Molecular Design of Cytocompatible Amphiphilic Redox-active Polymers for Efficient Extracellular Electron Transfer

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Abstract

For electrochemical regulations of the intracellular metabolisms, lipophilic electron mediators with cell membrane permeability have been conventionally used. We have recently developed amphiphilic, cell-membrane permeable polymer composed of hydrophilic 2-methacryloyloxyethyl phosphorylcholine and hydrophobic redox-active units as a new category of electron mediator. The advantage of the redox active polymer is that we can obtain appropriate molecules in a synthetic bottom-up manner. Here we report that the rate of the extracellular electron transfer (EET) through the redox active polymer can be regulated by sophisticated molecular design of the polymers. It was also shown that the cellular metabolism of yeast *Saccharomyces cerevisiae* was regulated by using the polymer with the highest EET rate.

Keywords

extracellular electron transfer, electron mediator, polymer, metabolism

1. Introduction

Cellular metabolism consists of numerous biological redox reactions. It is widely known that the intracellular redox state in living cells has a critical role for their metabolism [1–6]. For example, high mitochondrial NADH/NAD⁺ ratio in yeast cells is known to trigger ethanol production [7]. Moreover, it was suggested that the intracellular NADH/NAD⁺ ratio increases with aging, which could cause age-related metabolic diseases [8]. Thus, the development of techniques for regulating the intracellular redox state are demanded in various fields such as bio-energy and medical treatments. As redox reactions can be controlled by altering the electrochemical conditions, a number of studies have examined the electrochemical regulation of cellular metabolism using lipophilic electron

mediators with cell membrane permeability [9–12]. However, lipophilic electron mediators generally exhibit low water solubility and high cytotoxicity [13–15]. These disadvantageous factors limit the potential of lipophilic electron mediators for use in living systems, even when combined with water-soluble electron mediators in double-mediator systems [11,15,16]. To overcome the limitations associated with conventional electron mediator systems, we recently synthesized a cytocompatible redox-active polymer (Schematic 1, hereafter called pMFC) that is able to cross bacterial cell membranes and exhibits cytocompatibility [17].

pMFC is an amphiphilic, cell-membrane permeable polymer composed of hydrophilic 2-methacryloyloxyethyl phosphorylcholine (MPC) [18] and hydrophobic vinyl ferrocene (VFc), which has electron-mediating functions attributable to the $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox couple of the ferrocene unit (Schematic 1). The MPC-based amphiphilic polymers, including pMFC, possess several advantageous characteristics originating from the unique properties of the MPC unit. First, MPC is able to solubilize large amounts of hydrophobic units, such as VFc and thereby enhance electron flux to/from electrodes. Second, MPC-copolymers have low cytotoxicity due to their protein adsorption-resistant property [19,20]. Finally, the redox potential of these polymers can be tuned based on the selection of the redox-active units. Due to these unique properties, pMFC was successfully used for the electrochemical regulation and detection of the cyanobacterial circadian clock [21,22] and the electrochemical enhancement of bacterial polyhydroxybutyrate production [23]. Although pMFC has the potential to be applied in various bioelectrochemical technologies in the fields of bio-energy and medical care, that concerning with cell metabolism, further improvement of the controllability of electron transportation by this polymer is required.

2. Material and methods

2.1. Synthesis and characterization of pMFCs

MPC was synthesized by NOF Co., Ltd., (Tokyo, Japan). VFc and α , α' -azobisisobutyronitrile (AIBN) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan) and Kanto Chemical Co., Inc. (Tokyo, Japan), respectively. The other organic reagents and solvents used in this study were commercially available reagents of extra-pure grade and were used without further purification. pMFC was synthesized using MPC and VFc by free radical polymerization with AIBN as an initiator (total monomer concentration: 1.0 mol/L). Polymerization was conducted in a test tube at 60 °C for 48 h under the argon gas atmosphere. After the reaction the polymer solution was precipitated to a mixed solvent composed of diethyl ether/ chloroform (90/10, v/v). The precipitation of polymer was filtered and dried in vacuum for 10 min. The polymer was then dissolved into distilled water and dialysis was performed for 4 days. The polymers were then freeze-dried and the resulting yellow powder were used for experiments. The molecular weight of polymers was measured by gel permeation chromatography in a mixed solution of water and methanol (30/70, v/v) containing 10 mM lithium bromide. The surface tension of each polymer was measured in distilled water using the Wilhermy method with Sigma701 (Biolin Scientific, Sweden) and a platinum rod at room temperature. The molecular properties of the synthesized pMFCs (pMFC-1, pMFC-2, and

pMFC-3) are summarized in Table 1.

2.2. Culture conditions and medium

Escherichia coli K12 was aerobically pre-cultured in Luria–Bertani (LB) medium with shaking at 37 °C. *Saccharomyces cerevisiae* was aerobically pre-cultured in YPDA medium with shaking at 30 °C. YPDA medium was composed of 20 g glucose, 20 g Bacto peptone, 10 g Bacto yeast extract, and 40 mg adenine (per liter). Defined medium for *E. coli* was composed of 7 g K₂HPO₄, 3 g KH₂PO₄, 1 g (NH₄)₂SO₄, 0.1 g MgSO₄·7H₂O, 10 g NaCl, and 0.1 g Bacto yeast extract (per liter). Synthetic defined (SD) medium for *S. cerevisiae* was composed of 6.7 g Difco yeast nitrogen base w/o amino acids, 6.8 g KH₂PO₄, 40 mg adenine sulfate, 20 mg uracil, 40 mg l-tryptophan, 30 mg l-leucine, 20 mg l-histidine, 20 mg l-lysine, and 33.3 mg l-tyrosine (per liter). The pH of SD medium was adjusted to 6.0 by 1 mol/L NaOH.

2.3. Electrochemical experiments

A single-chamber, three-electrode system was used for all electrochemical measurements. The system was equipped with a glassy carbon (GC) electrode (3 cm²) at the bottom of the reactor as the working electrode, and Ag/AgCl (saturated KCl) and platinum wire were used as the reference and counter electrodes, respectively. To conduct electrochemical measurements, 4 mL of the specific medium used for each microbe was supplemented with glucose (20 mM for *E. coli* and 50 mM for *S. cerevisiae*) and pMFC, and was then injected into the system to give a final VFc concentration of 2 mM. Oxygen was removed from the system by nitrogen purging for 15 min. *E. coli* and *S. cerevisiae* cells were collected by centrifugation for 10 min at 6000 × g, washed three times with the specific medium used for each microbe, then injected into the system. The optical density at 600 nm (OD₆₀₀) of microbes in the medium was adjusted to 1.0 for all measurements, which were conducted without agitation at 37°C for *E. coli* and at 30°C for *S. cerevisiae*.

2.4. Effects of EET on metabolism

S. cerevisiae was inoculated (initial OD₇₃₀ = 0.01) into a glass vial (2 mL) containing SD medium supplemented with and without oxidized forms of pMFC. The pMFC concentration was adjusted to give a final VFc concentration of 4 mM. The medium was electrochemically oxidized at +0.6 V (vs. SHE) for 2 days, and was then supplemented with 20 mM glucose. The vial (2 mL) was tightly sealed with an aluminum cap and was then purged with nitrogen for 15 min to remove oxygen. *S. cerevisiae* was cultivated anaerobically at 30 °C without shaking for 48 h. Cell growth was quantified by measuring the OD₇₃₀. The concentrations of ethanol in the medium were determined using F-kits for ethanol (J.K. International).

3. Results and Discussion

3.1. Characterization of pMFcs

As the interior region of the phospholipid bilayer membrane is hydrophobic, hydrophilic molecules do not readily cross cell membranes [24–26]. Due to this property, an electron mediator must have sufficient hydrophobicity to permeate cell membranes [15,27]. However, because highly hydrophobic molecules have the potential to become trapped in the cell membrane, electron mediators require moderate amphiphilicity to effectively permeate the cell membrane. Theoretical calculations have also revealed that there is an optimized ratio between hydrophilic and hydrophobic units (R) for maximizing cell membrane permeability [25,26]. Cell membrane permeability has also been predicted to increase with decreasing molecular weight (M_w) [26]. Based on the theoretical calculations, we synthesized pMFC polymers with three different R values (the ratio of VFc in the pMFC polymer) (Table 1).

Measurements of the surface tension of the three pMFC solutions in distilled water revealed that the surface tension decreased with increasing concentration of pMFC polymers. The surface tension of pMFC-2 and pMFC-3 remained constant at concentrations over 0.5 mg/mL, whereas the value of pMFC-1 didn't reach to a constant value (Fig. 1). These results indicated that pMFC-2 and pMFC-3 formed stable aggregates in aqueous solution at concentrations above 0.5 mg/mL, a property that is critical for cell membrane permeability [24]. In addition, it was confirmed that as R increased, the surface tension decreased. Thus, pMFC-2,3, which had lower surface tension, would be expected to have the higher cell membrane permeability.

3.2. Investigation of relationship between the properties of pMFC and EET rates.

For extracellular electron transfer (EET) reactions via small lipophilic molecules, the penetration of cell membranes is known to be a step determining the EET rate [28,29]. First, we tried to verify whether this is also the case for the pMFC-mediated EET. As shown in Fig. 2, the EET current for *E. coli* at a poised potential of +0.6 V (vs. SHE), at which pMFC can be electrochemically oxidized and accept intracellular electrons, was increased by the addition of ethylene diamine tetra acetic acid (EDTA) that is known to be a reagent for increasing the cell-membrane permeability of gram-negative bacteria including *E. coli*. Notably, EDTA did not affect the redox activity of pMFC (Fig. S1) and such increase was not observed in the absence of pMFC (Fig. S2). These results clearly indicated that cell membrane permeability also strongly influences the pMFC-mediated EET rate.

We next evaluated the effects of three pMFC polymers on EET in solutions of *E. coli* and *S. cerevisiae* cells, which were cultured using glucose as the sole carbon and electron source. The different pMFC polymers were added to the electrolyte solution at concentrations that equivalent to 2 mM VFc to exclude the possibility that differences in the VFc concentration were responsible for variation in the EET current. The electrochemical behaviors of pMFcs were investigated by CV in media used for electrochemical cultivation of *E. coli* and *S. cerevisiae* (Fig. 3a). The decreased peak intensity in the CVs of pMFC-2,3 compared to pMFC-1 (about 20 %) can be attributed to the more stable aggregate formation and lower accessibility of VFc units to electrodes of pMFC-2,3. Representative EET current

curves for *E. coli* at a poised electrode potential of +0.6 V are shown in Fig. 3b. After the *E. coli* cells were injected into the electrochemical chamber (arrow 1), the EET current gradually and monotonically increased with time and became saturated at approximately 15~20 h. Such a current was not observed in the absence of pMFC (Fig. S3) or microorganisms [17], indicating the EET was mediated by pMFC. The EET current increased in the order of pMFC-1 < pMFC-2 < pMFC-3, although pMFC-2 and -3 had lower reactivity against electrodes than pMFC-1, as shown in Fig. 3a. These results indicated that cell membrane permeability was more critical for determining pMFC-mediated EET current, compared with the reactivity against electrodes. Comparisons of the EET current profiles in solutions supplemented with pMFC-1 and pMFC-2 indicated that R is a more critical parameter than M_w for determining EET rate. However, comparison of the EET current profiles associated with pMFC-2 and pMFC-3 suggested that the EET rate increases with decreasing M_w at similar R values.

3.3. Metabolic modifications in *S. cerevisiae* induced by EET using different pMFCs.

Based on the above results, we attempted to clarify the relationship between EET rate and the metabolic changes induced by pMFC-mediated EET. We assumed the different rate of EET would induce different metabolic changes in a living cell. *S. cerevisiae* is a suitable model microbe to confirm our assumption, because some research groups reported its metabolic change induced by EET; extraction of electrons from *S. cerevisiae* via EET repressed its production of ethanol in the anaerobic condition [16,30]. As *S. cerevisiae* cannot utilize any electron acceptors besides oxygen, glycolysis is the only way to get energy from glucose in the anaerobic condition. In the anaerobic glycolysis, glucose is converted to pyruvate in a process that leads to the reduction of NAD^+ to NADH and production of ATPs, followed by the excretion of ethanol and the regeneration of NAD^+ . Therefore, the contribution of producing ethanol to NAD^+ -regeneration decreases, when EET functions as an artificial secondary pathway for NAD^+ -regeneration. The EET currents generated after injecting the *S. cerevisiae* cells into the electrochemical chambers (Fig. 4a). For the experiments, pMFC-1 and -3 that exhibited the lowest and largest EET current for *E. coli* cells were used. Just like the case of *E. coli* shown in Fig. 3b, pMFC-3 mediated larger EET current than pMFC-1, indicating the difference of the rate of EET between them. Such current generation was not observed in absence of pMFCs (Fig. S4).

To investigate the influence of the rate of EET on metabolism in *S. cerevisiae*, we anaerobically cultivated the cells in the absence and presence of pMFC-1 and pMFC-3, and then measured the produced ethanol after the 48-h cultivation. As expected, the ethanol concentration was approximately 20% lower in the presence of the oxidized form of the pMFC-3, whereas no significant differences in the ethanol concentration were observed in the presence of the reduced form of the pMFC-3 or the oxidized form of the pMFC-1 (Fig. 4b, Fig. S5). Notably, the color of the medium supplemented with pMFC-3 changed from dark green (oxidized form of pMFC) to yellow (reduced form), whereas the color of pMFC-1-supplemented medium did not significantly change, indicating that the rate of EET using pMFC-1 was slower (Fig. 4d). Although the above results indicated that the rate of EET via pMFC-3 was fast enough to change the metabolism of *S. cerevisiae*, the possibility that pMFC-3 lead to unexpected de-functionalization of biological processes associated with the ethanol production could not be excluded. To exclude the possibility, an

optical density of the medium, which indicates a number of *S. cerevisiae* cells, was measured after the 48-h cultivation (Fig. 4c). We measured the optical density not at 600 nm but at 730 nm (OD_{730}), as pMFC absorbs light with the wave length of 600 nm. It was clarified that the cell growth of *S. cerevisiae* supplemented with the oxidized form of pMFC-3 was improved rather than repressed, indicating that the pMFC-3 did not inhibit the biological processes associated with the ethanol production. We speculate that the pMFC-mediated EET activated the mitochondrial electron transporter chain (ETC) in *S. cerevisiae* by acting as an electron acceptor, leading to the enhanced production of ATP (Schematic 2), hence to the improved cellular growth. Our supposition is in a good agreement with other results, which demonstrate that electrons originating from yeast mitochondria [31].

4. Conclusions

In the present study, we demonstrated that EET rate can be controlled through the sophisticated molecular design of pMFC, which functions as a cell membrane-permeable electron mediator. As M_w and R are controllable properties of the family of pMFC polymers described here, this approach may be applicable to control the EET rate of similar systems. We anticipate that these findings will provide new insights into developing effective strategies for the electrochemical regulation of cellular metabolism, which may have impacts in bioelectrochemical technologies in the fields of bio-energy and medical care.

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Table 1 Properties of the pMFc polymers synthesized in this study.

	in feed	in copolymer ^[b]	AIBN ^[c]	<i>M_w</i> ^[d] (kDa)	<i>M_w</i> / <i>M_n</i> ^[c]
	MPC/VFc (mol %) ^[a]	MPC/VFc (mol %) & <i>R</i>	(mmol/L)		
pMFc-1	60/40	51/49, <i>R</i> = 0.49	50	5.1	1.8
pMFc-2	40/60	37/63, <i>R</i> = 0.63	6.3	7.6	1.6
pMFc-3	40/60	34/66, <i>R</i> = 0.66	50	2.4	2.3

[a] MPC; 2-methacryloyloxyethyl phosphorylcholine, VFc; vinyl ferrocene. Total concentration of monomers was 1.0 mol/L in ethanol.
 [b] Determined from UV-vis.
 [c] AIBN; α, α'-azobisisobutyronitrile
 [d] Number-average (*M_n*) and weight-average (*M_w*) molecular weights measured by gel permeation chromatography using poly(ethylene glycol) standards.

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Schematic 1. Structure of amphiphilic redox-active polymers; poly(MPC-*co*-VFc) (pMFC) used in this study. MPC; 2-methacryloyloxyethyl phosphorylcholine, VFc; vinyl ferrocene.

Figure 1. Surface tension of pMFc solutions (black circle: pMFc-1, blue square: pMFc-2, red triangle: pMFc-3) as a function of pMFc concentrations in distilled water.

Figure 2. Microbial current generation during the electrochemical cultivation of *E. coli* at +0.6 V vs. SHE in the presence of pMFC. Arrows 1 and 2 indicate the point at which *E. coli* cells ($OD_{600} = 1.0$) and EDTA (5 mM), respectively, were injected into the reactors. The sole carbon and electron source was glucose 20 mM.

Figure 3. (a) Cyclic voltammograms (CVs) of pMFC-1 (black curve), pMFC-2 (blue curve), or pMFC-3 (red curve) in DM for *E. coli*. The scan rates are 50 mV/s. (b) Microbial current generation during the electrochemical cultivation of *E. coli* at +0.6 V vs. SHE in the presence of pMFCs. The arrow indicates the point at which *E. coli* cells were injected into the reactors.

Figure 4. (a) Microbial current generation during the electrochemical cultivation of *S. cerevisiae* at +0.6 V vs. SHE in the presence of pMFC-1 (black curve) or pMFC-3 (red curve). The arrow indicates the point at which *S. cerevisiae* cells were injected into the reactors. (b) Ethanol productions and (c) Optical densities at 730 nm of *S. cerevisiae* after 48h anaerobic cultivations with and without PMFs. Error bars represent standard errors (n=3). (d) Photographs of the SD medium containing oxidized PMFs before and after cultivation of *S. cerevisiae*. Statistical significance: *p < 0.05, **p < 0.01.

Schematic 2. Schematic illustration of (a) anaerobic glycolysis of *S. cerevisiae* and (b) the proposed effect of the EET via pMFc.