A Novel Spin Probe with Long Life *in Vivo* for ESR Imaging

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A dextran-bonded nitroxide radical (TEMPO-DX) was synthesized to obtain a radical with long life *in vivo* for ESR imaging. TEMPO-DX was injected intravenously into a rat tail vein and the decrease in ESR intensity in the collected, circulating blood was followed. The result showed that the half life of TEMPO-DX *in vivo* was 30 min, the longest value reported so far and more than 30 times longer than the corresponding radicals of the six-membered piperidine ring, which means that the bonding of a radical to the polymer greatly prolonged life. The stabilities of TEMPO-DX against the reduction with L-ascorbic acid and the rat liver homogenate were also examined and compared with those of the 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxy (CPROXYL) known as a radical stable *in vivo*. TEMPO-DX was shown not to be as stable as CPROXYL, thus *in vivo* stability of TEMPO-DX arises from the fact that it is slowly absorbed into the tissues where the radicals are quenched. An ESR image of the mouse head domain was obtained only by an intravenous injection of TEMPO-DX solution into the tail vein.

Key words ESR imaging; dextran-bonded nitroxide radical; L-band ESR

Electron spin resonance (ESR) imaging to measure the spatial distribution of radicals was first performed by Ohno on an irradiated sample.^{1,2)} This method led to an interest in the radical distribution in living bodies as well as in organs where the radicals are metabolized. Berliner *et al.* succeeded in *in vivo* ESR imaging of a mouse in which melanoma cells had been implanted using an L-band ESR spectrometer, which enables measurement of a sample containing a large amount of water.³⁾ The sensitivity of the apparatus is not yet high enough to measure directly the signal of the radicals formed *in vivo*, therefore, the images are generally obtained after administering nitroxide radicals known as stable free radicals to a living body. In this case, the distribution of the radicals and the clearance as the result of the metabolism are of interest.

To obtain satisfactory ESR images, variation of the radical concentration must be kept at a minimum throughout the experiment, and this requires the use of radicals with long life *in vivo*. The lives of the nitroxide radicals examined to date, however, are not long enough *in vivo*, so that radicals must be continuously administered during the period of image measurement. For example, concentrated radical solution was injected intraperitoneally into a rat and the radicals were absorbed slowly from there and circulated in the blood vessel.⁴⁾ In this case, since absorption into the vein balances with the clearance, the concentration of the radicals was kept constant. With this method, however, large amounts of radicals are consequently absorbed and metabolized during the experiment, which poses a large burden on a living body and occasionally results in death. It, therefore, seems desirable to develop non-toxic radicals with long life *in vivo*. In this experiment, we selected polymer-bonded nitroxide radicals as an ESR imaging agent, expecting that the lives of these radicals would be prolonged *in vivo* by combining them with polymers.

MATERIALS AND METHODS

Synthesis of Dextran-Bonded Nitroxide Radical (TE-

MPO-DX) Dextran (M.W. = 100000—200000) was purchased from Wako Pure Chemicals. Spin labeling agent, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyloxy)-chloroacetamide (abbreviated Cl-TEMPO) was synthesized from 2,2,6,6-tetramethyl-4-aminopiperidine by the method reported by McConnell *et al.*⁵⁾ Six hundred mg of dextran was dissolved in 6 ml of anhydrous dimethylsulfoxide (DMSO). A concentrated aqueous NaOH solution containing NaOH molar equivalent to Cl-TEMPO was added to the solution and emulsified with a vortex-mixer. One hundred fifty mg of Cl-TEMPO was dissolved in 1 ml of DMSO and the solution was added to the dextran mixture. The mixture was stirred at room temperature for a day and then was dialyzed repeatedly with distilled water until no ESR signals of Cl-TEMPO were observed in the dialyzing water. Through this operation, other low molecular weight materials such as DMSO and NaOH were also eliminated. After the dialysis, the solution was freeze-dried and white cotton-like spin-labeled dextran (TEMPO-DX) was obtained. The structure of TEMPO-DX is shown in Fig. 1.

Measurement of the Stabilities against L-Ascorbic Acid (VC) and Tissue Homogenate The stabilities against the reduction with VC were examined as follows; TEMPO-DX and CPROXYL were dissolved in physiological salt solution (saline) to a concentration of 1×10^{-3} M, and VC was prepared as 1×10^{-1} M solution. The radical and the VC solutions were mixed rapidly in the cavity using syringes and the ESR intensity change was followed. The liver and kidney were homogenized as 10% (w/v) saline solution and stored by cooling with ice throughout the experiment. TEMPO-DX or CPROXYL solutions were mixed with the same volume of homogenate solutions and put into capillary tubes for ESR measurements. The effect of the storage of the homogenate solutions was checked by repeating the ESR measurements, and it was found that the storage had not affected the reactivities of the homogenates toward the two radicals.

Measurements of the Spectra ESR spectra were measured by a JEOL RE3X spectrometer (X-band), and

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Fig. 1. Structures of the Radicals Used for *in Vivo* ESR Study

TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy; A-TEMPO, 4-amino-2,2,6,6-tetramethylpiperidinyloxy; PCA, 3-carboxy-2,2,5,5-tetramethylpyrrolidin-1-yloxy; CPROXYL, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxy; TEMPO-DX, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)acetamidyl-dextran.

the two-dimensional ESR image was calculated from eighteen L-band spectra which were measured from each 10 degree step direction under a field gradient of 1 mT/cm, microwave frequency to 0.7 GHz, and magnetic field scanning range of 15 mT, using a home-made spectrometer. The spectral deconvolution was performed in Fourier space and the reconstruction of the two-dimensional image was carried out by filtered back projection.⁶⁾

RESULTS AND DISCUSSION

The combination of nitroxide radicals and polysaccharides, namely spin-labeling of carbohydrates, has rarely been reported⁷⁾ except for a few cases.⁸⁾ This is because polysaccharides do not have the reactive amino, thiol and carboxyl groups generally found in proteins, and this fact makes the combining of polysaccharide with the radicals difficult. We tried the spin-labeling of dextran with Cl-TEMPO. The problems of this synthesis are,

- 1) Cl-TEMPO is usually used as a spin-labeling agent for a thiol group and its reactivity toward a hydroxyl group is low; dextran, in particular, has no primary hydroxyl groups, so a strong base is required for the elimination of HCl.

- 2) If the reaction is carried out with dissolved dextran in the aqueous solution, most of the Cl-TEMPO is hydrolyzed.

We found that DMSO is a good aprotic organic solvent to dissolve dextran and Cl-TEMPO, and that the condensation reaction between them was carried out efficiently using an emulsion of the concentrated aqueous NaOH solution as the base, as shown in Materials and Methods section.

The nitroxide radicals shown in Fig. 1 have been tried for use as imaging agents. Couet *et al.* showed that the stability of the five-membered pyrrolidine ring against the

reduction with ascorbic acid was larger than that of the six-membered piperidine ring.⁹⁾ The stabilities of the pyrrolidine ring toward the liver and the kidney homogenates of a rat were also larger than those of the piperidine ring. Chen and Swartz examined the reduction of the nitroxide radicals to the corresponding hydroxylamines with mouse thymus-bone marrow cells and revealed that the stability of the pyrrolidine ring is large than that of the piperidine ring.¹⁰⁾

When the radicals are used as an ESR imaging agent and injected into the whole body, the rate of clearance from various tissues becomes important. Couet *et al.* reported that PCA has low toxicity, and that the half life of the metabolism was 22 min in dogs.¹¹⁾ Ishida *et al.*¹²⁾ and Tsuchihashi¹³⁾ measured the half lives of a few nitroxide radicals in rats by measuring the L-band ESR intensity of the head domain after intravenously administering radicals dissolved in saline solution into the tail vein. The half lives of TEMPOL and A-TEMPO were less than 1 min, but that of CPROXYL which possessed pyrrolidine ring was 7–8 min. On the other hand, Utsumi *et al.*¹⁴⁾ examined the spin clearance of CPROXYL and TEMPOL administered into the tail vein in hepatic and bladder regions of mice. The results obtained were almost the same as those reported by Ishida *et al.*¹²⁾ and Basic *et al.*¹⁵⁾ Sotgiu and his colleagues determined the half life of PCA administered to a mouse was 14 min.¹⁶⁾ According to these reports, the half lives of the nitroxide radicals in living bodies depend on the kind of animal as well as the structure of the radical, but they seem to be around 10 min even for stable radicals such as CPROXYL or PCA when rats and mice are used.

Polymeric drugs synthesized by combining low molecular weight drugs with polymers have been used for drug delivery systems.¹⁷⁾ For example, mitomycin C known as an anti-cancer drug was covalently bonded to dextran through a spacer.¹⁸⁾ This mitomycin C–dextran

complex was shown to retain its high concentration in the tumor, compared with the case of non-treated mitomycin C. We used polymer-bonded nitroxide radicals for ESR imaging, expecting that the life of the radicals *in vivo* would be prolonged by combination with the polymer, and also by its characteristic distribution in the living body. Dextran is a natural polysaccharide composed of D-glucose with mainly $\alpha(1,6)$ bonds but branching is observed with $\alpha(1,3)$ bonds. This polymer is non-toxic, biodegradable, and non-accumulative, and is used popularly as a blood

substitute. Therefore, dextran seems to be one of the most bio-adaptable polymers and usable for an ESR imaging agent.

Figure 2 shows the ESR spectra of TEMPO-DX in the saline solution and in blood after administration to a rat, as well as the spectrum of the aqueous Cl-TEMPO solution. The lines, especially the high-field ones, of the triplet spectra of TEMPO-DX became broader than those of Cl-TEMPO in the aqueous solution, showing that the mobility of the radical was largely diminished in TEMPO-DX as a result of the bonding with the polymer. By comparing ESR intensities and the molarities of these solutions, the ratio of the bonded TEMPO radical to the glucose residues could be calculated. It was found that one radical was attached to every 44 sugar residues on average.

The saline solution of TEMPO-DX was injected intravenously into the rat tail vein, then a medical silicon tube was cannulated into the jugular vein and blood was drawn from there at arbitrary time intervals. ESR intensity of the collected blood was measured as a function of time after collection was carried out. The intensity at the time of the collection was obtained by extrapolating the values to time zero. This value must be same as the intensity in the circulating blood at that time. Thus, it is able to know the rate of decrease in ESR intensity in the living body after intravenous injection. The results are shown in Fig. 3. At first glance, it is noticed that the life of TEMPO-DX is very long in the collected blood, whereas it is far shorter in the circulating blood. The same phenomenon was found earlier by Utsumi *et al.*¹⁴⁾ This is explained by the idea that the clearance occurs in other tissues rather than in the blood. The half life of TEMPO-DX in the living body is about 30 min, which is the longest value reported so far and is more than 30 times longer than those of the radicals possessing the same piperidine ring; this confirms that the life was prolonged by bonding the radical with the polymer.

The stability against the reduction with VC is shown in Fig. 4. TEMPO-DX is far weaker than CPROXYL, though *in vivo* stability of TEMPO-DX is about three times greater than that of CPROXYL. By comparing

Fig. 2. X-Band ESR Spectra of the Aqueous Cl-TEMPO Solution (A), Saline Solution of TEMPO-DX (B), TEMPO-DX in the Collected Blood after Administration to a Rat (C), L-Band Spectra of TEMPO-DX in the Mouse Head Region Measured by Applying Homogeneous Magnetic Field (D), and Field Gradient (1 mT/cm) (E)

Fig. 3. ESR Intensity Changes in the Blood Collected at 0.5 min (A), 20 min (B), 40 min (C) and 80 min (D) after Injecting 1 ml of Saline TEMPO-DX Solution (20 wt%) into a Rat Tail Vein

Solid line indicates the intensity change in the circulating blood (see text).

Fig. 4. ESR Intensity Change of TEMPO-DX and CPROXYL after Mixing with VC Solution

Fig. 5. ESR Intensity Change of TEMPO-DX, TEMPOL and CPROXYL after Mixing with Liver Homogenate

Fig. 6. ESR Intensity Change of TEMPO-DX, TEMPOL and CPROXYL after Mixing with Kidney Homogenate

Fig. 7. ESR Image of the Mouse Head Region Measured after Injecting 1 ml of 20% TEMPO-DX Solution

this result with that reported by Couet *et al.*,⁹⁾ it is seen that the stability of the piperidine ring radical was not changed by the combination with dextran in contrast to the *in vivo* case.

Figure 5 shows the stabilities of TEMPO-DX and CPROXYL and TEMPOL against the liver homogenate. The stability of TEMPO-DX is close to that of TEMPOL and is also far smaller than that of CPROXYL as in the case of the reduction with VC. Figure 6 shows their stabilities against the kidney homogenate. The result was almost same as that of liver homogenate.

From these results, we concluded that the prolongation of the *in vivo* life of TEMPO-DX was not achieved by the chemical stabilization, but by the prolonged capture of the radical with the tissues. In other words, combination with dextran causes the radical to escape from the tissues which charges clearance.

An ESR image of the mouse head region could be obtained only by an injection of TEMPO-DX saline solution into the tail vein as expected. The image is shown in Fig. 7.

These results and discussion have shown that polymer-bonded nitroxide radicals seem promising as ESR imaging agents. In this work, piperidine ring radical was bonded to the polymer. It is anticipated to use chemically more stable pyrrolidine ring radicals which would enable to prolong more the *in vivo* life. However, 3-amino-2,2,5,5-tetramethylpyrrolidin-1-yloxy (3-amino-PROXYL) for the synthesis is not commercially available. In addition, other polymers may be more useful than dextran. Examination of these points remains for the future.

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