Oxidative stress in vivo
The progress of aging and life process are controlled by several factors. While one of these factors is genetic regulation [1], oxidative stress is known to be one of the biggest external factors [2]. An animal takes in oxygen and uses it for the oxidation of glucose; the oxidation process involves the use of the mitochondrial electron transport system [3]. At the same time, the animal gets energy to live. In the glucose-oxidation process, oxygen is converted into superoxide, hydrogen peroxide, hydroxyl radical and water [4]. These oxidative compounds are known as reactive oxygen species (ROS). ROS function as important energy-transfer systems. Furthermore, a neutrophile and a macrophage produce and release ROS during emergencies, such as the approach of a foreign enemy, bleeding and inflammation, in order to maintain and restore normal body functions. However, excessively generated ROS trigger the unregulated oxidation of the living body and often cause various diseases. In particular, oxidation of lipids, protein and DNA is known to occur frequently and to cause a significant increase in oxidative stress; for example, guanosine in DNA is easily oxidized by ROS and produces 8-deoxyguanosine [5]. Guanosine is, therefore, used as an oxidative stress marker. The accumulation of oxidative stress combined with molecular level dysfunction causes functional disorders. Furthermore, the accumulation is thought to be associated with the age-related decline in body functions, asthenia and the risk of aging-related diseases.

ROS, which cause oxidative stress, are known to be produced not only by an endogenous system, but also via exogenous pathways such as chemical substances, foods, UV light and radiation [6]. The oxidative damage of the living body is governed by the balance between the production of ROS and the production of antioxidant-protective systems (glutathione, ascorbic acid, super oxide dismutase [SOD], a catalase and a glutathione peroxidase) and also by the restoration system (DNA injury-restoration systems) [7]. Although the oxidative stresses that exceed endogenous restoration systems are controlled by administering ascorbic acid and various antioxidants, there are problems with the use of exogenous antioxidants; for example, problems relating to their aqueous solubility and ease of metabolism and triggering of side reactions in the entire body. Recently, our group has been focusing on a new design for a redox polymer drug [8]. We have also been focusing on antioxidative stress nanotherapy using self-assembling polymers possessing nitroxide radicals [9,10]. This article discusses the present status and future prospects of antioxidant treatment with new nanoparticles along with self-assembling redox polymers.
**Scavenging of ROS by nitroxide radicals**

Nitroxide radicals are unstable chemical species similar to conventional radicals. However, cyclic nitroxide compounds possessing bulky substituents are highly stable. For example, 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) (Figure 1) is known as a stable radical and can be sublimated without employing any decomposition reaction. Because of its moderate stability and reactivity, TEMPO is employed in organic synthesis as a catalyst for the oxidation of primary alcohols to aldehydes. Cyclic nitroxides are, thus, known as versatile catalytic reactants. Because nitroxide radicals also have the ability to react and/or interact with a free radical, they have been employed as a biophysical tool for many years.

Owing to the ability of the radicals to degrade superoxide, inhibit Fenton reactions and undergo radical–radical recombination reactions, biological reactions involving these radicals have recently been used in novel therapies. Numerous in vitro cell experiments have confirmed that nitroxide radicals effectively scavenge ROS and regulate redox conditions, which improve cell viability and biofunctions.

**Equation 1**

\[
[RR'NO'] + O_2 \rightarrow RR'NO' + O_2
\]

**Equation 2**

\[
RR'NO' + O_2 + 2H^+ \rightarrow [RR'NO'] + H_2O_2
\]

Both TEMPO and hydroxylamine react with radical species according to the following equations:

**Equation 3**

\[
RR'NO' + X \rightarrow RR'NOX
\]

**Equation 4**

\[
RR'NOH + X \rightarrow RR'NO' + XH
\]

Thus, TEMPO behaves like SOD and scavenges ROS effectively. This ability to participate in redox reactions is the reason for nitroxide compounds being used for protection against oxidative damage in several models, ranging from models of cell systems to isolated organs to whole animals.

Alternatively, nitroxide radicals have for long been utilized as tools for ESR spectroscopic studies; for example, to study spin label/oxymetry and spin trapping, because of their stability and their paramagnetic nature in vitro and in vivo. Recently, several groups have stepped up research on in vivo ESR imaging. Some groups have reported on pH-sensitive nitroxide radicals of the imidazolidine type or the imidazoline type for the noninvasive measurement of pH under in vivo conditions, however, such low-molecular-weight nitroxide radicals pose several problems, such as nonspecific dispersion in normal tissues, preferential renal clearance and rapid reduction of the nitroxide radical to the corresponding hydroxylamine.

To solve these problems, we have designed and developed nitroxide radical-containing polymers, which have the tendency of self-assembling and forming nanoparticles (nitroxide radical-containing nanoparticles [RNPs]) because RNPs confine nitroxide radicals to their core, their antioxidative nature weakens significantly when they have a spherical form. Since the nanoparticles also change its biodistribution, their characteristics vary significantly in comparison with low-molecular-weight antioxidants. Thus, the RNPs are thought to have the potential to be used as high-performance bio-nanoparticles, which can be used in vivo. In the following section, we discuss their toxicity and circulation in the blood stream in view of their use in vivo.
Manufacture of radical-containing polymers & particles

Before introducing the characteristics of RNPs in vivo, the production of nitroxide radical-containing polymers on a large scale is discussed. Because the nitroxide radical can react with a free radical and acts as an inhibitor of radical polymerization, it is difficult to introduce it in polymers by using the radical polymerization technique. Nesvabda et al. reported group transfer polymerization of a methacrylate monomer possessing a nitroxide radical moiety [23]. Oyaizu et al. reported anionic polymerization of the same monomer [24]. After the reduction of the nitroxide radical to hydroxylamine, it can be polymerized by the radical polymerization technique. However, it is hard to synthesize these monomers on a large scale because of the requirement of a sophisticated technique. Yoshida et al. have reported a block copolymer possessing nitroxide radicals as a side chain of a segment. They prepared polystyrene-\textasciitilde poly(chloromethylstyrene) by a living radical polymerization technique, and this was followed by the introduction of hydroxyl-TEMPO via Williamson ether synthesis [25]. Sato et al. reported the introduction of hydroxyl-TEMPO in poly(vinyl alcohol) via an ester exchange reaction [26]. Because poly(chloromethylstyrene), poly(vinyl alcohol) and TEMPO derivatives are commercially available, these polymer modification reactions are promising methods for the large-scale stable production of polymers containing nitroxide radicals.

We have synthesized a nitroxide radical-containing polymer from poly(ethylene glycol)-\textasciitilde poly(chloromethylstyrene) (PEG-b-PCMS) in a manner similar to that of Yoshida and colleagues. For example, when PEG-b-PCMS is mixed with 4-hydroxyl-TEMPO (TEMPOL) in the presence of sodium hydride in dimethyl sulfoxide, poly(ethylene glycol)-\textasciitilde poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)oxyethylstyrene] (PEG-b-PMOT) is obtained almost quantitatively, as shown in Figure 3. When 4-aminotemPO is used instead of TEMPO, PEG-b-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)aminomethylstyrene] (PEG-b-PMNT) is obtained.

Nitroxide radicals in polymer

Recent developments in molecular-targeted therapy have drastically changed the concept of drug design. Drugs have evolved from traditional low-molecular-weight synthetic compounds to high-molecular-weight compounds, such as enzymes and antibodies. Even in the case of a molecular-targeted drug, the amount of drug accumulated in the diseased area is less than a few percentage of the injected dose. Other drugs get metabolized or spread nonspecifically to normal organs, which causes severe dysfunction in the living body. In order to improve the delivery efficiency of drugs, the precise control of pharmacokinetics is crucial. Drug delivery by nanoparticles such as polymeric micelles and liposomes can control the drug pharmacokinetics, and some of them have already been commercialized for anticancer therapy. An amphiphilic block copolymer spontaneously forms core–shell-type polymer micelles, which are extremely stable, because of the entanglement of hydrophobic chains in the core. This is the reason that polymer micelles are suitable for circulation in the blood stream in vivo, characterized by extremely dilute conditions. Low-molecular-weight surfactant micelles cannot be utilized under the same conditions below a critical micelle concentration. Polymeric micelles containing anticancer drugs were originally developed by Kataoka’s and Kabanov’s groups independently [28,29]. Anticancer drugs are incorporated into micelles via physical entrapment or chemical conjugation. A number of micelles are being assessed in clinical trials [30], and polymer micelle systems are being improved. Nishiyama et al. reported the development of a cisplatin-loaded micelle [31], in which platinum...
is coordinated by carboxylate groups in block copolymers consisting of PEG and polyaspartate.

Nanoparticles are known to accumulate in specific regions because of changes in the specific vascular microenvironment, for example, since neovascularity is leaky and immature lymphatic systems develop in tumor regions, high-molecular-weight compounds, such as proteins and nanoparticles, tend to accumulate in these regions (the so-called enhancement permeation and retention [EPR] effect). The EPR effect works well and highly dispersible and biocompatible nanoparticles amounting to up to 10% of the injected dose tend to accumulate in the tumor regions. More than 90% of the nanoparticles, however, spread nonspecifically in vivo after systemic administration, even in the case of nanoparticle systems. Active targeting is one of the challenges in improving nanoparticle accumulation in specific disease areas; however, to date, no remarkable effect the ligand on its biodistribution has been reported. When ligands with high specificity are incorporated on the carrier surface, the ease with which they circulate with the blood often decreases owing to the lowered colloidal stability. Even if the ligand-incorporated carrier works well in vitro, it is very difficult to effectively work under in vivo conditions. One promising way to improve the efficiency of nanotherapy is to use ‘on–off regulation’, whereby the nanoparticle is dormant in a non-target tissue and is activated in the target area, thus, improving treatment outcomes and decreasing side effects. Bae et al. developed a novel method for the conjugation of doxorubicin with PEG-b-poly(aspartic acid) diblock copolymers via a hydrazine linkage [32]; the conjugation facilitates the release of doxorubicin in acidic environments, for example, in the case of acidosis and endocytosis. Thus, the conjugation facilitates the stimuli-responsive delivery As stated above, we have developed amphiphilic block copolymers possessing nitroxide radicals as a side chain of the hydrophobic segment (Figure 3). The nanoparticles formed by these block copolymers, which are 40 nm in diameter (RNPs) [8], show unique ESR spectra. In contrast with the free-TEMPO signal shown in Figure 4B, which is a clear triplet signal corresponding to an interaction between 14N nuclei and the unpaired electron in the dilute solution, the nanoparticle signal broadens (Figure 4A). The broadened ESR signal of the nanoparticle is attributable to the restricted mobility of the nitroxide radicals incorporated in the solid core of the nanoparticles. These results confirm that nitroxide radicals are present in the hydrophobic solid core of the nanoparticles. The confinement of catalytic species in the core of polymer micelles reduces their side effect and unwanted reactions under spherical form (Figure 5).

TEMPO derivatives are known to be biologically active substances [15,33]. Suy et al. have reported that one of the physiological activities of nitroxide radicals in cancer cells is the promotion of apoptosis [34]. We have evaluated the toxicity of TEMPO derivatives and RNPs. Figure 6 shows the dose-dependent cell viabilities in colon cells treated with low-molecular-weight TEMPO, 4-amino-TEMPO, and RNPs prepared from PEG-b-PMNT (RNP®). The colon cell viabilities are progressively reduced after 24 h with an increase in the concentrations of low-molecular-weight TEMPO and amino-TEMPO. The median inhibitory concentrations (IC50) of TEMPO and 4-amino-TEMPO are 8.3 and 4.8 mM, respectively. Because amino groups generally show high toxicity, the cytotoxicity of 4-amino-TEMPO might be higher than that of TEMPO. In contrast, the RNPN induces no change in the cell viability up to a concentration of 8-mmol N/l though PEG-b-PMNT possesses a polyamine segment. The extremely low toxicity of the RNPN is probably owing to the fact that the outer PEG layer constitutes an excellent stealth shield around the amino–TEMPO moieties in the RNP core. The in vivo toxicity is evaluated using Institute of Cancer Research mice. After the administration of RNPs in the approximate range of 0–300 mg/kg, no dead mice were observed for 2 weeks. Even

Key Term

Cancer: A large group of different diseases, all involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, which invade nearby parts of the body or spread to more distant parts of the body through the lymphatic system or blood stream.
at 600 mg/kg (concentration of amino-TEMPO moieties: 960-µmol N/kg), 60% of the mice are alive. Stefano et al. have reported that the LD$_{50}$ of poly(1-lysine) with a molecular weight of 28,000–42,000 (polymerization degree: 135–203) is between 15 and 30 mg/kg (concentration of amino groups: 72.5–145-µmol N/kg) [35]. The extremely low toxicity of the RNP, viz. IC$_{50}$ > 8-µmol N/l and LD$_{50}$ > 600 mg/kg (> 960-µmol N/kg), is considered to be because of the confinement of the polyamine segment of the block copolymer in the RNP core. The antioxidant character of the RNP might also have contributed to the reduced toxicity.

The circulation of nitroxide radical compounds with the blood stream is of interest, which is monitored by ESR spectra. The ESR signals in the blood stream are hardly observed even after 2 min, when low-molecular-weight TEMOL is administered via tail-vein injection (Figure 7). The half-life of TEMOL in blood has been reported to be approximately 15 s [36]. On the contrary, when RNP prepared from PEG-b-PMNT and PEG-b-PMOT (RNP$^0$) are administered, the ESR signal in the blood stream is observed even 2 h after the tail-vein injection of RNP. The half-life of the RNP is 60-times longer (15 min) than that of low-molecular-weight TEMOL. RNP$^0$s show much longer circulation. Actually, the half-life of the RNP$^0$ is 600 min, which is 2400-times longer than that of low-molecular-weight TEMOL. The rapid clearance of low-molecular-weight TEMOL from the blood stream is probably a result of the preferential renal clearance and the rapid reduction of the nitroxide radical to the corresponding hydroxylamine in the blood. Compartmentalization of nitroxide radicals in the core of RNP must be the reason for improvement in the observed reduction resistance of RNP. Actually, the ESR signals of blood are broad singlet similar to Figure 4, a proof that the nitroxide radical moieties are still located in the solid core of the RNP. Thus, self-assembling structure of amphiphilic block copolymer, which confines nitroxide radicals in the solid core is one of the important factors to improve the retention of nitroxide radicals in the blood stream.
Use of RNPs as a redox imaging tool

Poly[2-(N,N-diethylamino)ethyl methacrylate] (PEAMA) homopolymer exhibits precipitation above pH 7.5, owing to the deprotonation of the amino groups of the PEAMA [37]. Using this pH-dependent phase transition character, unique pH-sensitive nanoparticle can be prepared. Poly(ethylene glycol)-b-PEAMA block copolymer forms core–shell type polymer micelle above pH 7.5, while it disintegrates under pH 7.4 [38,39]. Thus, polyamine with appropriate hydrophobicity can be employed for pH-sensitive material design [40,41]. Because the PMNT segment possesses both a hydrophobic phenyl group and an amino group in each repeating unit, a similar phase-transition phenomenon is shown in response to acidic pH environments, owing to the protonation of the amino groups. In fact, the pKa value of PMNT is approximately 7.0, meaning that it is precipitate above pH 7.0, while it is soluble below pH 7.0. From the dynamic light scattering investigation, the scattering intensity drastically decreases at pH values below 6.0 in the case of RNPNs, whereas it does not decrease at all in the case of RNPO, which has no amino group in hydrophobic segment as shown in Figure 8 [27]. These results clearly indicate that the RNPN disintegrates at pH values below 6.0.

As stated above, ESR spectra are known to give information on the local environment around the spin probe [42]. The ESR measurement of the polymers carrying nitroxide radicals thus gives us information on the dynamics of the polymer chain [25,43]. Since the assembly of PEG-b-PMNT depends on the protonation of the amino groups, the ESR spectra should be influenced by the environmental pH. Figure 9 shows the ESR spectra of the RNPN for various pH values, from 4.0 to 8.2. In contrast to the clear triplet signals of low-molecular-weight TEMPO derivatives, broad signals of the RNPN are observed at pH values above 6.0, similar to the ESR signal obtained after dialysis. The ESR spectra of the RNPN at pH values above 6.0 correspond to the confinement of the nitroxide radicals in the solid core of the RNPN. With decreasing pH, the ESR signals gradually change, and typical triplet signals are observed under acidic pH (pH < 6.0) conditions, which is consistent with the disintegration region of the RNPN. Since the spin probe is covalently conjugated in the PMNT segment of the PEG-b-PMNT block copolymer, the relative anisotropy observed in the ESR spectrum is directly related to the rotational mobility of the probe. The rotational correlation time $\tau_c$ is approximately $8.0 \times 10^{-10}$ s under neutral conditions. Its abrupt decrease is observed around the phase transition region (approximately at pH = 6.0). $\tau_c$ is in the approximate range $3 \times 10^{-10}$ to $4 \times 10^{-10}$ s at pH values below 6.0. A lower $\tau_c$ indicates the higher mobility of the nitroxide radical moieties, which is certainly

**Figure 7.** Blood circulation of nitroxide radical compounds administered from tail vein (Institute of Cancer Research male mice; 24 g). Data of RNPO from [Unpublished data]. Data of RNPN and TEMPOL from [10]. RNPN: RNPs prepared from PEG-b-PMNT; RNPO: RNPs prepared from PEG-b-PMOT; TEMPO: 2,2,6,6-tetramethylpiperidine-1-oxyl. Reprinted with permission from [10] © American Chemical Society (2009).

**Figure 8.** Change in light scattering intensity of nitroxide radical-containing nanoparticle solution as a function of pH. RNPN: RNPs prepared from PEG-b-PMNT; RNPO: RNPs prepared from PEG-b-PMOT. Reproduced with permission from [27] © Elsevier (2011).
correlated with the disintegration of the RNPN. Crucially, the mobility of the TEMPO moieties as side chains of the PMNT segments increased with the disintegration of the RNPN core, and the distance between the nitroxide radicals increases because of the electrostatic repulsion between the protonated amino groups.

In conjunction with the phase transition, a change in the ESR signal height is observed. Indeed, a significant increase in the signal height is observed at pH values below 6.0, which confirms that the RNPN shows a phase transition at a specific pH (~pH = 6.0). This phenomenon is also observed in the L-band ESR spectra. L-band ESR imaging systems operating in low-frequency microwave bands (less than 1.2 GHz), where the dielectric losses are lower, have been developed and utilized for the in vivo imaging of animals by using a spin probe [44]. Figure 9B shows phantom images of RNP in a glass vial at pH 5.6 and 7.4. An appropriate threshold (cutoff 40%) is used for deionizing water solution. The phantom images clearly show a remarkable on–off regulation in the capillary glass tube. Since RNP can be delivered to tumor and inflammation sites effectively, the RNPN might emit strong ESR signals in vivo by disintegration in low pH conditions. This is our strategy for imaging under low pH conditions by using pH-sensitive RNPNs. From these results, the RNPN might be used as a nanoprobe for in vivo ESR imaging under low pH conditions.

**Effectiveness of RNPNs for ischemia-reperfusion injuries**

Ischemic cardiovascular disease is the second leading cause of death in the world owing to the disruption of blood supply to the vital organs [45]. It is known that arterial recanalization achieved by thrombolysis and intravascular intervention are the main treatment strategies to restore blood supply in ischemic stroke and heart attack patients [46]. However, reperfusion can lead to ischemia-reperfusion (IR) injury, which is caused by ROS, and the injury can increase the extent of the damaged area [47–52]. Protection of organs that can be affected by ROS has been shown to prevent the increase in the extent of the damaged area associated with the arterial occlusion. The nitroxide radical can catalytically react with ROS as stated in the section titled 'Scavenging of ROS by nitroxide radicals', and it could improve the functional outcome of patients with ischemic brain and heart diseases by scavenging free radicals at the IR injury site [53–57]. However, these low-molecular-weight nitroxide radical compounds have not been used clinically because they are inactivated by strong reduction by antioxidant systems, such as catalase and glutathione peroxidase in vivo [54,56,58]. Nanoparticle therapy based on RNPNs is effective for the accumulation of the nanoparticles in the IR area, and it helps avoid problems related to the bioavailability and biocompatibility of the nanoparticles.

In order to confirm the effectiveness of RNPNs for treating IR injury, the mouse renal IR model is employed. The ex vivo ESR assessment is utilized after intravenous administration of RNPNs to mice subjected to IR injury. Figure 10 shows the ESR spectra of the blood and kidney fields.

**Key Term**

Ischemia-reperfusion injury: Tissue damage caused when blood supply returns to the tissue after a period of ischemia or lack of oxygen. Ischemia-reperfusion injury is caused by reactive oxygen species and can enhance the damage sustained in the ischemic organ.
oxidized by the addition of K$_3$[Fe(CN)$_6$], which is converted from the hydroxylamine form to the nitroxide radical form. Figure 10A & B shows time profiles of the ESR signals in the blood and kidney. The 4-amino-TEMPO and TEMPOL signals completely disappeared within a minute in blood, while both RNPO and RNPN showed long circulation in blood, which is similar to the case of normal mice. A similar observation is made for the injured kidney. The low-molecular-weight TEMPO compounds disappeared for several hours in the injured kidney, while RNPs were observed for half to one day. The RNPO shows the longest circulation in both the blood and kidney. Figure 10C–J shows the ESR spectra of the TEMPO compounds and RNPs in the blood and kidney. Low-molecular-weight TEMPO derivatives show a clear triplet signal, as shown in Figure 10C–F, both in the blood and kidney. In contrast, the observed ESR signals of the RNPO and RNPN are broad singlets in blood, as shown in Figure 10G & I, suggesting that the nitroxide radicals are still located in the solid core of the polymer micelles in the blood stream. Figure 10H & J show the ESR spectra of the injured renal region. It is interesting to note that the administration of RNPO’s resulted in the triplet ESR signal in the IR kidney, whereas the RNPN did not show any change from the singlet spectra. This result strongly suggests the disintegration of RNPO’s at the injured renal lesion resulting from the lowered pH, accompanied with delivery of RNP in injured renal area.

The therapeutic effect of RNPs on acute kidney injury (AKI) is monitored by considering parameters that reflect renal functioning, such as the blood urea nitrogen (BUN) and creatinine (Cr) levels. 24 h after the reperfusion, the RNPN-treated mice show extremely low BUN and Cr levels as compared with both non-treated mice and low-molecular-weight TEMPO compounds (Figure 11). It is interesting to note that the therapeutic effect of RNPO’s is greater than that of RNPN, though the circulation of RNPO’s is much longer than that of RNPN. The fact strongly suggests that the disintegration of the RNPO’s improves its therapeutic efficiency. As shown in Figure 11C, hematoxylin and eosin (HE) staining reveals that tubular cell swelling, interstitial edema, and tubular dilatation are much more severe in the vehicle-treated IR mice than in the sham-operated mice. Compared to other treatments, the treatment with RNPN’s results in significantly less damage, consisting with low blood concentration of BUN and Cr.

The antioxidative effect of RNPs is confirmed on the basis of the detection of ROS and lipid peroxidation. As shown in Figure 12A & B, the
production of ROS in the kidney is significantly inhibited by the treatment with RNPNs, in comparison to other treatments. Compared with the other treatments, the treatment with RNPNs results in a significant reduction of the thiobarbituric acid reactive substance (TBARS) level, which serves as an index of lipid peroxidation in the kidney, and the generation of inflammatory cytokine in plasma, as can be seen in Figure 12C & D, respectively. In particular, the TBARS level of the RNPN-treated group is statistically lower than that of the other groups, as shown in Figure 12C. On the basis of these evidence, the prolonged retention of nitroxide radicals in the kidney might be an important factor in the protection of renal function after IR because excess ROS gradually continue to be generated after reperfusion [59]. In addition, the site-specific disintegration of RNPNs in the targeted organs would lead to improved therapeutic effect on Ischemia reperfusion – acute kidney injury, as compared with the case of RNPNs, regardless of longest distribution time of RNPNs in ischemic renal injury.

Though a mechanism is not clear, nitroxide radical compounds are known to show dose-related antihypertensive action accompanied by reflex tachycardia, increased skin temperature and seizures [60]. Hypotensive action would thus, lead to serious complications during treatment and surgery. For example, 4-amino-TEMPO and TEMPOL drastically decrease the mean arterial blood pressure as shown in Figure 13, which was measured 15 min after the intravenous administration of drugs. In contrast, both RNPNs inhibit any decrease in the arterial blood pressure. There are no significant differences between the non-treated, RNPN and RNPO groups. Although the reason for this observation is unknown, it is thought that the reduction in the arterial blood pressure after the administration of low-molecular-weight TEMPO derivatives is a consequence of an increase in the endogenous nitric oxide (NO), resulting in vasodilatation.

**Figure 11. Therapeutic effect of nitroxide radical-containing nanoparticles on ischemia reperfusion – acute kidney injury.** (A) BUN and (B) Cr in plasma of mice at 24 h after reperfusion following 50 min ischemia (values expressed as mean standard error *P < 0.0001 as compared with IR veh. **P < 0.005 as compared with IR veh. ***P < 0.05 as compared with IR veh. n = 7, ANOVA). (C) Representative photomicrographs (hematoxylin and eosin staining, magnification x200) of renal cortex of the kidneys in mice at 24 h after reperfusion following 50 min ischemia.

BUN: Blood urea nitrogen; IR: Ischemia reperfusion; IR/AT: 4-amino-TEMPO treated group; IR/HT: 4-hydroxy-TEMPO treated group; IR/RNPN: RNPN-treated group; IR/RNPO: RNPO-treated group; IR/Veh: Vehicle-treated group; RNPN: RNPs prepared from PEG-b-PMNT; RNPO: RNPs prepared from PEG-b-PMOT; Sham/Veh: Sham-operated and vehicle-treated group; TEMPO: 2,2,6,6-tetramethylpiperidine-1-oxyl.

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These results demonstrate that RNPs protect against blood-pressure fluctuations, because of the encapsulation of nitroxide radical moieties in the hydrophobic core of the nanoparticles in the bloodstream.

A similar effect has been confirmed in the case of cerebral ischemia reperfusion injury [63]. Intravenous administration of RNPs to transient middle-cerebral-artery-occlusion-model rats confirms that the infarct volume in the RNP group is significantly smaller than that in saline, micelle, and low-molecular-weight TEMPO groups (Figure 14A). Comparison of neurological symptom scores shows that the RNP group has a significantly lower score than the saline, micelle, and TEMPO groups (P<0.05, P<0.01 and P<0.05, respectively) as observed in Figure 14B. These data revealed that the RNPs could limit the extent of cerebral infarction caused by IR injury and improve neurological deficits.
Future perspective
Excessively generated ROS trigger unregulated oxidation of the living body and often cause various diseases, as stated above. The nitroxide radical works effectively as a catalytic redox reactant. Low-molecular-weight compounds, however, lead to several problems such as ther- apid metabolism, excretion and reduction of the compounds in living systems and triggering of side reactions. The confinement of nitroxide radicals in the core of the nanoparticle reduces its toxicity and improve the ease with which it can circulate with the blood in the blood stream. The disintegration of RNPs in acidic ischemia regions renders the RNPs an effective antioxidant spatio-temporally; the RNPs are inert when nonspecifically distributed to other areas. This is a promising strategy for the realization of a novel therapeutic system.

Because nanoparticles with high dispersion tend to permeate leaky neovascular regions, such as tumor and inflammation sites (the so-called EPR effect), they are thought to accumulate in these areas (passive targeting). Because the living body obtains energy via the mito- chondrial electron transport system, an anti-oxidative drug must not suppress this regular system. Because the nanoparticle distribution in vivo varies with the size of the nanoparticles, it is believed that they do not suppress the normal oxidation reaction in mitochondria. This may be one of the reasons for the extremely low toxicity of RNPs in vivo. Although it is clear that one of the reasons is the compart- mentalization of the redox center (nitroxide radicals) in the core of the nanoparticle, the controlled biodistribution of the RNPs might play an important role.

RNPs effectively scavenge unwanted ROS in the case of IR injuries without any significant side effect. They suppress the generation

Figure 13. The change in blood pressure at 15 min after the administration of drugs. Mean arterial pressures were measured at 15 min after bolus injection of RNPN, RNPO, 4-amino-TEMPO (AT) and TEMPOL (HT) in Institute of Cancer Research mice. Nitroxide radical concentration of all substances was 75 mmol/kg (Values expressed as mean ± standard error. *P < 0.05 as compared to PBS. n = 5, ANOVA).

PBS: Phosphate buffer saline; RNPN: RNPs prepared from PEG-b-PMNT; RNPO: RNPs prepared from PEG-b-PMOT TEMPO: 2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPOL: 4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl.


Figure 14. (A) Cerebral infarct volumes and (B) neurological symptom score in each groups. Infarct volume in RNP group was significantly smaller than that in saline, micelle and TEMPOL groups (A). Score of RNP group was significantly lower than that of other groups (B).

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RNP: Nitroxide radical-containing nanoparticles; RNPN: RNPs prepared from PEG-b-PMNT; TEMPOL: 4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl.
of inflammatory cytokines and quench ROS production. Similar observations have been made in the case of other disease models. RNPs suppress excessive inflammation after cerebral hemorrhage induced by focusing ultrasound beam radiation coupled with fluorocarbon microbubbles [64]. Considering both scavenging of ROS in penumbra areas and suppression of intracerebral hemorrhage, RNP nanotherapy is an ideal therapy for cerebral infarction. This redox nanotherapeutic strategy can be extended further to Alzheimer’s disease models in vitro [65]. Recently, the administration of RNPs to tumor-bearing mice was found to increase the effectiveness of the anticancer drug Adriamycin in subcutaneously inoculated cancer-bearing mice [66]. Since RNPs suppress the side reactions triggered by Adriamycin, especially in the heart and liver, they may be a suitable adjuvant in cancer therapy [67]. RNPs also improve the gene transfection efficiency associated with a polyplex system [68].

One of the potential research areas in redox nanotherapy is to find new routes for nanoparticle administration. If nanoparticles can be administered orally, they can be used for treating chronic diseases. Orally administered RNPs are captured by the colon mucous membrane without requiring their entry into the blood stream; this fact makes RNPs a promising candidate for the treatment of bowel inflammatory disease. In fact, RNPs have been found to effectively suppress inflammation in a DSS-induced ulcerative colitis mouse model [69]. Since RNPs do not enter the blood stream, there are no side reactions. Administration through nasal, pulmonary and other routes can help in the development of new redox nanotherapies, because oxidative stress causes many diseases.

On the basis of the obtained data, self-assembling redox nanoparticle, RNP, is confirmed to show high efficiencies to versatile disease, which is mainly accomplished by the delivery and on–off regulation by pH. Recently, it comes to be known that gaseous molecules such as carbon monoxide, nitrogen monoxide, hydrogen sulfide and hydrogen play important roles in vivo. These gaseous molecules were involved to versatile redox reactions in vivo to maintain or destruct homeostasis of living body. However, it is important to precisely control delivery of these gaseous molecules to specific region in order to utilize them for therapy because it has strong bioactivity. Several works on the control of confinement and controlled delivery of these gaseous molecules are reported. Hasegawa et al. reported carbon monoxide-releasing polymer micelle for immunotherapy [70]. We prepared a nanoparticle, which releases nitrogen oxide by photo irradiation [71]. Controlled delivery, generation and scavenging of these gaseous molecules including ROS are promising technology for next generation for radical delivery of nanotherapeutic systems.

Executive summary

- We have designed and developed nitroxide radical-containing polymers, which have the tendency of self-assembling and forming nanoparticles (nitroxide radical-containing nanoparticles [RNPs]).
- Scavenging of reactive oxygen species (ROS) by nitroxide radicals
  - Nitroxide radical reacts with ROS effectively, which can be regarded as a new drug for redox antioxidant. Nitroxide compounds possessing controlled reactivity can be synthesized chemically.
- Manufacture of radical-containing polymers and particles
  - Because many types of nitroxide compounds are very stable, it is easy to install versatile types of polymers.
- Nitroxide radicals in polymer
  - Because RNPs confine nitroxide radicals to their core, their antioxidative nature weakens significantly when they have a spherical form, the character of which reduces their toxicity and suppresses the side effects.
- Use of RNPs as a redox imaging tool
  - pH-responsive RNP (RNP9) disintegrates at pH below 7.0 to expose nitroxide radicals, which are monitored by both X- and L-band ESR spectra. Thus, it is anticipated as pH-dependent new ESR imaging tools.
- Effectiveness of RNPs for ischemia-reperfusion injuries
  - Since the nanoparticles also change its biodistribution, their characteristics vary significantly incomparisonto low-molecular-weight antioxidants. Long blood-circulation time improves their therapeutic efficiency. RNP9 effectively scavenge ROS in ischemia-reperfusion sites and can be utilized as a new redox nanomedicine. RNPs are also effective several disease models in animals such as, intracerebral hemorrhage, ulcerative colitis and cancer, thus promising as novel type redox nanomedicine.
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References
Papers of special note have been highlighted as:

* of interest

** of considerable interest


• Publication of therapeutic effect of RNP for cerebral ischemia-reperfusion injury.


• Publication of therapeutic effect of RNP for Alzheimer’s disease model in vitro


