

Interaction of Zn(II) and Cu(II) with ϵ -poly-L-lysine and properties of the complexes

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Abstract. ϵ -Poly-L-lysine (ϵ -PL) is a natural food preservative with high safety to humans and a wide antimicrobial spectra. It was found that ϵ -PL could form precipitates when it contacted to Cu(II) and Zn(II). Thus in this study, two complexes of ZnCl₂ and CuCl₂ with ϵ -poly-L-lysine having molecular weights between 2 and 5 kDa were prepared and characterized by elemental analysis (EA), atom absorption spectrometry (AAS), chlorine assay, etc. The results show that the compositions of two complexes can be expressed as $[-NH-(CH_2)_5-CO-]_{2n} \cdot (ZnCl_2)_n \cdot 2nH_2O$ and $[-NH-(CH_2)_5-CO-]_{2n} \cdot (CuCl_2)_n \cdot 2nH_2O$ ($n=6-18$), respectively. The results of FT-IR reveal that the formation of Zn-N and Cu-N coordination bonds. X-RD patterns indicate that the two complexes are new phases different from the raw ϵ -PL material. On the basis of TG-DTA curves, the complexes have a coordination reaction. From the results of the bacteriostatic test, ϵ -PL-Zn(II) and ϵ -PL-Cu(II) have similar antibacterial activity to ligand ϵ -PL.

1 Introduction

ϵ -Poly-L-lysine (ϵ -PL), an unusual cationic, natural homopolyamide of L-lysine, is characterized as being edible, water soluble, stable at high temperatures and nontoxic to humans due to its biodegradability. Moreover, it exhibits antimicrobial activities against a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, yeasts, molds, and even viruses^[1]. Due to these properties, its use as a natural food preservative has attracted much attention in recent years. Thus, ϵ -PL has been authorized as a natural food preservative to apply in food in Japan, South Korea, the USA, China and other countries^[2,3].

Amino acids and peptides contain two coordination groups, $-COOH$ and $-NH_2$, which make them ideal complexing ligands to coordinate with many transition metals^[4]. These coordination complexes have tremendous application prospects in foodstuffs, medicine and cosmetics as nutrient additives^[5]. For example, the α -PL-copper(II) complex could catalyze the oxidation of 3,4-dihydroxyphenylalanine^[6]. Moreover, the complex of α -PL-Au(II) was used as probes for differential detection of glycosaminoglycans and phosphoproteins in the predentine and dentine of rat incisor^[7].

The ϵ -PL molecule contains many $-COOH$ and $-NH_2$ groups and can form complexes with many transition metals. Usually, the food system contains a variety of nutrients including trace elements. For instance, some canned fruits, seafood bread and dairy foods contain Zn²⁺ or Cu²⁺^[8,9]. If ϵ -PL is used as a preservative, the complexation between ϵ -PL and transition metals will

be considered. In addition, precipitation sometimes occurs when we add ϵ -PL to the trace element stock solution of Zn²⁺ and Cu²⁺. This phenomenon may be related to the coordination of ϵ -PL. Up to now, no report has been involved in the investigation of the coordination of ϵ -PL with transition metals^[10,11]. Therefore, complexes of ϵ -PL with Zn²⁺ or Cu²⁺ were prepared and characterized herein, respectively. The coordination interaction between ϵ -PL and Zn²⁺ or Cu²⁺ was investigated. Meanwhile, antimicrobial activities of the complexes were also assayed.

2 Material and methods

2.1 Materials

The negatively charged polysulfone flat membrane used for ultra-filtration with molecular weight cut-offs of 2 to 5 kDa were purchased from Alfa Laval Ltd. (Lund, Sweden). The strains used herein were provided by the Project of Public Service Platform for Excellent Industrial Microbial Strain Screening and Fermentation Technology, including *Bacillus subtilis* (CMCC 63501), *Staphylococcus aureus* (ATCC 63589), *Escherichia coli* (ATCC 25922), *Saccharomyces cerevisiae* (ATCC 26785).

2.2 Methods

2.2.1 Pretreatment of ϵ -poly-L-lysine

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The ϵ -PL (Zhejiang Yinxiang Bioengineering Co., Ltd., Tiantai, China) was classified by ultrafiltration to obtain a component having molecular weight of 2-5 kDa. The 2-5 kDa retentate was desalted with Sephadex G-25 (PHARMACIA, Sweden), reducing its conductivity to 3-5 $\mu\text{S}/\text{cm}$ (conductivity meter, FE30, METTLER TOLEDO instrument Shanghai Co., Ltd). The effluent was collected, concentrated by a rotary evaporator and then freeze-dried using Christ Alpha 1-4 LD (CHRIST, Germany). ϵ -PL powder was obtained and used to prepare complexes^[12].

2.2.2 Reaction of $\text{Zn}^{2+}/\text{Cu}^{2+}$ with ϵ -PL solution at different pH

The 10.0 g of purified ϵ -PL powder and 5.0 g of ZnCl_2 (or 6.0 g of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$) were dissolved in 100 mL of deionized water (conductivity less than 0.1 $\mu\text{S}/\text{cm}$) and treated with 2.0 mol/L NaOH and HCl solution. The pH of the ϵ -PL solution was adjusted to 3, 5, 7, 9, 11 and 13, respectively. The CuCl_2 or ZnCl_2 solutions that prepared above were added to ϵ -PL solutions of different pH, and the phenomenon was observed.

2.2.3 Preparation of complexes

The ϵ -PL and CuCl_2 (or ZnCl_2) solutions were prepared as described above and uniformly mixed. The mixture was adjusted to pH 8.0 with 2.0 mol/L NaOH and HCl solution to produce the corresponding precipitate of complex. The precipitate was filtered through a Buchner funnel and washed with deionized water. It was then placed in a desiccator and dried to constant weight at room temperature. The dried complex precipitate was ground to a homogeneous powder and used for the following measurements.

2.2.4 Solubility experiments of complexes at different pH

The two complexes were weighed in 0.05 g to 20 mL of deionized water, and the pH was adjusted to 3, 5, 7, 9, 11 and 13, respectively. The mixture was shaken at a constant temperature of 30°C for 4 h with a water bath shaking table (THZ-82, Changzhou Champion Instrument Manufacturing Co., LTD, China). Then the mixture was transferred into colorimetric tube for observation.

The two complexes solutions of pH 7 above were centrifuged at 1500 \times g. The 5.0 mL of the supernatant was added to a pre-weighed dry petri dish (W_0). Subsequently, the petri dish containing the supernatant was reweighed (W_1) and heated to constant weight (W_2) at 60°C. The solubility (S) of the two complexes were calculated from the following equation:

$$S(\text{g}/100\text{mL}) = (W_2 - W_0) / (W_1 - W_0) \times 100\% \quad (1)$$

Each experiment was repeated three times, and the average was taken.

2.2.5 Elemental analysis (EA) and atom absorption spectrometry (AAS).

The determination of C, H, and N content of the complexes were carried out by a Vario EL-III elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

The Zn^{2+} and Cu^{2+} were determined with atom absorption spectrometry method. Analytical grade chemicals and deionized water with a conductivity < 0.1 $\mu\text{S}/\text{cm}$ were used throughout the metal ions concentration assay. Each sample was transferred into TeflonTM vessels and digested by 10 mL HNO_3 and 2 mL HClO_4 on an electric furnace to be colorless or light yellow transparent. The digested solutions were diluted to an appropriate volume and analyzed by flame atomic absorption spectrophotometer (Shimadzu AA-6800, Kyoto, Japan) for the determination of Zn^{2+} and Cu^{2+} .

The determination of chlorine in complexes was conducted based on the Volhard's method in this study^[13]. In order to reduce the interference of Cu^{2+} , the sample solution was diluted with deionized water appropriately. Each experiment was repeated three times.

2.2.6 Analysis of Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra was determined using a Magna-560 FTIR spectrometer (Nicolet Co., Madison, Wisconsin, USA) with KBr pellet method^[14]. The scanning range was 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and measured at room temperature.

2.2.7 Thermogravimetric and differential thermal analysis (TG-DTA)

The TG-DTA analyses were recorded on a thermogravimetry analyzer Q50 (TA[®] Instruments Inc., New Castle, Delaware, USA). The samples were put into aluminum pans and measurements were carried out under air atmosphere at a heating rate of 10 °C/min from 25°C up to 800°C. The reference is α - Al_2O_3 .

2.2.8 Analysis of X-ray diffraction (XRD)

X-ray powder diffraction patterns were collected by means of a diffractometer of D8 Advance (Bruker, munich, Germany), with $\text{K}\alpha$ radiation of Cu ($\lambda = 0.154$ nm). Patterns were collected in the 2θ range of 5-80° with a stage size of 0.02° and a rate of 2° per minute.

2.2.9 Assay of antimicrobial activities(MIC)

In this study, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae* were selected as indicative bacteria to examine the antimicrobial activities of ϵ -PL, Zn^{2+} , Cu^{2+} , ϵ -PL-Zn(II) and ϵ -PL-Cu(II). The minimum inhibition concentration (MIC) of the five substances was determined on the basis of the liquid medium dilution method reported by Wiegand, Hilpert and Hancock^[15]. The bacteriostatic

agent of 10240-1.25 µg/mL was prepared by the "two-fold broth dilution method". The 1 mL of bacteriostatic agent and 100 µL indicator broth (10⁸ cfu/mL) were added to a test tube containing 9 mL of LB medium (cultured bacteria) or YEPD medium (cultured yeast). The culture was incubated 12-24 h at 37°C (culture bacteria) or 30°C (culture yeast) and 200 rpm in the shaker. At the same time, the blank control and three parallel experiments were performed for each concentration. The concentration of the lowest concentration test tube without bacterial growth is the MIC of the bacteria.

3 Result and discussion

3.1 Reaction of Zn²⁺/Cu²⁺ with ε-poly-L-lysine at different pH

In order to view the coordination of ε-PL with Cu²⁺, the CuCl₂ solution was dropped into the transparent ε-PL (100 g/L) with pH of 3, 5, 7, 9, 11, 13, respectively. It could be seen that obvious blue complex appeared at the pH of 7, 9, 11, 13 (Figure 1a). After the shock, for the test tube with pH of 7, 9, 11 and 13, the color of precipitates became deep blue or blue-purple with the increase of pH. The results are shown in Figure 1b. The deep blue or blue-purple color is the characteristic color of complex of Cu²⁺ with amine or amino acids^[16]. Thus, the appearance of deep blue precipitates in neutral and alkaline condition may be attributed to the formation of ε-PL-Cu(II). The similar situation was occurred for Zn²⁺. The results are shown in Figure 1c and Figure 1d. The white floc of complex of ε-PL with Zn²⁺ appeared in the range of pH 7 to 11. The floc dissolved after shaking at a pH of 13, which may be due to the amphoteric nature of the formed ZnO₂²⁻^[17].

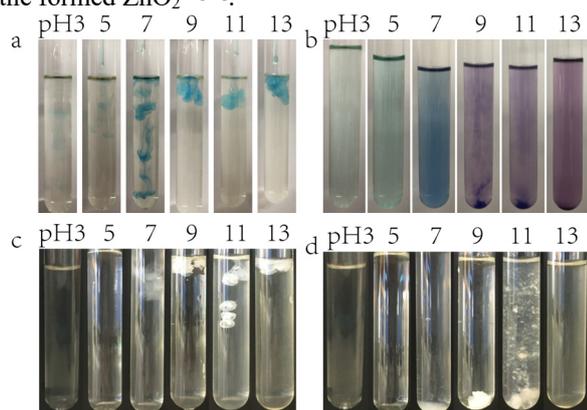


Figure 1. CuCl₂/ZnCl₂ was added dropwise to the ε-PL solution of different pH and allowed to stand (a)/(c) and shake (b)/(d).

Based on the above results, ε-PL can form a precipitate with Zn²⁺ and Cu²⁺ at pH of 7 to 11. Therefore, in the application of high concentration stock solutions of ε-PL and trace elements^[18], mixing should be avoided, and the method of adding ε-PL to foods should be considered.

3.2 The solubility of the prepared complexes at different pH

The prepared complex ε-PL-Zn(II) is pale white crystal, and ε-PL-Cu(II) is blue crystal. Both complexes were completely dissolved in water at pH 3 and 5. For ε-PL-Zn(II), it did not dissolve in the pH range of 7 to 11. However, it was dissolved at pH of 13, which may be due to the amphoteric nature of zinc formed ZnO₂²⁻ (Figure 2a). For ε-PL-Cu(II), the color changes with pH., The ε-PL-Cu(II) dissolved under acidic conditions, and the solution was light blue. At pH 7, however, a distinct dark blue precipitate was observed. And as the pH increased, the precipitate turned into blue-violet solid (Figure 2b). This is the characteristic color of the complex of Cu²⁺ with an amine or an amino acid. Considering that ε-PL is usually applied under neutral pH conditions, the solubility of the complexes under neutral conditions was measured in this experiment: the solubility of ε-PL-Zn(II) is 0.16 g/100mL, and the solubility of ε-PL-Cu(II) is 0.06 g/100mL.

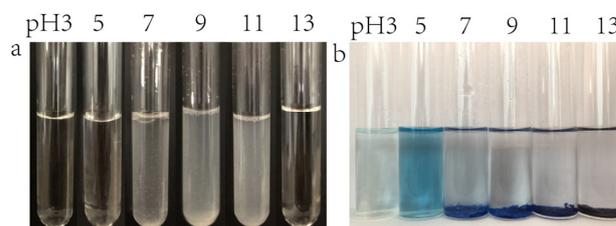


Figure 2. Different pH aqueous solutions of ε-PL-Zn(II) (a) and ε-PL-Cu(II) (b).

3.3 Elemental analysis of complexes

In this study, the contents of C, H, N in the complexes were determined by EA, Cl⁻ was determined by AAS, and Zn²⁺ and Cu²⁺ were determined by flame detector. The contents of the main constituent elements in the two complexes are shown in Table 1. The empirical formula was derived from the percentage of the element content in the complexes. Based on the empirical formula, the element content of each complex basically conforms to the calculated element content, indicating that Zn²⁺ (or Cu²⁺) can coordinate with the two side chains -NH₂. The

Table 1. The results of EA, AAS and chlorine assay of complexes.

Complex	Empirical formula	Content (found/calcd.,%)				
		C	H	N	Zn/Cu	Cl
ε-PL-Zn(II) ^a	(C ₁₂ H ₃₀ Cl ₂ N ₄ O ₄ Zn) _n	32.99/33.46	7.24/7.02	12.83/13.01	15.06/15.19	15.84/16.46
ε-PL-Cu(II) ^b	(C ₁₂ H ₃₀ Cl ₂ N ₄ O ₄ Cu) _n	33.06/33.61	6.98/7.05	12.87/13.06	15.01/14.82	15.73/16.53

^a [NH-(CH₂)₅-CO] _{2n}. (ZnCl₂)_n. 2nH₂O, n = 6-18.

^b [NH-(CH₂)₅-CO] _{2n}. (CuCl₂)_n. 2nH₂O, n = 6-18.

composition of the two complexes can be expressed as $\text{[NH-(CH}_2\text{)}_5\text{-CO]}_{2n}\cdot(\text{ZnCl}_2)_n\cdot 2n\text{H}_2\text{O}$ and $\text{[NH-(CH}_2\text{)}_5\text{-CO]}_{2n}\cdot(\text{CuCl}_2)_n\cdot 2n\text{H}_2\text{O}$ ($n=6-18$), respectively.

3.4 FT-IR spectra of ϵ -poly-L-lysine and complexes

To further investigate the chemical structure of complexes, FT-IR spectra of the three species was examined. The results are shown in Figure 3 and Table 2. Compared with the ligand, obvious changes occurred in the feature and fingerprint regions of the FT-IR spectra of complexes. The characteristic absorptions of the ligand were similar to those reported previously^[12,19,20].

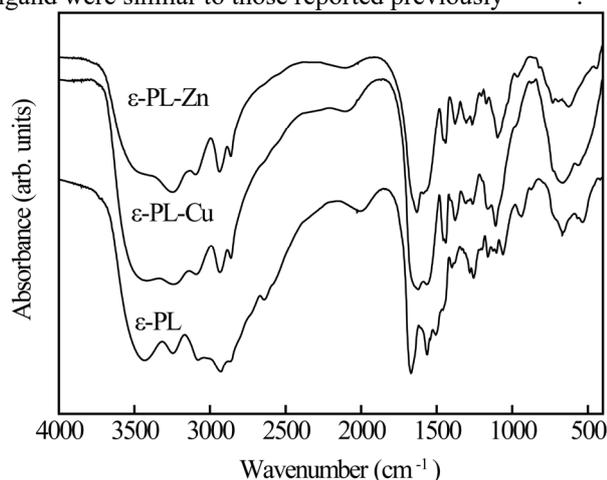


Figure 3. FT-IR spectra of ϵ -PL-Zn(II), ϵ -PL-Cu(II) and ϵ -PL.

The -NH_2 asymmetry vibrations in ϵ -PL-Zn(II) and ϵ -PL-Cu(II) shifted toward the low frequencies compared with those in ϵ -PL ligand. However, the N-H stretching (Amide A and B) and deformation (Amide II) bands shifted to the high frequencies due to their coordination with Zn^{2+} and Cu^{2+} . The N-H stretching absorptions were observed at $3,251\text{ cm}^{-1}$ and $3,096\text{ cm}^{-1}$ for ϵ -PL-Zn(II), and for ϵ -PL-Cu(II), observed at $3,254\text{ cm}^{-1}$ and $3,091\text{ cm}^{-1}$. The N-H deformation vibration absorption peaks of the two complexes were $1,589\text{ cm}^{-1}$ and $1,567\text{ cm}^{-1}$, respectively. The absorption peaks at $1,632\text{ cm}^{-1}$ and $1,623\text{ cm}^{-1}$ were attributed to the stretching vibration of C=O groups (Amide I). Compared with the ligand, they appeared at lower frequencies. The $1,506\text{ cm}^{-1}$ deformational mode bands of charged side chain amine groups, -NH_3^+ in the ligand disappeared in the complexes. On the contrary, in the fingerprint, the peaks at 627 cm^{-1} for ϵ -PL-Zn(II) and 567 cm^{-1} for ϵ -PL-Cu(II) appeared, which might be due to the Zn-N and Cu-N stretching vibrations, respectively. In general, the infrared absorption peaks of crystal water appear at $3,300$

cm^{-1} and $1,630\text{ cm}^{-1}$. In this study, they may coincide with the absorption peaks of Amide A and Amide I^[21]. When Belfiore and McCurdie characterized the complexes formed by α -PL with Co^{2+} , Ni^{2+} and Zn^{2+} , respectively, they speculated from their infrared spectra that the oxygen on the carboxyl group of the α -PL main chain participated in the complexation reaction. When the transition metal ions was combined with C=O, the hydrogen bond bonded to the amino group is destroyed, thereby reducing the glass transition point^[22]. Consequently, it can be concluded that α -amino and C=O groups in ϵ -PL were coordinated with Zn^{2+} and Cu^{2+} . That is, the coordination bonds Zn-N and Cu-N are formed.

3.5 Thermal behavior of ϵ -poly-L-lysine and the complexes

In order to obtain information on the composition, the TGA and DTA curves of the complexes were determined and given in Figure 4. The thermogram of ϵ -PL-Zn(II) shows a sharp endothermic peak at 172.8°C in DTA curve. Correspondingly, there was a weight loss of 7.25% from 25°C to 210°C in TGA curve, and this stage was attributed to the dehydration process (the calculated weight loss of this stage was 8.36%). Two exothermic peaks appeared at 351.1°C and 536.8°C , respectively. Accordingly, there were two drastic weight losses from 210°C to 440°C and 440°C to 670°C , respectively, and these were the thermal decomposition processes of the complex. The total weight loss of three stages was 87.51%. The residual may be ZnO ^[23]. And for ϵ -PL-Cu(II), the dehydration process took place from 25°C to 230°C with 9.60% of weight loss in TGA curves accompanied with a sharp endothermic peak at 183.3°C in DTA curve. The two weight loss stages in the ranges of 230°C to 450°C and 450°C to 710°C were the thermal decomposition process, and two exothermic peaks appeared at 385.5°C and 474.8°C , respectively, in DTA curve. The total weight loss was 87.36%. The residual may be CuO ^[24]. But the residual weights of ϵ -PL-Zn(II) and ϵ -PL-Cu(II) were lighter than the calculated residual (the calculated residual weights were 15.18% and 14.82%, respectively), which may be due to the simultaneous formation of ZnCl_2 or CuCl_2 and volatilization as the temperature increases^[25].

Similar to the report by Shima et al., ϵ -PL begins to soften and decompose at 300°C without melting^[3]. That the two compounds behaved differently in TG-DTA curves from the ligand indicates the formation of the complexes.

Table 2. Observed FT-IR frequencies (cm^{-1}) of ϵ -PL and the complexes.

Sample	νNH_2	Amide A	Amide B	νCH_2	Amide I	Amide II	$\delta\text{-NH}_3^+$	$\nu\text{M-N}$
ϵ -PL	3,431	3,242	3,079	2,929	1,670	1,563	1,504	
ϵ -PL-Zn(II)	3,411	3,251	3,096	2,937	1,632	1,589		627
ϵ -PL-Cu(II)	3,418	3,254	3,091	2,934	1,623	1,567		567

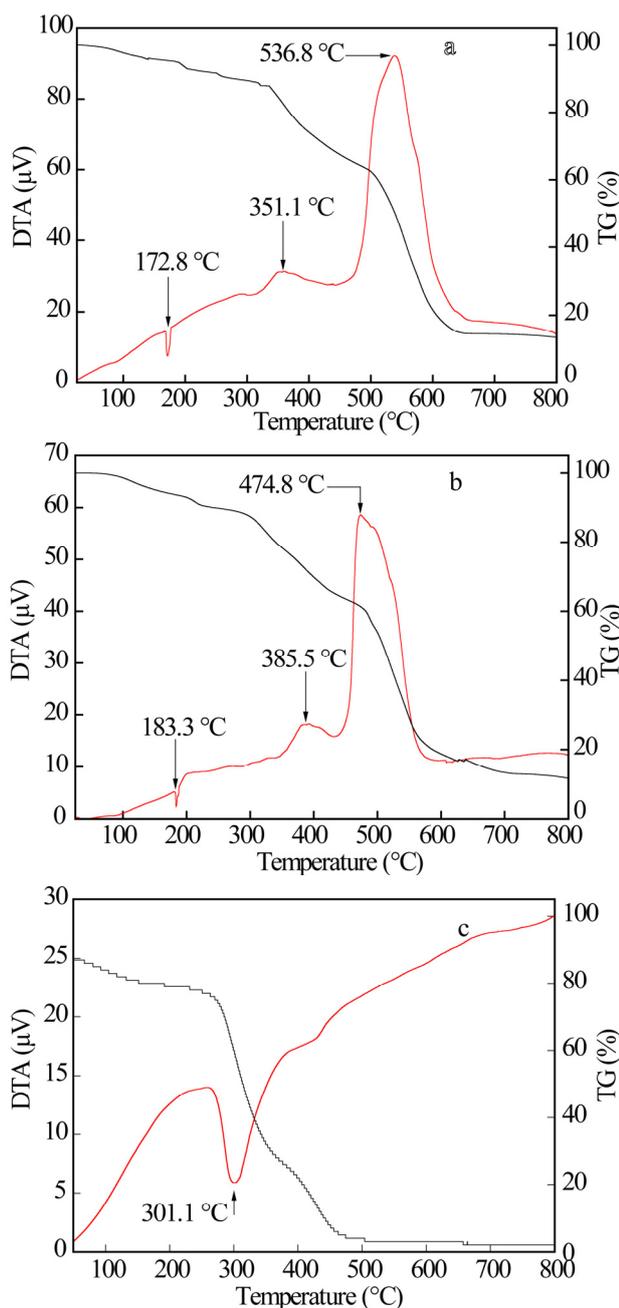


Figure 4. The TGA and DTA curves for ϵ -PL-Zn(II) (a), ϵ -PL-Cu(II) (b) and ϵ -PL (c).

3.6 X-ray diffraction (XRD) of ϵ -poly-L-lysine and complexes

The crystal structure analysis of the sample can be carried out by XRD. In this study, the XRD of ϵ -PL, ϵ -PL-Zn(II) and ϵ -PL-Cu(II) was detected to compare the changes in their crystal structure. The XRD patterns of them are shown in Figure 5.

There are significant differences between ϵ -PL and the complexes. The main peak appeared at 24.81° of 2θ for ϵ -PL, however, the corresponding peaks for ϵ -PL-Zn(II) and ϵ -PL-Cu(II) appeared at 22.24° and 22.31° , respectively. In addition, the newly appeared diffraction peaks at 11.60° for ϵ -PL-Zn(II) and 12.59° for ϵ -PL-Cu(II), respectively, different from ϵ -PL, which indicates that ϵ -PL-Zn(II) and ϵ -PL-Cu(II) are new compounds different from the ligand^[26].

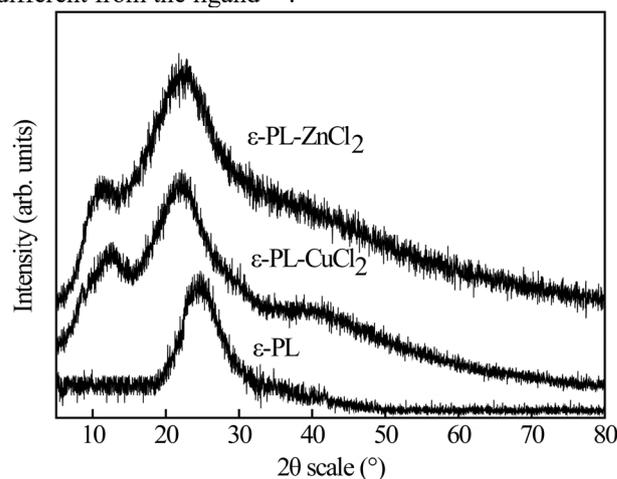


Figure 5. XRD patterns of ϵ -PL-Zn(II), ϵ -PL-Cu(II) and ϵ -PL.

3.7 Antimicrobial activities of ϵ -poly-L-lysine and complexes

ϵ -PL has a extensive spectrum of inhibition, and the metal ions also have bacteriostatic. In order to investigate the effect of two metals ions on the bacteriostatic activity of ligand ϵ -PL, this study examined the MIC of ϵ -PL, two metal ions and two complexes. The results in Table 3 suggest that zinc ions are not effectively inhibited by *Saccharomyces cerevisiae* in the tested concentration range, and the remaining bacteriostatic agents can inhibit the growth of the indicator strain. The antibacterial activities of ϵ -PL-Zn(II) and ϵ -PL-Cu(II) were significantly better than the two metal ions and similar to ϵ -PL. This is because that in the five substances of the same mass, a small part of the ϵ -PL in the complexes was

Table 3. Antibacterial activities of ϵ -PL ligand, Cu^{2+} , Zn^{2+} and complexes.

Indicator	MIC ($\mu\text{g/ml}$)				
	ϵ -PL	Zn(II)	Cu(II)	ϵ -PL-Zn(II)	ϵ -PL-Cu(II)
<i>Bacillus subtilis</i>	2	128	256	4	4
<i>Staphylococcus aureus</i>	8	64	256	16	16
<i>Escherichia coli</i>	16	128	256	32	32
<i>Saccharomyces cerevisiae</i>	256	-	1024	256	256

Note: "-" indicates that the bacteriostatic agent has no bacteriostatic activity against the indicator bacteria in the concentration range tested.

replaced by metal ions, resulting in the content of ϵ -PL slightly lower than the pure ϵ -PL. It can also speculate that the coordination bonds Zn–N and Cu–N formed by the complexes affect their own charge, resulting in a slight decrease in bacteriostatic activity^[27]. The overall data display that the antibacterial activity of the complexes ϵ -PL–Zn(II) and ϵ -PL–Cu(II) was similar to that of the ligand ϵ -PL. The application of the complexes is for further study.

4 Conclusions

In order to explain the phenomenon that ϵ -PL can form precipitates with Cu(II) and Zn(II) in its application, the two complexes of ϵ -PL with ZnCl₂ and CuCl₂ were prepared respectively. The compositions of two complexes can be expressed as $[-NH-(CH_2)_5-CO-]_{2n} \cdot (ZnCl_2)_n \cdot 2nH_2O$ and $[-NH-(CH_2)_5-CO-]_{2n} \cdot (CuCl_2)_n \cdot 2nH_2O$ (n=6-18), respectively, based on EA, AAS and chlorine assays of complexes. The results of FT-IR reveal the coordination information for the ϵ -PL–Zn(II) and ϵ -PL–Cu(II) that their -NH₂ asymmetry stretching and C=O stretching (Amide I) vibrations shifted toward the low frequencies compared with the ϵ -PL ligand. Meanwhile, the N-H stretching (Amide A and B) and deformation (Amide II) bands shifted to the high frequencies due to their coordination with Zn²⁺ and Cu²⁺. The peaks at 627 cm⁻¹ for ϵ -PL–Zn(II) and 567 cm⁻¹ for ϵ -PL–Cu(II) indicate the formation of Zn–N and Cu–N coordination bonds. XRD patterns show that the two complexes are new phases different from the raw ϵ -PL material. TG-DTA curves show that both the two complexes have different thermal behavior from ϵ -PL, indicating that the complexes have a coordination reaction. The antibacterial tests prove that the complexes have similar inhibitory activities to the ligand ϵ -PL.

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