to dryness under reduced pressure to yield a yellow viscous solid. The product was dissolved in a small amount of methanol, followed by precipitation twice in cold diethyl ether. To synthesize PEI-SS, the thiolated PEI (0.5 g) was dissolved in DMSO (50 mL) and stirred at room temperature for 48h. The reaction solution was purified by dialysis (MWCO 3,500) and then lyophilized to obtain PEI-SS. The $^{1}$H NMR spectra of the PEI-SS was obtained using a Bruker Avance III HD NMR spectrometer at 400MHz. The molecular weight and polydispersity index of the PEI-SS was confirmed using gel permeation chromatography (GPC) (solvent: acetic acid and sodium acetate buffer; concentration: 0.5 mol/L; instruments: Waters 1515 GPC).

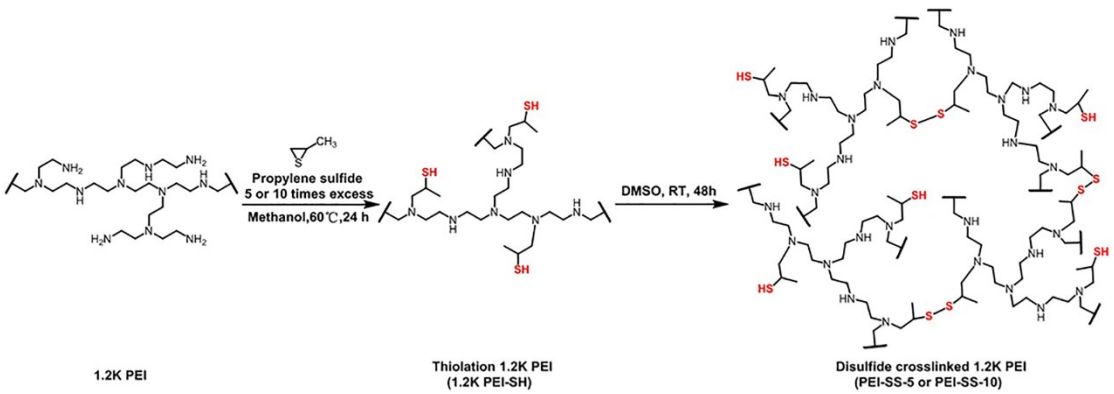


**Figure S1.** Synthesis procedure of PEI-SS

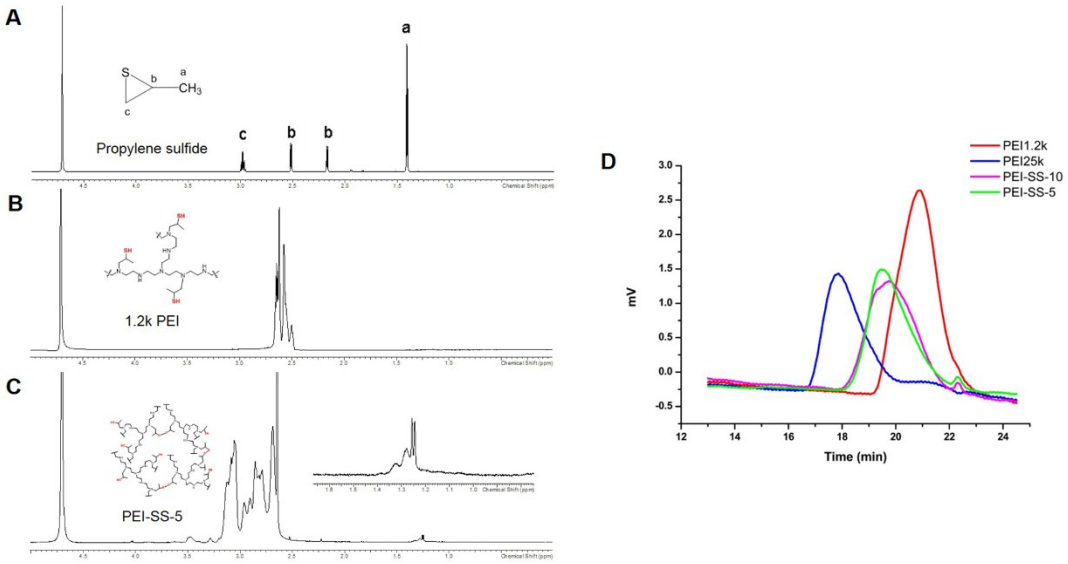


**Figure S2.** Characterizations of PEI-SS

**Table S1.** Characterizations of PEI-SS

| | Mw (Da) | Polydispersity |
|---|---|---|
| PEI-SS-5 | 10,524 | 1.0971 |
| PEI-SS-10 | 11,545 | 1.0967 |

## 2. Characterization of D/siRNA-NGel

### 2.1 Determination of gelation temperature and time

The gelation temperature of the drug-loaded gel was determined by the tube inversion method[2]. Briefly, 1 mL of the test solution was placed in a 10 mL test tube which was placed in a constant temperature water bath and gradually heated at 1°C intervals. The temperature was held for 5 minutes after reaching the specified temperature. The test tube was tilted at regular time intervals, and the temperature at which the test solution was converted to a gel and ceased to flow with no change in meniscus upon tilting up to 90° was recorded. The gelation time of the drug-loaded hydrogel was determined by the same method. The test tube containing the test solution was placed in a constant temperature water bath at 37°C, and the final gelation time was recorded when the test solution ceased to flow when tilting to 90°. The measurement was performed 3 times in parallel, and the mean value ± SD was calculated.

2.2 Determination of viscosity of *in situ* gel

The viscosity of the different F127 and F68 based formulations was determined using a rotary rheometer. Briefly, an appropriate amount of sample was dropped in the middle of the Pel plate and scanned for viscosity, using a 2° cone angle measuring head with a diameter of 40 mm, a test temperature of 25°C, and a shear rate of 0-120 $s^{-1}$.

$$\text{Viscosity (Pa.s)} = \frac{\text{Shear stress(Pa)}}{\text{Shear rate(1/s)}}$$

**Table S2.** Properties of different combination of pluronic 127 (F127) and pluronic 68 (F68) *in situ* hydrogel systems and the effect of shear force on viscosity of F127/F68 based *in situ* hydrogel formulations. Results are expressed as mean values ± SD, (n = 3).

| Formulation code | F127 (%W/V) | F68 (%W/V) | Gelation temperature(°C) | Gelation time (s) | Viscosity (Pa.s) |
|---|---|---|---|---|---|
| Gel 1 | 28 | 12.5 | 35.5 ± 1.0 | 21.31 ± 4.48 | 0.5753 |
| Gel 2 | 28 | 15 | 34.5 ± 0.5 | 20.43 ± 1.25 | 0.5401 |
| Gel 3 | 27 | 10 | 33.0 ± 0.5 | 12.31 ± 2.50 | 0.3964 |
| Gel 4 | 26 | 10 | 36.0 ± 1.0 | 37.56 ± 5.31 | 0.3536 |

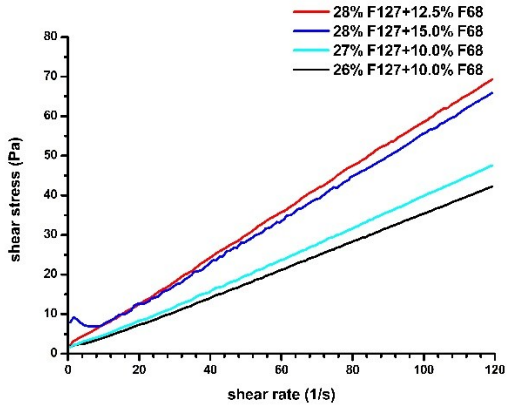


**Figure S3.** Shear stress-shear rate curve for different hydrogel formulations.

## 3. TEM scan of release medium

The drug-loaded temperature-sensitive gel mixture solution was directly added to the release vial without a dialysis bag, and then the release vial was placed in a 37°C constant temperature water bath to cause gelation. The pH 7.4 PBS was then added to the release vial containing the temperature-sensitive gel. The vials were carefully placed in a shaking bath with constant temperature (37°C) and shaking (100 rpm). The medium was completely removed at predetermined time points and

replenished with fresh medium. The release medium removed at each time was examined by TEM to observe whether the nanoparticles was present in the release medium.

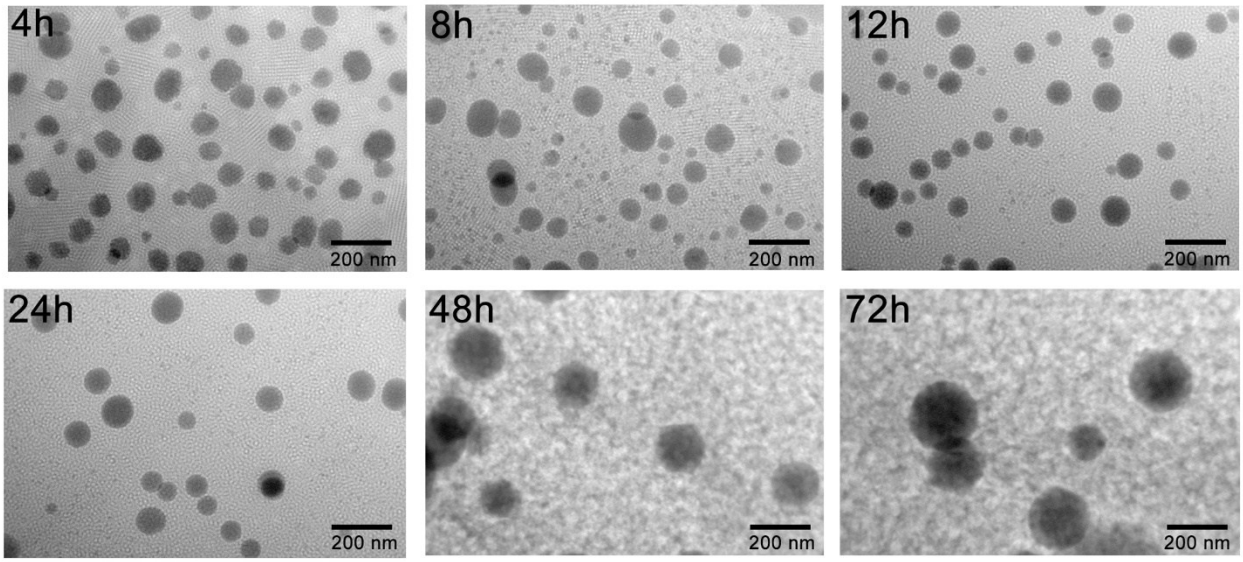


**Figure S4.** Transmission electron microscope (TEM) scanning images of the D/siRNA-NGel release medium at different time points. Scale bar, 200 nm.

## 4. Hydrogel biodistribution in inflamed joints

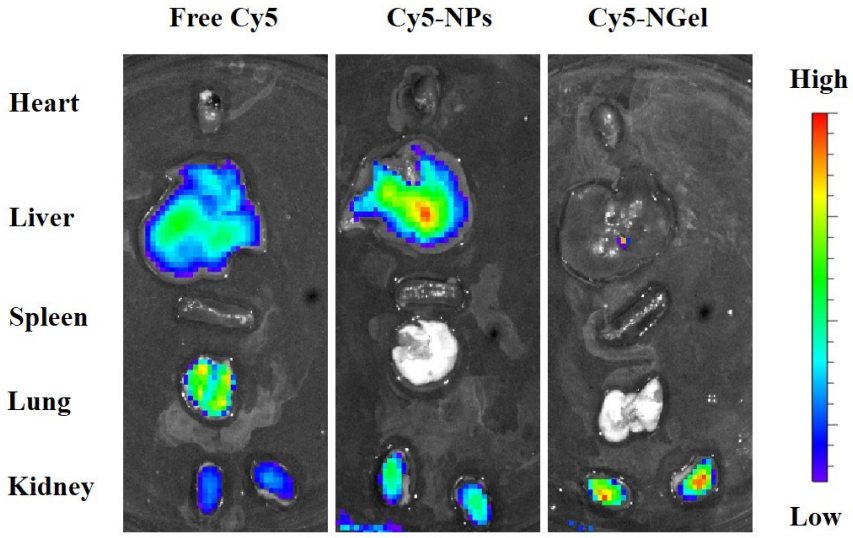


**Figure S5.** *Ex vivo* imaging of major organs at 24h after intra-articular injection.

## 5. TEM scan of D/siRNA-NGel

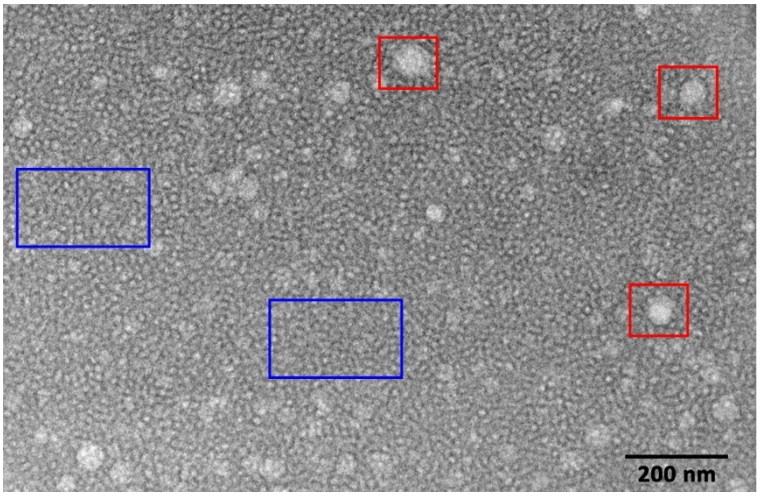


**Figure S6.** The transmission electron microscopy image of D/siRNA-NGel after 100 times dilution. Micellar aggregates (inside the blue frames) and some larger particles (inside the red frames) were shown.

## 6. Stability of D/siRNA-NGel

**Table S3.** *In vitro* stability of D/siRNA-NGel at 4°C.

| Time | 2h | 4h | 8h | 12h | 24h | 48h |
|---|---|---|---|---|---|---|
| Appearance | Light yellow, clear and transparent solution | Light yellow, clear and transparent solution | Light yellow, clear and transparent solution | Light yellow, clear and transparent solution | Light yellow, clear and transparent solution | Light yellow, clear and transparent solution |
| Gelation temperature | 33.0°C | 33.2°C | 33.1°C | 33.0°C | 32.9°C | 33.1°C |

## References

1. S. Son, K. Singha and W. J. Kim, *Biomaterials*, 2010, **31**, 6344-6354.
2. Z. M. A. Fathalla, A. Vangala, M. Longman, K. A. Khaled, A. K. Hussein, O. H. El-Garhy and R. G. Alany, *European Journal of Pharmaceutics and Biopharmaceutics*, 2017, **114**, 119-134.