Free Radical Research, November–December 2011; 45(11–12): 1325–1332

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ORIGINAL ARTICLE

A novel ascorbic acid-resistant nitroxide in fat emulsion is an efficient brain imaging probe for *in vivo* EPR imaging of mouse

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(Received date: 30 May 2011; Accepted date: 25 August 2011)

Abstract
The loss of paramagnetism of nitroxide radicals due to reductant reactions in biological systems, places a fundamental time constraint on their application as an imaging probe in *in vivo* EPR imaging studies. However, *in vitro* studies of the newly synthesized tetraethyl-substituted piperidine nitroxide radical demonstrated high resistivity to paramagnetic reduction when exposed to ascorbic acid, a common reduction agent in biological systems. In this work we investigated the use of these nitroxides as an imaging probe in EPR imaging of small rodents. 2,2,6,6-Tetraethyl-piperidine nitroxide (TEEPONE) is not highly soluble in aqueous media, thus a lipid-based emulsion system of lecithin was used to solubilize TEEPONE. The obtained solution was homogenous and with low viscosity, allowing smooth intravenous injection into mice tail vein. Acquired three dimensional (3D) EPR images of mouse head clearly showed TEEPONE distributed in all tissues including brain tissues, with an average measurable signal half-life of more than 80 min, thus demonstrating high resistivity to reduction due to ascorbic acid in *in vivo* animal studies, and the potential for use of this compound in *in vivo* studies of animal model systems.

Keywords: *EPR, imaging, nitroxide, ascorbic acid, in vivo, oxidative stress, redox status*

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Introduction

Nitroxides are stable, nontoxic free radicals, with a characteristic single unpaired electron. Due to the sensitivity of their electron paramagnetic resonance (EPR) spectra to their microenvironment and their molecular motion of enzymes, proteins and DNA, they have been utilized in many types of biological studies since the first demonstration in enzyme-substrate systems [1]. Nitroxide radicals are also widely used as versatile reporter probes, assessing oxygen concentration, reactive oxygen species (ROS) and redox status under oxidative stress *in vivo* [2,3].

In addition to many useful EPR studies with nitroxides *in vivo* and *ex vivo,* recently nitroxides have been reported as redox-sensitive contrast agents both in MRI and EPR imaging studies [4–7]. Since nitroxides have a single unpaired electron, they are capable of providing image contrast by shortening the relaxation time, T1, of water molecules, similar to gadolinium ions and manganese ions commonly used in MRI [9]. The redox-sensitivity of nitroxides means they can undergo oxidation by various oxidants, and can also be reduced to hydroxylamine by antioxidant materials. The reduction process of nitroxides leads to a decrease in their paramagnetic properties, resulting in observable changes in their EPR spectra and in T1 values of water molecules. However, the rapid decrease in paramagnetism due to reductants such as ascorbic acid (AsA) in biological systems, has until now limited their use as potential contrast agents in MRI. But with the development of novel piperidine nitroxide radicals with resistance to the reduction reaction by

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2011.618499

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bio-reductants such as AsA, there is a greater potential for their use as both imaging probes in EPR and contrast agents in MRI.

One potential method for increasing the resistance of nitroxides against reduction reaction by AsA is through the introduction of imidazole [10] and isoindoline [11] nitroxides with ethyl groups proximal to the N-O moiety. Kinoshita et al. [12–13] also reported the synthesis of tetraethyl-substituted nitroxide radicals, which exhibit remarkable resistivity toward AsA reduction during *in vitro* testing. Although the exact mechanism for this stability is not clearly understood, it could be explained by the steric hindrance the ethyl groups offer the N-O moiety. Recently, Yamasaki et al. described about the relationship between electrochemical properties and computed structural properties of nitroxides [14]. The introduction of four bulky ethyl groups into piperidine nitroxide compounds successfully provided increased resistivity toward AsA, however, the compound was found to have poor solubility in aqueous media. Its maximum concentration in saline was around 10 mM, and despite the introduction of a mixed solvent with organic solvent ethanol and DMSO, the concentration of the obtained solution remained too dilute for *in vivo* EPR imaging experiments of small animals.

In this study, we aimed to address this issue by developing two novel formulations of tetraethyl-substituted piperidine nitroxide derivatives using both emulsification and inclusion techniques, for intravenous (i.v.) injection in mice. The emulsification technique promotes solubilization through a lipid-based emulsion system [15], whereas the inclusion technique utilizes hydroxypropyl-β-cyclodextrin, allowing inclusion of intended hydrophobic nitroxide molecules within the center of cyclodextrin molecules [16]. After *in vivo* EPR imaging trials of mouse heads using nitroxide solutions prepared with both methods, we found the lipid-based emulsion system to be the most suitable, providing a high concentration, homogenous solution for tetraethyl-substituted nitroxide compounds with a viscosity that facilitates i.v. injection via tail. The acquired EPR images demonstrate that tetraethyl-substituted nitroxides can be successfully used as AsA-resistive, blood brain barrier permeable imaging probes, for *in vivo* imaging of mouse heads.

Materials and methods

Materials

3-Hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (HMP) was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). 3-Carboxy-2,2,5,5-tetrametyl-pyrrolidine- 1-oxyl (COP), and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl (carbamoyl-PROXYL) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid and hydroxylpropyl-β-cyclodextrin were purchased from Wako Pure Chemical Industries (Osaka, Japan). INTRAFAT INJ20% was from Takeda Pharmaceutical Co., Osaka, Japan. All nitroxide solutions were prepared in phosphate-buffered saline (PBS). Deionized water was used for all experiments. Other materials used in this work were of analytical grade.

Synthesis of 2,2,6,6-tetraethyl-4-piperidone-N-oxyl (TEEPONE)

TEEPONE was synthesized according to the previously reported method [12–13]. The molecular structure of TEEPONE is shown in Figure 1.

Formulation of TEEPONE as imaging probes

Two methods were employed to prepare aqueous solutions of TEEPONE: (i) lipid-based emulsion system, and (ii) inclusion system.

(i) Lipid-based emulsion system:
Two different methods were employed:

(a) Use of INTRAFAT: The nitroxide compound, TEEPONE, was directly dissolved in 20% fat emulsion, INTRAFAT. The mixture was stirred for more than 2 h at room temperature.
(b) Use of liposome: Liposome was prepared according to the general reported method [16–17], and nitroxide TEEPONE was added to the liposome system. Briefly, phosphatidylcholine and cholesterol were dissolved in chloroform/methanol (2/1) mixture, and nitroxide was added to the solution. The solvent was then evaporated under vacuum to form a lipid film. The film was hydrated with PBS, and the mixture was sonicated with Sonifier US-102 (SND Co. Ltd., Nagano, Japan).

(ii) Inclusion system
In this method, 0.5 mL of TEEPONE (5 mM in PBS) was added under vigorous stirring to 0.5 mL of a hydroxypropyl-β-cyclodextrin solution (60% w/v in PBS). For the preparation of higher concentration of TEEPONE, 50 mM nitroxide solution in 20% DMSO aqueous solution was added to hydroxypropyl-β-cyclodextrin solution (60% w/v in PBS).

Animals

The protocol for all animal experiments was approved by Sapporo Medical University Animal Care Committee according to the National Institutes of Health Animal Care and Use Protocol (NIH, Bethesda, MD, USA). The approved study number was 08-081. Nine male ICR mice (6 to 7 weeks of age, weighing 25 to

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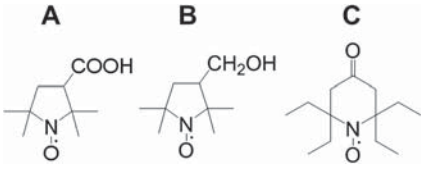

Figure 1. Molecular structure of nitroxide compounds used in this study. A: COP (3-Carboxy-2,2,5,5-tetrametyl-pyrrolidine- 1-oxyl), B: HMP (3-Hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl), C: TEEPONE.

28 g) were obtained from Japan SLC Inc. (Shizuoka, Japan), and were used for EPR imaging experiments. Mice were housed three per cage in climate-controlled, circadian rhythm-adjusted rooms, and were allowed access to food and water *ad libitum*. Three mice were used for *in vivo* EPR imaging experiments with each nitroxide (TEEPONE, HMP, or COP).

Reaction of nitroxide materials with AsA

The EPR spectra of nitroxide materials in PBS or INTRAFAT were recorded using an X-band ESR spectrometer (JEOL, Akishima, Japan). The solution of nitroxide (50 μM) and AsA (1 mM) was prepared and the signal intensity of the low-field peak was measured as a function of time. The experimental condition for X-band EPR spectrometer was used with the following parameters: microwave power 6 mW, modulation amplitude 0.06 mT, time constant 0.1 sec, scanning time 2 min.

EPR Spectrometer and Imager

An in-house built 750-MHz CW-EPR imager [18] was used in the animal experiments. A reflection-type microwave bridge was used with the microwave synthesizer (Agilent Technologies, N5181A, Santa Clara, CA, USA). A multi-coil parallel-gap resonator was used in the bridge [19]. The sample space was 22 mm in diameter and 30 mm in length. Data acquisition was controlled by a LabVIEW-based program on MacOS 10.3 and a PowerMac G5 computer. Based on the method of filtered back-projection, image reconstruction from EPR spectra was carried out with a PowerMac G5 computer.

In vivo EPR imaging of mouse heads

Mice were anesthetized with isoflurane (4%) in air at 250 mL/min, and were positioned onto the bed of the imager. The tail vein was cannulated with a 27G needle for the injection of nitroxides. A solution of nitroxide, 0.7 μmol/g of body weight, was injected by tail vein cannulation over 30 s. The body temperature of mice was 36.5 ± 0.5°C during the imaging experiments. The experimental condition for *in vivo* EPR imaging studies was used with the following parameters: field scanning 6 mT, field scan time 0.3 sec, microwave power 5 mW, modulation amplitude 0.2 mT, field gradient 0.6 mT/cm, number of projections 246.

MRI Scanner for Mice

MR images were acquired using MRmini (MR Technologies, Tsukuba, Japan) consisting of 0.5 Tesla permanent magnet and a solenoid MRI coil with an inner diameter of 22 mm. A spin-echo multi-slice T1-weighted sequence was used with the following parameters: repetition time (TR), 450 ms; echo time (TE), 12 ms; FOV, 60 mm × 30 mm; matrix, 256 × 128; number of excitations, 5; slice thickness, 1 mm.

Statistical Analysis

The statistical analysis was performed using the two-tailed *t*-test in GraphPad Prism (GraphPad Software, Inc., CA, USA). All data are presented as mean ± s.d. of 3 independent experiments. Significance was defined as $p < 0.05$.

Results

Lipid-Based Emulsion System

Obtaining sufficient signal intensity during *in vivo* scanning of mice with a 750 MHz 3D EPR imaging scanner typically requires 0.5 μmol of nitroxide compound per gram of mouse weight. To administer imaging probes intravenously to mice through a tail vein, the bolus volume of nitroxide solution should be less than 150 μL for adult mice. Therefore, the concentration of TEEPONE in the 150 μL solution should be high enough to deliver the required quantity of spin probes, as determined by the mouse weight. To fulfill this experimental requirement, we tried to develop the novel formulations of TEEPONE, which as previously mentioned, were both homogenous and low in viscosity, to facilitate i.v. injection via tail vein.

Lipid-based system

A lipid-based emulsion system using INTRAFAT was incorporated. TEEPONE was solubilized into the lipid emulsion, INTRAFT. The solution was found to be homogenous for at least a week after preparation, with a maximum concentration of TEEPONE of approximately 300 mM. A second method for lipid-based solubilization of TEEPONE using liposome was also tested. However, despite being satisfactory for i.v. injection, the homogeneity of the solution was found to deteriorate 2–4 h after preparation.

Inclusion system

The second novel formulation we tested was an inclusion system using hydroxypropyl-β-cyclodextrin.

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Using this method, a maximum concentration of approximately 50 mM of TEEPONE was achieved. However, the relatively high viscosity of the solution at this concentration prevented further efforts to obtain higher concentrations of TEEPONE, and was deemed to be unsuitable for i.v. injection.

Based on the observed homogeneity and viscosity of the developed solutions, the lipid-based emulsion system using INTRAFAT was chosen to be the most suitable method for the nitroxide carrier system throughout this study.

Reactivity of TEEPONE to Ascorbic Acid (AsA) in Lipid-based Emulsion System

Reactivity of TEEPONE to AsA was examined by X-band EPR spectroscopy both in a lipid-based emulsion system and PBS. Signal intensities of EPR spectra of TEEPONE were plotted as a function of time in the both systems after the addition of AsA (Figure 2). The signal intensity of TEEPONE remained at more than 92% of maximum signal intensity for up to 10 min in both systems (PBS or INTRAFAT) with AsA, demonstrating the high level of resistivity of TEEPONE to AsA. To examine whether the lipid-based emulsion system itself acted to change the resistivity of common nitroxide compounds toward AsA, a similar experiment was carried out using carbamoyl-PROXYL in both the emulsion and PBS systems. It is well known that carbamoyl-PROXYL has higher resistivity toward reduction reaction by AsA in PBS than most of piperidine nitroxyl radicals [20]. However, the observed EPR signal intensity time course of carbamoyl-PROXYL in the presence of AsA (Figure 2) shows that the signal decay of carbamoyl-PROXYL in the emulsion system was rather accelerated compared to the decay in PBS, and carbamoyl-PROXYL in the emulsion system was no more resistive to reduction by AsA. Table I summarized the pseudo-first-order rate constant of examined nitroxides, TEEPONE and carbamoyl-PROXYL, in both systems. These results indicate that the lipid-based emulsion system itself does not act to protect nitroxide compounds against the reduction reaction by AsA, and also that TEEPONE is still resistive to AsA in the emulsion system.

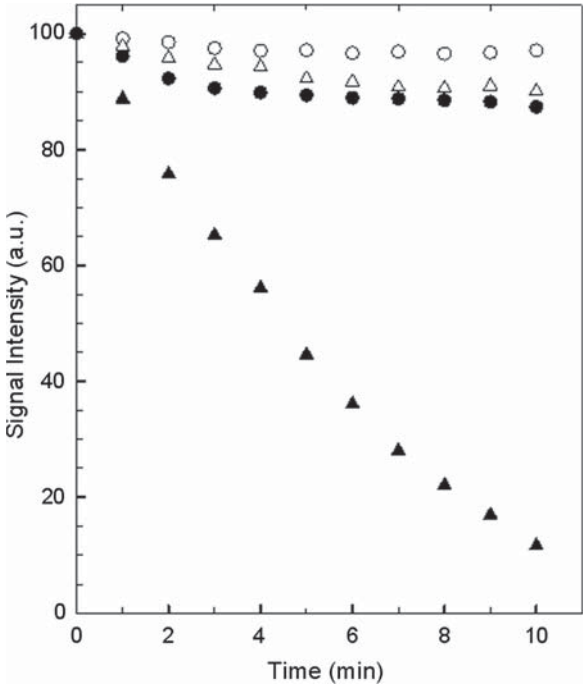

Figure 2. The changes in EPR signal intensities of nitroxide compounds in PBS and INTRAFAT system. Nitroxide materials (50 μM) were mixed with AsA (1 mM) in both PBS and INTRAFAT systems. EPR signal intensities of TEEPONE was measured by X-band EPR spectroscopy in PBS (△) and INTRAFAT (○). Carbamoyl-PROXYL was also measured in PBS (●) and INTRAFAT (▲).

In vivo EPR Imaging

To examine the potential for use of TEEPONE as an AsA-resistive spin probe for *in vivo* EPR imaging at 750 MHz, a solution of TEEPONE in the emulsion system was injected into mice via the tail vein. The signal intensities of EPR spectra of a mouse head doped with TEEPONE were followed time-dependently (Figure 3); with a maximum signal intensity recorded approximately 10 min after i.v. injection of TEEPONE, after which a very gradual decrease in signal intensity occurred, maintaining a measured intensity of 80% maximum up to one hour after i.v. injection. Further analysis of the averaged half-life of TEEPONE obtained from three mice was 81.1 ± 0.7 min. The high level of *in vivo* AsA resistivity observed with TEEPONE is far greater than the previously used hydroxyl-TEMPO spin probe [21].

In order to compare the differences in the distribution of injected nitroxides, EPR images of three different mouse heads were measured using different spin probes, HMP and COP. Figure 4A shows the mouse head orientation during imaging experiment, and Figure 4B shows axial (YZ plane) and sagittal (XY plane) MR images of an examined mouse head for anatomical reference. Figure 4C, D, E show 3D surface-rendered EPR images and 2D slice images of mouse heads 10 min after injection with the cell-impermeable nitroxide COP (C), cell-permeable nitroxide HMP (D), and TEEPONE (E), respectively.

Table I. Pseudo-first order decay rate constant (min^{-1}) of nitroxide compounds of the reaction with ascorbic acid both in PBS and INTRAFAT emulsion system.

Nitroxide compound	Pseudo-first order rate constant (min^{-1})
TEEPONE in PBS	5.7×10^{-3}
TEEPONE in INTRAFAT	1.4×10^{-2}
Carbamoyl-PROXYL in PBS	3.4×10^{-2}
Carbamoyl-PROXYL in INTRAFAT	2.1×10^{-1}

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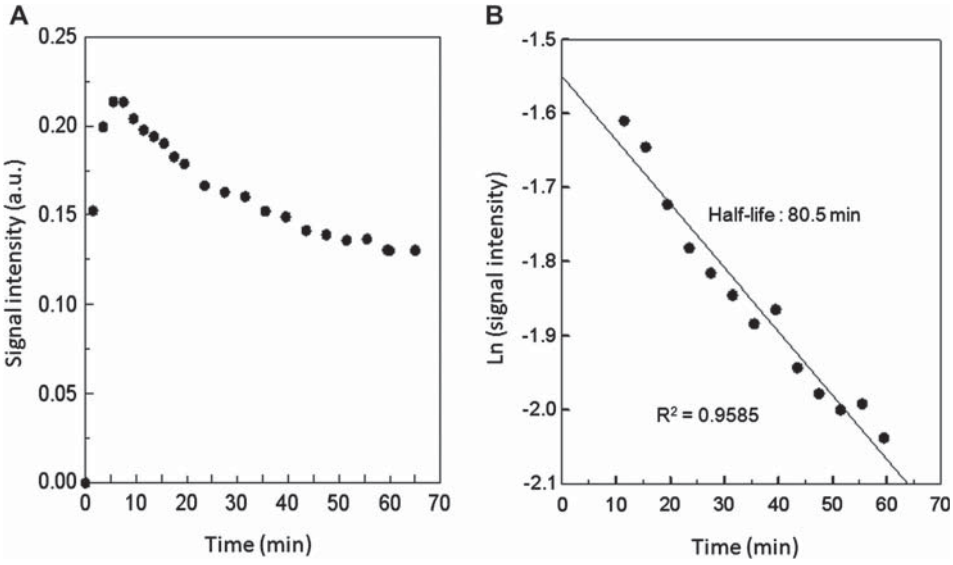

Figure 3. *In vivo* EPR signal of a mouse head after intravenous injection of TEEPONE. (A) The signal intensity of the center field resonance signal in the triplet TEEPONE signals was recorded time-dependently to follow TEEPONE decay *in vivo*. (B) The signal decay rate in a mouse head. The signal intensity of TEEPONE was plotted as a function of time, and the pseudo-first order rate constant and its half-life were obtained from the slope.

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With COP, some parts of the mouse head lacked nitroxide compounds (3D and 2D images in Figure 4C), but these parts were filled with blood brain barrier (BBB)-permeable nitroxide, HMP (Figure 4D). These results indicate that COP does not enter the mouse brain, which is further confirmed by MR images of mouse heads shown in Figure 4B. This is consistent with the previous studies [6,8,22] that HMP distributes in all regions of the mouse head but BBB-impermeable COP is distributed in only non-brain regions of mouse heads. When TEEPONE in INTRAFAT was intravenously injected, the obtained 3D and 2D EPR images were shown in Figure 4E, where the distribution of TEEPONE is the same as that of HMP but not COP. From these results, TEEPONE, like HMP, is distributed throughout the brain tissues and the entire mouse head regions.

A series of 3D surface-rendered EPR images of a mouse head were periodically acquired over a 40 min period after injection of TEEPONE, from which 2D slice images (XY plane) were reconstructed (Figure 5). By visual comparison of time-dependent 3D (Figure 5A) and 2D images (Figure 5B) acquired over 40 min after injection of TEEPONE, it is apparent that the first 3D and 2D images taken 10 min after injection of TEEPONE are remarkably similar to those at 40 min after injection of TEEPONE.

Based on the MRI anatomical information of the examined mouse shown in Figure 4B and 2D slice images obtained for BBB-impermeable COP in Figure 4C, the region of interest (ROI) corresponding to the mouse brain was selected from the 2D EPR images in Figure 5B, according to the previously reported method (8), and was highlighted as in the inset of Figure 6. The averaged image intensities within the selected ROI were obtained from four images shown in Figure 5B, and were summarized in Figure 6. In the same ROI, nitroxide signal was not detected in the case of the BBB-impermeable COP. The image intensity change for TEEPONE in Figure 6 shows that the image intensity remained 65.6% when the first image at 10 min was compared with that at 40 min after addition of TEEPONE, indicating that TEEPONE is stable within the brain tissues compared to other nitroxide compounds [6–8].

Discussion

Nitroxides are utilized as an imaging probe in EPR imaging studies assessing redox status, pO_2, pH and other physicochemical properties [2–3]. Previous research exploring the use of nitroxide compounds as T1 contrast agents in MRI found them to be less than optimal, due to their rapid *in vivo* reduction properties; a phenomenon which also places limitations on their use in *in vivo* EPR imaging studies. In an attempt to overcome this problem and to enable the use of a variety nitroxides as imaging probes in both EPR imaging and MRI, recent attempts have been made to develop nitroxide probes with different reactivity and improved resistivity to biological reductants such as AsA. As such, a number of techniques have been developed using liposome, polysaccharide and proteins to preserve the paramagnetic properties of nitroxides in biological systems, which are typically lost during the nitroxide reduction process [23–26]. Despite some successes, there remain a number of issues such as line-broadening, cell-permeability change and adsorption of examined nitroxides due to

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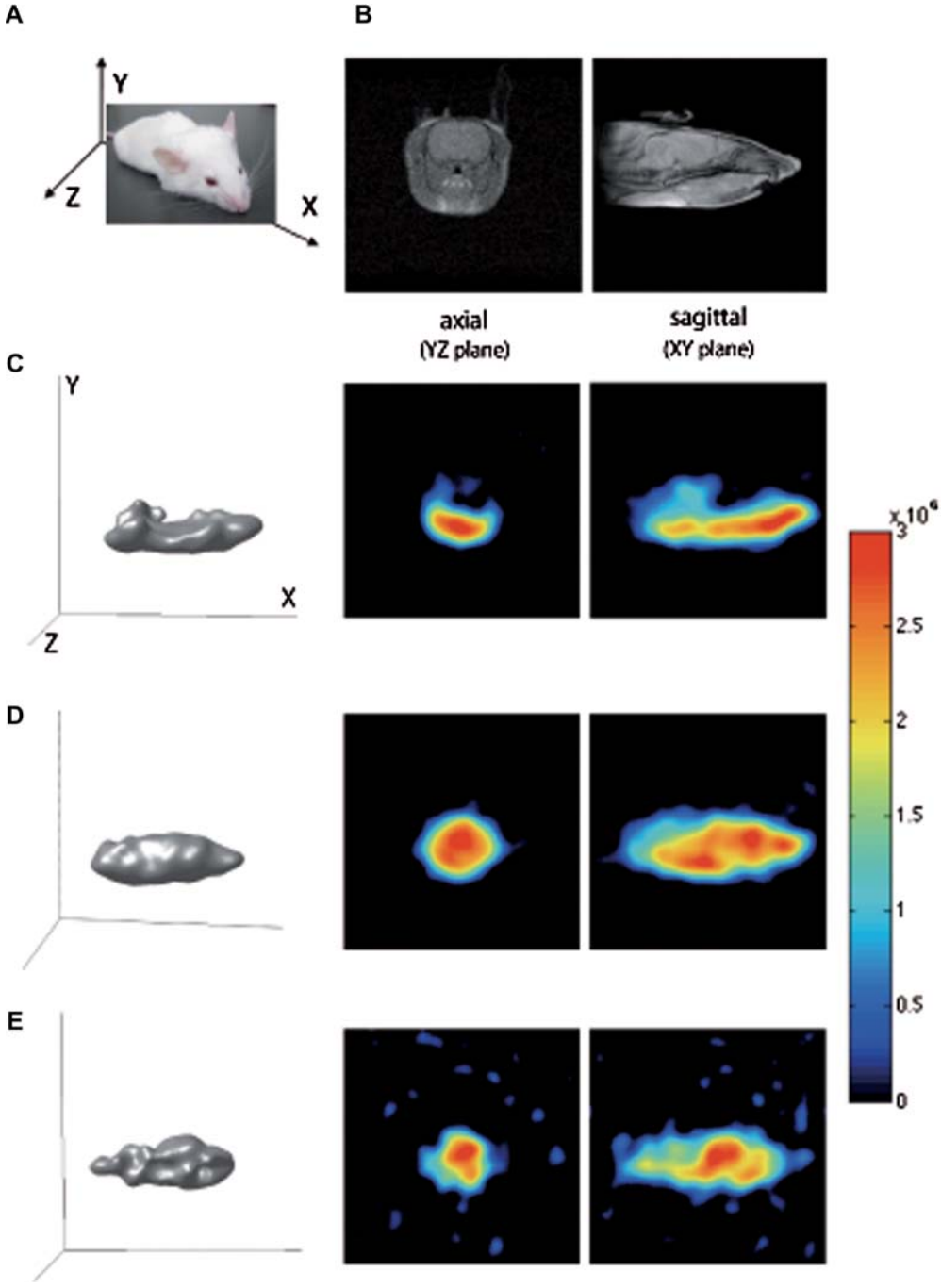

Figure 4. Distribution of TEEPONE in mouse head. A: photograph of a mouse head. B: T1-weighted MR image of a mouse head (left: axial direction, right: sagittal direction). C, D, E: three dimensional (3D) surfaced-rendered images and 2D slice images of a mouse head using cell-impermeable COP (C), cell-permeable HMP (D), and TEEPONE (E). 3D EPR images were obtained under field scanning of 0.3 s and 246 projections.

the modification of such nitroxide molecules, which need to be addressed.

To overcome such problems, and to preserve nitroxide paramagnetic properties in the presence of AsA *in vivo*, Kinoshita et al. developed tetraethyl-substitute piperidine nitroxides [12–13]. Recently, Yamasaki et al. reported that the reactivity toward AsA correlates with the redox potential of 2,5-substituted piperidine nitroxide's derivatives [14]. These nitroxide compounds display different reactivity toward AsA, depending on their chemical structures, and have the potential to be used as both antioxidant and contrast agents. When the tetraethyl-substitute piperidine nitroxide compounds are used as spin probes for *in vivo* EPR experiments, relatively high concentrations of nitroxide solution are required. Although TEEPONE has relatively poor solubility in aqueous media, the lipid-based emulsion system employed in this study demonstrated that it can be solubilized up to concentrations of around 300 mM. Additionally, this solution was found to be highly

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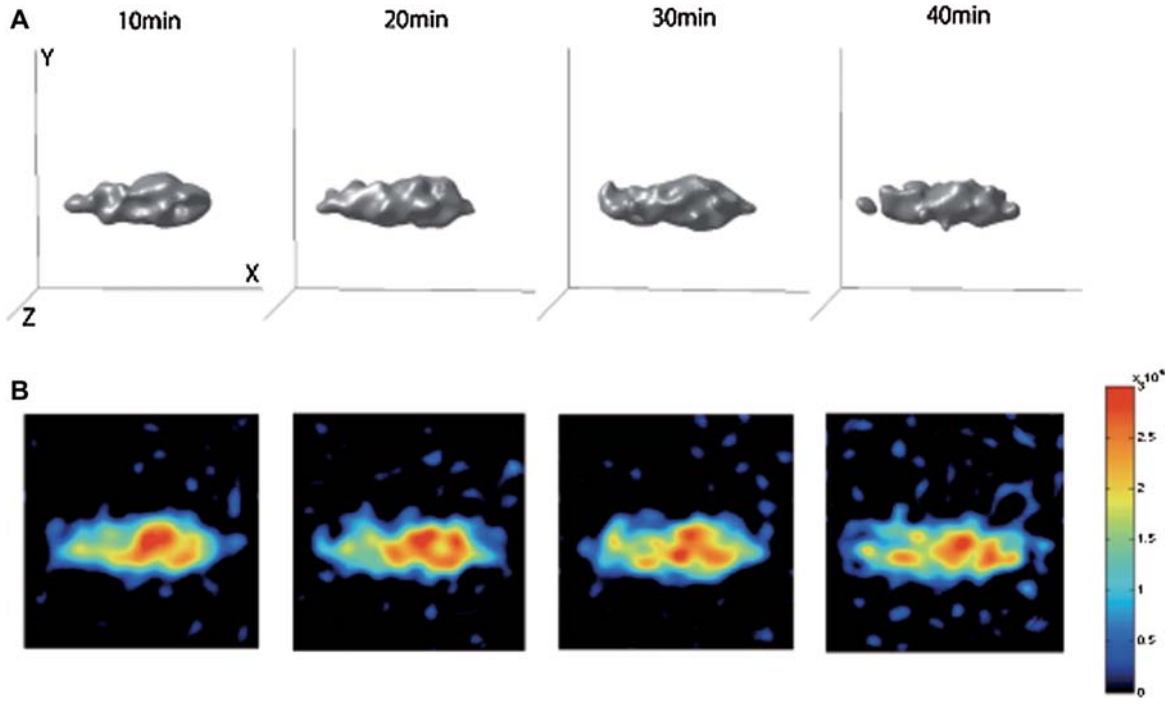

Figure 5. Temporal changes in 3D surface-rendered EPR images (A) and 2D slice images (B) of TEEPONE in mouse heads. EPR images were obtained at 10, 20, 30, 40 min after i.v. injection of TEEPONE.

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homogenous for periods up to at least a week, without any precipitation or separation of chemicals, and due to its low viscosity it was found to be ideal for i.v. injection to small animals.

AsA is recognized as the main antioxidant responsible for nitroxide reduction *in vivo*. This view was also supported by our experimental findings, whereby nitroxide reduction observed in healthy control mice was markedly suppressed when using AsA-resistive nitroxides, suggesting that AsA is primarily responsible for the reduction of administrated nitroxides in healthy animals. However there are a number of other antioxidants, perhaps most specifically glutathione, which despite not acting directly as a nitroxide reductant in *in vitro* systems, may indirectly contribute to nitroxide reduction in *in vivo* systems, particularly in disease states such as inflammation, ischemia and cancer, where the levels of *in vivo* antioxidant materials such as AsA and glutathione are known to change [6–8,27,28]. Therefore, by using AsA-resistive TEEPONE in disease model animals, it may be possible to clarify the importance of AsA *in vivo*.

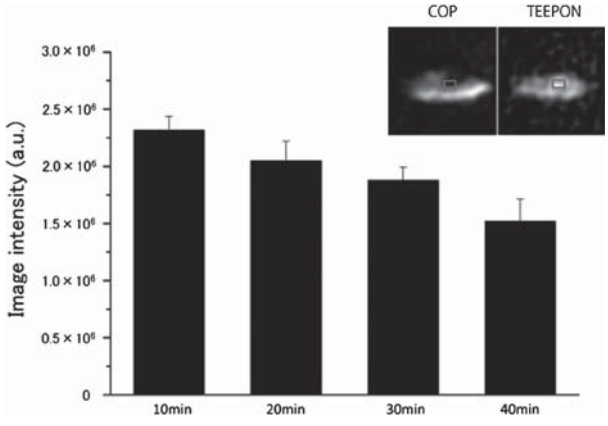

Figure 6. Time-dependent changes in the image intensities of TEEPONE in the brain tissues of mouse heads. Averaged image intensities in ROI selected in the brain tissues of mouse heads were shown as a function of time after i.v. injection of TEEPONE (n = 3).

In order to obtain tissue-specific information from nitroxide molecules distributed in biological specimens, both cell-permeable and cell-impermeable nitroxide compounds are required. In the case of brain tissues, BBB-permeable nitroxide compounds are needed. The previously developed BBB-permeable imaging probes and contrast agents, HMP and 3-methoxycarbonyl-2, 2,5,5-tetramethyl- pyrrolidine-1-oxyl (MC-PROXYL) were utilized in *in vivo* EPR imaging and MRI studies [6,8,29], but are susceptible to rapid reduction when exposed to AsA. However, as demonstrated in this EPR imaging study, TEEPONE is both a BBB-permeable and AsA-resistive imaging probe, which sustains its paramagnetic properties and therefore EPR signal intensity for considerably longer periods than the previously mentioned spin probes, and may therefore be a more effective MRI contrast agent than previously tested spin probes. In addition, the resistivity to AsA in TEEPONE can be modulated by replacing the alkyl group at 2,6-position of piperidine nitroxide [12–13], which would then make it feasible to evaluate and map the amount of ascorbic acid in brain tissues using these nitroxide compounds.

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Acknowledgements

We thank Yosuke Yamazaki for providing technical support and Dr. Jonathan Goodwin for a critical reading of the manuscript.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing the paper. The work was supported by a grant from the Japan Society for the Promotion of Science (19390322 to HF) and SENTAN, JST. HH was supported by NEXT program (LR002), Japan Society for the promotion of Science.

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This paper was first published online on Early Online on 29 September 2011.

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