1 **Suppression of NSAID-induced Small Intestinal Inflammation by Orally**

2 **Administered Redox Nanoparticles**

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2

1   **Abstract**

2   Patients regularly taking non-steroidal anti-inflammatory drugs (NSAIDs) such as

3   indomethacin (IND) run the risk of small intestinal injuries. In this study, we have

4   developed an oral nanotherapeutic by using a redox nanoparticle (RNP^O), which is

5   prepared by self-assembly of an amphiphilic block copolymer that possesses nitroxide

6   radicals as side chains of hydrophobic segment via ether linkage, to reduce

7   inflammation in mice with IND-induced small intestinal injury. The localization and

8   accumulation of RNP^O in the small intestine were determined using fluorescent-labeled

9   RNP^O and electron spin resonance. After oral administration, the values of area under

10   the concentration-time curve of RNP^O in both the jejunum and ileum tissues were about

11   40 times higher than those of low-molecular-weight nitroxide radical compounds. By

12   this specific accumulation of RNP^O in small intestine, RNP^O remarkably suppressed

13   inflammatory mediators such as myeloperoxidase, superoxide anion, and

14   malondialdehyde in the small intestines of IND-treated mice. Compared to

15   low-molecular-weight nitroxide radical compounds, RNP^O also significantly increased

16   the survival rate of mice treated daily with IND. On the basis of these results, RNP^O is

17   promising as a nanotherapeutic for treatment of inflammation in the small intestine of

18   patients receiving NSAIDs.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin (IND) are the most commonly prescribed drugs for their antipyretic, analgesic, and anti-inflammatory effects. The total consumption of NSAIDs is increasing in accordance with the increase in the incidence of orthopedic and cardiovascular diseases [1-3]. However, it has been reported that the use of NSAIDs causes severe adverse effects including ulcers, erosions, bleeding, perforation, and strictures in the gastrointestinal (GI) tract such as stomach and small intestine [4-9]. The absolute number of patients with serious NSAIDs-induced GI complications is increasing due to the expansion of long-term NSAIDs treatment. Though the etiology and pathogenesis of NSAIDs-induced inflammation are not well understood [10], several studies have reported that overproduction of reactive oxygen species (ROS) and an imbalance of important antioxidants exist in the intestine of patients receiving repeated doses of NSAIDs, leading to oxidative damage [8, 11-14]. Self-sustaining cycles of oxidant production may amplify inflammation and mucosal injury. Thus far, it has been reported that antioxidant compounds and free radical scavengers heal NSAIDs-induced inflammation [15, 16]. However, orally administered low-molecular-weight (LMW) compounds are not sufficiently effective due to their

1   non-specific distribution to the entire body, metabolism in the GI tract, low retention in

2   the lesion area, and undesired adverse effects.

3   To address these issues, we have developed a newly designed oral

4   nanotherapeutic using redox nanoparticles (RNP^O) with ROS scavenging potential of

5   nitroxide radicals for treatment of inflammation in the GI tract. RNP^O is a

6   core-shell-type polymeric micelle with approximately 40 nm in diameter, prepared by

7   the self-assembly of methoxy-poly(ethylene

8   glycol)-*b*-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)oxymethylstyrene]

9   (MeO-PEG-*b*-PMOT), which is an amphiphilic block copolymer possessing nitroxide

10   radicals as side chains of hydrophobic segment via ether linkages (Figure 1a). Thus far,

11   we have found that orally administered RNP^O specifically accumulates in the colonic

12   mucosa and effectively suppresses inflammation in mice with colitis [17]. In addition,

13   we have previously confirmed that, as RNP^O is not absorbed into the bloodstream via

14   the mesentery, it does not cause the adverse effects of nitroxide radicals in the entire

15   body [17].

16   The objective of this work was to confirm the protective effect of RNP^O on

17   NSAIDs-induced small intestinal inflammation in mice. The accumulation tendency of

18   orally administered RNP^O and the suppression of ROS and inflammation in the small

1   intestine by RNP^O were investigated in detail (Figure 1b).

2

3   **2. Materials and methods**

4   *2.1. Preparation of RNP^O*

5   RNP^O was prepared by the self-assembly of an amphiphilic block copolymer

6   (MeO-PEG-*b*-PMOT) composed of the hydrophilic PEG segment and the hydrophobic

7   poly(4-methylstyrene) segment possessing nitroxide radicals as side chains via ether

8   linkages, according to our previous study [18]. Briefly, poly(ethylene

9   glycol)-*b*-poly(chloromethylstyrene) (MeO-PEG-*b*-PCMS) was synthesized by the

10   radical telomerization of chloromethylstyrene using MeO-PEG-SH (Mn = 5000; NOF

11   corporation, Tokyo, Japan) as a telogen. The chloromethyl groups were converted to

12   2,2,6,6-tetramethylpiperidinyl-1-oxyls (TEMPOs) via a Williamson ether synthesis of

13   benzyl chloride in the MeO-PEG-*b*-PCMS block copolymer with the alkoxide of

14   4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL). To prepare RNP^O, the

15   MeO-PEG-*b*-PMOT was dissolved in *N,N*-dimethylformamide (Wako Pure Chemicals,

16   Osaka, Japan), and transferred into a membrane tube (SpectraPor, molecular weight

17   cut-off size: 3,500 Da, Spectrum Laboratories Inc., Savannah, GA, USA) and then

18   dialyzed for 24 h against 2 L of distilled water, which was changed after 2, 4, 8, 12, and

1 20 h. After dialysis, the diameter of the obtained particles was determined using

2 dynamic light scattering measurements (Zetasizer Nanoseries ZEN3600, Malvern

3 Instruments Ltd., Worcestershire, UK).

4

5 *2.2. Animal preparation*

6   All experiments were carried out using 6-week-old male ICR mice

7 (approximately 30 g) purchased from Charles River Japan, Inc. (Yokohama, Japan).

8 Mice were maintained in the experimental animal facilities at the University of Tsukuba.

9 All experiments were performed according to the Guide for the Care and Use of

10 Laboratory Animals Resource Center of the University of Tsukuba.

11

12 *2.3. Localization of RNP^O in the small intestine*

13   The localization of RNP^O in the small intestine was determined by fluorescent

14 rhodamine-labeled RNP^O. Rhodamine-labeled RNP^O was prepared via a thiourethane

15 bond between MeO-PEG-*b*-PMOT possessing reduced TEMPO moieties and

16 rhodamine B isothiocyanate in the presence of sodium hydride. One milliliter of

17 rhodamine-labeled RNP^O (5 mg/mL) was orally administered to mice, and the mice

18 were sacrificed at 0.5, 1, 4, and 12 h after oral administration. Residues in the ileum

1 were gently removed with phosphate buffer (pH 7.4), and 7-μm–thick sections of ileum

2 were prepared. Localization of rhodamine-labeled RNP^O was recorded using a

3 fluorescent microscope (FL-III; Leica, Tokyo, Japan).

4

5 *2.4. Biodistribution of RNP^O*

6 One milliliter of RNP^O (13.34 mg/mL) or LMW TEMPOL (2.49 mg/mL) was

7 orally administered to mice, and the mice were sacrificed at 0.25, 0.5, 1, 4, and 12 h

8 after oral administration. It should be noted that the concentrations of nitroxide radicals

9 in RNP^O and TEMPOL were equivalent, and were adjusted by measurements of

10 electron spin resonance (ESR) spectra. The jejunum, ileum, and blood were isolated

11 after operation. The jejunum and ileum tissues were gently washed with 0.9% normal

12 saline to remove any residual food. About 1 g jejunum or 0.5 g ileum tissues were

13 homogenized in 0.5 mL of phosphate buffer (pH 7.4) containing potassium ferricyanide

14 (200 mM) on ice. The ESR signal intensities in homogenized samples were analyzed

15 using an X-band ESR spectrometer (JES-TE25X; JEOL, Tokyo, Japan) at room

16 temperature under the following conditions: frequency, 9.41 GHz; power, 10.00 mW;

17 center field, 333.3; sweep width, 5 mT; sweep time, 0.5 min; modulation, 0.1 mT; time

18 constant, 0.1 s.

1   The accumulation of RNP^O in the mucosa and muscle layers of jejunum and

2   ileum was also determined by the ESR assay. To separate the mucosa and muscle of

3   both jejunum and ileum, the methods of Rang et al. [19] and Patonet et al. [20] were

4   employed. Briefly, 1 mL of RNP^O (13.34 mg/mL) or LMW TEMPOL (2.49 mg/mL)

5   was orally administered to mice, and the mice were sacrificed at 0.5 h after

6   administration. The intestinal content was gently flushed with 0.9% normal saline. Then,

7   the jejunum and ileum were longitudinally opened and the mucosa was scraped with a

8   blunt spatula.

9

10   *2.5. Experimental design using IND-induced small intestine inflammation model in mice*

11   Small intestine inflammation in mice was induced by the oral administration of

12   IND (10 mg/kg body weight [BW]; Wako Pure Chemicals), which was suspended in

13   distilled water with 1% carboxymethylcellulose (CMC; Wako Pure Chemicals) and use

14   immediately. All mice were fasted for 24 h before the experiment and divided into the

15   following 4 groups of 5 animals each.

16   Group I (Control): mice were received with distilled water 1 h before the oral

17   administration of CMC solution (1% w/v).

18   Group II (IND): mice were received with distilled water 1 h before the oral

1   administration of IND.

2   Group III (IND + TEMPOL): mice were treated with TEMPOL (18.67 mg/kg

3   BW) 1 h before the oral administration of IND.

4   Group IV (IND + RNP^O): mice were treated with RNP^O (100 mg/kg BW with

5   same amount of TEMPOL in RNP^O) 1 h before the oral administration of IND.

6   The animals were given *ad libitum* access to food and water after the

7   administration of IND. Twelve hours after the administration of IND or CMC, the

8   animals were sacrificed by cervical dislocation and the small intestine tissues were kept

9   on ice. The intestine was gently washed with 0.9% normal saline to remove any residual

10   food. The whole small intestine was separated into the duodenum, jejunum, and ileum.

11   Five-centimeter lengths of the jejunum and ileum were isolated for hematoxylin and

12   eosin (H&E) staining. The remaining tissues were used for measurements of

13   myeloperoxidase (MPO) activity, superoxide anion production, and malondialdehyde

14   (MDA) level.

15

16   *2.6. H&E staining*

17   Jejunum and ileum segments of mice were opened along the antimesenteric

18   border, gently rinsed to remove fecal contents, fixed on a 4% (v/v) buffered formalin

1   solution and embedded in paraffin for use in histopathological examination.

2   Seven-micrometer-thick sections were cut, deparaffinized, hydrated and stained with

3   H&E. The histology of the small intestine was evaluated using a microscope (DM

4   RXA2; Leica, Tokyo, Japan).

5

6   *2.7. Measurement of MPO activity*

7   MPO activity was measured according to the method of Bradley et al. [21].

8   Samples of intestinal tissue from treated mice were excised and homogenized in cold 50

9   mM phosphate buffer (pH 6.0) containing 0.5% (w/v) hexadecyltrimethylammonium

10   bromide. Supernatants were collected by centrifugation for 10 min at 10,000 rpm at 4°C

11   and kept at -80°C until use. The enzymatic reaction was carried out in a 96-well plate by

12   adding 190 μL of 50 mM phosphate buffer (pH 6), 5 μL of 0.5% (w/v) *o*-dianisidine

13   hydrochloride, 10 μL of the supernatant sample, and 5 μL of 20 mM H_2O_2. After

14   allowing the reaction to proceed for 30 min at room temperature, the absorbance at 460

15   nm was measured using a plate reader (Varioskan Flash; Thermo Scientific, Tokyo,

16   Japan). MPO activity was determined by comparison to a standard MPO curve (Sigma

17   Chemical Co., St. Louis, MO, USA). The protein concentration of the supernatant

18   sample was measured using a BCA kit (Thermo Scientific Pierce Protein Research

1   Products, Rockford, IL, USA). Values are expressed as MPO units/µg protein.

2

3   *2.8. Measurement of production of superoxide anion*

4   Superoxide anion generation was determined by the nitro blue tetrazolium (NBT)

5   assay [22]. Samples of intestinal tissue from treated mice were collected, homogenized

6   in cold 10 mM phosphate buffer (pH 7.4), and centrifuged at 10,000 rpm at 4°C for 15

7   min to obtain the supernatant. The reaction was carried out in a 96-well plate by adding

8   the supernatant sample, 10 mM boric acid-sodium hydroxide buffer, and the enzymatic

9   reaction mixture consisting of 10 mM phosphate buffer (pH 7.4) containing 0.1 mM

10   xanthine, 0.1 mM ethylene diaminetetraacetic acid, 0.1 mM NBT, and 0.1 unit xanthine

11   oxidase in a final volume of 1 mL. After 10 min at room temperature, the absorbance at

12   560 nm was measured using a plate reader. The amount of superoxide anion was

13   calculated per wet tissue weight.

14

15   *2.9. Measurement of lipid peroxidation*

16   Lipid peroxidation was evaluated by measuring the amount of MDA, following

17   the method of Ohkawa et al. [23]. Samples of intestinal tissue from treated mice were

18   collected, homogenized in cold 0.1 mM phosphate buffer (pH 7.4), and centrifuged at

1   10,000 rpm at 4°C for 15 min. An aliquot of the supernatant was added to the reaction

2   mixture containing 8% (w/v) sodium dodecyl sulfate, 20% (v/v) acetic acid, 0.8% (w/v)

3   thiobarbituric acid, and distilled water. After incubation at 95°C for 1 h, the amount of

4   MDA formed in the reaction mixture was measured using a plate reader at an

5   absorbance of 532 nm. 1,1,3,3-tetramethoxypropane was used as the standard. The

6   protein concentration of the supernatant sample was measured using the BCA kit as

7   well as the MPO assay.

8

9   *2.10. Survival rate experiment*

10   The survival rate of mice was determined by orally administering IND (10 mg/kg

11 BW) daily for 7 d. LMW TEMPOL (18.67 mg/kg BW) and RNP^O (100 mg/kg BW)

12 were also orally administered daily at 1 h before IND administration until 7 d, and the

13 number of surviving mice was counted for 7 d.

14

15 *2.12. Statistical analysis*

16   All data are expressed as mean ± SEM. from 5 mice per group. Statistical analysis

17 using SPSS (IBM Corp., NY, USA) was performed using one-way analysis of variance,

18 followed by Tukey's post-hoc test. A *P*-value of less than 0.05 was considered

1   significant for all statistical analyses.

2

3   **3. Results and Discussion**

4   *3.1. The specific accumulation of RNP^O in small intestine*

5   The accumulation of nanoparticles in the small intestine is one of the most

6   important features for an effective nanotherapeutic against small intestinal injury. We

7   have previously confirmed that a fairly large amount of RNP^O (ca. 15% of injected dose)

8   accumulated in the colonic mucosa by oral administration. In order to confirm the effect

9   of RNP^O on NSAID-induced injury, we examined the localization of RNP^O in the small

10   intestine using fluorescent rhodamine-labeled RNP^O. When LMW rhodamine was

11   administered orally, almost no fluorescent signal was observed at 1 h (Figure 2a). On

12   the contrary, a strong fluorescent signal of RNP^O in the mucosa area of ileum was

13   observed (Figures 2b–f). In addition, the fluorescent signal was observed until 12 h after

14   oral administration of rhodamine-labeled RNP^O, indicating that the effective

15   accumulation of RNP^O in the small intestinal area continued for at least half a day.

16   To obtain quantitative information on the tendency of RNP^O to accumulate in the

17   small intestinal areas, ESR analysis was carried out in comparison with LMW

18   TEMPOL. At 0.5 h after administration of LMW TEMPOL to mice, 4.2 and 1.4% of

1   the initial dose was observed in the jejunum and ileum areas, respectively. At 1 h after

2   administration, however, almost no ESR signal was observed, as shown in Figures 3a

3   and b. In contrast, when RNP^O was orally administrated to mice, a considerably higher

4   accumulation of RNP^O in the small intestine was observed. The values of area under the

5   concentration-time curve (AUC), an important parameter in biopharmaceuticals and

6   pharmacokinetics, of RNP^O were 3.3 and 2.1 mg·h/mL in the jejunum and ileum areas,

7   respectively, which were significantly higher than those of LMW TEMPOL (0.09 and

8   0.05 mg·h/mL) (Table 1).

9   The morphology of RNP^O in the small intestine was determined by ESR spectra.

10   At 0.5 h after oral administration, the ESR signals of LMW TEMPOL in the ileum

11   showed a sharp triplet due to an interaction between the ^{14}N nuclei and the unpaired

12   electron (Figure 3c). In contrast, the ESR signals of RNP^O even in the ileum were

13   consistently broad (Figure 3d), and this signal was observed until 4 h after oral

14   administration, indicating that the TEMPO radicals are still located in the solid core of

15   the polymeric micelles for a long time, even in the terminal portion of small intestine.

16   This might be one of the reasons preventing its uptake into blood via the mesentery.

17   The distribution of RNP^O in the small intestine region was further investigated in

18   detail. For this objective, the mucosa and muscle of both jejunum and ileum were

1   separated, and RNP^O localization was investigated by ESR measurement. As shown in

2   Table 2, at 0.5 h after oral administration, the amounts of RNP^O in the jejunum mucosa,

3   jejunum muscle, ileum mucosa, and ileum muscle were 30.6, 7.4, 15.3, and 4.1% of the

4   initial dose, respectively. The amounts of RNP^O in the mucosa layer were about 4 times

5   higher than those in the muscle layer in both jejunum and ileum. These results confirm

6   that our nanoparticle specifically accumulated in the intestinal mucosa preferentially.

7   Lamprecht et al. reported that the size determines the accumulation of polystyrene

8   nanoparticles in the intestinal mucosal layer [24]. However, in the harsh environment of

9   the GI tract, which includes gastric juices with strong acid, digestive enzymes, and bile

10   acid, these polystyrene nanoparticles do not always stably maintain their sizes. Block

11   copolymers can self-assemble to form a polymeric micelle structure with a dense PEG

12   brush on the surface [25]. The optimal structure allows RNP^O to easily diffuse in the

13   mucosa and inflammatory areas in GI tract. Because RNP^O did not disintegrate even in

14   the intestinal mucosa, its nanosize of 40 nm prevented the uptake of RNP^O into the

15   bloodstream [17], suggesting a lack of systemic adverse effects, such as hypertension

16   [26]. Furthermore, the covalent conjugation of nitroxide radicals to the backbone of

17   polymeric micelles is another important strategy to suppress the toxicity of nitroxide

18   radicals and deliver them to a target area *in vivo* without drug leakage. Based on these

1 reasons, RNP^O is the ideal material for accumulation in the small intestine.

2

3 *3.2. The protective effect of RNP^O on IND-induced small intestinal inflammation*

4   Since it has been confirmed that orally administered RNP^O significantly

5 accumulated in the intestinal area, the ROS scavenging efficiency of RNP^O in

6 IND-induced small intestinal inflammation was examined. We have previously

7 confirmed that non-disintegrated RNP^O has a definite ROS scavenging ability *in vitro*

8 [18]. Here, the protective effects of RNP^O on IND-induced inflammation in mice were

9 investigated. In this experiment, the encapsulation of IND into the hydrophobic core of

10 RNP^O in GI tract is ignorable, because almost IND exists outside of RNP^O in GI tract,

11 which is based on the maximum IND encapsulation capacity in the hydrophobic core of

12 RNP^O (6.7 wt%) (see Supporting information). After the treatments shown in the

13 experimental section, histological assessments were performed in both the jejunum and

14 ileum areas. As shown in Figures 4b and f, gross damage, such as focal and upper

15 villous necrosis, was observed in both the jejunum and ileum of mice treated with IND,

16 compared to those of control mice (Figures 4a and e). When LMW TEMPOL was orally

17 administered prior to IND-treated mice, the jejunum area was almost similar to that of

18 control mice (Figure 4c); however, a part of a necrotic villus was observed in the ileum

1   (see arrow in Figure 4g). Since LMW TEMPOL is absorbed from the upper GI tract,

2   most of it might not reach to the ileum area. On the contrary, the histology of the small

3   intestine from RNP^O-treated mice showed almost no damage and was similar to that

4   from control mice in both the jejunum and ileum areas (Figures 4d and h).

5   The different efficiencies of pretreatment with TEMPOL and RNP^O on

6   IND-treated mice were further investigated by measuring the levels of inflammatory

7   mediators, such as MPO, superoxide anion, and MDA, which indicate the extent of

8   neutrophil invasion, oxidative stress, and lipid oxidation, respectively. As shown in

9   Figure 5a, MPO activity significantly increased in small intestine of IND-treated mice,

10   especially in ileum tissues, compared to those of control mice, indicating the increase in

11   neutrophil invasion especially in the ileum region. When LMW TEMPOL was orally

12   administered to IND-treated mice, the MPO level decreased effectively, both in jejunum

13   and in ileum (Figure 5a). However, it was not suppressed to the controlled level in the

14   ileum, indicating that LMW TEMPOL could not suppress neutrophil invasion

15   completely. On the contrary, the level of MPO decreased significantly and was almost

16   the same level as that of control both in jejunum and in ileum of mice pretreated with

17   RNP^O, indicating that RNP^O could suppress neutrophil invasion almost completely both

18   in jejunum and in ileum regions. As shown in Figure 5b, the superoxide level increased

1   significantly by IND-treatment both in jejunum and in ileum. Since the MPO level in

2   the jejunum did not increase to the level in the ileum, the higher superoxide level in the

3   jejunum might be due to the direct toxicity of IND in addition to neutrophil invasion.

4   When LMW TEMPOL was administered to the IND-treated mice, the production of

5   superoxide anion decreased in the jejunum area but not in the ileum (Figure 5b),

6   suggesting that the most of LMW TEMPOL might not reach to the ileum area,

7   consistent with the result of H&E staining, as can be seen in Figures 4c and g. When

8   RNP^O was used, the amount of superoxide anion both in the jejunum and in ileum was

9   significantly suppressed, compared to that of IND-treated mice (Figure 5b), indicating

10 that RNP^O effectively scavenged overproduced superoxide anion in IND-treated mice.

11 We also confirmed lipid oxidation by measuring the amount of MDA. As shown in

12 Figure 5c, higher amounts of oxidative products were observed in both the jejunum and

13 ileum by IND treatment. LMW TEMPOL did not decrease the amounts of these

14 oxidative compounds well, especially in the jejunum area. On the other hand, all

15 oxidative stress markers both in jejunum and in ileum tissues of mice pretreated with

16 RNP^O were significantly decreased compared to those of mice treated with IND only.

17 Importantly, these oxidative stress markers both in jejunum and in ileum tissues of mice

18 pretreated with RNP^O also showed the statistically significant differences from those

1   pretreated with LMW TEMPOL. Taken together, these results clearly indicate that

2   pretreatment with RNP^O effectively prevented IND-induced inflammation by ROS

3   scavenging, unlike LMW TEMPOL.

4   Finally, we confirmed the consequences of pretreatment with TEMPOL and

5   RNP^O in IND-treated mice by a survival experiment. It is noted that mortality was

6   induced by daily administration of IND to mice for 7 d, and TEMPOL and RNP^O were

7   administered daily, 1 h before IND administration. As shown in Figure 6, the daily

8   administration of IND caused severe damage to the small intestines of mice, leading to a

9   significant decrease in survival rate (28.6%) after 7 d treatment. However, the

10   administration of LMW TEMPOL did not result in any improvement (14.3%),

11   indicating the low efficiency of TEMPOL in reducing the IND-induced the intestinal

12   inflammation. In contrast, the survival rate of mice treated daily with RNP^O (57.1%)

13   was remarkably increased. It is emphasized that the high accumulation and long

14   retention of RNP^O in the intestinal area are critical factors to reduce IND-induced small

15   intestinal inflammation.

16

17   **4. Conclusions**

18   This study demonstrates the protective effect of orally administered RNP^O on

IND-induced small intestinal inflammation in mice. Compared to LMW nitroxide

radical compounds, RNP^O showed remarkable accumulation and long retention in the

jejunum and ileum, especially in the mucosa layer, resulting in effective scavenging of

ROS and suppression of inflammation in the small intestines of IND-treated mice. On

the basis of these results, we believe that the oral administration of RNP^O, prior to the

oral administration of NSAIDs, might become an important approach for the treatment

of small intestinal injury in patients regularly taking NSAIDs.

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9

10

11   **Figure Legends**

12   **Figure 1.** Schematic illustration of RNP^{O} and nanotherapeutics for the treatment of

13   NSAID-induced small intestinal inflammation in mice. (a) RNP^{O} is prepared by

14   self-assembly of a methoxy-poly(ethylene

15   glycol)-*b*-poly(4-[2,2,6,6-tetramethylpiperidine-1-oxyl]oxymethylstyrene)

16   (MeO-PEG-*b*-PMOT) block copolymer possessing nitroxide radicals as side chains of

17   hydrophobic segment. (b) After oral administration, RNP^{O} accumulates in the mucosa

18   of small intestine and scavenges ROS effectively.

1

Figure 2. Localization of RNP^O in the ileum was determined with rhodamine-labeled

RNP^O. Mice were sacrificed 0.5, 1, 2, 4, and 12 h after the oral administration of 1 mL

of rhodamine-labeled RNP^O at a dose of 5 mg/mL (n = 3 mice per group), and ileum

sections were cut circularly. The localization of rhodamine-labeled RNP^O in the ileum

was analyzed by fluorescent microscopy. Scale bars = 200 μm. Lu and Se in figure

indicate lumen and serosa, respectively.

8

Figure 3. (a) Accumulation of LMW TEMPOL (open circle) and RNP^O (closed circle)

in the jejunum. After the oral administration of LMW TEMPOL or RNP^O with

equivalent molar amount of nitroxide radicals (14.5 μmol), the amount of nitroxide

radicals was measured using electron spin resonance (ESR). Data are expressed as mean

± SEM. from 5 mice per group. (b) Accumulation of LMW TEMPOL (open circle) and

RNP^O (closed circle) in the ileum. Data are expressed as mean ± SEM. from 5 mice per

group. (c) The ESR spectrum of LMW TEMPOL in the ileum homogenate at 0.5 h after

oral administration. (d) The ESR spectrum of RNP^O in the ileum homogenate at 0.5 h

after oral administration.

18

1   **Figure 4.** Histological assessments by hematoxylin and eosin (H&E) staining. At 12 h

2   after treatment, the jejunum and ileum were collected, and 7-μm-thick sections of

3   jejunum and ileum were prepared. Sections of the jejunum and ileum were stained by

4   H&E and assessed histologically. *Red arrows* indicate the lesion areas. Scale bars = 200

5   μm.

6

7   **Figure 5.** Protective effect of RNP^O on IND-induced small intestinal inflammation in

8   mice. At 12 h after treatment, jejunum (open bar) and ileum (closed bar) homogenates

9   were prepared, and myeloperoxidase (MPO) activity, superoxide anion generation, and

10   malondialdehyde (MDA) levels were measured. (a) MPO activity was determined by a

11   colorimetric assay using o-dianisidine hydrochloride and H_2O_2 as substrates. (b) The

12   generation of the superoxide anion was determined by the NBT assay. (c) Lipid

13   peroxidation was measured by MDA formation in intestinal tissue homogenates. Data

14   are expressed as mean ± SEM. #$P < 0.05$ vs. control, *$P < 0.05$ vs. IND, and ¶$P < 0.05$

15   vs. TEMPOL from 5 mice per group.

16

17   **Figure 6.** RNP^O increased the survival rate of mice with IND-induced small intestinal

18   inflammation. The survival rate of mice was determined following the oral

1   administration of IND (10 mg/kg BW) daily for 7 d. LMW TEMPOL (18.67 mg/kg

2   BW) and RNP^O (100 mg/kg BW) were orally administered 1 h before IND daily for 7 d,

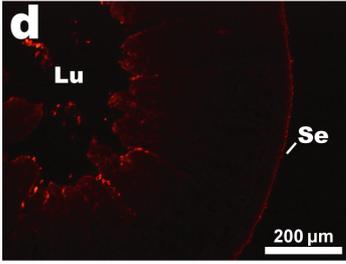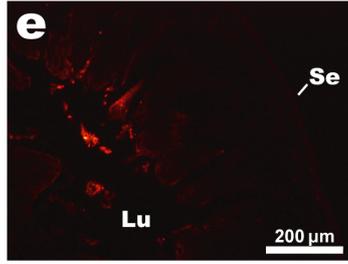3   and the number of surviving mice was counted for 7 d. n = 7 mice per group.

4

a

PEG: poly(ethylene glycol)
PMOT: poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)oxymethylstyrene]

b

Oral administration of Redox nanoparticles(RNP^O)

5

6

a Se, Lu, 200 µm
1 h Rhodamine

b Se, Lu, 200 µm
0.5h Rhodamine-RNP°

c Se, Lu, 200 µm
1 h Rhodamine-RNP°

d Lu, Se, 200 µm
2 h Rhodamine-RNP°

e Se, Lu, 200 µm
4 h Rhodamine-RNP°

f Lu, 200 µm
12h Rhodamine-RNP°

1

2

a

b

c

d

1

2

	Control	IND	TEMPOL+IND	RNPᴼ+IND

Jejunum

Ileum

a

b

c

1