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Catechol-bearing block copolymer micelles: Structural characterization and antioxidant activity

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ABSTRACT

Oxidative stress caused by uncontrolled production of reactive oxygen species (ROS) has been linked to the initiation and progression of some diseases. Therefore, the use of antioxidants capable of scavenging ROS has attracted growing interest. We recently reported antioxidant micelles having catechol moieties, a structural motif found among natural antioxidants. These micelles showed a strong inhibitory effect in ROS-mediated angiogenesis as compared to the small catecholic compound dopamine. Here, we aim to explain this interesting function of the micelles by correlating the effects of self-assembled structures on the auto-oxidation stability and anti-angiogenic activity. The micelles were prepared from a hydrophilic poly(*N*-acryloyl morpholine) and a hydrophobic catechol-bearing block with different catechol content. The micelles with higher catechol content formed stable spherical micelles and showed increased stability against auto-oxidation, while micellization did not affect the redox potential of catechol moieties. Furthermore, the micelles with higher catechol content showed a stronger anti-angiogenic activity.

1. Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), are constantly produced as a consequence of aerobic metabolism in the human body [1,2]. Despite their important roles in cellular metabolism and signal transduction, uncontrolled production of ROS induces tissue damage and inflammation, which is often associated with the initiation and progression of cancer and inflammatory diseases [3,4]. Therefore, scavenging of endogenous ROS is of interest for treating these conditions.

Polyphenols, a class of compounds having multiple phenolic hydroxyl groups, are among the most abundant antioxidants found in foods and beverages [5]. These compounds are known to scavenge ROS and thereby prevent cellular damage in plants. Inspired by their protective role against oxidative stress, their effects on human health have attracted growing attention. Recent reports showed that these compounds have preventive effects against oxidative stress-related diseases, particularly cardiovascular diseases and cancer [6,7]. Although polyphenols showed promise in treatment of some diseases, their application has been limited due to low oxidation stability and poorly controlled pharmacokinetics in the human body.

We recently reported a polymeric micelle bearing catechol moieties, one of the common structural units in natural polyphenols [8]. We prepared block copolymers by reversible addition fragmentation (RAFT) polymerization [9] of a protected glycine monomer using a poly(ethylene)glycol (PEG) polymer as a RAFT agent. After deprotection of the carboxylic acid groups and dopamine conjugation these block copolymers self-assembled to form micelles. These micelles showed high oxidation stability under aerobic conditions compared to the low molecular weight catecholic compound, dopamine (DA) [10,11]. Furthermore, the micelles exerted anti-angiogenic activity by scavenging endogenous ROS in endothelial cells.

In this study, we explore the mechanism of the enhanced oxidation stability and anti-angiogenic activity of the catechol-bearing micelles. We synthesized amphiphilic catechol-bearing block copolymers with poly(N-acryloyl morpholine) (PAM) instead of PEG as the hydrophilic block. We prepared polymers with different catechol content that formed micelles by self-assembly. These micelles were characterized by dynamic light

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scattering (DLS), atomic force microscopy (AFM), transmission electron microscopy (TEM) and size exclusion chromatography-low angle laser light scattering (SEC-LALS). The oxidation stability, electrochemical properties as well as their inhibitory effects on tube formation in endothelial cells were evaluated to elucidate the effects of micellar structures on the antioxidant activity.

2. Experimental

2.1. Materials

N-acryloyl morpholine, 2,2′-azobis(2-methylpropionitrile) (AIBN), *N*-hydroxysuccinimide (NHS), *N*,*N*'-dicyclohexylcarbodiimide (DCC), dopamine hydrochloride (DA·HCl), diethyl ether (Et_2O), ninhydrin, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-dimethylaminopyridine (DMAP) and 1-(4,5-dimethyl-2-thiazolyl)-3,5-diphenylformazan (MTT) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Dichloromethane (CH_2Cl_2), triethylamine (TEA), *N*,*N*-dimethylformamide (DMF), 1,4-dioxane, methanol (MeOH), calcium hydride, acetate buffer (0.1 M, pH 5.0) and phosphate buffer (0.1 M, pH 7.0) were purchased from Nacalai Tesque (Kyoto, Japan). $CDCl_3$ and d_6-DMSO were purchased from Cambridge Isotope Laboratories (Massachusetts, USA). Aluminum oxide, 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid, polyethylene oxide (PEG) standard and 1-ethylpiperidine hypophosphite (EPHP) were purchased from Sigma–Aldrich (Missouri, USA). Amplex red assay kit, calcein AM and Hank's balanced salt solution (HBSS) were purchased from Life Technologies Corporation (California, USA). Normal human umbilical vein endothelial cells (HUVECs) and EBM-2 medium containing 2% FBS were purchased from Lonza (New Jersey, USA). Growth factor-reduced (GFR) Matrigel was purchased from BD Biosciences (California, USA). Slide-A-lyzer G2 (MWCO 20 kDa) was purchased from Thermo Fisher Scientific. 96 well plates were purchased from Iwaki (Tokyo, Japan). μ-Slide Angiogenesis was purchased from Ibidi (Martinsried, Germany). *N*-acryloyl morpholine was passed through plug of aluminum oxide to remove inhibitor. AIBN was recrystallized from MeOH and stored at −20 °C. TEA was distilled over ninhydrin. 1,4-dioxane was distilled over calcium hydride under argon. *N*-acryloyl glycine *tert*-butyl ester was synthesized as reported previously [8].

2.2. Instrumentation

2.2.1. ^1H NMR

Spectra were acquired on a Bruker DPX400 NMR spectrometer at room temperature with the residual undeuterated solvent signal as reference (2.50 d_6-DMSO and 7.26 $CDCl_3$).

2.2.2. Size exclusion chromatography (SEC)

Elution profiles of the polymers (10 mg/mL) were collected on a Shodex KD-803 column at 50 °C equipped with a Tosoh differential refractometer and photodiode array detector. Dimethylformamide (DMF) containing 100 mM LiCl (flow rate of 1.0 mL/min) was used as the eluant. The polydispersity index (PDI) of the polymers was calculated based on the elution time of polyethylene glycol standard polymers.

2.2.3. Dynamic light scattering (DLS)

Hydrodynamic diameter of the micelles were measured on an Otsuka ELSZ machine. For the micelles the mean diameter (Z-average of D_h) and polydispersity index (PDI $= \mu_2/\Gamma^2$) were calculated by the cumulant method. For PAM-PDA_{18}, D_h was calculated by the CONTIN method.

2.2.4. Size exclusion chromatography-low angle light scattering (SEC-LALS)

The micelles were dispersed in acetate buffer (0.1 M, pH 5.0) at 1.0 mg/mL and filtered through a cellulose acetate syringe filter (pore size: 0.45 μm). The sample (100 μL) was analyzed on a Tosoh chromatography system connected to a Malvern Viscotek 305 TDA. A TSK gel G4000SWXL (Tosoh) was used as a SEC column. Acetate buffer (0.1 M, pH 5.0) was used as an eluant at a flow rate of 1.0 mL/min. The average molar mass of the micelles was determined using OmniSEC software.

2.2.5. Transmission electron microscopy (TEM)

Carbon coated 250 mesh copper grids were prepared under glow discharge. The micelle solution was placed onto the grid and then dried by blotting the side of the grid with a filter paper. The grids were negatively stained with 2 wt% sodium phosphotungstate solution. Images were acquired on a HITACHI H-7700 TEM.

2.2.6. Atomic force microscope (AFM)

The micelles were adsorbed onto a fresh mica surface and air dried. Images were acquired on a Seiko SPA400 in dynamic mode using a Si probe (SI-DF20, Seiko).

2.2.7. Critical micelle concentration (CMC) measurement

The micelle solution in milliQ water at different concentrations (2 μL) was dropped onto Parafilm and the contact angle was measured on a Kyowa Interface Science Drop Master DM 300 to determine the critical micelle concentration.

2.2.8. UV–VIS and fluorescence spectrometry

Spectra were obtained on a Nanodrop instrument or a Tecan infinite M200 well plate reader using black polystyrene well plates.

2.2.9. Cyclic voltammetry (CV)

DA and the PAM-PDA micelles were dissolved in 0.5 mL water at 2.0 mM of catechol moieties and mixed with 9.5 mL acetate buffer (0.1 M, pH 5.0). The solutions were degassed by bubbling argon and kept at 25 °C using a thermostatic bath. Cyclic voltammograms of these solutions were recorded on an ALS 600E electrochemical analyzer equipped with a CS-3A Cell Stand and a three-electrode set up: an Ag/AgCl/saturated KCl reference electrode, a glassy carbon (0.19 cm^2) working electrode and a platinum wire counter electrode. The scan rate was 100 mV/s.

2.2.10. Fluorescence microscopy

Images were collected on an Olympus BX51WI fluorescence microscope.

2.3. Synthesis of poly(N-acryloyl glycine tert-butyl ester) (PAG, 3)

N-acryloyl glycine *tert*-butyl ester (926 mg, 5 mmol), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (2) (36.3 mg, 0.1 mmol) and AIBN (3.29 mg, 0.02 mmol) were placed in a Schlenk tube and dissolved in 1,4-dioxane (final volume: 2.5 mL). Oxygen was removed from the solution by five freeze–pump–thaw cycles using argon and the solution was heated at 70 °C for 24 h. The polymerization was stopped by placing the Schlenk tube in liquid nitrogen and opening to the air. After warming the mixture to RT, the resulting polymer was precipitated in *n*-hexane (1000 mL) to yield a yellow solid (924.3 mg, 96%). The degree of polymerization was calculated from the ^1H NMR spectrum using the integral values of the $-C_{11}H_{22}-C\underline{H}_3$ and ($-C\underline{H}_2-C\underline{H}$ + $-C-$ ($C\underline{H}_3$)$_3$) signals (Figure S1 in Ref. [12]).