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Cyclodextrin Polymers Functionalized with Histidine and Carcinine as Chelating Therapeutics for Copper Dyshomeostasis

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In recent years, cyclodextrin polymeric nanoparticles have been designed to introduce new properties and extend their medical applications. Based on the features of cyclodextrins, we derivatized cross-linked cyclodextrin polymers with histidine or carcinine moieties. We found that amylases do not hydrolyze cyclodextrin polymers. The new polymers can form copper(II) complexes and may act as nanochelators to counteract copper(II) dyshomeostasis-related diseases. Furthermore, the copper(II) complexes show superoxide dismutase activity, similar to free carcinine and histidine complexes. The antioxidant biological activity of the copper(II) complex formed in situ may protect cells from oxidative damage related to copper dyshomeostasis.

Introduction

Chelation therapy has been widely applied in clinical practice since the first use of British Anti-Lewisite (BAL).[1] Many metal chelators have been designed for metal poisoning and biometal overload.[2] Low-molecular-weight ligands have been typically used, such as deferiprone, deferoxamine, clioquinol, PBT2, EDTA, penicillamine, DOTA and 2,3-dimercaptosuccinic acid.[3] These chelators are generally intravenously or parenterally administrated. Particular attention has been paid to biometal overloads like iron and copper.[2,4] The chelation of biological redox metals is a mechanism by which chelators can protect the biological targets from oxidative stress preventing the deleterious Fenton reaction.[5]

Copper is an essential metal involved in a variety of biological functions. Its concentration is carefully controlled in cells and copper dyshomeostasis has been related to many neurodegenerative diseases, including Alzheimer's (AD), Parkinson's (PD), Wilson's (WD), and Menkes's diseases (MD).[6,7] The effects of copper overload have been mainly analyzed in WD, a rare autosomal recessive disorder related to a mutation of the ATPase 7B gene.[8] The mutation reduces copper(II) excretion from cells and leads to long-term damage.[9] Chelation therapy is a therapeutical approach currently available to reduce copper excess stored in various tissues, particularly the liver and the brain.[9] A low-copper diet is also suggested to reduce the metal supply in the case of WD.[8] Antioxidant therapy can also improve clinical outcomes.[10]

Even though small chelators can manage the metal excess,[11,12] their systemic use should aim for better-defining dosing. Their use has some limitations associated with severe side effects and neurological deterioration.[13] Polymers orally administrated may reduce the side effects of systemic chelation therapy because they keep the effect limited to the gastrointestinal tract.[14] In general, a variety of polymeric systems have been widely used in clinical care to bind small molecules and ions, such as phosphate and potassium.[15]

Sodium polystyrene sulfonate (Kayexalate) was the first synthetic polymeric sequestrant used for hyperkalemia.[16] Polyallylamine cross-linked polymers, such as Sevelamer (trade name Renagel), have also found therapeutic applications by oral administration as phosphate binders in hyperphosphatemia for kidney diseases.[17] Colesevelam hydrochloride (trade name WelChol) has been used as a bile acid binder for reducing cholesterol levels.[18] Although there is considerable interest in transition metal chelating therapies, few examples of chelating polymers are used in clinical. Some examples have been only studied for bioanalytical applications.[19,20] Emerging data point toward polymer-based chelation therapies to reduce the side effects of low molecular weight chelators.[14]

Based on the proven advantage of polymeric systems for sequestering cations and the interest in transition metal chelation, herein we designed new chelator polymers based on cyclodextrin (CyD) chemistry. CyDs are cyclic oligosaccharides of α(1→4) glucose. They have appealing applications in numerous fields, such as supramolecular, bioinorganic, organic, cosmetic, pharmaceutical and material chemistry.[21–23] They have been used as excipients in drug delivery.[24,25]

Moreover, different studies have shown that β- and γ-CyDs are biocompatible and their toxicity has been evaluated.[26] They can be administrated orally without significant degradation or

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absorption in the stomach and small intestine.[27,28] Therefore, their oral bioavailability is very low in animals and humans. β-CyD levels orally administrated in tissues and serum are $<1\%$. β-CyD toxicity has been recently re-evaluated, and an acceptable daily intake (ADI) of 5 mg/kg body weight per day has been confirmed.[26]

More recently, many CyD polymers have also been synthesized and modified to introduce new properties that increase CyD applications.[29–36] Many families of CyD polymers have been investigated with applications in food and pharmaceutical science.[37–39] Some of them are currently in Phase I and II clinical trials as nano-prodrugs.[40] Although a number of metal complexes based on CyDs[41] have been characterized, the potential of chelating CyD-based polymers has been little exploited for their application in biological systems.

In this context, we functionalized cross-linked CyD polymers with histidine (His) or carcinine (Carc, β-alanyl-histamine) moieties (Figure 1) with high functionalization degree. His and Carc are bioligands in mammals with important physiological roles[42,43] and their copper complexes have been well characterized.[44–46]

The CyD polymers can exploit the complexing ability of the moieties and form copper(II) complexes. We also studied the superoxide dismutase (SOD) activity of the new copper(II)-polymer systems compared to His and Carc complexes. Oxidative stress is associated with neurological diseases AD, PD and WD. The antioxidant activity can protect cells subjected to copper dyshomeostasis. The copper complex formed in situ can perform SOD biological activity and protect cells from oxidative damage.

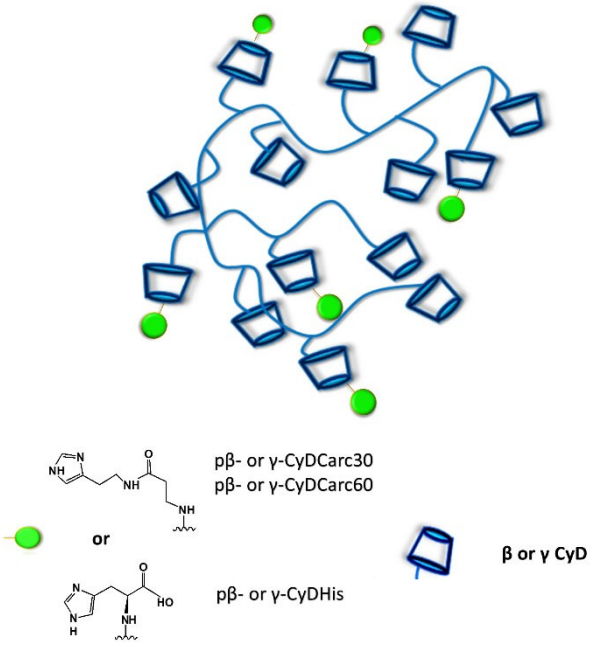

Figure 1. Schematic structure of cyclodextrin polymers studied in this work.

Results and Discussion

Synthesis and characterization

We functionalized epichlorohydrin cross-linked β- or γ-CyD polymers[47] derivatized with carboxylate groups (pCyDA). The functionalization of the polymers with His or Carc was performed through a condensation reaction in DMF with EDC and HOBt. The functionalization degree was modulated using different amine amounts in the condensation reaction. In the case of His-based polymers, we obtained a functionalization degree higher than Carc derivatives when a stoichiometric amount of amine was used. We added an excess of Carc to obtain a high functionalization degree.

^{1}H NMR spectra of all derivates (Figure 2, 1S–8S) show common patterns. In the spectra, the peaks are broad due to the high molecular mass of the polymers. In the spectra, in addition to the peak due to the CyD protons, the signals of the moieties can be identified. As for pCyDCarc, the CH_2 of beta-alanine residue resonates at about 2.4 ppm and CH_2 in alpha to imidazole ring resonate at 2.8 ppm. Imidazole protons are evident in the aromatic region. As for His derivatives, the signals of the ABX spin system can be seen in the spectra (Figure 2).

The number of Carc or His units can be calculated from the integration ratio between the imidazole and CyD H-1 signal. pβCyDCarc and pγCyDCarc spectra showed that pCyDAs were functionalized at 30% (pβCyDCarc30 and pγCyDCarc30) or 60% (pβCyDCarc60 and pγCyDCarc60) of the pCyDA cavities with Carc units.

We determined the functionalization degree of pCyDHis from signal integration values. Both pβCyDHis and pγCyDHis were modified in about 60% of the cavities with His.

All the polymers form nanoparticles (Figures 9S and 10S). pβCyD systems show higher hydrodynamic diameters than pγCyD polymers, in keeping with the different molecular weights of the native polymers. The functionalization modified the size slightly and pCyDCarc systems are bigger than the corresponding pCyDA.

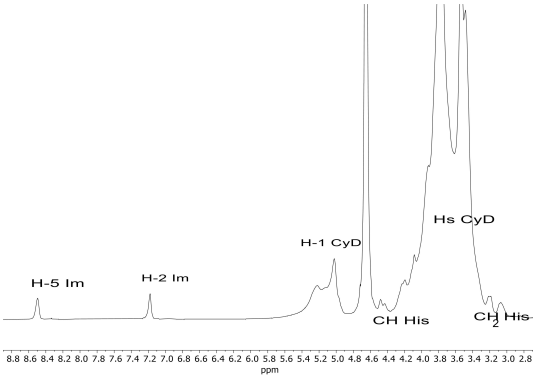

Figure 2. ^{1}H NMR spectrum (D_2O, 500 MHz) of pγCyDHis.

Amylase cleavage assay

Preliminary experiments at pH 7.4, 4.0 and 3.0 were carried out to study the stability of the polymers towards amylase enzymes, compared to free β- and γ-CyD. Data showed that the polymers, in our experimental conditions at pH 7.4, were not hydrolyzed in smaller units. However, a slight degradation was observed in the presence of amylase only for free γ-CyD at all the pH values, in keeping with the literature data.[27,48] In particular, it has been reported that only γ-CyD can be slightly hydrolyzed by amylase for its size and flexibility. In the case of the pγCyD polymer, the network stabilizes the CyD structure and we did not find hydrolysis products. At acid pH (3.0 and 4.0), after 4 h, the polymers alone or in the presence of amylase showed only a slight degradation. The presence of amylase did not influence this trend. These data support the potential of the new CyD nanochelators to survive in the bowel and perform their actions.

Metal complexes

Cu^{2+}complexes of pCyDCarc60 or pCyDHis were studied with UV-vis spectroscopy in HEPES buffer solution at pH 7.4 in molar ratio 1:2 M/L, L being Carc or His moiety. HEPES buffer was used because it is commonly labeled as non-coordinating species with metal ions. The UV-Vis spectra showed weak absorption bands in the Vis region (Figure 11S) due to the d-d transitions. The λ value for the Cu^{2+}-pCyDCarc60 system (650 nm) is slightly higher than those reported for the free Cu^{2+}-Carc complex (628 nm).[46] A similar trend was found for the Cu^{2+}-pCyDHis systems: the d-d band is at 666 nm, higher than that for the Cu^{2+}-His complex (642 nm).[45,49] Such a difference could reasonably be due to the metal coordination environments in the pCyD polymers. In particular, the amino group of the free ligands (Carc or His) became an amide group in pCyDHis and pCyDCarc. This modification can give different coordination properties as found for N-acetyl-Carc or -His[46,50]

SOD activity of Copper (II) complexes.

Many CyD functionalized metal complexes have been investigated as SOD mimetics. It was reported that the CyD scaffold improved SOD activity compared to free moiety complexes.[41] This improvement was explained to be due to the CyD cavity microenvironment (dielectric constant, rigidity of the complex). Similar behavior has also been found for CyDs conjugated with Carc.[51]

We studied the SOD activity of Cu^{2+} complexes of pCyDCarc60 and pCyDHis to investigate the role of the polymer network. The SOD activity of the new complexes was determined with an indirect assay.[52] Superoxide anion was generated using a non-enzymatic source Phenazine methosulfate (PMS)-NADH, and 4-Nitro blue tetrazolium chloride (NBT) reduction was followed by UV-Vis spectroscopy. The concentration of the complexes that inhibit the NBT reduction by 50% was determined (IC_{50}). The IC_{50} value is the concentration for $V_0 = 2V_c$ (Table 1). The data representation is reported in Figures 3 and 12S. Carc and His complexes were also investigated for comparison.

The ligands did not show any activity as expected. All the systems (pH 7.4, molar ratio 1:2 M/L, L is Carc or His moiety) showed a high SOD activity. Cu^{2+}-pγCyDCarc60 and pβCyD-Carc60 systems showed SOD activity with an IC_{50} value lower than that of the free Carc complex. The pCyDHis complexes showed an antioxidant activity better than pCyDCarc systems. The IC_{50} values were slightly higher compared to the free Cu^{2+}-His system.

The different coordination environment of copper in the polymers, due to the formation of the amide from the amino group, may partly explain the differences from Carc and His complexes. However other effects may occur, such as the polymer network, unfunctionalized COOH groups and different stability constant values that can modify the speciation of the systems.

Table 1. IC_{50} values (μM) for SOD activity of Cu^{2+}-pCyD polymers (Cu/L 1:2, L is the chelating moiety Carc or His, pH 7.4 HEPES buffer). His and Carc complexes are reported for comparison.

Complexes	IC_{50} (M) × 10^7
Cu^{2+}-pβCyDHis	1.7 ± 0.2
Cu^{2+}-pγCyDHis	3.1 ± 0.3
Cu^{2+}-His	0.4 ± 0.4
Cu^{2+}-pβCyDCarc60	3.4 ± 0.4
Cu^{2+}-pγCyDCarc60	3.7 ± 0.5
Cu^{2+}-Carc	6.0 ± 0.7
Cu^{2+}	1.2 ± 0.2

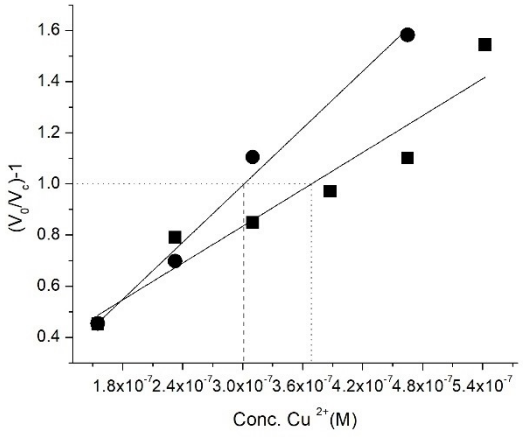

Figure 3. Superoxide dismutase activity assay: V_0 is the NBT reduction rate and V_c is the NBT reduction rate in the presence of Cu^{2+}-pγCyDHis (·) or pγCyDCarc60 (●). The IC_{50} value is the complex concentration for which $(V_0/V_c)-1=1$

Conclusions

New cyclodextrin-based polymers were functionalized with carcinine or histidine as potential copper(II) nanochelators. Carcinine and histidine are well-known bio-ligands. The polymers can form copper(II) complexes similar to free carcinine or histidine. The chelating polymers orally administrated may bind copper(II) after its release from the food during digestion and prevent its uptake in the enterocytes. Furthermore, we found that alpha-amylase does not degrade cyclodextrin polymers, which may exploit chelating activity in the bowel and counteract copper(II) dyshomeostasis. Furthermore, copper complexes show SOD activity likewise to the carcinine and histidine complexes. Therefore the nanochelator could reduce oxidative stress via copper(II) chelation and SOD activity of the final complex.

Experimental Section

Materials

Hydroxybenzotriazole (HOBt), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), L-Histidine Methyl ester dihydrochloride (HisOCH$_3$), α-amylase of hog pancreas were purchased from Merck, Carc were purchased from Baker. The water-soluble EPI (epichlorohydrin) cross-linked polymer pβCyDA (84 kDa, 54 CyD cavities, the average number of COOH groups for a cavity is 3) and pγCyDA (54 kDa, 28 CyD cavities, the average number of COOH groups for a cavity is 3) were purchased from Cyclolab. TLC (Thin layer chromatography) was carried out on silica gel plates (Merck 60-F254). Carbohydrate derivatives were detected on TLC with the anisaldehyde test. Membrane Dialysis was carried out with a molecular weight cut-off of 3 kDa was used.

Synthesis of pγCyDHis. HisOCH$_3$ Dihydrochloride was converted to the base form using a DEAE-Sephadex column (form OH^-). HOBt (14.0 mg, 0.103 mmol) and EDC (16.0 mg, 0.103 mmol) were added to pγCyDA (200 mg, 3.7 μmol) in DMF. After 10 min, HisOCH$_3$ (18 mg, 103 mmol) was added. The reaction mixture was stirred at room temperature for 24 h. The product was isolated by a Sephadex G-15 column. The methyl ester of His was hydrolyzed by a solution of NaOH 1% (1 mL) for 2 h. The final product was purified using CM Sephadex C-25 (NH_4^+ form) and water as the eluent. Yield: 20% ^{1}H NMR: (D_2O, 500 MHz) δ(ppm): 3.1 (m, CH_A His); 3.2 (m, CH_B His); 3.2–4.4 (m, H-3, -4, -5, -6 of CyD), 4.5 (m, CH_X His); 4.9–5.5 (m, H-1 of CyD), 7.2 (s, H-2, Im), 8.5 ppm (s, H-5, Im). ^{13}C NMR (D_2O, 125 MHz) δ(ppm): 27.3 (CH_2 His), 60.4 (C-2 CyD), 62.4 (C-3 CyD), 67.0–76.0 (C-6, C-5, C EPI), 80 (C-4 CyD), 100.0 (C-1 CyD); 116.8 (C-3 Im), 129.7 (C-2 Im); 132.9 (C-4 Im); 133.2 (C-5 Im); 175.8 (CONH); 178.0 (COOH). Size (DLS, Z Average):14 ± 2 nm.

Synthesis of pβCyDHis. The synthesis was carried out as reported for pγCyDHis, starting from pβCyDA (200 mg, 2.3 μmol), HOBt (17.4 mg, 129 mmol), EDC (20 mg, 129 mmol) and HisOCH$_3$ (22 mg, 129 mmol). Yield: 25% ^{1}H NMR: (D_2O, 500 MHz) δ(ppm): 3.1 (m, CH_A His); 3.2 (m, CH_B His); 3.2–4.4 (m, H-3, -4, -5, -6 of CyD, H EPI), 4.5 (m, CH_X His); 4.9-5.5 (m, H-1 of CyD), 7.2 (s, H-2, Im), 8.5 ppm (s, H-5, Im). ^{13}C NMR (D_2O, 125 MHz) δ(ppm): 27.4 (CH_2 His), 60.3 (C-2 CyD), 53.3 (CH His); 62.4 (C-3 CyD), 67.0–76.0 (C-6, C-5, C EPI), 80.0 (C-4 CyD), 100.4 (C-1 CyD); 116.8 (C-3 Im), 129.7 (C-2 Im); 132.9 (C-4 Im); 133.4 (C-5 Im); 175.9 (CONH); 178.2 (COOH). Size (DLS, Z Average): 19 ± 2 nm.

Synthesis of pγCyDCarc. HOBt (14.0 mg, 0.100 mmol) and EDC (16.0 mg, 0.100 mmol) was added to pγCyDA (200 mg, 3.7 μmol) in DMF. After 5 min, Carc (20.0 mg, 0.100 mmol) was added. The reaction mixture was stirred at room temperature for 24 h. The product was isolated with a Sephadex G-15 column using water as the eluent and was dialyzed against water. **pγCyDCarc30**: Yield 43% ^{1}H NMR: D_2O, 500 MHz) δ(ppm): 1.9 (bs, H-2 Ala); 2.3 (bs, H-2 Histamine (Hm)); 3.5-4.2 (m, H -3, -6, -5, -2, -4 γCyD, H-1 Ala and Hm and H EPI); 5.0–5.6 (m, H-1 γCyD); 7.2 (s, H-2, Im); 8.5 (s, H-5, Im). ^{13}C NMR (D_2O, 125 MHz) δ(ppm): 24.0 (C-2 hm), 35.0 (C-2 Ala), 35.5 (C-1 Ala); 38.1 (C-1 Hm); 60.4 (C-2 CyD), 63.0 (C-3 CyD), 67.0-76.0 (C-6, C-5, C EPI), 80 (C-4 CyD), 100.0 (C-1 CyD); 117.0 (C-3 Im), 130.0 (C-2 Im); 132.0 (C-4 Im); 133.0 (C-5 Im); 173.8 (CONH); 177.0 (COOH).

The synthesis was carried out with a higher Carc/pCyD molar ratio in order to obtain a higher degree of substitution. After the activation step, Carc (40 mg, 0.200 mmol) was added to the reagents. **pγCyDCarc60**: Yield 54% ^{1}H NMR: D_2O, 500 MHz) δ(ppm): 2.0 (bs, H-2 Ala); 2.2 (bs, H-2 Histamine); 3.6–4.2 (m, H-3, -6, -5, -2, -4 γCyD, H-1 Ala and Hm); 5.1–5.6 (m, H-1 γCyD); 7.2 (s, H-2, Im); 8.6 (s, H-5, Im). ^{13}C NMR (D_2O, 125 MHz) δ(ppm): 24.4 (C-2 Hm), 35.0 (C-2 Ala), 35.8 (C-1 Ala); 38.2 (C-1 Hm); 60.4 (C-2 CyD), 63.0 (C-3 CyD), 67.0-76.0 (C-6, C-5, C EPI), 80 (C-4 CyD), 100.0 (C-1 CyD); 117.0 (C-3 Im), 130.0 (C-2 Im); 132.5 (C-4 Im); 133.7 (C-5 Im); 173.9 (CONH); 177.2 (COOH). Size (DLS, Z Average): 44 ± 4 nm.

Synthesis of pβCyDCarc. The synthesis was carried out as reported for pγCyDCarc starting from pβCyDA (200 mg, 2.3 μmol), HOBt (17.4 mg, 129 mmol), EDC (20 mg, 129 mmol) and Carc (24 mg, 129 μmol). **pβCyDCarc30**: Yield 56%. ^{1}H NMR: (D_2O, 500 MHz) δ(ppm): 2.3 (bs, H-2 Ala); 2.6 (bs, H-2 Hm); 3.25-4.5 (m, H-3, -6, -5, -2, -4 CyD, H-1 Ala and Hm); 5.0-5.6 (m, H-1 CyD); 7.2 (s, H-2, Im); 8.55 (s, H-5, Im).

The synthesis was carried out with a higher Carc/pCyD molar ratio in order to obtain a higher degree of substitution. After the activation step, Carc (50 mg, 0.258 mmol) was added to the reagents. **pβCyDCarc60**: Yield 50% ^{1}H NMR: (D_2O, 500 MHz) δ(ppm): 2.3 (bs, H-2 Ala); 2.6 (bs, H-2 Hm); 3.5–4.5 (m, H-3, -6, -5, -2, -4 CyD, H-1 Ala and Hm); 5.0-5.6 (m, H-1 CyD); 7.2 (s, H-2, Im); 8.6 (s, H-5, Im). ^{13}C NMR (D2O, 125 MHz) δ(ppm): 24.5 (C-2 hm), 35.3 (C-2 Ala), 36.0 (C-1 Ala); 38.9 (C-1 Hm); 60.4 (C-2 CyD), 63.5 (C-3 CyD), 67.5-76.2 (C-6, C-5, C EPI), 80.5 (C-4 CyD), 100.0 (C-1 CyD); 117.0 (C-3 Im), 130.2 (C-2 Im); 132.5 (C-4 Im); 133.0 (C-5 Im); 174.0 (CONH); 177.5 (COOH). Size (DLS, Z Average): 49 ± 5 nm

Instrumentation

NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 25 °C with a Varian UNITY PLUS-500 spectrometer at 499.9 and 125.7 MHz, respectively, using standard pulse programs from the Varian library. 2D experiments (COSY, TOCSY, HSQC, HMBC) were acquired using 1 K data points, 256 increments.

DLS. Dynamic light scattering (DLS) measurements were performed at 25 °C with a Zetasizer Nano Z.S. (Malvern Instruments, Oxford, U.K.) operating at 633 nm (He–Ne laser). The mean hydrodynamic diameter (d) of the NPs was calculated from intensity measurement. The samples at about mM concentration were diluted in HEPES buffer (50 mM, pH 7.4) prepared in ultrapure water filtered (0.2 μm).

UV-Vis spectroscopy. UV-Vis spectra were recorded with Agilent Cary 8500 spectrophotometer equipped with a Peltier cell holder. The samples of ligands and complexes were diluted in HEPES buffer (50 mM, pH 7.4).

Amylase cleavage assay. α-Amylase hydrolysis tests were carried out at 37 °C in buffer at pH 7.4 (phosphate buffer 50 mM) or pH 4.0 (acetate buffer 50 mM). The polymers at 10 mM at two concentrations (2.5 mg/ml and 5.0 mg/ml) were incubated under stirring alone and in the presence of α-amylase of hog pancreas. TLC monitored hydrolysis at different times, at regular intervals up to 24 h. β- and γ-CyD were used for comparison. TLC was eluted with PrOH/AcOEt/H_2O/NH_3 5:1:3:3.

SOD activity. The reaction mixture was composed of 4-Nitro blue tetrazolium chloride (NBT, 200 μM), Phenazine methosulfate (PMS, 6.2 μM), NADH (312 μM) in HEPES buffer (50 mM, pH 7.4). During the experiment, the solutions of reagents were kept cool in an ice bath. The complexes were prepared in HEPES buffer at M/L (L is Carc or His moiety) 1:2 molar ratio. The reaction started when the PMS solution was added to the mixture in the cuvette, under stirring. The absorbance of the NBT was monitored at 560 nm every 30 sec. for 5 min at 25 °C. All tests were carried out in triplicate. A graphical representation of experimental data was obtained by plotting the V_0/V_c-1 against the complex concentration, yielding a straight line. V_0 is the uninhibited reduction rate of NBT and V_c is the reduction rate of NBT in the presence of the complex. The IC_{50} value is the complex concentration for which $V_0=2V_c$, $(V_0/V_c)-1=1$.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: biometal · chelation · copper · SOD mimetics, Wilson's disease

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