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Electrochemical Evaluation of the Controlled Release Behaviors of the Cyclodextrin Inclusion Complexes on the Drug Deliveries to Microbial Cell

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A mediated electrochemical method was applied for *in-vivo* evaluation of the controlled release effects of the mediator-β-cyclodextrin complexes to microbial delivery. Single hydrophilic mediator (ferricyanide) system or three double mediator systems (lipophilic mediator plus ferricyanide) were employed to detect the intracellular redox activities of the yeast (*Saccharomyces cerevisiae*). The responses from the double mediator systems were much higher than that of from the single mediator system, which proved that the responses from the double mediator were originated from the reduction of the lipophilic mediator by the biological reducing equivalents inside the yeast cells. The apparent equilibrium constants of the three lipophilic mediator-β-CD inclusions were 240.24, 564.50 and 1013.24 $dm^3 \cdot mol^{-1}$, respectively, for menadione-β-CD, lawsone-β-CD and 2,6-dichlorophenolindophenol-β-CD. The electrochemical responses were reduced by 3.2%, 34.5% and 43.5% for the menadione/ferricyanide, lawsone/ferricyanide and DCPIP/ferricyanide mediator system, respectively, as the β-CD/lipophilic mediator ratio was up to 65.5. The decrease in the electrochemical response was an indication of the controlled release effects of the mediator-β-CD inclusion complexes. A significant positive correlation was observed between the sustained release effects of the three mediator-β-CD complexes with the apparent equilibrium constants of the complexes. The purposed method can be recommended as the appropriate and convenient method for evaluating the controlled delivery effects of the drug-CDs complexes to microbial cells.

Keywords: Double-mediator system; Lipophilic mediators; *Saccharomyces cerevisiae*; Controlled release assay

1. INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides containing six (α-CD), seven (β-CD) or eight (γ-CD) α-1,4-linked glucopyranose units with a hydrophilic hydroxyl group on their outer surface and a hydrophobic cavity in the center. The hydrophilic exterior of the CD molecules can make them water soluble, but the hydrophobic cavity provides an environment for appropriate sized non-polar molecules. In aqueous solution CDs are capable of forming inclusion complex with many molecules by taking up a whole molecule or some part or it, into the cavity. These non-covalent complexes offer a variety of physicochemical advantages over uncomplexed molecules including increased water solubility and stability [1]. CDs can function as antimicrobial delivery systems as they can release antioxidant and antimicrobial compounds (guest molecules) as the complexation is in touch with the cell membrane of the microbial cells. During the interactions between the lipid bilayer of the bacterial membrane and the complexation, the host-guest interactions are weakened; consequently, the antimicrobial molecule is released and should protect the product against the microbial growth [2].

In the literature, some CDs associated with biocides are described as active or inactive on bacterial growth [3]. As example, Schmidt *et al*. showed that encapsulated organic biocides are significantly more active than the biocides alone [4]. Simpson reports a total inhibition of the biocidal activity of complexed benzethonium chloride [5]. As a compromise, some authors disclose that CDs reduce the antimicrobial activity of a range of biocides to some extent [6]. The controlled release property of the complexation is one of the most important factors which influence the antimicrobial activities of the inclusion complexes. However, so far, no appropriate methods have been proposed for quantitatively evaluating the controlled release properties of the inclusion complexes to microbial delivery, which is important to evaluate the bactericidal effects of the inclusion complexes [2].

In the present paper, a mediated electrochemical method has been purposed for quantitatively evaluating the controlled release property of the drug-CDs inclusion complexes. The method was demonstrated by using three redox mediators including 2-methyl-1,4-naphthoquinone (menadione), 2-hydroxy-1,4-naphthoquinone (lawsone) and 2,6-dichlorophenolindophenol (DCPIP) as the model drugs and, *Saccharomyces cerevisiae* (the baker's yeast) as the model microorganism. It is well known that the catabolism in bacteria could be monitored by replacing oxygen with redox mediators that capture electrons from a redox molecule in the electron transport chain [7-10]. An oxidized mediator is reduced in its interaction with a reduced electron transport chain molecule and the reduced mediator is then quantified by an electrochemical method such as amperometry, voltammetry or coulometry [10].

Mediators include hydrophilic mediators and lipophilic mediators. Hydrophilic mediators are soluble in the aqueous environment of the cell but do not cross the cell membrane to enter the cytoplasm [7]. Examples include ferricyanide, futhenium hexamine *etc.,* which can only interact with the redox components located at the outer surfaces of the membrane [**9**]. Lipophilic mediators are soluble in the cell membrane and can enter the cytoplasm to interact with electron transport chains located within internal membranes. Examples of lipophilic mediators include menadione, benzoquinone, DCPIP, and Lawsone *etc* [11]. Moreover, combination of mediators, *e.g.,* application of both hydrophilic and lipophilic mediators together (ferricyanide and menadione or DCPIP), gives a significant increase of the electrochemical response [7, 10]. The function of the lipophilic mediator

(e.g. menadione) was to shuttle electrons from intracellular redox sites to the cell surface for reaction with the hydrophilic mediator ferricyanide (Fig.1). It is expected that the formation of lipophilic mediator-β-CDs complex in the double mediator detection systems will influence the electrochemical response to some extent (Fig.2). The controlled release properties of the lipophilic mediator-β-cyclodextrin complex in microbial delivery could be drawn from the variance in the electrochemical response. The results of this study suggested that electrochemical measurements can form the basis for a new and rapid method for *in vivo* testing of the controlled release behaviors of the drug-cyclodextrin complex to the microbial deliveries.

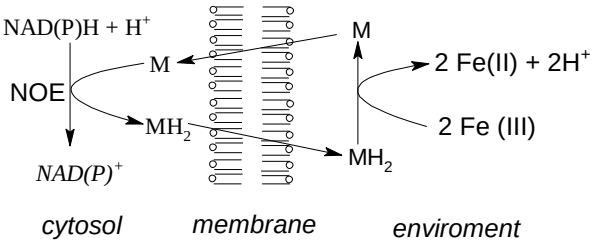

Figure 1. Detection of intracellular redox activities with the use of double mediator system. The lipophilic mediator M diffuses into a yeast cell, where it is reduced to MH_2 and then MH_2 diffuses out of the cell and react with ferricyanide, labeled for simplicity, Fe^{3+}, converting it into ferrocyanide, labeled Fe^{2+}, and regenerating the lipophilic mediator M to repeat the cycle. Ferrocyanide accumulations arising from the above reaction cycle was assayed by voltametric measurement. "NOE" represents the NAD(P)H oxidising enzymes**.**

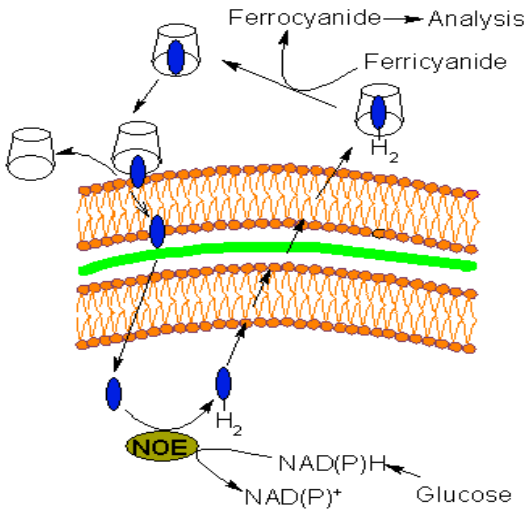

Figure 2. Schematic model of the electrochemical method for quantitative determination of the controlled release effects of the mediator-β-CD inclusion complex to microbial delivery. M-β-CD complex diffuses onto the surface of yeast cells, dissociates and leaves M penetrate into a cell, where it is reduced to MH_2, MH_2 then diffuses out of the cell and forms MH_2-β-CD with β-CD, MH_2-β-CD reacts with ferricyanide, and regenerating the M-β-CD inclusion to repeat the cycle. "M" represents the lipophilic mediators, "NOE" represents the NAD(P)H oxidising enzymes.

2. EXPERIMENTAL PARTS

2.1 Chemicals and reagents

β-cyclodextrin was obtained from sigma corporation. 2-Methyl-1,4-naphthoquinone (menadione), 2-hydroxy-1,4-naphthoquinone (lawsone), and 2,6-dichlorophenolindophenol (DCPIP) were purchased from Acros Organics. Other chemicals were of analytical grade. All solutions were prepared with double-distilled water, which was purified with a Milli-Q purification system (Branstead, USA) to a specific resistance of >18 MΩ cm^{-1}, and stored in refrigerator at 4 ºC.

2.2 Isothermal titration calorimetry

Titration calorimetric measurement was performed by a 201 nanowatt scale isothermal titration microcalorimeter supported by Thermal Activity Monitor TAM 2277 (Thermometric, Sweden), which was controlled by Digitam 4.1 software [12]. In the experiment, the reaction cell and reference cell of the calorimeter were initially loaded with 500 μL guest solution (lipophilic mediators) and 750 μL pure water, respectively. The concentration of β-CD as titrant was 2.0 mol L^{-1}. The titrant solution was injected into the stirred sample vessel in 25 aliquots of 15 μL using a Hamilton syringe controlled by a 612 Lund Pump. The initial concentrations of the guest molecules were 5.0×10^{-4}, 5.7×10^{-4} and 6.8×10^{-4} mol L^{-1}, respectively, for menadione, lawsone and DCPIP. The interval between two injections was 40 min, which was sufficiently long for the signal to return to the baseline. The system was stirred 30 rpm with a gold propeller. All experiments were performed at the temperature of 298.15±0.01 K and were started after the baseline became stable so that the heat produced by stir can be automatically deduced [13].

The enthalpy of 1:1 complex formation (ΔH^o) and the apparent equilibrium constant (also called binding constant *K*) were calculated simultaneously and fitting the experimental data to a theoretical titration curve using standard instrument software [12]. To obtain more accurate thermodynamic data for low affinity systems the complex stoichiometry determined independently was fixed at known value during the fitting. The other thermodynamic parameters such as the free energy ($\Delta_c G^o$) and the entropy ($\Delta_c S^o$) of complex formation were estimated on the basis of well-known thermodynamic equations:

$$\Delta_c G^o = -RT \ln K$$
$$\Delta_c G^o = \Delta_c H^o - T\Delta_c S^o$$

2.3 Microorganism cultivation and preparation

Saccharomyces cerevisiae was maintained on yeast extract peptone dextrose (YEPD) agar at 4 ºC. Yeast cultures for experimental use grown in 50 ml YEPD broth in shake flasks rotated at 180 rpm for 16 h at 30 ºC. Cells were harvested by centrifugation at 8,000 g for 5 min at 4 ºC. The cells were washed twice in phosphate buffer (PB, 0.05 M K_2HPO_4/KH_2PO_4, pH 7.0) and re-suspended in

phosphate buffered saline (PBS, 0.05 M K_2HPO_4/KH_2PO_4, pH 7.0, 0.1 M KCl). Cell density was adjusted to an optical density at 600 nm (OD_{600}) of 9.0 using a 722 E spectrophotometer.

2.4 Mediators and substrate

Potassium ferricyanide was dissolved in distilled water to give a 0.3 M solution, filter-sterilised and stored at 4 ºC. Each lipophilic mediator was dissolved in 96% ethanol to give a 20 mM solution, filter-sterilised and stored at 4 ºC. Glucose, 0.5 M in distilled water was sterilized at 121 ºC for 15 min and stored at 4 ºC.

2.5 Incubation of cells with mediator (s) and substrates

A total volume of 20.0 ml experimental sample was prepared for each trial. The standard incubation suspension comprised: 12 ml of cell suspension, 3.0 ml of ferricyanide solution, 100 μl of lipophilic mediator solution (final concentration 100 μM) or 100 μl PBS, 300 μl of glucose solution, and appropriate volumes of PBS to constitute the mixture to 20 ml. In the trials with the aim to evaluate the controlled delivery behaviors of the mediator-β-cyclodextrin inclusion, increasing concentrations up to 7.0 mM β-cyclodextrin was added to the incubation mixtures. The experimental samples were incubated at 30 ºC with oxygen-free nitrogen sparging for 2.0 h. At the completion of incubation, the yeast cells were pelleted by centrifugation (6,000 rpm, 4 ºC, 10 min) and the supernatants were taken for analysis.

2.6. Electroanalysis of the supernatant

Steady-state voltammetry was conducted using an electrochemical working station (CHI 760, Shanghai, China) controlled by CHI 760 software. Steady-state voltammograms were obtained at a scan rate of 10 mV s^{-1} scanning from 500 to 100 mV versus saturated Ag/AgCl reference electrode. A 15 μm diameter Pt disk electrode was used as the working electrode and a Pt gauze auxiliary electrode was used to complete the three-electrode electrochemical cell. The microelectrode was pretreated by polishing with 0.05 μm alumina/water slurry on a flocked twill polishing cloth. The steady-state current at 500 mV was measured, which was proportional to the concentration of the ferrocyanide produced.

3. RESULTS AND DISCUSSION

A variety of non-covalent forces, such as van der Waals forces, hydrophobic interactions and other forces are responsible for the formation of the stable complex. Inclusion complex formation can be regarded as ‘encapsulation’ of the drug molecule, or at least the labile part of the molecule [14]. Complexation of the drug (D) to cyclodextrin (CD) occurs through a non-covalent interaction between

the molecule and the CD cavity. This is a dynamic process whereby the drug molecule continuously associates and dissociates from the host CD. Assuming a 1:1 complexation, the interaction will be as follows.

$$D_{free} + CD_{free} \overset{k}{\rightleftharpoons} DCD_{complex}$$

The apparent equilibrium constant (*K*) of the above inclusion reaction is an indication of the stability of the inclusion complex, the higher value of the K, the more stable of the inclusion complex is [14].

Table 1 shows the thermodynamic parameters of the complex formation of three lipophilic mediators with β-CD, as determined from the calorimetric measurements. From the values of *K*, it could be observed that the inclusion complex of DCPIP-β-CD is much more stable than that of menadione-β-CD and Lawsone-β-CD, with the stability in order was: DCPIP-β-CD> Lawsone-β-CD> menadione-β-CD. It was commonly believed that the higher the *K* value of the drug-β-CD inclusion complex is, the more perfect controlled delivery behaviors is be expected. As depicted in Fig.2, the formation of the mediator-β-CD inclusion complex would delay the release of the lipophilic mediators to *S. cerevisiae* lipid bilayer, and then result in the decrease in ferrocyanide accumulations, which could be measured by the microelectrode steady-state voltammetry. In this respect, the variance in the electrochemical responses by the presence of increasing concentrations of β-CD in the incubation mixtures would indicate the controlled release effects of the inclusion complex to microbial lipid bilayer.

Table.1. Thermodynamic characteristics of complex formation of β-CD with menadione, lawsone and DCPIP in water at 298.15 K

Complex	K ($dm^3 \cdot mol^{-1}$)	$\Delta_c G^o$ ($kJ \cdot mol^{-1}$)	$\Delta_c H^o$ ($kJ \cdot mol^{-1}$)	$T\Delta_c S^o$ ($kJ \cdot mol^{-1}$)
β-CD/M	240.24	-13.60	-10.40	3.20
β-CD/L	564.50	-15.71	-31.09	-15.41
β-CD/DCPIP	1013.24	-17.16	-4.45	12.71

The use of voltammetric microelectrodes has been shown to be a simple, reliable and rapid method for determing the concentration of electroactive species such as ferrocyanide. Diffusion limiting currents (quantitatively related to concentration) can be established in second time scale with virtually zero destruction of analyte [15]. The ferrocyanide accumulations arising from the lipophilic mediators mediated reduction of ferricyanide by yeast cells were indicative of the intracellular redox activities of the yeast [7]. Fig.3. shows the steady-state voltammogram of ferrocyanide accumulations after a 2.0 h incubation of the yeast suspensions with each of three double mediator systems or the single ferricyanide system. The steady-state anodic plateau current measured at *E* =500 mV was used as a relative measure of the amount of ferrocyanide produced. Before incubation, the mediator is fully in the oxidized form, and the steady-state anodic plateau current measured at *E* = 500 mV was about

zero (data was not shown). After incubation for 2.0 h with the yeast cells, the plateau currents shifted up the current axis. The limiting oxidative currents are 1.53, 56.07, 14.34 and 23.58 nA, respectively, for the single ferricyanide mediator, menadione/ferricyanide, lawsone/ferricyanide and DCPIP/ferricyanide mediator system. From this it can be seen that the steady-state current is dependent on the type of mediator and of the way the mediator solution is prepared. Compared with three double mediator systems, the single ferricyanide mediator gave a nearly negligible electrochemical response, which confirmed the way by which the double mediator systems can be employed in detecting the intracellular redox activities of living bacterial cells (Fig.2). Although menadione has a very similar molecular structure to that of lawsone, the electrochemical response from the menadione/ferricyanide system was three times higher than that from the lawsone/ferricyanide system. This is most probably due to a higher diffusion rate of uncharged hydrophobic menadione molecule across the lipid bilayer. Thus the major contribution in NAD(P)H oxidising enzyme catalyzed reduction of menadione in intact cells comes from the bioavailability of the mediator, which is a function of its hydrophobicity [16, 17].

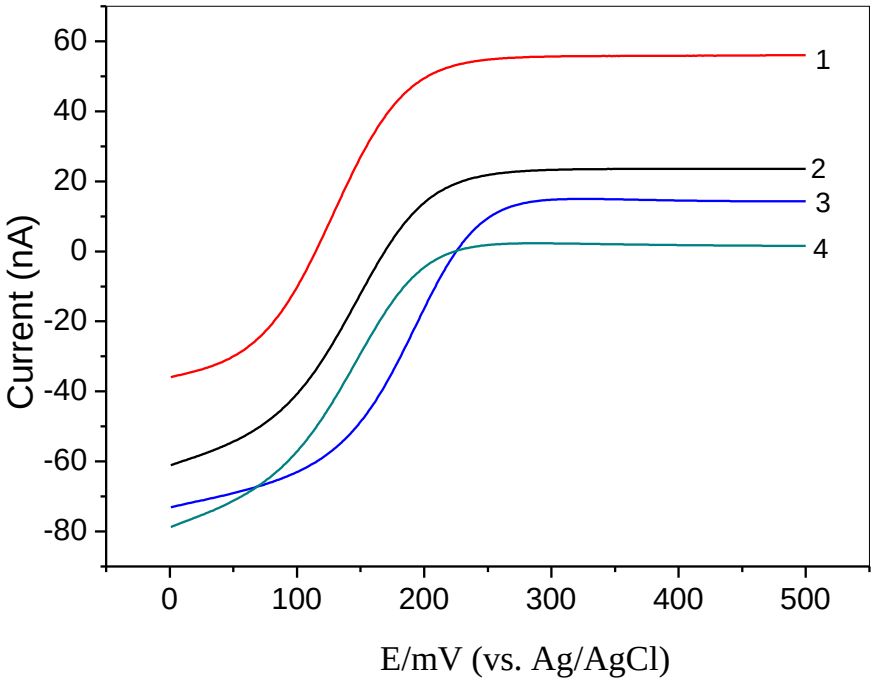

Figure 3. Steady-state voltammograms of the supernatants of the experimental samples consisting of fresh *S. cerevisiae* cells with single ferricyanide or double mediator systems for 2 hours incubation: Menadione-ferricyanide (curve 1), DCPIP-ferricyanide (curve 2), lawsone-ferricyanide (Curve 3), and single ferricyanide mediator (Curve 4). Ferricyanide concentration, 45 mM, lipophilic mediators, 100 μM, *S. cerevisiae cell*, OD_{600}=5.0, glucose concentration, 1.0 g/L.

A biological membrane is an enclosing or separating amphipathic layer that acts as a barrier within or around a cell. It is, almost invariably, a lipid bilayer, composed of a double layer of lipid-class molecules, specifically phospholipids, with occasional proteins intertwined, some of which

function as channels. The membrane acts as a selective permeability barrier to bulky or highly charged molecules, which cannot easily pass through the hydrophobic interior of the lipid bilayer. Organic molecules must possess some lipophilicity to be able to permeate biological membranes, including biological barriers [18]. Some studies on organic adsorption by microbial surfaces have shown that organic molecule uptake by microbial cells is not a metabolic process, but is a partitioning process [18]. According to the partitioning approach, it was clear that the higher hydrophobicity the organic molecules are, the faster the molecules are adsorbed onto microbial surfaces.

The proposed controlled release mechanism for the guest-β-CD complexes is based on the partitioning process of lipophilic drugs into the lipid bilayer of microorganisms with four-steps: (a) the complex diffuses in the solution; (b) the guest-β-CD complex is in touch with the bacterial membrane surface by random collision, (c) the complex is dissociated and the lipophilic guest molecular diffuse into the bacterial membrane, and (d) the interactions between the lipophilic guests and intracellular targets.

There is a competitive adsorption affinity between β-CD and microbial surfaces for drug, and which will determine the release property of the drug-CDs complexes in microbial delivery. The binding affinity of the drug-β-CDs complex can be indicated by the apparent equilibrium constants of the inclusion complexes; and the adsorption affinity between drug and microbial surfaces can be indicated by the lipophilicity of the drugs. It has been reported that the formation of inclusion complexes between CDs and organic biocides will reduce the antimicrobial activities of the biocides, and the magnitude of the inactivation is positively related with the apparent equilibrium constants of the inclusion complexes [2].

Based on the above discussions, the purposed electrochemical method depicted in Fig.2 would objectively indicate the controlled delivery effects of the mediator-CDs inclusion complexes in microbial delivery. Fig.4. shows the effect of β-CD on the electrochemical responses from three mediator systems by the presence of *S. cerevisiae* cells. The electrochemical responses from the menadione/ferricyanide system remained nearly unchanged with the increasing concentrations of β-CD, which indicated that the formation of the menadione-β-CD inclusion complex did not affect the release property of menadione in *S. cerevisiae* delivery.

By contrast, the electrochemical responses from the other two mediator systems continuously decreased by the presence of increasing concentrations of β-CD, which indicated that the drug delivery rates of the two mediators were retarded by the formation of the mediator-β-CD inclusion complexes (Fig.4). The electrochemical responses were reduced by 34.5% and 43.5% for the lawsone/ferricyanide mediator system and the DCPIP/ferricyanide mediator system, respectively, as the β-CD/lipophilic mediator ratio was up to 65.5. As discussed above, the best candidate for the controlled delivery system was the DCPIP-β-CD complex, followed by the lawsone-β-CD complex and the menadione-β-CD complex, with the latest has only slight controlled release effect in the presence of microorganisms. It was interesting that a significant positive correlation was observed between the sustained release effects of the three mediator-β-CD complexes with the apparent equilibrium constants of the complexes (Table 1, Fig.4).

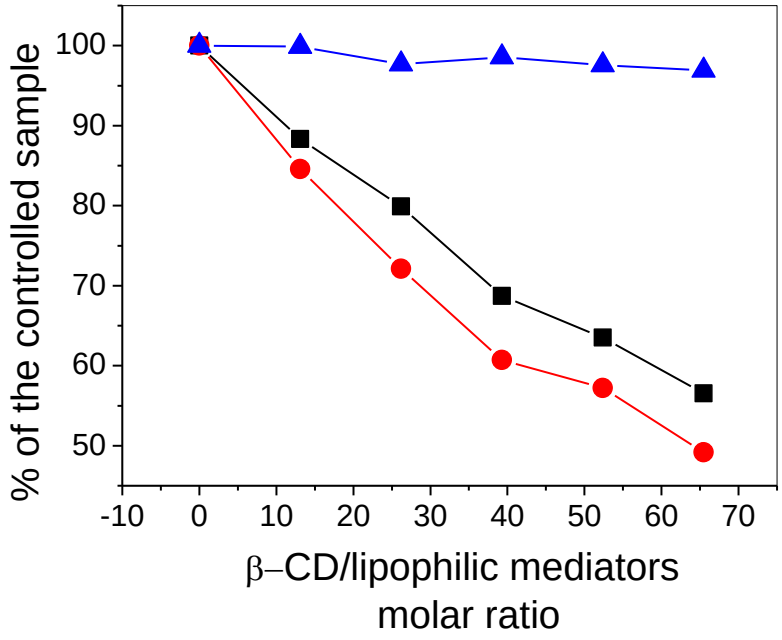

Figure 4. Influence of β-cyclodextrin complexation on menadione (▲), lawsone (■) and DCPIP (●) mediated reduction of ferricyanide by *S. cerevisiae* suspensions.

Thus, the electrochemical analysis of the intracellular redox activities of *S. cerevisiae* cells by the presence of lipophilic mediator-CDs complexes has allowed revealing the controlled delivery effects of some inclusion complexes to microbial cells. It has to be noted that the purposed electrochemical method is only applicable to some electrochemical mediator-CDs inclusion complexes. Even though, the controlled delivery effects of none mediator-CDs complexes to microbial cells, such as some drug-CDs complexes, could also be evaluated by referring to the controlled delivery effects of proper mediator-CDs inclusion complexes, which have comparable apparent equilibrium constants with that of the studied drug-CDs complexes. Many lipophilic low molecules have been used for microbial intracellular redox assay, including 2,6-dichlorophenolindophenol, phenazine and phenoxazine derivatives, benzoquinone and quinine derivatives, *etc.*, the inclusion complexes formed between which and CDs have been studied either, which provide more candidates for comparison purpose in learning the controlled delivery effects of the drug-CDs complexes.

Based on this study, the mediated electrochemical method can be recommended as the appropriate and convenient method for evaluating the controlled delivery effects of the drug-CDs complexes to microbial cells.

4. CONCLUSION

In this work, a mediated electrochemical method employing double mediator systems has been purposed for *in vivo* study of the controlled release effects of the mediator-β-CD inclusion complex to yeast delivery. In addition, we demonstrated that the apparent equilibrium constant of the inclusion

complex is the crucial parameter influencing the controlled delivery effects of the complex to yeast delivery. The assay method would be also applicable to the study of some non mediator-CDs complexes (e.g. drug-CDs) by means of selecting proper mediator-CDs complex which has comparable apparent equilibrium constant with the studied one.

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