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A MICROCALORIMETRIC STUDY OF THE TOXICITY OF TWO COBALT COMPOUNDS ON *ESCHERICHIA COLI* DH5α GROWTH

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Microcalorimetry was applied to study the toxic action of two cobalt compounds such as bis(salicylideniminato-3-propyl)methylaminocobalt(II) (denoted as Co(II)) and Co(III) sepulchrate trichloride (denoted as $Co(sep)^{3+}$) on (*E. coli*) DH5α. The power–time curves of the *E. coli* DH5α growth were determined, and the thermokinetics parameters such as the growth rate constant k, the maximum power output P_m and the time (t_m) corresponding to the P_m were obtained. The half-inhibitory concentrations (IC_{50}) of Co(II) and $Co(sep)^{3+}$ to *E. coli* DH5α were 15 and 42.1 mg mL^{-1}, respectively.
The experimental results revealed that the toxicity of the Co(II) compound was larger than that of $Co(sep)^{3+}$. On the other hand, the scanning electron microscopy (SEM) demonstrated that the two cobalt compounds had the same toxic mechanism on *E. coli* DH5α, which was attributed to the damage of cell wall of the bacteria caused by both Co(II) and $Co(sep)^{3+}$. Furthermore, accumulation of intracellular cobalt of *E. coli* DH5α, due to the interaction of Co(II) or $Co(sep)^{3+}$ and *E. coli* DH5α, has been found by using inductively coupled plasma (ICP) analytical technique.

Keywords: *Co compounds, E. coli DH5α, inhibition, microcalorimetry, toxicity*

Introduction

Cobalt is a biologically essential trace element in humans and animals, but some common used chemical reagents containing Co may lead to toxicological problems and harmful to human health. Some of Co chemicals are even classified as carcinogenic compounds by the International Agency in Cancer Research [1, 2]. Therefore, it is necessary to understand the compound's toxicity and detailed information about the toxic mechanism. The accurate toxicological effects in the rational use of these chemical compounds, however, are still not fully clarified. Hence, it has attracted many researchers' attention to study the toxicology of these Co compounds [3–5].

The bis(salicylideniminato-3-propyl)methylaminocobalt(II) (denoted as Co(II) in the following text) is a Schiff base which possesses a unique structure of $R_1R_2C{=}NR_3$ (R_3 stands for a phenyl or alkyl group). It is used in the study of reversible binding of dioxygen in biological system [6]. The cobalt(III) sepulchrate trichloride (denoted as $Co(sep)^{3+}$ in the following text), with a cage ion, has currently been applied in electrochemistry [7, 8].

All biosystem metabolism processes are going with the generation of thermal effects, and the amount of heat evolved during metabolic processes is strictly proportional to metabolic activities [9–11]. Thus, microcalorimetry provides a direct route to investigate the microbial growth process and the biological activity of a living system by exploring the thermal effect [12–15]. It has been widely used in clinical analysis, pharmacology, ecology, biotechnology and agriculture due to abundant thermodynamic and kinetic information [16–22]. In addition, it can monitor the processes continuously and reveal temporal details, which cannot be observed by other techniques [23].

The aim of this work is to study the toxic effects of two Co compounds (Co(II) and $Co(sep)^{3+}$) on *E. coli* DH5α growth by using microcalorimetric technique. The power–time curves of the *E. coli* DH5α growth was determined and the parameters such as the growth rate constant k, the maximum power output P_m and the time (t_m) corresponding to the P_m were gained. From the relationships between k, P_m, t_m and C, we can estimate the toxicity of the two Co compounds on *E. coli* DH5α. Meanwhile, scanning electron microscopy (SEM) and inductively coupled plasma (ICP) were carried out to give internal information about the action mechanism of Co compounds on the *E. coli* DH5α bacteria.

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Experimental

Materials

E. coli DH5α was used as the test organism, and was provided by Biomass Conversion Technology Group (Dalian Institute of Chemical Physics, CAS, Dalian 116023, P. R. China). This strain of *E. coli* DH5α was routinely cultivated on Luria–Bertani (LB) culture medium which consisting of tryptone 1 mass%, yeast extract powder 0.5 mass%, NaCl 1 mass% (pH=7.0–7.2), and the procedures have been reported previously [24]. The LB culture medium was sterilized by autoclaving at 121°C for 20 min prior to the experiment.

The bis(salicylideniminato-3-propyl)methylaminocobalt(II) (Co(II)), 97% and the cobalt(III) sepulchrate trichloride ($Co(sep)^{3+}$), 95%, were purchased from Aldrich Chemical Company. Their structures are shown in Fig. 1. All reagents were used as received without further purification.

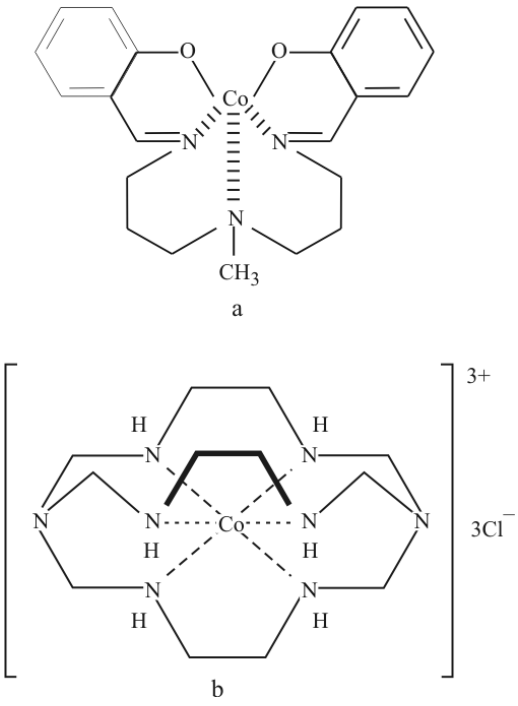

Fig. 1 The structures of the two Co compounds: a – Co(II), b – $Co(sep)^{3+}$

Methods

Microcalorimetric experiments were carried out with the 8-channel isothermal microcalorimeter TAM Air (Thermometric AB, Sweden). Measurements were performed in sealed 20 mL glass ampoules. The signal generated was recorded in-situ by a computer. The structure and operation of the instrument have been described in detail by Wadsö and our previous publications [19, 21, 25].

For the poor water solubility of Co(II), it was freshly dissolved in sterilized N,N-dimethylformamide (DMF) prior to use. The $Co(sep)^{3+}$ was also freshly prepared in the sterilized distilled water before experiment.

The ampoule method was used in the microcalorimetric measurement. For these determinations, the glass ampoules were sterilized before they were charged with 10 mL Co compound-containing LB culture medium. Thereafter, 200 μL *E. coli* DH5α suspension (optical density is about 0.6 at λ=600 nm) were inoculated into each ampoule. Then the ampoules were sealed with a cap and placed into the microcalorimeter. The operating temperature was maintained at 37°C and the power–time signals were recorded at an interval of 1 min.

When the *E. coli* DH5α grew to its stationary phase, the cells were rinsed with PBS and fixed with glutaraldehyde solution, dehydrated by a series of increasing concentration of tertiary butanol (50, 70, 80, 90, 100%). Finally, the specimens were coated with a gold layer to improve their conductivity and were observed by SEM (JEM-1200EX).

Before the ICP analysis, the samples were centrifuged, collected and washed by the ultra-pure water, and then the washed cells were lysed in HNO_3 and H_2O_2. Finally, the solution was diluted to the certain volume of the bacteria solution, and put into ICP (IRIS Intrepid II) to determine content of the Co.

Thermokinetics

In the logarithmic phase of growth, the relationship between the cell number n and the constant of cell growth rate k obeys the exponential equation [26]:

$$n_t = n_0 \exp(kt) \quad (1)$$

where t represents the incubation time, n_0 and n_t are the cell number at time 0 and t, respectively. If we denote the power output of each cell as P_w, then:

$$n_t P_w = n_0 P_w \exp(kt) \quad (2)$$

P_0 and P_t are defined as the power output at time 0 and t, respectively. Then Eq. (2) becomes:

$$P_t = P_0 \exp(kt) \quad (3)$$

or

$$\ln P_t = \ln P_0 + kt \quad (4)$$

Equation (4) is the simplest equation for calculating the rate constant of cell growth. Therefore, using the experimental data of P_t and t obtained from the bacterial growth curves, the rate constant k of cell growth was calculated, its values and the corresponding correlated coefficients r are summarized in Tables 1 and 2.

Table 1 Parameters of *E. coli* DH5α growth at different concentrations of Co(II)

C/μg mL^{-1}	k/10^{-3} min^{-1}	r	P_m/μW	I/%	IC$_{50}$/μg mL^{-1}
0.0	1.85	0.9997	542.7	0	
1	1.38	0.9957	405.1	25.4	
5	0.94	0.9942	496.8	49.2	
15	0.91	0.9993	517.0	50.1	15
20	0.89	0.9981	497.9	51.9	
25	1.23	0.9965	552.8	33.5	
30	1.32	0.9994	389.7	28.6	
40	0	–	–	100	

k – growth rate constants, r – correlated coefficients for k, P_m – maximum power output, P_m, I – inhibitory ratio

Table 2 Parameters of *E. coli* DH5α growth at different concentrations of Co(sep)$^{3+}$

C/μg mL^{-1}	k/10^{-3} min^{-1}	r	I/%	IC$_{50}$/μg mL^{-1}
0.0	1.85	0.9997	0	
11.9	1.47	0.9995	20.5	
23.8	1.09	0.9929	41.1	
47.7	0.8	0.9957	56.8	
95.4	0.61	0.9982	67.0	42.1
143.1	0.45	0.9992	75.7	
190.7	0.52	0.9992	71.9	
238.5	0.75	0.9985	59.5	
286.1	0.74	0.9733	60.0	
332.5	0	–	100	

There is another necessary parameter in evaluating the toxicity of the Co compound, i.e., inhibitory ratio (I), and it can be defined as:

$$I=[(k_0-k_c)/k_0]100\%$$

k_0 and k_c representing the rate constant of *E. coli* DH5α growth without and with effect of the Co compound. When the inhibitory ratio (I) is 50%, the corresponding half-inhibitory concentration of the Co compound is expressed as IC$_{50}$. It can be regarded as the inhibiting concentration causing a 50% decrease of the *E. coli* DH5α growth rate constant. The calculated results are also exhibited in Tables 1 and 2.

Results and discussion

The toxicity of the Co(II)

Parts of the power-time curves of the *E. coli* DH5α growth affected by Co(II) with different concentrations are displayed in Fig. 2a. From the power-time curves of the *E. coli* DH5α, it can be seen that all the curves were of the same basic shapes, and the heat generation time prolonged with increasing Co(II) concentrations. The results presented in Table 1 and Fig. 3 also reveal that the Co(II) has a toxic action on the growth of *E. coli* DH5α. The rate constant (k) of the growth of *E. coli* DH5α declines gradually with the increase of the concentration, as the Co(II) concentration is below 20 μg mL^{-1}. But when the Co(II) concentration is above 20 μg mL^{-1}, the rate constant has a slight increase. The most possible explanation for this behavior could be that the bacteria was depressed at the beginning of the experiment, then it may adjust itself and adopt the environment with the lapse of time. However, if the Co(II) concentration was so high, then most of the bacteria were inhibited, and they could not restore to its control level. The following equation would provide us a better understand for the relationship between k and C [21]:

$$k=(-2.083\text{E-}4)C^3+0.01263C^2-0.2051C+1.714$$
$$R^2=0.9027$$

The lethal dose of Co(II) is 40 μg mL^{-1}. For this concentration, the heat production of *E. coli* DH5α was below the detection limit of the calorimeter and no growth was observed in the experimental period.

As shown in Fig. 4, the time (t_m) corresponding to the P_m increases as the Co(II) concentration goes up. The t_m increases slowly as the concentration is in the range of 0–15 μg mL^{-1}. But the t_m increases

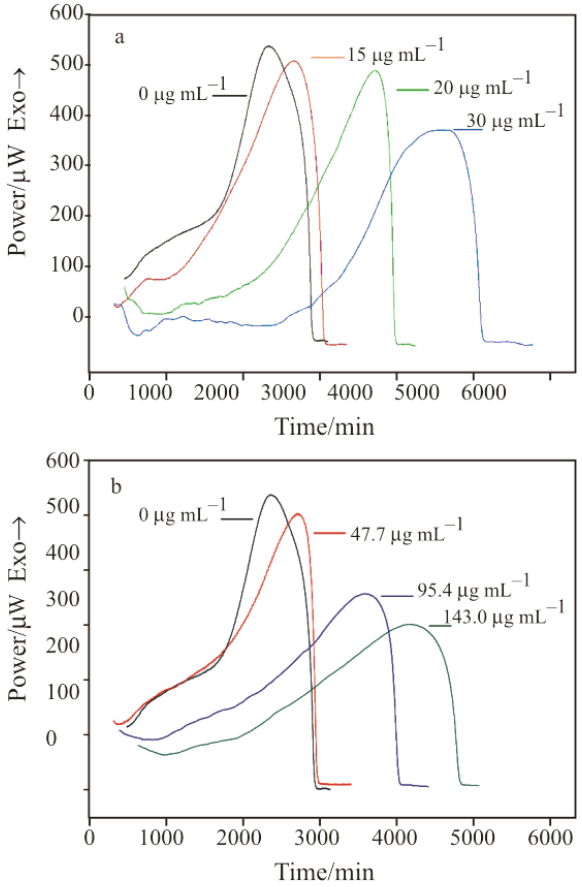

Fig. 2 Power–time curves of *E. coli* DH5α growth in the presence of Co compounds at various concentrations: a – Co(II), b – $Co(sep)^{3+}$

steeply as the concentration reaches 20 μg mL^{-1}. The equation of t_m-C can be described as:

$$t_m=2.271C^2+11.23C+2290$$
$$R^2=0.9659$$

The toxicity of the Co(sep)$^{3+}$

From Table 2 and Fig. 2b, one can distinctly know that the $Co(sep)^{3+}$ has toxic on *E. coli* DH5α. During the concentration range of 0.0–143.1 μg mL^{-1}, the value of k decreases as the C increases. When the concentration reaches 190.7 μg mL^{-1}, k has a little augmentation. At the concentration of 332.5 μg mL^{-1}, the *E. coli* DH5α enters a lethal state and no heat released from metabolism is detected. Moreover, the change tendency of k–C for the $Co(sep)^{3+}$ is resemblant to that of Co(II), indicating that their toxicological mechanism is similar. The following SEM also confirmed this point. This relationship of k–C is schematically illustrated in Fig. 5:

$$k=(-2.595E\text{-}7)C^3+(1.504E\text{-}4)C^2-0.02534C+1.750$$
$$R^2=0.9686$$

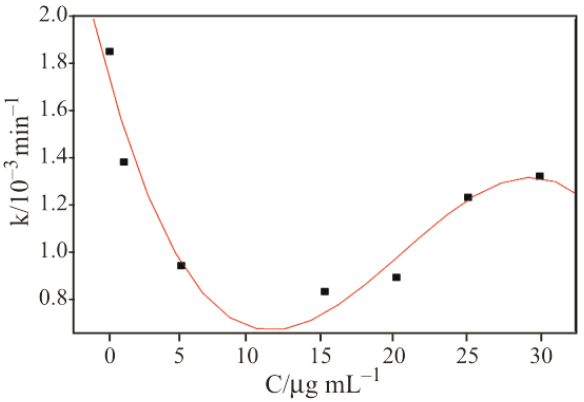

Fig. 3 The curve of k–C for the Co(II) effects on *E. coli* DH5α

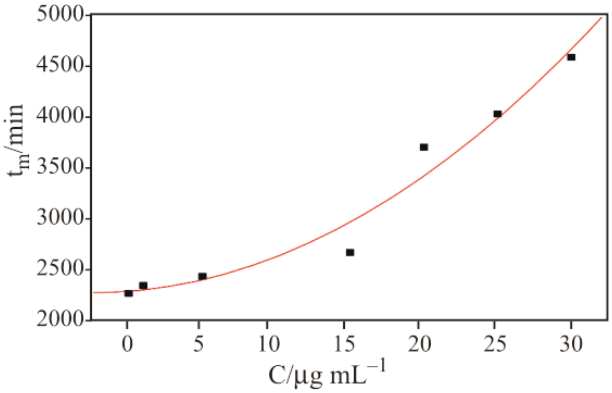

Fig. 4 The curve of t_m–C for the Co(II) effects on *E. coli* DH5α

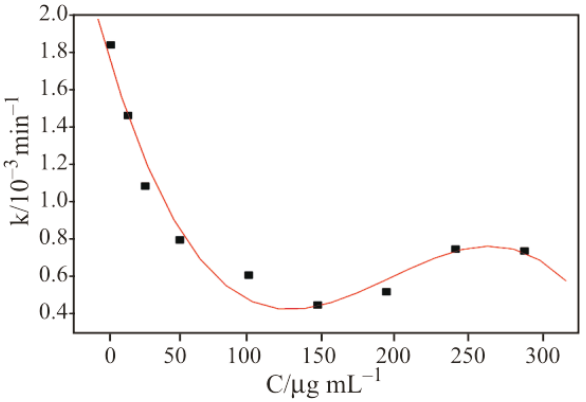

Fig. 5 Plot of k for the growth of *E. coli* DH5α *vs.* C for $Co(sep)^{3+}$

As indicated in Fig. 6, with increasing of the $Co(sep)^{3+}$ concentration, the P_m is at first higher than the control level and then decrease to a lower level. The possible reason could be that when the $Co(sep)^{3+}$ was at low concentration, the cells may demand an additional energy supply under adverse conditions to maintain and reorganize their structure and functions, so the energy metabolism was activated and correlated with a greater heat production. While with the $Co(sep)^{3+}$

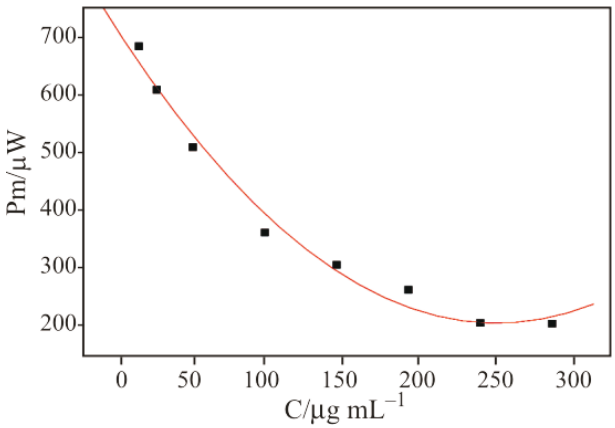

Fig. 6 Plot of P_m for the growth of *E. coli* DH5α *vs*. *C* for Co(sep)$^{3+}$

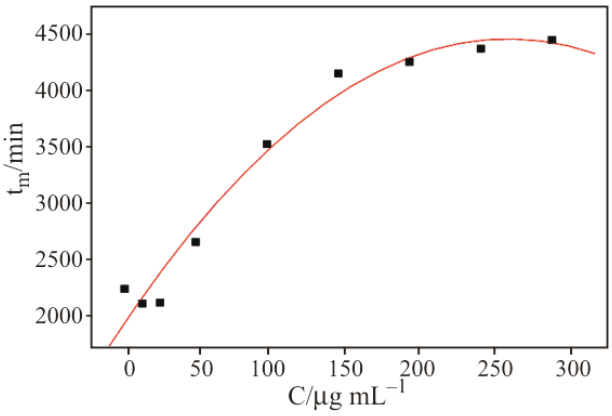

Fig. 7 Plot of t_m for the growth of *E. coli* DH5α *vs*. *C* for Co(sep)$^{3+}$

concentration increasing, more and more cells were damaged and needed high energy for their survival, which was reflected in a decreased heat production, especially when the concentration is 95.4 μg mL^{-1}, the P_m decreased suddenly. The equation of P_m–C may express as:

$$P_m=0.007950C^2-3.965C+700.5$$
$$R^2=0.9857$$

The results in Fig. 7 suggest that the t_m delayed regularly when the concentration increased except for the concentration of 95.4 μg mL^{-1}, and the t_m prolonged sharply at this concentration, and this phenomenon is alike to that of P_m change. One can obtain the t_m–C equation as:

$$t_m= -0.03768C^2+19.33C+1973$$
$$R^2=0.9731$$

SEM analysis

In order to study the inhibition mechanism of Co compounds on the *E. coli* DH5α growth, the morphology of *E. coli* DH5α grown at different conditions were observed by SEM. The SEM image in Fig. 8A is the *E. coli* DH5α cell grown without presence of Co(II) and Co(sep)$^{3+}$. It can be seen that the native cell wall was integrity and there was no damage. Figures 8b and c show the cells grown in the presence of Co(II), with a concentration of 15 μg mL^{-1} and Co(sep)$^{3+}$ with a concentration of 143.1 μg mL^{-1}, respectively. Figure 8b illuminates that the Co(II) destroys the cell wall of *E. coli* DH5α cell, and inhibits *E. coli* grown accordingly. The damage caused by the Co(sep)$^{3+}$ is more seriously as shown in Fig. 8c. The results are consistent with those obtained from the microcalorimetry, that is, the Co(sep)$^{3+}$ with a concentration of 143.1 μg mL^{-1} corresponded to the inhibitory ratio of 75%, and the Co(II) with a concentration of 15 μg mL^{-1} has a lower inhibitory ratio of

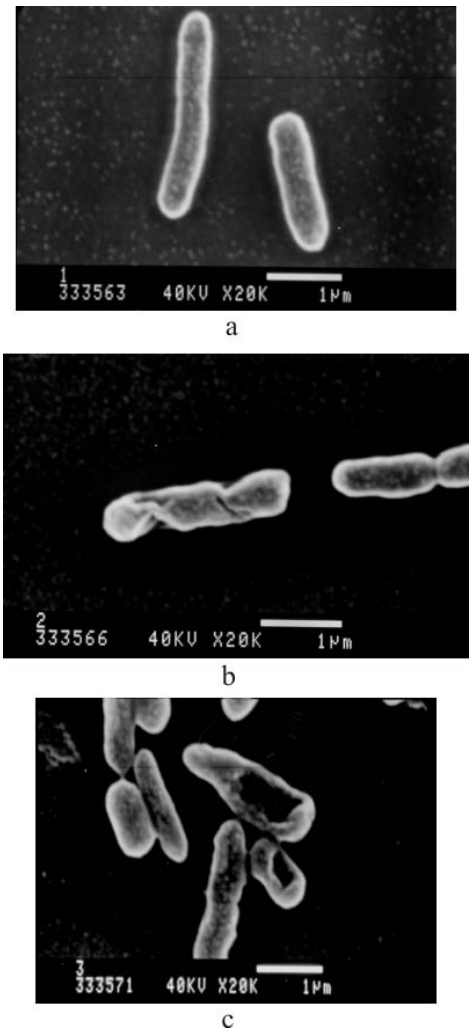

Fig. 8 SEM micrographs of the *E. coli* DH5α: a – the native cells, b – the cells grown in the presence of Co(II) with a concentration of 15 μg mL^{-1}, c – the cells grown in the presence of Co(sep)$^{3+}$ with a concentration of 143.1 μg mL^{-1}

50%. Thus, we conclude that the Co(II) and $Co(sep)^{3+}$ have the same toxic mechanism on *E. coli* DH5α, and the cellular damage is more severe under the higher inhibitory ratio.

ICP analysis

The total intracellular cobalt in the *E. coli* DH5α cell was estimated by ICP. The intracellular cobalt concentrations for Co(II) and $Co(sep)^{3+}$ in the *E. coli* DH5α are 8.6 and 182.9 μg mL^{-1}, respectively. And we did not found any cobalt in the blank. The results indicated that the Co(II) and $Co(sep)^{3+}$ compounds could be absorbed by the bacteria.

Conclusions

The present investigation reports the toxicity effects of two kinds of Co compounds on bacterial growth by applying a microcalorimetric technique. The experimental results show that the IC_{50} for Co(II) and $Co(sep)^{3+}$ are 15 and 42.1 μg mL^{-1}, and their lethal dose are 40 and 332.5 μg mL^{-1}, respectively. These values suggest that the toxic effects produced by Co(II) are stronger than that by $Co(sep)^{3+}$. Combining with the microcalorimetry, SEM and ICP results, it is no doubt that the compounds have the physiological functions and biological effects on the bacteria. Furthermore, these two compounds have a common mechanism of toxicity in inhibiting the *E. coli* DH5α growth, which can be attributed to the destroying of cell wall.

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