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Preparation and Mimic Enzyme Catalytic Kinetics of Dendritic Copper Complex

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A new kind of dendritic-salicylaldehydeimine ligand was synthesized using 1.0G polyamidoamine and salicylaldehyde as raw materials through Schiff reaction and further formed dendritic copper complex by Cu complex ion. The structures of dendritic ligand and its copper complex were characterized by elemental analysis, IR, NMR, UV and XRD. With H_2O_2 as the oxidant, the oxidation reaction performance of dendritic copper complex catalyzing ascorbic acid was determinated by spectrophotometry and catalytic kinetic model was established. The influences of catalytic reaction order, pH value of buffer and complexes concentration on catalytic reaction rate constant were investigated. Results showed that the dendritic copper complex had good mimic enzyme activity. The catalytic reactions had the same features of pseudo-first-order reaction to enzymatic reaction. The catalytic reaction rate obviously imcreased under the conditions of higher concentration of mimic enzyme complex and pH = 7-7.5.

Keywords: Dendrimer, Schiff reaction, Mimic enzyme activity, Catalytic kinetics.

INTRODUCTION

Dendrimer is a new synthetic polymer with very ordered and exquisite structure, whose molecular volume, shape and function can be accurately controlled. Poly(amidoamine) is a typical dendrimer which has drawn widespread interest from the domestic and overseas academic circles at present. Due to its vast internal empty cavity and high density peripheral group, it can be used as an ideal mimic enzyme of protein, enzyme and virus. It has found wide application in the field of biology[1,2], medicine[3-5], catalyst[6,7] as its peripheral groups can be easily modified.

Enzymatic reaction is noticeable due to its high effectiveness, specificity and mild application conditions. However, there are so much difficulties in acquisition, purification and preservation that it's hardly used in practice. Thus the development of artificial enzyme has become an important research subject in modern chemistry and bioic technology fields. Study on the structure, chemical property and application of peroxidase, especially for horseradish peroxidase, has been reported[8-10]. Researches on mimic enzyme is vitally significant as horseradish peroxidase is expensive, easy to lose activity.

Based on the advantages of poly(amidoamine) in mimic enzyme and our previous work[11], dendritic copper complex was synthesized by introducing salicylaldehyde to end groups of poly(amidoamine) firstly and further complexing with copper, in order to give mimic enzyme with better effects. At the same time, performance and catalytic reaction kinetics of dendritic copper complex catalyzing ascorbic acid were investigated. The structure of dendritic-salicylaldehyde-imine ligand and dendritic copper complex (Fig. 1).

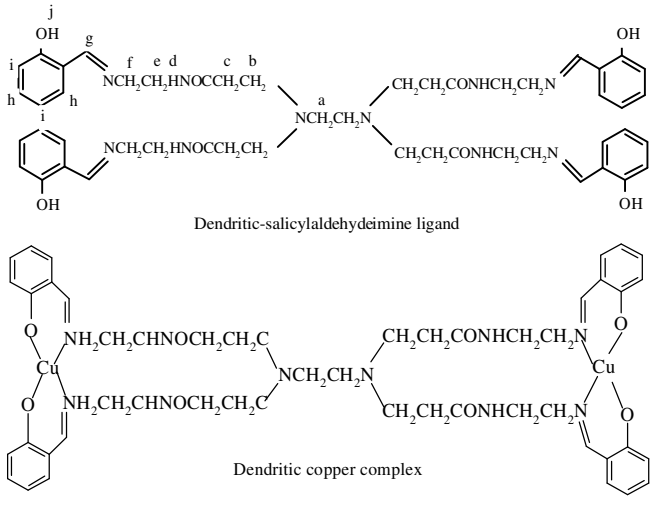


Fig. 1. Dendritic-salicylaldehydeimine ligand and dendritic copper complex

EXPERIMENTAL

The anhydrous methanol and alcohol are analytical reagents purchased from Shenyang Xinxing and Shenyang Donghua chemical plant, respectively. The salicylaldehyde is chemically pure purchased from Guoyao Chemical Co., Ltd.. The copper acetate (AR) are provided by Beijing Shuanghuan

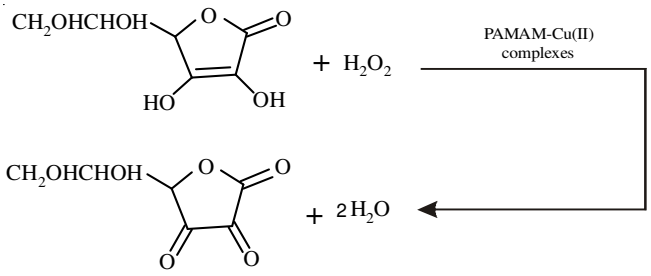
chemical reagent factory. Anhydrous sodium sulfate purchased from Harbin chemical plant. 1.0 G poly(amidoamine) was synthesized in a method similar to that referred in one literature[12]. Deionized water was secondary distillation before use.

4.6 g (8.91 mmol) 1.0 G poly(amidoamine) was resolved in 80 mL anhydrous alcohol and added into a flask which has magnetic stirrer, reflux condensation tube and thermometer. In addition, 3 g anhydrous sodium sulfate was added under N_2 and refluxed for 10 min with efficient stirring at 78 °C. Then 8.6 g (0.07 mol) salicylaldehyde was added gradually into the flask. The mixture was stirred for 12 h at 78 °C and filtered, standing for 36 h to give a yellow crystal. The crude product was washed 3 times with anhydrous alcohol. Then the crystal was recrystallized from anhydrous alcohol and dried under vacuum at 50 °C to obtain dendritic-salicylaldehydeimine ligand. The yield was 86.7 % and the melting range was 152-153 °C. Anal. calcd. for ($C_{50}H_{64}O_8N_{10}$): C, 64.38; H, 6.87; N, 15.02. Found: C, 64.16; H, 6.17; N, 15.09 %.

0.8 g (0.86 mmol) ligand was resolved in 25 mL anhydrous methanol and added into a flask which has magnetic stirrer, reflux condensation tube and thermometer. The ligand methanol solution was stirred for 10 min under N_2 at 25 °C. 25 mL copper acetate-anhydrous methanol solution (0.344 g, 1.72 mmol) was added gradually into the flask. After 24 h reaction, the mixture was put into ice-water, pumping filtrating, washing 3 times with anhydrous methanol and drying under vacuum at 70 °C to give a dark green solid (dendritic copper complex, 88.2 % yield). The yield was 88.2 % and the melting range was 250-251 °C. Anal. calcd. for ($C_{50}H_{60}N_{10}O_8Cu_2$): C, 56.87; H, 5.69; N, 13.27. Found: C, 55.83; H, 5.87; N, 12.90 %. The analysis to copper complex found that metal ions/ligand ratio was 2, which was also to say metal complex was formed with one ligand molecular and two Cu(II).

Mimic enzyme activity is determinated by spectrophotometry. Ascorbic acid (Vit. C) has absorption peak in 265 nm, which disappears when it is oxidezed by H_2O_2. Therefore, the variation of absorbance value ΔA in 265 nm could be used to represent the activity of mimic enzyme complex, namely large ΔA illustrates high activity.

Phosphoric acid buffer solution (2 mL), mimic enzyme complex in DMF (0.1 mL) and ascorbic acid solution (0.5 mL) were orderly added to 10 mL colorimetric tube with deionized water diluting to graduation line. Then the solution was removed to quartz colorimetric dish, absorbance initial value (A_1) in 265 nm was determinated by spectrophotometry. 5 μL H_2O_2 solution (0.3 %) was added under constant temperature for 10 min, absorbance terminal value A_2 was determinated in the same way and insteaded of mimic enzyme, H_2O was used in blank experiment. $\Delta A = A_1 - A_2$, catalytic reaction equation as follows:



Phosphoric acid buffer solution (2 mL, pH = 7), mimc enzyme complex in DMF (0.1 mL, 1.99×10^{-4} mol L^{-1}), ascorbic acid solution (0.5 mL) and 5 μL H_2O_2 solution (0.3 %) were orderly added to 10 mL colorimetric tube with deionized water diluting to graduation line and absorbance A in 265 nm was determined every 2 min, the realationship of $\ln(C_0/C_t)/t$ *vs*. ascorbic acid concentration was determined.

Phosphoric acid buffer solution (2 mL), mimic enzyme complex in DMF (0.1 mL, 1.99×10^{-4} mol L^{-1}), ascorbic acid solution (0.5 mL, 5×10^{-4} mol L^{-1}) and 5 μL H_2O_2 solution (0.3 %) were orderly added to 10 mL colorimetric tube with deionized water diluting to graduation line and absorbance A in 265 nm was determinated every 2 min. The relationship of $\ln(C_0/C_t)/t$ *vs*. pH of phosphoric acid buffer solution was calculated.

Phosphoric acid buffer solution (2 mL, pH = 7), mimic enzyme complex in DMF (0.1 mL), ascorbic acid solution (0.5 mL, 5×10^{-4} mol L^{-1}) and 5 μL H_2O_2 solution (0.3 %) were orderly added to 10 mL colorimetric tube with deionized water diluting to graduation line and absorbance A in 265 nm was determinated every 2 min, The realationship of $\ln(C_0/C_t)/t$ *vs*. concentation of mimic enzyme complex was investigated.

Detection method: IR, ^{1}H NMR spectroscopy, UV spectrum, elemental analysis and XRD analysis were used to establish the structures of ligand and metal complex. FTIR spectra was detemined using Nicolet FT-IR750 infrared spectrometer of America. Elemental analysis was determined with Heraeus element analyzer of Germany. Absorbency was determined using UV-1700 UV-visible spectrophotometer. NMR spectra was recorded with American Varian NOVA400 MHz nuclear magnetic resonance apparatus ($CDCl_3$ as solvent, TMS as internal standard). XRD analysis was determined using X-ray diffraction of Japan (CuK_α radiation, tube voltage 40 kV, tube current 30 mA, scan rate 100/min, scanning scope 2θ = 100-800).

RESULTS AND DISCUSSION

Infrared spectra of dendritic-salicylaldehydeimine ligand and copper complexe: As is shown in Fig. 2, 1638 cm^{-1} was ascribed to the vibration of C=N which can prove the occurrence of shiff base reaction between 1.0G poly-(amidoamine) and salicylaldehyde[13]. The band around 3411 cm^{-1} which is due to the ν(-OH) stretching frequency of ligand was broad and weak. The blue-shift of the absorption may be attributed to the intra molecular hydrogen bond formed between the -OH of benzene ring and the N atom of >C=N-. After coordination, the absorption peak of ν(OH) near 3400 cm^{-1} disappears, which shows that the hydrogen atom lost by -OH participates in coordination. The coordination between copper ion and nitrogen atom after complexing of metal and ligand saps the strength of double bond C=N, enhances the C-H bond bending vibration of carbon atom and enables the absorption peak of ν(C=N) to move to lower wavenumber. Therefore, the wavenumber at the adsorption peak of C=N of metal complex is less[14]. The phenomenon that the stretching vibration of ν(Ar-O) moves to high wave also shows that -OH participates in coordination, which causes planar failure to benzene ring. The phenomenon that the absorption peak of

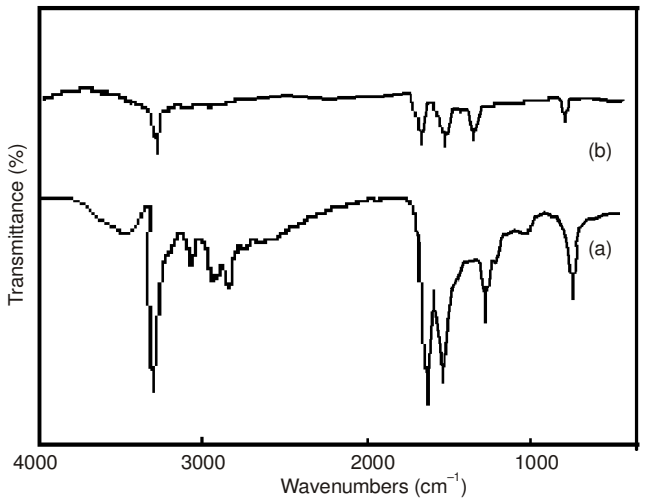


Fig. 2. Infrared spectrum of ligand and copper complex (a) ligand, (b) copper complex

ν(C=C) moves to lower wavenumber shows that C=N participates in coordination, which embodies the effects of the change in substituent on benzene ring. Meanwhile, there is N-H stretching vibration peak for amide in both ligand and complex in 3300-3250 cm^{-1} region.

^{1}H NMR analysis of ligand: The ^{1}H NMR spectrogram for synthetic dendritic salicylaldimine ligand are given in Fig. 3. All the H atoms in the synthetic ligand are reasonably assigned. The analysis results are shown in Table-1.

TABLE-1
^{1}H-NMR (δ, ppm) OF LIGAND

Compound	H_a	H_b	H_c	H_d	H_e
Shift (δ, ppm)	2.33	2.56	2.23	7.20	3.47-3.51
Compound	H_f	H_g	H_h	H_i	H_j
Shift (δ, ppm)	3.66-3.69	8.30	7.30-7.20	6.91-6.84	13.26

As shown in Fig. 3, 2.23-2.56 and 3.47-3.70 are the characteristic proton peaks on the methylene in ligand skeleton; due to strong electron withdrawing property, the density of electron cloud around the proton at position d becomes less, so δ gradually moves to low magnetic field. Position j is the hydroxyl H atom of ph-OH and its δ value is 13.26 ppm, which is greater. Therefore, it is possible that the density of electron cloud around ^{1}H nucleus in hydrogen bond decreases, ^{1}H is under low magnetic field and δ value increases, due to the formation of intra-molecular hydrogen bond between the hydroxyl H atom of ph-OH and the N atom of >C=N- and the electrostatic interaction. This happens to correspond to wide and weak band spectrum of stretching absorption peak of -OH peak of ligand in infrared spectrogram at 3411 cm^{-1}. There is no H atom on the adjacent carbon in H_g atom and H_j atom and the two H atoms at position H_e have the same chemical environment, without coupling, so all appear in the form of single peak[11]. Unlike H_g and H_j, multiplets are formed by a phenomenon called spin-spin coupling splitting in H_h and H_i due to the interference of adjacent hydrogen nucleus. Meanwhile, the overlapped effects of the substituents -C=N- and -OH on benzene ring and H_h and H_i decrease the density of the electron cloud at the *ortho*- and *para*- positions relative

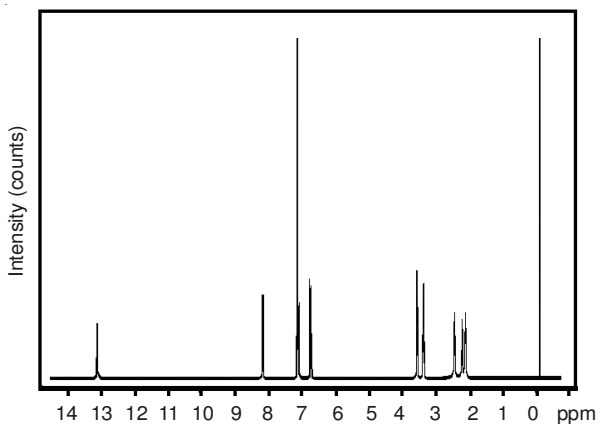


Fig. 3. ^{1}H NMR of ligand

to -C=N- on benzene ring, increase δ_{Hh} value, increase the density of the electron cloud at meta-position and decrease δ_{Hi} value. From the diagram, we can also calculate the peak area ratio of 10 kinds of H atom as S_a: S_b: S_c: S_d: S_e: S_f: S_g: S_h: S_i: S_j = 1: 2.19: 2: 1.20: 2.08: 2.08: 1.01: 2.20: 2.06: 1 (theoretical value is 1: 2: 2: 1: 2: 2: 1: 2: 2: 1), which is basically consistent with theoretical value. Meanwhile, the sum of the integral curve area ratios corresponding to all the H atoms is about 16 and the total number of molecular hydrogen nucleus is 64, which happens to be 4 times as great as the sum of integral areas. It can be inferred that this molecule has high symmetry.

UV spectrum analysis of dendritic copper complexes: Using dimethyl sulfoxide as solvent, the UV spectrum was determined (Table-2). The absorption at 252 and 323 nm shows ligand-field transition. In metal complex, the first transition position stays unchanged basically, but the second position has significant red shift. In ligand, the strong absorption peak at 218 and 247 nm are band E_2 of benzene ring and band K arising from the conjugation of benzene ring and the C=N. Band B of benzene ring is hidden by band K. Band R of $n\rightarrow\pi^*$ transition for >C=N- appears at 323 nm. After coordination between ligand and copper ion, band R red-shifts to 336 nm. This shows that N atom participates in coordination, which enhances delocalized conjugation and causes red shift to absorption spectral peak. Band E_2 and band K has slight change.

TABLE-2
UV-VISIBLE SPECTRA OF LIGAND AND COPPER COMPLEX

Product	λnm (ε/L mol^{-1} cm^{-1})	
Ligand	252 (2.350×10^7)	323 (3.012×10^7)
Copper complex	253 (2.669×10^7)	336 (3.439×10^7)

XRD analysis of dendritic copper complex: XRD spectra of copper acetate, ligand and copper complex were shown in Fig. 4. Contrasting the XRD spectra of ligand and copper complex, we could find that ligand in 21^0 for 2θ has stronger diffraction peak and very weak in copper complex, which suggests that the complexing with ligand and copper ion in the copper acetate makes crystalline structure of copper complex more disorderly and crystal particle more tiny. It is disorderly structure that makes active components stable and sufficient dispersed in ligand molecular.

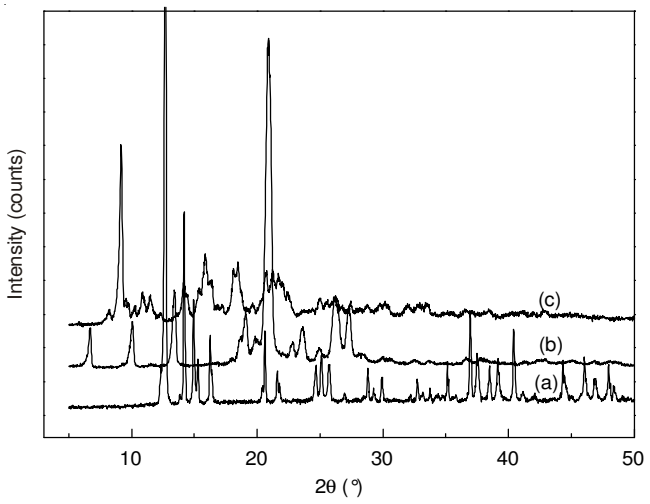
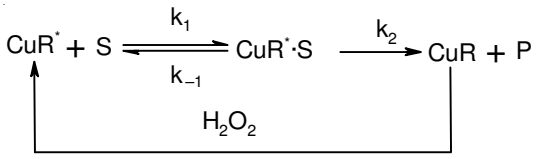


Fig. 4. XRD spectra (a) copper acetate, (b) ligand, (c) copper complex

Kinetic model: Similar to enzymatic reaction, intermediate is firstly formed with mimic enzyme complex and substrate by reversible combining and then decomposes into product[15]. It is different that we propose that the active species (CuR^*) is firstly formed with copper complex (CuR) (reaction is completed instantly) when CuR is oxidated by H_2O_2 and generates intermediate ($CuR^*{\cdot}S$) by combining ascorbic acid (S), decomposes into product, then CuR^* is reduced to initial CuR which is the rate determining step, finally CuR is oxidaded repeatedly into CuR^*. Therefore, the kinetic model as follows in the catalytic system:



k_1, k_{-1} are the positive and negative reaction rate separately and k_2 is first-class rate constant of rate determining step. As $CuR^*{\cdot}S$ is formed in the process, the reaction of H_2O_2 oxidizing ascorbic acid was changed into electronic transfer reaction of intramolecular, which reduces activation energy. CuR^* is namely mimic enzyme E, $CuR^*{\cdot}S$ for ES.

In certain concentration range, as long as the concertration of substrate S increases, the reaction rate increases, which illustrates that the reaction rate comforms to the Michaelis-Menten equation to some extent.

$$r_p = \frac{r_{p,\max} \cdot [s]}{k_s + [s]} \tag{1}$$

among r_p is the biggest generation rate of p.

G.E. Briggs and J.B. Haldane revised the hypothesis of Michaelis-Menten equation, proposing "quasi-steady" hypothsis. It believes that the concentration of S is much higher than E in the system and the E decomposed by ES can instantly combine with S, so the concentration of ES keeps unchanged nearly, namely the concentration of ES is no longer changed as time.

$$\frac{d[ES]}{dt} = 0 \tag{2}$$

$$k_1 \cdot [E] \cdot [S] - (k_{-1} + k_2) \cdot [ES] = 0 \tag{3}$$

Eliminating ES gives

$$r_p = \frac{k_2 \cdot [E_0] \cdot [S]}{\frac{k_{-1} + k_2}{k_1} + [S]} = \frac{r_{p,\max} \cdot [S]}{k_m + [S]} \tag{4}$$

K_m is Michaelis-Menten constant(mol/L).

Relationship of k_m and k_s gives

$$k_m = k_s + \frac{k_2}{k_1} \tag{5}$$

K_2 expresses that the number of molecule is catalyzed by an enzyme, so it reflects the enzyme reaction ability and its value is varied as different enzyme catalysis.

$$r_s = r_p \quad r_{s,\max} = r_{p,\max} = r_{\max} \tag{6}$$

when $[S] << k_m$, reaction rate and the concentration of the substrate is approximate proportional, so enzyme catalysis can be approximately regarded as pseudo-first-order reaction.

$$r_s = \frac{r_{\max} \cdot [S]}{k_m} = k \cdot [S] \tag{7}$$

K is pseudo-first-order reaction constant of total reaction.

This is because the most of enzymes are in free state and the amount of [ES] are very tiny, when the value of k_m is very big. Only by increasing the value of [E] and further increasing the value of [ES], we may be to improve the reaction rate. So the reaction rate mainly depends on the change of substrate concentration now.

$$\text{Since} \quad r_s = -\frac{d[S]}{dt} \tag{8}$$

$$\text{Thus} \quad -\frac{d[S]}{dt} = k[S] \quad \text{or} \quad \frac{-d[S]}{[S] \cdot dt} = k \tag{9}$$

$$r_s = \frac{-d[S]}{dt} = k[S] \quad \text{or} \quad \ln\frac{[S_0]}{[S]} = kt \tag{10}$$

$$-\int_{[S]}^{[S]} \frac{1}{[S]} \cdot d[S] = k\int_0^t dt \tag{11}$$

$$\frac{-d[S_0]}{[S]} = kdt \tag{12}$$

S_0: initial concentration of substrate

When [S] is determinated using standard curve method by spectrophotometry, it is consistent with Lambert-Beer's Law.

$$A = \log\left(\frac{I_0}{I_t}\right) = \varepsilon bc \quad \text{Be} : A \propto c \tag{13}$$

A: absorption ε: molar absorption coefficient b: thickness of liquid layer (optical path length) c: molar concentration of liquid

$$\frac{-d[A_0]}{[A]} = kdt \tag{14}$$

The eqn. (14) indicates that catalytic performance of mimic enzyme complex can be evaluated by UV spectrophotometry.

Catalytic performance of mimic enzyme complex on H_2O_2 oxidizing ascorbic acid

Catalytic comparison of copper(II) complexes with horseradish peroxidase: Catalytic activity of two kinds of salicylaldehydeimine-Cu(II) complexes and horseradish peroxidase to H_2O_2 oxidizing ascorbic acid is gived in Table-3. (1) deionized water instead of mimic enzyme in blank experiment; (2) A is ethylenediamine-Cu(II) [Cu(Salen) and B as dendritic copper complex].

TABLE-3
CATALYTIC COMPARISON OF Cu(II) COMPLEXES AND HORSERADISH PEROXIDASE

Compd.	A	B	Horseradish peroxidase	Blank
$c/10^{-3}$ g mL^{-1}	1.0	1.0	1.0	0
t/min	5	5	5	5
ΔA	0.153	0.174	0.180	0.013

As shown in Table-3, both of Cu(II) complexes have horseradish peroxidase activity and dendrimer-Cu(II) complex is better which activity is equivalent to horseradish peroxidase, so dendrimer-Cu(II) complex is likely to be as an ideal mimic enzyme. On the one hand, the latter has two hydrophobic cavity in intramolecular consisting N, N, O, O and strong compatibility ept to combine substrate, so it may reduce the activation energy by substrte'directionality and distortion and deformation of band. On the other hand, the periphery of Cu(II) complex especially connecting high generation dendrimer have high density functional group, which makes it having multiple activity center and certain slow-releasing effect, so dendritic copper complex also shows unique dendritic effect.

Effect of pH of buffer solution on catalytic activity: Fig. 5 shows influence of buffer in pH 6-8.5 on oxidized property of ascorbic acid by mimic enzyme complex catalyzing. When pH is 6-7.5, the activity of mimic enzyme complex little changes and maximum of activity appears in approximately 7 in pH which is the optimal pH value in reaction system. But pH more than 7.5, enzyme activity decreases rapidly, which is same to the influence of pH on natural peroxidase[16].

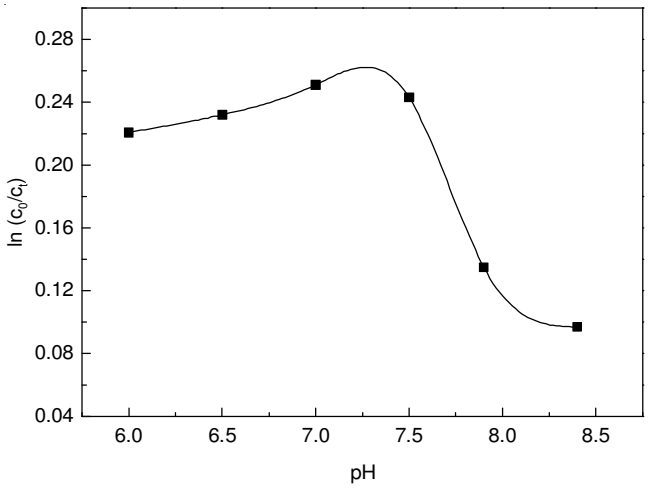


Fig. 5. Effect of pH of buffer on catalytic activity

Determination of orders of catalytic reaction: $\ln(C_0/C_t)/t$ changed in different concentration of ascorbic acid is shown in Fig. 6. $\ln(C_0/C_t)/t$ is nearly linear in different concentration of ascorbic acid and the correlation coefficients R are above 0.95, which accords with pseudo-first-order reaction characteristics. So the catalysis of mimic enzyme complex can be described by eqns. 12 or 14. Results show that the mimic enzyme displays obviously pseude-first-order reaction characteristic of enzymatic reaction.

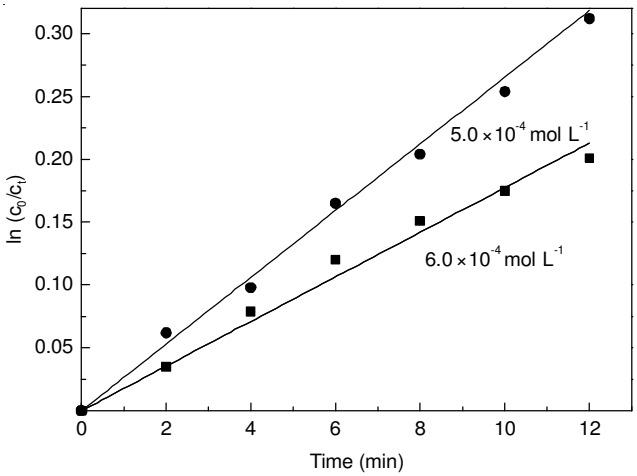


Fig. 6. Plots of $\ln(C_0/C_t)/t$ in different V_C concentration

Effect of pH of buffer on reaction rate constant: $\ln(C_0/C_t)/t$ changed in different concentration of ascorbic acid is shown in Fig. 7. The catalytic reaction rate constant is most when pH in 7-7.5 of buffer, which is also consistent to the conclusion of Fig. 5.

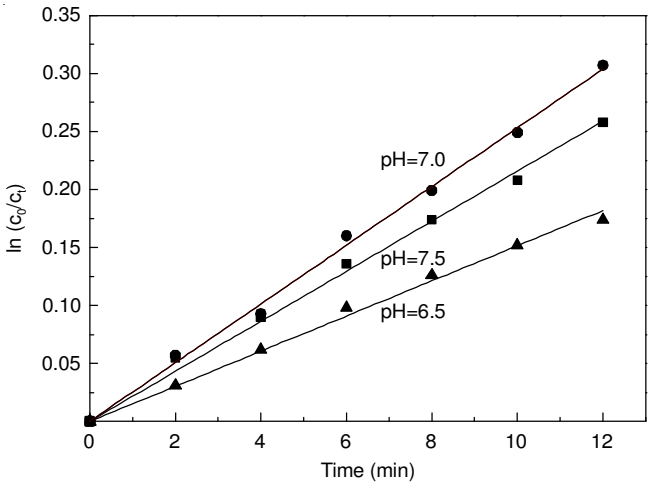


Fig. 7. Plots of $\ln(C_0/C_t)/t$ in different pH of buffer

Effect of mimic enzyme concentration on reaction rate constant: In Fig. 8, with the concentration of mimic enzyme complex decreasing, catalytic reaction rate constant reduces. This is because catalyst can reduce reaction activation energy (E_a) and the less concentration of catalyst declines, the less activation energy decreases, which is identical with Arrhenius equation $k = Ae^{\frac{-E_a}{RT}}$ (A: preexponential factor, R: molar gas constant).

The research on mimic enzyme with multiple recognition function, has become a challenging new topic in mimic enzyme

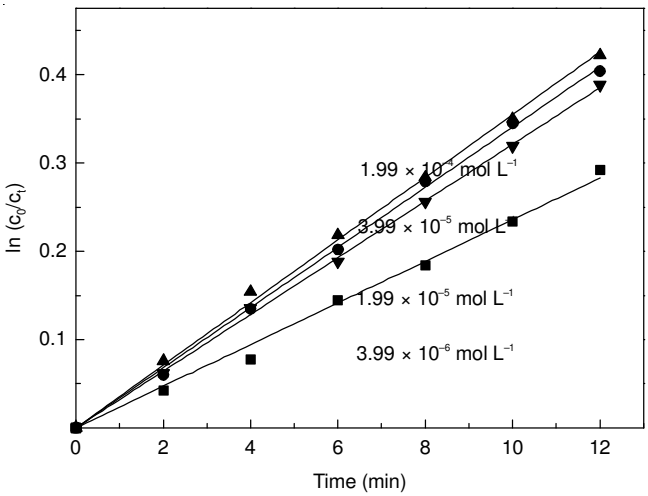


Fig. 8. Plots of $\ln(C_0/C_t)/t$ in different concentration mimic enzyme

field. In view of metal complexes with multidentate Schiff base ligands have mimic enzyme activity and play an important role in the synthesis and study of mimic enzyme catalysts, especially for the dendritic ligands with structure of salicylaldehydeimine-multidentate-schiff base and hydrophobic stereo-cavity, so it can be used to build mimic enzyme with the performance of coordination, identification and catalysis. Dendritic copper complex not only has good natural enzyme characteristic such as hydrophilic amino acids long chain, slight reaction conditions (high catalytic activity just by room temperature), efficiency and durability, but also has unique characteristic such as hydrophobic cavity, not easy to deactivation and reuse for good dendritic effect. Although the catalytic performance of mimic enzyme is not yet fully able to achieve the order of natural enzyme, it is still a kind of good, practical mimic enzyme of horseradish catalase.

Conclusion

In summary, dendritic-salicylaldehydeimine ligand and its copper complex were synthesized and the structures of ligand and complexes were established by elemental analysis, IR, NMR, UV and XRD. Elemental analysis, IR and NMR results have showed that salicylaldehydeimine ligand is synthesized by introducing salicylaldehyde to 1.0G dendrimer and the intramolecular hydrogen bond is formed in ligand. Ultraviolet and XRD have indicated that ligand and Cu(II) with the proportion of 1:2 formed metal complex. The mimic enzyme property of dendritic copper complex was investigated. Catalytic kinetic model of mimic enzyme complex was established, The catalytic reactions had the same features of pseudo-first-order reaction to enzymatic reaction. The catalytic reaction rate obviously increased under the conditions of higher concentration of mimic enzyme complex at pH = 7-7.5. The mimic enzyme complex has both of good natural enzyme characteristic and unique positive dendritic effect, which have shown that it's suitable to be used as an excellent mimic enzyme of horseradish catalase.

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