Suppression of NSAID-induced small intestinal inflammation by orally administered redox nanoparticles

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Suppression of NSAID-induced Small Intestinal Inflammation by Orally Administered Redox Nanoparticles

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Abstract

Patients regularly taking non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin (IND) run the risk of small intestinal injuries. In this study, we have developed an oral nanotherapeutic by using a redox nanoparticle (RNP\textsuperscript{O}), which is prepared by self-assembly of an amphiphilic block copolymer that possesses nitroxide radicals as side chains of hydrophobic segment via ether linkage, to reduce inflammation in mice with IND-induced small intestinal injury. The localization and accumulation of RNP\textsuperscript{O} in the small intestine were determined using fluorescent-labeled RNP\textsuperscript{O} and electron spin resonance. After oral administration, the values of area under the concentration-time curve of RNP\textsuperscript{O} in both the jejunum and ileum tissues were about 40 times higher than those of low-molecular-weight nitroxide radical compounds. By this specific accumulation of RNP\textsuperscript{O} in small intestine, RNP\textsuperscript{O} remarkably suppressed inflammatory mediators such as myeloperoxidase, superoxide anion, and malondialdehyde in the small intestines of IND-treated mice. Compared to low-molecular-weight nitroxide radical compounds, RNP\textsuperscript{O} also significantly increased the survival rate of mice treated daily with IND. On the basis of these results, RNP\textsuperscript{O} is promising as a nanotherapeutic for treatment of inflammation in the small intestine of 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
non-specific distribution to the entire body, metabolism in the GI tract, low retention in
the lesion area, and undesired adverse effects.

To address these issues, we have developed a newly designed oral
nanotherapeutic using redox nanoparticles (RNP\textsuperscript{O}) with ROS scavenging potential of
nitrooxide radicals for treatment of inflammation in the GI tract. RNP\textsuperscript{O} is a
core-shell-type polymeric micelle with approximately 40 nm in diameter, prepared by
the self-assembly of methoxy-poly(ethylene

glycol)-\textit{b}-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)oxymethylstyrene]
(MeO-PEG-\textit{b}-PMOT), which is an amphiphilic block copolymer possessing nitrooxide
radicals as side chains of hydrophobic segment via ether linkages (Figure 1a). Thus far,
we have found that orally administered RNP\textsuperscript{O} specifically accumulates in the colonic
mucosa and effectively suppresses inflammation in mice with colitis [17]. In addition,
we have previously confirmed that, as RNP\textsuperscript{O} is not absorbed into the bloodstream via
the mesentery, it does not cause the adverse effects of nitrooxide radicals in the entire
body [17].

The objective of this work was to confirm the protective effect of RNP\textsuperscript{O} on
NSAIDs-induced small intestinal inflammation in mice. The accumulation tendency of
orally administered RNP\textsuperscript{O} and the suppression of ROS and inflammation in the small
intestine by RNP<sup>O</sup> were investigated in detail (Figure 1b).

2. Materials and methods

2.1. Preparation of RNP<sup>O</sup>

RNP<sup>O</sup> was prepared by the self-assembly of an amphiphilic block copolymer (MeO-PEG-<i>b</i>-PMOT) composed of the hydrophilic PEG segment and the hydrophobic poly(4-methylstyrene) segment possessing nitroxide radicals as side chains via ether linkages, according to our previous study [18]. Briefly, poly(ethylene glycol)-<i>b</i>-poly(chloromethylstyrene) (MeO-PEG-<i>b</i>-PCMS) was synthesized by the radical telomerization of chloromethylstyrene using MeO-PEG-SH (Mn = 5000; NOF corporation, Tokyo, Japan) as a telogen. The chloromethyl groups were converted to 2,2,6,6-tetramethylpiperidinyl-1-oxyls (TEMPOs) via a Williamson ether synthesis of benzyl chloride in the MeO-PEG-<i>b</i>-PCMS block copolymer with the alkoxide of 4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL). To prepare RNP<sup>O</sup>, the MeO-PEG-<i>b</i>-PMOT was dissolved in <i>N</i>,<i>N</i>-dimethylformamide (Wako Pure Chemicals, Osaka, Japan), and transferred into a membrane tube (SpectraPor, molecular weight cut-off size: 3,500 Da, Spectrum Laboratories Inc., Savannah, GA, USA) and then dialyzed for 24 h against 2 L of distilled water, which was changed after 2, 4, 8, 12, and
20 h. After dialysis, the diameter of the obtained particles was determined using
dynamic light scattering measurements (Zetasizer Nanoseries ZEN3600, Malvern
Instruments Ltd., Worcestershire, UK).

2.2. Animal preparation

All experiments were carried out using 6-week-old male ICR mice
(approximately 30 g) purchased from Charles River Japan, Inc. (Yokohama, Japan).
Mice were maintained in the experimental animal facilities at the University of Tsukuba.
All experiments were performed according to the Guide for the Care and Use of
Laboratory Animals Resource Center of the University of Tsukuba.

2.3. Localization of RNP$^O$ in the small intestine

The localization of RNP$^O$ in the small intestine was determined by fluorescent
rhodamine-labeled RNP$^O$. Rhodamine-labeled RNP$^O$ was prepared via a thiourethane
bond between MeO-PEG-b-PMOT possessing reduced TEMPO moieties and
rhodamine B isothiocyanate in the presence of sodium hydride. One milliliter of
rhodamine-labeled RNP$^O$ (5 mg/mL) was orally administered to mice, and the mice
were sacrificed at 0.5, 1, 4, and 12 h after oral administration. Residues in the ileum
were gently removed with phosphate buffer (pH 7.4), and 7-µm–thick sections of ileum
were prepared. Localization of rhodamine-labeled RNP was recorded using a
fluorescent microscope (FL-III; Leica, Tokyo, Japan).

2.4. Biodistribution of RNP

One milliliter of RNP (13.34 mg/mL) or LMW TEMPOL (2.49 mg/mL) was
orally administered to mice, and the mice were sacrificed at 0.25, 0.5, 1, 4, and 12 h
after oral administration. It should be noted that the concentrations of nitroxide radicals
in RNP and TEMPOL were equivalent, and were adjusted by measurements of
electron spin resonance (ESR) spectra. The jejunum, ileum, and blood were isolated
after operation. The jejunum and ileum tissues were gently washed with 0.9% normal
saline to remove any residual food. About 1 g jejunum or 0.5 g ileum tissues were
homogenized in 0.5 mL of phosphate buffer (pH 7.4) containing potassium ferricyanide
(200 mM) on ice. The ESR signal intensities in homogenized samples were analyzed
using an X-band ESR spectrometer (JES-TE25X; JEOL, Tokyo, Japan) at room
temperature under the following conditions: frequency, 9.41 GHz; power, 10.00 mW;
center field, 333.3; sweep width, 5 mT; sweep time, 0.5 min; modulation, 0.1 mT; time
constant, 0.1 s.
The accumulation of RNP$^O$ in the mucosa and muscle layers of jejunum and ileum was also determined by the ESR assay. To separate the mucosa and muscle of both jejunum and ileum, the methods of Rang et al. [19] and Patonet et al. [20] were employed. Briefly, 1 mL of RNP$^O$ (13.34 mg/mL) or LMW TEMPOL (2.49 mg/mL) was orally administered to mice, and the mice were sacrificed at 0.5 h after administration. The intestinal content was gently flushed with 0.9% normal saline. Then, the jejunum and ileum were longitudinally opened and the mucosa was scraped with a blunt spatula.

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

Small intestine inflammation in mice was induced by the oral administration of IND (10 mg/kg body weight [BW]; Wako Pure Chemicals), which was suspended in distilled water with 1% carboxymethylcellulose (CMC; Wako Pure Chemicals) and use immediately. All mice were fasted for 24 h before the experiment and divided into the following 4 groups of 5 animals each.

Group I (Control): mice were received with distilled water 1 h before the oral administration of CMC solution (1% w/v).

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

Group III (IND + TEMPOL): mice were treated with TEMPOL (18.67 mg/kg BW) 1 h before the oral administration of IND.

Group IV (IND + RNP$^O$): mice were treated with RNP$^O$ (100 mg/kg BW with same amount of TEMPO in RNP$^O$) 1 h before the oral administration of IND.

The animals were given *ad libitum* access to food and water after the administration of IND. Twelve hours after the administration of IND or CMC, the animals were sacrificed by cervical dislocation and the small intestine tissues were kept on ice. The intestine was gently washed with 0.9% normal saline to remove any residual food. The whole small intestine was separated into the duodenum, jejunum, and ileum. Five-centimeter lengths of the jejunum and ileum were isolated for hematoxylin and eosin (H&E) staining. The remaining tissues were used for measurements of myeloperoxidase (MPO) activity, superoxide anion production, and malondialdehyde (MDA) level.

### 2.6. H&E staining

Jejunum and ileum segments of mice were opened along the antimesenteric border, gently rinsed to remove fecal contents, fixed on a 4% (v/v) buffered formalin
solution and embedded in paraffin for use in histopathological examination.

Seven-micrometer-thick sections were cut, deparaffinized, hydrated and stained with H&E. The histology of the small intestine was evaluated using a microscope (DM RXA2; Leica, Tokyo, Japan).

2.7. Measurement of MPO activity

MPO activity was measured according to the method of Bradley et al. [21]. Samples of intestinal tissue from treated mice were excised and homogenized in cold 50 mM phosphate buffer (pH 6.0) containing 0.5% (w/v) hexadecyltrimethylammonium bromide. Supernatants were collected by centrifugation for 10 min at 10,000 rpm at 4°C and kept at -80°C until use. The enzymatic reaction was carried out in a 96-well plate by adding 190 µL of 50 mM phosphate buffer (pH 6), 5 µL of 0.5% (w/v) o-dianisidine hydrochloride, 10 µL of the supernatant sample, and 5 µL of 20 mM H₂O₂. After allowing the reaction to proceed for 30 min at room temperature, the absorbance at 460 nm was measured using a plate reader (Varioskan Flash; Thermo Scientific, Tokyo, Japan). MPO activity was determined by comparison to a standard MPO curve (Sigma Chemical Co., St. Louis, MO, USA). The protein concentration of the supernatant sample was measured using a BCA kit (Thermo Scientific Pierce Protein Research
2.8. Measurement of production of superoxide anion

Superoxide anion generation was determined by the nitro blue tetrazolium (NBT) assay [22]. Samples of intestinal tissue from treated mice were collected, homogenized in cold 10 mM phosphate buffer (pH 7.4), and centrifuged at 10,000 rpm at 4°C for 15 min to obtain the supernatant. The reaction was carried out in a 96-well plate by adding the supernatant sample, 10 mM boric acid-sodium hydroxide buffer, and the enzymatic reaction mixture consisting of 10 mM phosphate buffer (pH 7.4) containing 0.1 mM xanthine, 0.1 mM ethylene diaminetetraacetic acid, 0.1 mM NBT, and 0.1 unit xanthine oxidase in a final volume of 1 mL. After 10 min at room temperature, the absorbance at 560 nm was measured using a plate reader. The amount of superoxide anion was calculated per wet tissue weight.

2.9. Measurement of lipid peroxidation

Lipid peroxidation was evaluated by measuring the amount of MDA, following the method of Ohkawa et al. [23]. Samples of intestinal tissue from treated mice were collected, homogenized in cold 0.1 mM phosphate buffer (pH 7.4), and centrifuged at
10,000 rpm at 4°C for 15 min. An aliquot of the supernatant was added to the reaction mixture containing 8% (w/v) sodium dodecyl sulfate, 20% (v/v) acetic acid, 0.8% (w/v) thiobarbituric acid, and distilled water. After incubation at 95°C for 1 h, the amount of MDA formed in the reaction mixture was measured using a plate reader at an absorbance of 532 nm. 1,1,3,3-tetramethoxypropane was used as the standard. The protein concentration of the supernatant sample was measured using the BCA kit as well as the MPO assay.

2.10. Survival rate experiment

The survival rate of mice was determined by orally administering IND (10 mg/kg BW) daily for 7 d. LMW TEMPO (18.67 mg/kg BW) and RNP (100 mg/kg BW) were also orally administered daily at 1 h before IND administration until 7 d, and the number of surviving mice was counted for 7 d.

2.12. Statistical analysis

All data are expressed as mean ± SEM. from 5 mice per group. Statistical analysis using SPSS (IBM Corp., NY, USA) was performed using one-way analysis of variance, followed by Tukey’s post-hoc test. A P-value of less than 0.05 was considered
significant for all statistical analyses.

3. Results and Discussion

3.1. The specific accumulation of RNP\(^{O}\) in small intestine

The accumulation of nanoparticles in the small intestine is one of the most important features for an effective nanotherapeutic against small intestinal injury. We have previously confirmed that a fairly large amount of RNP\(^{O}\) (ca. 15% of injected dose) accumulated in the colonic mucosa by oral administration. In order to confirm the effect of RNP\(^{O}\) on NSAID-induced injury, we examined the localization of RNP\(^{O}\) in the small intestine using fluorescent rhodamine-labeled RNP\(^{O}\). When LMW rhodamine was administered orally, almost no fluorescent signal was observed at 1 h (Figure 2a). On the contrary, a strong fluorescent signal of RNP\(^{O}\) in the mucosa area of ileum was observed (Figures 2b–f). In addition, the fluorescent signal was observed until 12 h after oral administration of rhodamine-labeled RNP\(^{O}\), indicating that the effective accumulation of RNP\(^{O}\) in the small intestinal area continued for at least half a day.

To obtain quantitative information on the tendency of RNP\(^{O}\) to accumulate in the small intestinal areas, ESR analysis was carried out in comparison with LMW TEMPOL. At 0.5 h after administration of LMW TEMPOL to mice, 4.2 and 1.4% of
the initial dose was observed in the jejunum and ileum areas, respectively. At 1 h after
administration, however, almost no ESR signal was observed, as shown in Figures 3a
and b. In contrast, when RNP\(^\circ\) was orally administrated to mice, a considerably higher
accumulation of RNP\(^\circ\) in the small intestine was observed. The values of area under the
concentration-time curve (AUC), an important parameter in biopharmaceuticals and
pharmacokinetics, of RNP\(^\circ\) were 3.3 and 2.1 mg·h/mL in the jejunum and ileum areas,
respectively, which were significantly higher than those of LMW TEMPOL (0.09 and
0.05 mg·h/mL) (Table 1).

The morphology of RNP\(^\circ\) in the small intestine was determined by ESR spectra.

At 0.5 h after oral administration, the ESR signals of LMW TEMPOL in the ileum
showed a sharp triplet due to an interaction between the \(^{14}\)N nuclei and the unpaired
electron (Figure 3c). In contrast, the ESR signals of RNP\(^\circ\) even in the ileum were
consistently broad (Figure 3d), and this signal was observed until 4 h after oral
administration, indicating that the TEMPO radicals are still located in the solid core of
the polymeric micelles for a long time, even in the terminal portion of small intestine.

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

The distribution of RNP\(^\circ\) in the small intestine region was further investigated in
detail. For this objective, the mucosa and muscle of both jejunum and ileum were
separated, and RNP\textsuperscript{O} localization was investigated by ESR measurement. As shown in Table 2, at 0.5 h after oral administration, the amounts of RNP\textsuperscript{O} in the jejunum mucosa, jejunum muscle, ileum mucosa, and ileum muscle were 30.6, 7.4, 15.3, and 4.1% of the initial dose, respectively. The amounts of RNP\textsuperscript{O} in the mucosa layer were about 4 times higher than those in the muscle layer in both jejunum and ileum. These results confirm that our nanoparticle specifically accumulated in the intestinal mucosa preferentially.

Lamprecht et al. reported that the size determines the accumulation of polystyrene nanoparticles in the intestinal mucosal layer [24]. However, in the harsh environment of the GI tract, which includes gastric juices with strong acid, digestive enzymes, and bile acid, these polystyrene nanoparticles do not always stably maintain their sizes. Block copolymers can self-assemble to form a polymeric micelle structure with a dense PEG brush on the surface [25]. The optimal structure allows RNP\textsuperscript{O} to easily diffuse in the mucosa and inflammatory areas in GI tract. Because RNP\textsuperscript{O} did not disintegrate even in the intestinal mucosa, its nanosize of 40 nm prevented the uptake of RNP\textsuperscript{O} into the bloodstream [17], suggesting a lack of systemic adverse effects, such as hypertension [26]. Furthermore, the covalent conjugation of nitroxide radicals to the backbone of polymeric micelles is another important strategy to suppress the toxicity of nitroxide radicals and deliver them to a target area \textit{in vivo} without drug leakage. Based on these
reasons, RNP\textsuperscript{O} is the ideal material for accumulation in the small intestine.

3.2. The protective effect of RNP\textsuperscript{O} on IND-induced small intestinal inflammation

Since it has been confirmed that orally administered RNP\textsuperscript{O} significantly accumulated in the intestinal area, the ROS scavenging efficiency of RNP\textsuperscript{O} in IND-induced small intestinal inflammation was examined. We have previously confirmed that non-disintegrated RNP\textsuperscript{O} has a definite ROS scavenging ability \textit{in vitro} [18]. Here, the protective effects of RNP\textsuperscript{O} on IND-induced inflammation in mice were investigated. In this experiment, the encapsulation of IND into the hydrophobic core of RNP\textsuperscript{O} in GI tract is ignorable, because almost IND exists outside of RNP\textsuperscript{O} in GI tract, which is based on the maximum IND encapsulation capacity in the hydrophobic core of RNP\textsuperscript{O} (6.7 wt%) (see Supporting information). After the treatments shown in the experimental section, histological assessments were performed in both the jejunum and ileum areas. As shown in Figures 4b and f, gross damage, such as focal and upper villous necrosis, was observed in both the jejunum and ileum of mice treated with IND, compared to those of control mice (Figures 4a and e). When LMW TEMPO\textsubscript{L} was orally administered prior to IND-treated mice, the jejunum area was almost similar to that of control mice (Figure 4c); however, a part of a necrotic villus was observed in the ileum.
(see arrow in Figure 4g). Since LMW TEMPOL is absorbed from the upper GI tract,
most of it might not reach to the ileum area. On the contrary, the histology of the small
intestine from RNP\(^0\)-treated mice showed almost no damage and was similar to that
from control mice in both the jejunum and ileum areas (Figures 4d and h).

The different efficiencies of pretreatment with TEMPOL and RNP\(^0\) on
IND-treated mice were further investigated by measuring the levels of inflammatory
mediators, such as MPO, superoxide anion, and MDA, which indicate the extent of
neutrophil invasion, oxidative stress, and lipid oxidation, respectively. As shown in
Figure 5a, MPO activity significantly increased in small intestine of IND-treated mice,
especially in ileum tissues, compared to those of control mice, indicating the increase in
neutrophil invasion especially in the ileum region. When LMW TEMPOL was orally
administered to IND-treated mice, the MPO level decreased effectively, both in jejunum
and in ileum (Figure 5a). However, it was not suppressed to the controlled level in the
ileum, indicating that LMW TEMPOL could not suppress neutrophil invasion
completely. On the contrary, the level of MPO decreased significantly and was almost
the same level as that of control both in jejunum and in ileum of mice pretreated with
RNP\(^0\), indicating that RNP\(^0\) could suppress neutrophil invasion almost completely both
in jejunum and in ileum regions. As shown in Figure 5b, the superoxide level increased
significantly by IND-treatment both in jejunum and in ileum. Since the MPO level in
the jejunum did not increase to the level in the ileum, the higher superoxide level in the
jejunum might be due to the direct toxicity of IND in addition to neutrophil invasion.
When LMW TEMPOL was administered to the IND-treated mice, the production of
superoxide anion decreased in the jejunum area but not in the ileum (Figure 5b),
suggesting that the most of LMW TEMPOL might not reach to the ileum area,
consistent with the result of H&E staining, as can be seen in Figures 4c and g. When
RNP\(^O\) was used, the amount of superoxide anion both in the jejunum and in ileum was
significantly suppressed, compared to that of IND-treated mice (Figure 5b), indicating
that RNP\(^O\) effectively scavenged overproduced superoxide anion in IND-treated mice.
We also confirmed lipid oxidation by measuring the amount of MDA. As shown in
Figure 5c, higher amounts of oxidative products were observed in both the jejunum and
ileum by IND treatment. LMW TEMPOL did not decrease the amounts of these
oxidative compounds well, especially in the jejunum area. On the other hand, all
oxidative stress markers both in jejunum and in ileum tissues of mice pretreated with
RNP\(^O\) were significantly decreased compared to those of mice treated with IND only.
Importantly, these oxidative stress markers both in jejunum and in ileum tissues of mice
pretreated with RNP\(^O\) also showed the statistically significant differences from those
pretreated with LMW TEMPOL. Taken together, these results clearly indicate that pretreatment with RNP\(^O\) effectively prevented IND-induced inflammation by ROS scavenging, unlike LMW TEMPOL.

Finally, we confirmed the consequences of pretreatment with TEMPOL and RNP\(^O\) in IND-treated mice by a survival experiment. It is noted that mortality was induced by daily administration of IND to mice for 7 d, and TEMPOL and RNP\(^O\) were administered daily, 1 h before IND administration. As shown in Figure 6, the daily administration of IND caused severe damage to the small intestines of mice, leading to a significant decrease in survival rate (28.6%) after 7 d treatment. However, the administration of LMW TEMPOL did not result in any improvement (14.3%), indicating the low efficiency of TEMPOL in reducing the IND-induced the intestinal inflammation. In contrast, the survival rate of mice treated daily with RNP\(^O\) (57.1%) was remarkably increased. It is emphasized that the high accumulation and long retention of RNP\(^O\) in the intestinal area are critical factors to reduce IND-induced small intestinal inflammation.

4. Conclusions

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.

5. References


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**Figure Legends**

**Figure 1.** Schematic illustration of RNP$^O$ and nanotherapeutics for the treatment of NSAID-induced small intestinal inflammation in mice. (a) RNP$^O$ is prepared by self-assembly of a methoxy-poly(ethylene glycol)-$b$-poly(4-[2,2,6,6-tetramethylpiperidine-1-oxyl]oxymethylstyrene) (MeO-PEG-$b$-PMOT) block copolymer possessing nitrooxide radicals as side chains of hydrophobic segment. (b) After oral administration, RNP$^O$ accumulates in the mucosa of small intestine and scavenges ROS effectively.
Figure 2. Localization of RNP<sup>0</sup> in the ileum was determined with rhodamine-labeled RNP<sup>0</sup>. Mice were sacrificed 0.5, 1, 2, 4, and 12 h after the oral administration of 1 mL of rhodamine-labeled RNP<sup>0</sup> at a dose of 5 mg/mL (n = 3 mice per group), and ileum sections were cut circularly. The localization of rhodamine-labeled RNP<sup>0</sup> in the ileum was analyzed by fluorescent microscopy. Scale bars = 200 µm. Lu and Se in figure indicate lumen and serosa, respectively.

Figure 3. (a) Accumulation of LMW TEMPOL (open circle) and RNP<sup>0</sup> (closed circle) in the jejunum. After the oral administration of LMW TEMPOL or RNP<sup>0</sup> 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<sup>0</sup> (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<sup>0</sup> in the ileum homogenate at 0.5 h after oral administration.
Figure 4. Histological assessments by hematoxylin and eosin (H&E) staining. At 12 h after treatment, the jejunum and ileum were collected, and 7-µm-thick sections of jejunum and ileum were prepared. Sections of the jejunum and ileum were stained by H&E and assessed histologically. Red arrows indicate the lesion areas. Scale bars = 200 µm.

Figure 5. Protective effect of RNP\textsuperscript{O} on IND-induced small intestinal inflammation in mice. At 12 h after treatment, jejunum (open bar) and ileum (closed bar) homogenates were prepared, and myeloperoxidase (MPO) activity, superoxide anion generation, and malondialdehyde (MDA) levels were measured. (a) MPO activity was determined by a colorimetric assay using o-dianisidine hydrochloride and H\textsubscript{2}O\textsubscript{2} as substrates. (b) The generation of the superoxide anion was determined by the NBT assay. (c) Lipid peroxidation was measured by MDA formation in intestinal tissue homogenates. Data are expressed as mean ± SEM. \#\textit{P} < 0.05 vs. control, *\textit{P} < 0.05 vs. IND, and ¶\textit{P} < 0.05 vs. TEMPOL from 5 mice per group.

Figure 6. RNP\textsuperscript{O} increased the survival rate of mice with IND-induced small intestinal inflammation. The survival rate of mice was determined following the oral
administration of IND (10 mg/kg BW) daily for 7 d. LMW TEMPOL (18.67 mg/kg BW) and RNP⁰ (100 mg/kg BW) were orally administered 1 h before IND daily for 7 d, and the number of surviving mice was counted for 7 d. n = 7 mice per group.

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