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# Design of Polymeric Micelle with Stable Radicals in the Core from Acetal-Poly(ethylene glycol)-*b*-poly(chloromethylstyrene)

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Utilizing self-assembling core-shell type polymeric micelle technique, high-performance nanosphere possessing stable radicals in the core and reactive groups on the periphery was prepared. An anionic ring opening polymerization of ethylene oxide (EO) was carried out using potassium 3,3-diethoxypropanolate as an initiator, followed by the mesylation with methanesulfonyl chloride to obtain an acetal-poly(ethylene glycol)-methanesulfonate (acetal-PEG-Ms) **(1)**. **1** was reacted with potassium *O*-ethyldithiocarbonate, followed by the treatment with n-propylamine to obtain heterobifunctional PEG derivatives containing both mercapto and acetal terminal groups (acetal-PEG-SH) **(2)**. A poly(ethylene glycol)-*block*-poly(chloromethylstyrene) (acetal-PEG-*b*-PCMS) **(3)** was synthesized by free radical telomerization of chloromethylstyrene (CMS) using **2** as a telogen. The chloromethyl groups in the PCMS segment of the block copolymer **(3)** were quantitatively converted to 2,2,6,6-tetramethylpiperidinyloxys (TEMPOs) via an amination reaction of **3** with 4-amino-TEMPO in DMSO **(4)**. The obtained **4** formed a core-shell type nanosphere in aqueous media by a dialysis method, the cumulant average diameter of which was ca. 40 nm and the nanospheres showed intense electron spin resonance (ESR) signals. The nanospheres showed a reduction resistance of TEMPO radical even in the presence of 10 mM glutathione (GSH) and will be expected as high performance bionanospheres.

Key word: acetal-PEG-*b*-PCMS, free radical telomerization, polymeric micelle, ESR, bioimaging

## INTRODUCTION

Recently, in vivo bioimaging have attracted much attention in the field of bio-related science and technology such as biotechnology, medical therapy and diagnostics.[1-6] Among these bioimaging systems, magnetic resonance imaging (MRI) is one of the powerful tools to visualize specific tissue, e.g., cancer, though it is not perfect. The intensity and resolution are dependent on the accumulation time. A few acquisition counts observe small signal.[1-2] In order to improve both sensitivity and resolution, numerous efforts have been paid. $^{19}F$ probe is one of the ideas to improve the performances because of no endogenous signal *in vivo*, high signal intensity, etc.[7-8] Electron spin resonance (ESR) is known to have $10^3$ times higher sensitivity than that of nuclear magnetic resonance (NMR). Recently, study on the ESR imaging is thus increasing by several groups.[3-4] Using stable radical species such as 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) as a probe, ESR imaging is investigated. Under the reducing conditions in the body, however, such radical species are easily reduced.[3-4]

To solve these issues, we started to create a novel nanospheric ESR probe, which is stable under reducing *in vivo* conditions (Figure 1). This paper communicates synthesis, preparation and characteristics of a core-shell type nanosphere possessing stable TEMPO radicals in the hydrophobic core and reactive acetal groups on the periphery.

## EXPERIMENTAL SECTION

**Materials.** 3,3-Diethoxypropanol (Aldrich Chemical Co. INC., U.S.A.), tetrahydrofuran (THF), benzene (reagent grade; Kanto Chemical Co., INC., Tokyo, Japan) and ethylene oxide (EO) (100 %; Sumitomo Seika Chemicals Co. LTD., Hyogo, Japan) were purified conventionally.[9] A THF solution of potassium naphthalene was prepared according to the previous report.[10] 2,2'-Azobisisobutyronitrile (AIBN) (Kanto Chemical Co. INC., Tokyo, Japan) was purified by recrystallization form methanol. Chloromethylstyrene (CMS) (Seimi Chemical Co. LTD., Kanagawa, Japan) was purified by silica gel column to remove inhibitor , followed by the vacuum distillation under nitrogen atmosphere. 4-Amino-2,2,6,6-tetramethylpiperidinyloxy (4-amino-TEMPO) (Aldrich Chemical Co. INC., U.S.A.), 2-propanol, diethyl ether, potasium *O*-ethyldithiocarbonate, methanesulfonyl chloride, triethylamine, glutathione (GSH) (reduced form), dimethyl sulfoxide (DMSO) and N,N-dimethylformamide (DMF) (Kanto Chemical Co. INC., Tokyo, Japan) were used without further purification.

**Synthesis of acetal-PEG-SH (2).** According to our previous report,[11] PEG possessing an acetal group at α-chain end was prepared by anionic ring opening

polymerization of ethylene oxide using potassium 3,3-diethoxypropanolate (PDP) as an initiator as follows: a polymerization was performed in a 100-mL round-bottomed flask with a three-way stopcock. An inside of the reactor was degassed sufficiently and filled with nitrogen gas. The degassing-$N_2$-purge cycle was repeated three times. The dry THF (30 mL), 3,3-diethoxypropanol (2 mmol) and potassium naphthalene (2 mmol) were added to the flask to form potassium 3,3-diethoxypropanolate (PDP). After stirring for 10 min, liquid EO (209 mmol; cooled below 0 °C) was added via a cooled syringe to the mixture. The mixture was allowed to react for 2 d at room temperature. The alkoxide group at the ω-chain end was converted to the methanesulfonate group by the addition of an excess amount of methanesulfonyl chloride and triethylamine as follows: 30 mmol (4.18 mL) of triethylamine and 24 mmol (1.85 mL) of methanesulfonyl chloride were added to the mixture and stirred for 6 h. The polymer was recovered by precipitation into 1 L of cold 2-propanol (-15 °C), centrifuged for 30 min at 5,000 rpm (4,600 g) and then freeze-dried with benzene. The yield of the obtained polymer **(1)** was 97.8 % (9.0 g).

To convert the methanesulfonate group to the *O*-ethyldithiocarbonate group, **1** was reacted with potassium *O*-ethyldithiocarbonate in a dry THF/DMF (30:10) cosolvent (40 mL) as follows: to 9.0 g of the obtained dry **1** in THF (20 mL), the 8-fold amount of potassium *O*-ethyldithiocarbonate in THF/DMF (20 mL/10 mL) was added and stirred for 3 h at room temperature. The reaction mixture was then mixed with chloroform and washed with a saturated NaCl aqueous solution several times to eliminate impurities from the polymer sample. The organic phase was then concentrated by evaporation after drying with sodium sulfate. The polymer was recovered by precipitation into 1 L of cold 2-propanol (-15 °C), centrifuged for 30 min at 5,000 rpm (4,600 g) and then freeze-dried with benzene. The yield of the obtained polymer was 83.8 % (7.54 g).

To generate the mercapto group through the deprotection of the dithiocarbonate-end group, 7.0 g of the acetal-PEG-ethyldithiocarbonate was treated with 38 mmol (1.48 mL) of n-propylamine in THF (30 mL). The mixture was stirred for 12 h at room temperature. The polymer was recovered by precipitation into 1 L of cold 2-propanol (-15 °C), centrifuged for 30 min at 5,000 rpm (4,600 g) and then freeze-dried with benzene. The yield of the obtained polymer was 99.0 % (6.9 g). The obtained polymer contained the dimmer (acetal-PEG-S-S-PEG-acetal) due to the oxidation of mercapto groups. To reduce the disulfide of the dimer, 6.9 g of the obtained polymer was treated with 14 mmol (2.1 g) of 1,4-dithiothreitol (DTT) in THF (50 mL). The polymer was recovered by precipitation into 1 L of cold 2-propanol (-15 °C), centrifuged for 30 min at 5,000 rpm (4,600 g) and then freeze-dried with benzene. The yield of the obtained polymer **(2)** was 99.0 % (6.8 g).

$^1$H NMR ($CDCl_3$) δ 1.20 (t, 6H, C***H***$_3$CH$_2$O-), 1.89 (q, 6H, acetal-CH-C***H***$_2$-CH$_2$-O-), 2.70 (q, 2H, PEG-O-CH$_2$-C***H***$_2$-SH), 3.71 (s, 417H, PEG backbone), 4.64 (t, 1H, acetal-C***H***-CH$_2$-).

**Synthesis of acetal-PEG-*b*-PCMS (3).** After 0.02 mmol (92 mg) of acetal-PEG-SH **(2)** (Mn = 4,600) was weighed into the flask, the inside of the reactor was degassed sufficiently and filled with nitrogen gas. The degassing-$N_2$-purge cycle was repeated three times. One millimole (0.138 mL) of CMS, 0.01 mmol of AIBN and benzene (1 mL) were then added to the flask. A polymerization was conducted for 24 h at 60 °C in an oil bath. The reaction mixture was precipitated in hexane, followed by freeze-drying with benzene. The obtained polymer was washed with diethyl ether three times in order to eliminate PCMS homopolymer, followed by freeze-drying with benzene. The yield of the obtained polymer **(3)** was 55.1 % (134 mg).

**Preparation of acetal-PEG-*b*-PCMS containing TEMPO moieties (4).** After 5.2 μmol (40 mg) of the obtained acetal-PEG-*b*-PCMS **(3)** (Mn = 7,900) was weighed into a 10-mL flask, DMSO solution (2 mL) of 4-amino-TEMPO (520 μmol, 88 mg) was added to the flask and stirred for 5 h at room temperature. The polymer was recovered by precipitation into 10 mL of cold 2-propanol (-15 °C), centrifuged for 30 min at 5,000 rpm (4,500 g) and then freeze-dried with benzene. The yield of the obtained copolymer **(4)** was 74.6 % (44 mg).

**Preparation of the micelles by the copolymer (4).** According to the procedure reported previously, polymeric micelle by **4** was prepared.[12] Briefly, 20 mg of **4** was dissolved in 2 mL of DMF, and the polymer solution was transferred into a pre-swollen membrane tube (Spectra/Por molecular weight cutoff size 3,500), dialyzed against water for 24 h by changing water at 2, 5 and 8 h passage.

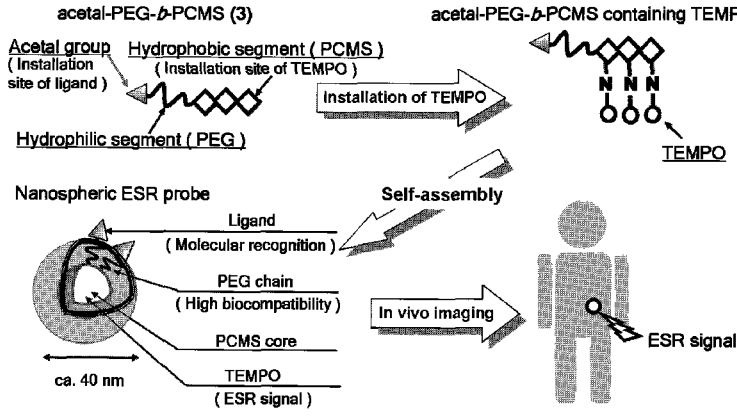
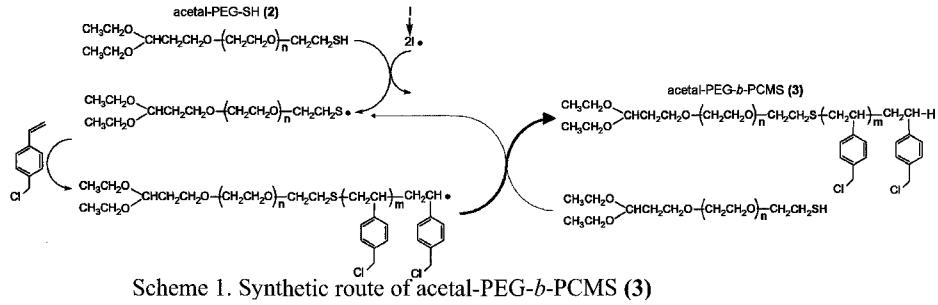



Figure 1. Schematic illustration of the nanospheric ESR probe based on the acetal-PEG-*b*-PCMS containing TEMPO moieties **(4)**




Scheme 1. Synthetic route of acetal-PEG-*b*-PCMS (**3**)

**Reduction resistance of TEMPO radical in the presence of GSH.** To evaluate the reduction resistance of TEMPO radical of the micelles by **4**, 64 μM of the micelle solutions in 900 mM of Britton-Robinson buffer at pH 7.2 were prepared, followed by measurement of the ESR spectra of the prepared micelle. After the addition of 10 mM GSH to the solution of the micelles, the mixture was stirred for 1h, followed by measurement of the ESR spectra of the solution. A free TEMPO was used as control.

**Instrument.** Size exclusion chromatography (SEC) measurements were carried out using a TOSOH HLC-8120 equipped with TSK gel columns (Super HZ4000 and Super HZ3000) and an internal refractive index (RI) and an ultraviolet (UV) detectors. THF with 5 % (v/v) triethylamine was used as eluent at a flow rate of 0.35 mL $min^{-1}$ at 40 °C. The $^1$H NMR spectra were obtained using chloroform-d with a JEOL EX270 spectrometer at 270 MHz. A light scattering spectrophotometer (Nano ZS (ZEN3600, Malvern Instruments, Ltd., UK)) equipped with a He-Ne laser that produces vertically polarized incident beams at a detection angle of 90 °at 25 °C was used in the present study for dynamic light scattering (DLS) measurements. ESR spectra were obtained using a Bruker EMX-T ESR spectrometer.

## RESULTS AND DISCUSSION

**Synthesis of acetal-PEG-*b*-PCMS (3).** Mercapto group is known to have a very high chain transfer constant in free radical polymerization reactions. If our synthesized acetal-PEG-SH (**2**) acts as a telogen without any side reaction, a novel polymeric design will be feasible. For example, if a telomerization of hydrophobic monomer can be done by **2**, new types of amphiphilic block copolymer will be synthesized. For the demonstration of radical telomerizations, CMS was employed because the anticipated **3** possesses high potential for modification of PCMS segment. A synthetic route to **3** is described in Scheme 1. Prior to the radical telomerization, **2** was synthesized, according to the experiment procedures. The total yield was 74.0 % (6.9 g). From a result of SEC of **2** thus synthesized, the Mn and the molecular weight distribution (MWD) were determined to be 4,600 and 1.04, respectively. The functionality of acetal and mercapto group at both ends was found to be 100 % and 95 %, respectively, which was determined by the $^1$H NMR spectroscopy based on the MW of PEG determined by the SEC. Utilizing the obtained **2**, radical telomerization of CMS was examined. Though a methine of an acetal group is known to be highly active toward radical attack, the radical

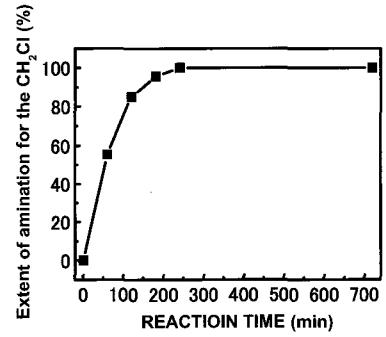



Figure 2. Reaction profile of 4-amino-TEMPO to the chloromethyl groups in the PCMS segment of the block copolymer (**3**) in DMSO. (temperature = 23 °C, number of scan = 64)

telomerization proceeded smoothly without any gel formation. The SEC profile of the reaction mixture after polymerization, however, showed bimodal distributions. In order to obtain information of the products, their $^1$H NMR spectra were measured after the two peaks were separated by SEC fractionation. From the $^1$H NMR analysis, the ratios of PEG vs. PCMS of the two fractions were the same. Based on the MW of **2** determined by SEC, the MWs of the PCMS segment for the two fractions were determined to be 3,300 and 6,600, respectively. Only different molecular weight with the same composition of two products means that the two products were formed by different termination reactions, *viz.*, disproportionation and recombination reactions. Thus, the products were mixture of diblock and triblock copolymers, possessing the same compositions.

**Synthesis of acetal-PEG-*b*-PCMS containing TEMPO moieties (4).** The chloromethyl groups in the PCMS segment of the block copolymer (**3**) were converted to stable radicals via an amination reaction of **3** with 4-amino-TEMPO in DMSO. The amination reaction proceeded smoothly in a homogeneous system. Figure 2 shows the extent of amination of the chloromethyl groups as a function of time, which was determined by the ratio of methylene of the chloromethyl group in the CMS unit vs. methylene in the EO unit using $^1$H NMR spectrum. As shown in Figure 2, the extent of amination of the chloromethyl groups of PCMS segment in **3** attained to 100 % for 5 h. The introduction of the TEMPOs in the side chain of the PCMS segment was confirmed by the $^1$H NMR spectrum after the TEMPO radicals were reduced by phenylhydrazine. After the reducing reaction, new singlet signal was clearly observed, which was attributable to the tetramethyl protons of TEMPO, while the signal for methylene of the chloromethyl group in the

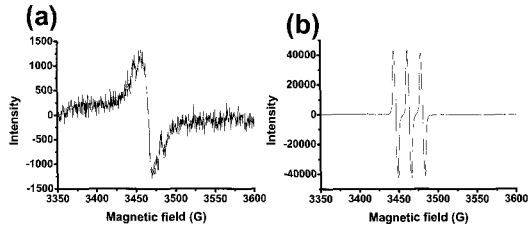



Figure 3. ESR spectra at pH 7.2. (a) micelles by the copolymer **(4)** (polymer concentration: 64 μM) (b) free TEMPO (concentration: 1.4 mM)

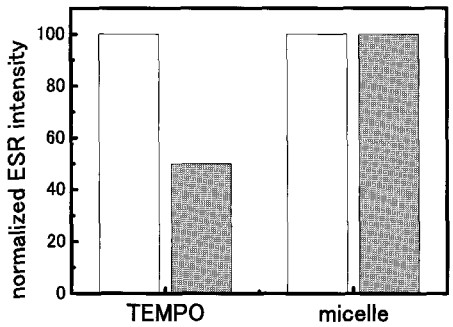



Figure 4. ESR signal intensities in the presence (□) and absence (■) of glutathione (10 mM). Left: TEMPO, Right: TEMPO in the core of the micelle

CMS unit was completely disappeared. Note that all their peaks including the end acetal methane proton at 1.2 ppm and 4.6 ppm remained intact. On the basis of these results, it was confirmed that the TEMPOs were successfully introduced to the polymer without any side reaction. Purification of **4** was carried out by SEC fractionation after crude polymer was obtained by precipitation. The complete removal of the unreacted 4-amino-TEMPO was confirmed by the ESR measurements of each fraction.

**Characterization of the micelles by the copolymer (4).** For the preparation of polymeric micelle, a dialysis method was employed. **4** was dissolved in a good solvent for both segments such as DMF and then dialyzed against water. The size of the obtained polmeric micelle was estimated by the DLS measurements. The micelles thus obtained possess unimodal distribution in histogram analysis. The average diameter and polydispersity factor determined by a cumulant method were ca. 40 nm and 0.228, respectively. The ESR signals of the obtained micelle were then analyzed by ESR measurements. Figure 3a shows the ESR spectrum of the micelles. Contrary to the free TEMPO signal shown in Figure 3b, which is clear triplet signal owing to an interaction between $^{14}N$ nuclei and unpaired electron in dilute solution, the micelle signal became broaden. The broaden ESR signal of the micelle is attributable to the shorten relaxation time ($T_2$) due to the restricted mobility of the TEMPO radicals incorporated in the solid core of the micelle.

**Reduction resistance of TEMPO radical in the presence of GSH.** The glutathione (GSH) exists in mammalian cells at the millimolar level (0.5 mM~10 mM), while the concentration in blood is 0.5 μM~10 μM. GSH acts as the most prevalent intracellular thiol, exhibiting strong reducing capability. Thus, TEMPO possessing a stable radical is known to be easily reduced to 2,2,6,6-tetramethylpiperidin-1-ol under reducing *in vivo* condition and rapidly disappear ESR signals. If our designed and prepared nanosphere maintain ESR signal under reducing *in vivo* condition for a long term due to a compartmentalized TEMPO in the core of the micelle, a novel design of ESR probe for a long-term measurement will be feasible. Figure 4 shows the reduction resistance of the TEMPO radical against GSH. When the free TEMPO solution was stirred in the presence of 10 mM GSH, the signal intensity decreased to half only for 1h. On the contrary, the ESR signal intensity in our prepared nanosphere was retained intact 1h after the addition of GSH as shown in Figure 4, indicating that our prepared nanosphere showed the reduction resistance of the TEMPO radical even in the presence of 10 mM GSH.

## CONCLUSIONS

In conclusion, acetal-PEG-*b*-PCMS **(3)** was synthesized by free radical telomerization of CMS using acetal-PEG-SH **(2)** as a telogen, followed by the conversion of the chloromethyl groups in the PCMS segment of the block copolymer **(3)** to stable radicals via an amination reaction of **3** with 4-amino-TEMPO. The high-performance nanosphere possessing stable radicals in the core and reactive groups on the peripheriy was prepared using acetal-PEG-*b*-PCMS containing TEMPO moieties **(4)** and showed intense ESR signal. The nanosphere showed the reduction resistance of TEMPO radical even in the presence of 10 mM GSH and will be expected as a high performance bionanosphere.

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