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A novel self-assembled nano micelle as a highly efficient artificial peroxidase based on hexadecyl trimethyl ammonium bromide and cytochrome c[1]

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Abstract. A novel artificial peroxidase (AP) with highly catalytic efficiency was designed using hexadecyl trimethyl ammonium bromide (CTAB, 3 mM) nano-micelles and bovine heart cytochrome c (Cyt *c*, 0.5 μM) in 100 mM, pH 8.0 phosphate buffer at 25°C. The catalytic rate (k_{cat}) and Michaelis-Menten (K_m) of the AP were determined to be 0.311 ± 0.013 s^{-1} and 8.64 ± 0.6 μM. The catalytic efficiency was 0.0360 ± 0.0020 $\mu M^{-1}s^{-1}$ (about 50% the efficiency of native horseradish peroxidase). The Ultraviolet–visible spectrophotometer and Circular Dichroism techniques were applied to study the properties of the CTAB-Cyt c nano-micelle. Designed AP can be applied instead of native horseradish peroxidase.

Keywords: Catalytic efficiency, artificial peroxidase, nano-micelle, cytochrome c, CTAB

1. Introduction

Recently, designs of heme-containing proteins are becoming research hotspots, in order to interpret the relationships between the function and structure of the proteins during their catalytic processes. Artificial peroxidases could be assembled not only for different applications, but to reveal the catalytic mechanisms [1-4].

Sodium dodecyl sulfate (SDS) is an amphipathic anion surfactant. It is being used widely in protein refolding [5-8], enzyme activation [9], stabilizing of the secondary structure of some proteins [10-11] and modeling membrane or other hydrophobic environments [10-12] at above critical micelle concentration (CMC). Cytochrome c (Cyt *c*) is a heme-containing protein, which has a 3 nm diameter [13], 104 amino acids and very low peroxidase activity [13-15]. In a previous study, a nano-artificial

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peroxidase was constructed by self-assembly with SDS and Cyt *c* in 50 mM, pH 10.5 sodium phosphate buffer. The catalytic efficiency of this AP can reach 30% of native horseradish peroxidase [16], however, it is worth considering how to reduce the optimum pH and increase the catalytic efficiency of the artificial peroxidiase for further various practical applications.

In this report, hexadecyl trimethyl ammonium bromide (CTAB), an amphipathic canion surfactant, was used to mix with Cyt *c* by self-assembly to design a novel nano structured artificial peroxidase with lower optimum pH and higher catalytic activity.

2. Experimental

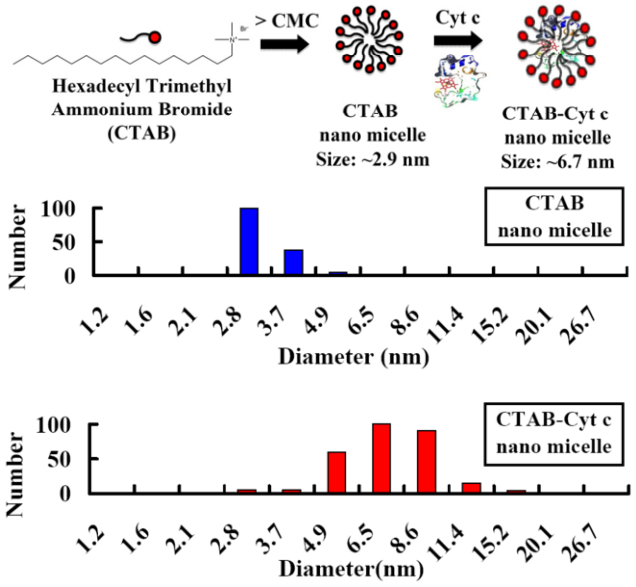
2.1. Reagents

Horseradish peroxidase (HRP), guaiacol, CTAB and bovine heart Cyt *c* were purchased from Sigma. Other chemicals were of analytical grade and used without further purification. Double distilled water was used throughout these studies.

2.2. Preparation of CTAB-Cyt c nano-micelle (AP) and the kinetic measurements

Artificial peroxidases (AP) could be constructed by self-assembly through addition of Cyt *c* into 100 mM phosphate buffer solution (PBS) containing 3 mM CTAB (above CMC) at different pH value, until the final concentration of Cyt *c* was 0.5 μM. The sizes of CTAB and CTAB-Cyt c nano micelle were determined to be 2.9 and 6.7 nm, respectively, using a Dynamic light scattering (DLS) instrument (Zeta Plus, Brookhaven Instruments Corporation, America) (see Scheme 1).

The assay of the peroxidase activity of the AP was similar to previous methods [16-18]. A spectrophotometer (Model TU-1901, Beijing Purkinje General Instrument Company, China), 1 cm path length cells equipped with a thermostat holder and an external temperature controller (Shanghai



Scheme. 1. Preparation process of CTAB-Cyt *c* nano micelle (AP).

Hengping Instrument Company, China) were applied at 25ºC. The catalytic reaction was started by the addition of hydrogen peroxide after an amount of guaiacol was added to the cell. The beginning concentrations of guaiacol and H_2O_2 were 3.0 mM and 1.0 mM, respectively. The initial guaiacol oxidation rate could be determined by the tetraguaiacol formation rate [16-18] at 470 nm in 100 mM PBS at a certain pH value (Eq. (1)).

$$4\text{Guaiacol} + 4H_2O_2 \xrightarrow{\text{AP}} \text{Teraguaiacol} + 8H_2O \quad (1)$$

Extinction coefficient of tetraguaiacol was 26.6 $mM^{-1}cm^{-1}$ at 470 nm. Ultraviolet–visible absorption and circular dichroism (CD) spectra of different components of AP were determined using aforementioned spectrophotometer and an Aviv 420SF (Lakewood, NJ) at 25ºC [19]. The concentration of Cyt *c* was 20 μM for CD spectra determinations.

3. Results and discussion

3.1. Influences of pH values and CTAB concentrations on the guaiacol oxidation rate

The influences of pH value on guaiacol oxidation rate are shown in Figure 1(A), the experiments were operated in PBS solution (100 mM) with 3 mM CTAB, 10 μM Cyt *c*, 3.0 mM guaiacol and 1.0 mM hydrogen peroxide at 25℃. The reaction rate of the CTAB-Cyt *c* nano micelle system increased rapidly with increase of pH value, until a maximum value (at pH 8.0). It should be mentioned that the optimum pH of CTAB-Cyt *c* nano micelle reduced significantly in comparison to that nano micelle of Cyt *c*-SDS [17]. However, the guaiacol oxidation rate for the natural Cyt *c* reaction system in these studies was very low.

Figure 1(B) indicates the influences of CTAB micelle concentrations on the activity of the peroxidase-like enzyme in 100 mM, pH 8.0 PBS containing 10 μM Cyt *c*, 3.0 mM guaiacol and 1.0 mM hydrogen peroxide at 25℃. The guaiacol oxidation rate of this system is dependent of CTAB concentrations above CMC and a platform was seen for CTAB at the concentration from 1.5 to 4.0 mM. In fact, CMC of CTAB was determined in this report to be 0.90 mM in 100 mM PBS at pH 8.0, similar to 0.98 mM in water [20].

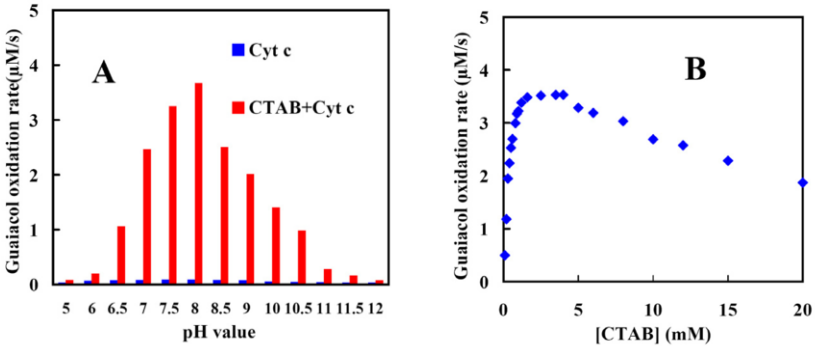


Fig. 1. Influences of pH values (A) (3 mM CTAB, 10 μM Cyt *c*) and CTAB concentrations (B) (10 μM Cyt *c*) on guaiacol oxidation rate in pH 8.0 PBS (100 mM) with 3.0 mM guaiacol and 1.0 mM hydrogen peroxide.

Table 1

Enzymatic kinetic parameters of different AP systems

AP systems	K_m (μM)	k_{cat} (s^{-1})	Catalytic Efficiency	Relative Catalytic Efficiency [17]	Refs.
CTAB-Cyt *c* nano micelle	8.64±0.6	0.311 ± 0.013	0.0360 ± 0.002	49.7 ± 1.5%	This work
Cyt *c*-SDS nano micelle	21.6±1.2	0.474 ± 0.013	0.0219 ± 0.002	30 ± 1.5%	[16]
Cyt *c*-NF nano cluster	2.5± 0.4	0.069± 0.001	0.028± 0.005	39± 5%	[19]
Cyt *c*-His-SDS reverse micelle	42	0.58	0.0173	24%	[21]
Gemini-SDS-Hemin-Imidazole nano vesicular	1.52	0.030	0.0198	27%	[22]
His-hematin-SDS nano micelle	3.31	0.0433	0.0131	18%	[18]
Native HRP	5800	420	0.0724	100%	[18]

Note: k_{cat}, catalytic rate constant; K_m, Michaelis- Menten constant.

3.2. Enzymatic kinetic parameters of AP

The enzymatic kinetic parameters of k_{cat}, K_m and k_{cat}/K_m of different AP reaction systems are shown in Table 1. Here, k_{cat} means the maximum moles of guaiacol turned into tetraguaiacol during unit time for one mole catalyst, and K_m indicates a function of guaiacol concentration for different AP models. The mentioned kinetic parameters could be determined from the linearized Lineweaver-Burk plot (Eq. (2)).

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \tag{2}$$

Where, V_0 is the initial guaiacol oxidation reaction rate. Then, k_{cat} and K_m of the AP were determined to be 0.311 ± 0.013 s^{-1} and 8.64 ± 0.60 μM, respectively. The catalytic efficiency (k_{cat}/K_m) of the AP was 0.0360 ± 0.0020 $\mu M^{-1}s^{-1}$. This value (49.7 ± 1.5% efficient of native HRP) means that the prepared AP could be used instead of HRP.

3.3. Ultraviolet–visible and CD spectra investigations on AP and natural Cyt c

Ultraviolet–visible spectra of AP (10 μM Cyt *c* + 3 mM CTAB) and native Cyt *c* (10 μM) in 100 mM PBS pH 8.0 are shown in Figure 2(A). It could be seen that the UV-vis spectra of 3 mM CTAB were very weak in the range from 300 to 500 nm. Moreover, the absorption peak wavelength for the heme group in the AP and natural Cyt *c* were 408 and 409 nm, respectively, at pH 8.0. The maximum wavelength (λ_{max}) and Abs (Abs_{max}) of the heme absorption peak depending on pH values were shown in Figures 2(a) and 2(b) (inset), respectively. It was worth noticing that the Abs_{max} for AP and Cyt *c* was decreased and increased, respectively, with increased pH value, and met at about pH 8.2, which maybe also the optimum pH value for the AP. Moreover, at pH 8.0, there is a Blue Shift (about 1 nm) for λ_{max} of AP respect to Cyt *c*. It appears that CTAB micelle offers Cyt *c* a hydrophobic situation, meanwhile, it also cause the exposure of the heme group [23].

Far-Ultraviolet, near and Soret CD spectra are very important in investigation on the secondary and

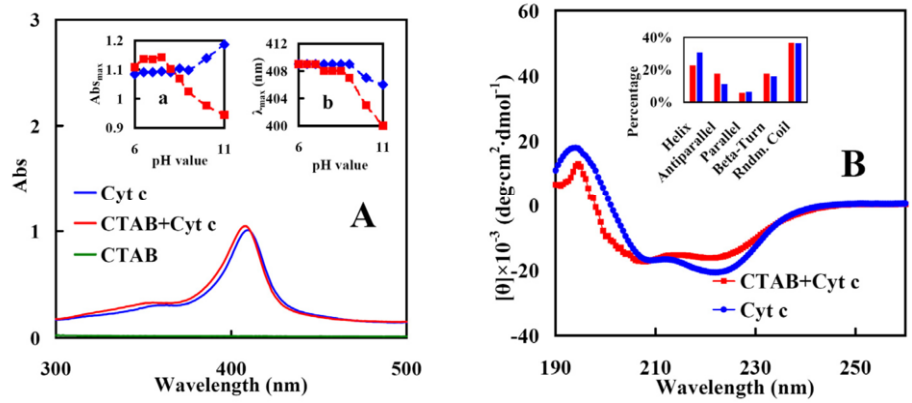


Fig. 2. (A) Ultraviolet–visible spectra of AP (10 μM Cyt *c* + 3 mM CTAB) and native Cyt *c* (10 μM) in 100 mM, pH 8.0 PBS. Insets show pH influences of (a) Abs_{max} and (b) λ_{max} of Soret Peak. (B) Far-Ultraviolet-CD spectra of AP (20 μM Cyt *c*+3 mM CTAB) and Cyt *c* (20 μM). Inset: contents of different secondary structures of AP (20 μM Cyt *c*+3 mM CTAB) and Cyt *c* (20 μM). The CD spectra were determined in 10 mM, pH 8.0 PBS at 25℃.

tertiary structure of a protein [21, 24]. The CD spectra of AP and Cyt *c* in pH 8.0 PBS (10 mM) is shown in Figure 2(B). Double minima could be seen at 222 nm and 208 nm for native Cyt *c*. For AP, the minima would become stronger at 208 nm and weaker at 222 nm, respectively. Moreover, at pH 8.0, prepared AP has a Blue Shift (about 1 nm) and stronger absorption peak in comparison to that of Cyt *c* in Soret and near CD spectra of AP (data not shown). The content of different secondary structure of AP and Cyt *c* at pH 8.0 was shown in Figure 2(B) inset, collected with a spectrum deconvolution software. It could be seen that the Cyt *c* and AP had different secondary structures, the content of the alpha helix and parallel components reduced from 30.5% and 6.3% of Cyt *c* to 22.5% and 5.8% of AP, respectively. Meanwhile, the contents of beta-turn and anti-parallel structures increased from 15.8% and 11.1% of Cyt *c* to 17.6% and 17.6%of AP, respectively. There is little difference between Cyt *c* and AP for Rndm coil structure. This might suggest that the alpha-helix structure in the AP might become into a folded structure [21, 25-26].

4. Conclusions

A CTAB-Cyt *c* nano-micelle structure with high peroxidase catalytic efficiency was designed when the Cyt *c* molecule was embraced by self-assembly in CTAB nano-micelle. The CTAB micelle could help Cyt *c* to generate hydrophobic patches near the heme group of Cyt *c* and lead the heme structure to more exposure, which make the Cyt *c* form an ordinary protein to a highly catalytic efficient AP.

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