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Original article

Comparative study of copper(II)–curcumin complexes as superoxide dismutase mimics and free radical scavengers

Atanu Barik [a], Beena Mishra [a], Amit Kunwar [a], Ramakant M. Kadam [b], Liang Shen [c], Sabari Dutta [d,*], Subhash Padhye [d], Ashis K. Satpati [e], Hong-Yu Zhang [c], K. Indira Priyadarsini [a,**]

[a] *Radiation and Photochemistry Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India*
[b] *Radiochemistry Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India*
[c] *Shandong Provincial Research Center for Bioinformatic Engineering and Technique, Shandong University of Technology, Zibo 255049, PR China*
[d] *Department of Chemistry, Pune University, Pune-411007, India*
[e] *Analytical Chemistry Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India*

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Abstract

Two stoichiometrically different copper(II) complexes of curcumin (stoichiometry, 1:1 and 1:2 for copper:curcumin), were examined for their superoxide dismutase (SOD) activity, free radical-scavenging ability and antioxidant potential. Both the complexes are soluble in lipids and DMSO. The formation constants of the complexes were determined by voltammetry. EPR spectra of the complexes in DMSO at 77 K showed that the 1:2 Cu(II)–curcumin complex is square planar and the 1:1 Cu(II)–curcumin complex is distorted orthorhombic. Cu(II)–curcumin complex (1:1) with larger distortion from square planar structure shows higher SOD activity. These complexes inhibit γ-radiation induced lipid peroxidation in liposomes and react with DPPH acting as free radical scavengers. One-electron oxidation of the two complexes by radiolytically generated azide radicals in Tx-100 micellar solutions produced phenoxyl radicals, indicating that the phenolic moiety of curcumin in the complexes participates in free radical reactions. Depending on the structure, these two complexes possess different SOD activities, free radical neutralizing abilities and antioxidant potentials. In addition, quantum chemical calculations with density functional theory have been performed to support the experimental observations.

Keywords: Cu(II)–curcumin complex; Superoxide radicals; Free radicals; Density functional theory

1. Introduction

Curcumin is a naturally occurring phytochemical found in the rhizomes of *Curcuma longa* or turmeric, used for centuries in a variety of pharmaceutical applications [1,2] including treatment for arthritis [3], as an anti-inflammatory agent [4] and as an orally available treatment for diabetes [5]. Curcumin fed amyloid-infused rats have shown significantly reduced levels of amyloid plaques [6]. Curcumin is a potent antioxidant [7], inhibiting lipid peroxidation [8] and effectively scavenging superoxide radical [9], the hydroxyl radical and nitrogen dioxide among other reactive

Abbreviations: DFT, density functional theory; DMSO, dimethyl sulfoxide; DPPH, 2,2′-diphenyl-1-picryl hydrazyl; EA, electron affinities; ECP, effective core potential; EPR, electron paramagnetic resonance; NHE, normal hydrogen electrode; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TD-DFT, time-dependent density functional theory; TE, total electronic energy; TEAP, tetraethyl ammonium perchlorate.

* Corresponding author. Present address: Department of Biochemistry and Molecular Biology, School of Medicine, Wayne State University, 540 E.Canfield, Detroit, MI-48201, United States. Tel.: +1 313 993 9113; fax: +1 313 577 2765.
** Corresponding author. Fax: +91 22 25505151.
E-mail addresses: sdutta@med.wayne.edu (S. Dutta), kindira@barc.gov.in (K. Indira Priyadarsini).

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oxygen species [10]. Curcumin is well absorbed, both *in vitro* [11] and *in vivo* [12] and has exceedingly low toxicity index [13]. It is a diferuloyl methane having two *o*-methoxy phenolic OH groups attached to the α,β-unsaturated β-diketone (heptadiene–dione) moiety, which can form chelates of the type 1:1 and 1:2 with copper, iron and other transition metals [14,15]. This property of binding of curcumin to metals like iron and copper is considered as one of the useful requirements for the treatment of Alzheimer's disease [6,16]. There also appears some correlations between drugs for Alzheimer's disease and the metal complexes acting as SOD mimics [15c,16].

Several metallocomplexes of curcumin have been synthesized, characterized and evaluated for various biological activities. Anti-arthritic properties of a five coordinate curcumin gold complex, $Au(cur)_2Cl$, in which curcuminate is bidentate were assessed in an adjuvant-induced rat polyarthritis model [17]. Greatly reduced paw swelling was seen after three weeks of $Au(cur)_2Cl$, 30 mg/kg/day by injection. Three manganese complexes with curcumin or one of two related compounds, diacetylcurcumin and 4-(4-hydroxy-3-methoxy-phenyl)-1-[7-(4-hydroxy-3-methoxy-phenyl)-[1,4]diazepam-5-ylidene]but-3-en-2-one [18], were evaluated for *in vitro* antioxidant properties and superoxide dismutase activity with IC_{50} values for the former in the range 6.3–26.3 μM and for the later 8.9–29.99 μM. All the three complexes were also tested *in vivo* for their potential as neuroprotective agents in vascular dementia; Mn(cur)(OAc) showed significant protective effects in a transient ischemia/repurfusion mouse model of neuronal damage [18]. Among a series of metal curcuminoids, the $Cu(curcumin)_2$ complexes were most cytotoxic in cultured L929 cells [19] and showed significant reduction in solid tumor volume in ascites tumor-bearing mice. Syntheses of a series of 1,7-diarylheptanoids and their VO(IV), Co(II), Ni(II) and Cu(II) complexes were reported [20]. The Cu(II) coordination complexes of these ligands had IC_{50} values of 6.6–11.1 μM in Erlich ascites tumor cells [20]. Testing for biological activity of the curcuminoid ligands and various metal complexes including both *in vivo* administrations of compounds (i.p.) to assess antidiabetic efficacy and *in vitro* cell studies of cytotoxic potentials have been extensively studied by Orvig et al. [21]. Recently Orvig et al. have reported vanadyl, gallium and indium curcumin complexes for medicinal applications, [22] corroborating the importance of curcumin's free phenolic OH groups for scavenging oxidants and correlated with reduced cytotoxic potential.

One of the interests of our group has been to develop new transition metal–curcumin complexes as SOD mimics. Recently we reported that the 1:1 complex of copper with curcumin exhibits SOD activity and *in vitro* antioxidant activity [23]. In this paper we compare some of these results of 1:1 complex along with additional new experiments with 1:2 complex of copper(II) and curcumin (Fig. 1) to understand the effect of stoichiometry and structural changes on their SOD activity, free radical reactions and antioxidant activity.

2. Results and discussion

2.1. Spectral and electrochemical properties of the complexes

The normalized ground state UV–VIS spectra of the complexes in DMSO are given in Fig. 2(a) and (b) for 1:1 and 1:2 complexes, respectively. The 1:1 Cu(II)–curcumin complex exhibits absorption maximum at 426 nm and two shoulders at 410 nm and 450 nm with extinction coefficients of 96700 $M^{-1}\,cm^{-1}$ at 426 nm and the 1:2 Cu(II)–curcumin complex exhibits maximum at 370 nm with extinction coefficient of 22125 $M^{-1}\,cm^{-1}$. The two complexes were insoluble in neutral water. The 1:1 Cu(II)–curcumin complex, however, could be solubilised in alkaline water, while the 1:2 Cu(II)–curcumin complex formed a precipitate. In DMSO, the 1:2 Cu(II)–curcumin complex is more soluble than the 1:1 Cu(II)–curcumin complex and both the complexes are soluble in lipids and Tx-100 micellar solutions. Aqueous solutions of lipids and micelles are micro heterogeneous systems and provide hydrophobic sites to solublise water insoluble compounds [24].

The electrochemical profiles of the complexes were recorded in DMSO solvent against Ag/AgCl reference electrode (voltammograms not shown). The $E_{1/2}$ values in the voltammograms for 1:1 and 1:2 complexes were found to be at 0.16 V and 0.20 V, respectively. The value for 1:1 agrees with that reported in our earlier reference [23]. After correcting the reference electrode as 0.222 V, the potentials for Cu^{+2}/Cu^{+} redox couple for 1:1 and 1:2 complexes were calculated to be 0.38 and 0.42 V vs. NHE, respectively. The data indicates that the reduction potential values of the complexes are not very much different and are within the range of compounds that are expected to show SOD activity [25].

To estimate the formation constant for the equilibrium between Cu(II) and curcumin as given in Eq. (1), electrochemical scanning of Cu(II) in copper salt solution in DMSO was carried out in presence of different concentrations of curcumin.

$$Cu^{2+} + p\,\text{Curcumin} \overset{K_f}{\rightleftharpoons} Cu(\text{Curcumin})_p \tag{1}$$

where K_f is the formation constant of the said complex and p is the coordination number or the number of molecules of curcumin attached to the copper ion. The shift in the half wave potential ($\Delta E_{1/2}$) of the Cu(II) in the absence and in the presence of different concentrations of curcumin was plotted against the logarithmic concentration of curcumin, according to Eq. (2).

$$\Delta E_{1/2} = -\frac{0.0591}{n}\log K_f - p\,\frac{0.0591}{n}\log C \tag{2}$$

Here n and C correspond to the number of electrons transferred in the redox process and concentration of the ligand (curcumin), respectively [26]. The data could be fitted to a linear plot, and the slope confirmed the formation of either 1:1 complex or 1:2 complex and the intercept gave the value

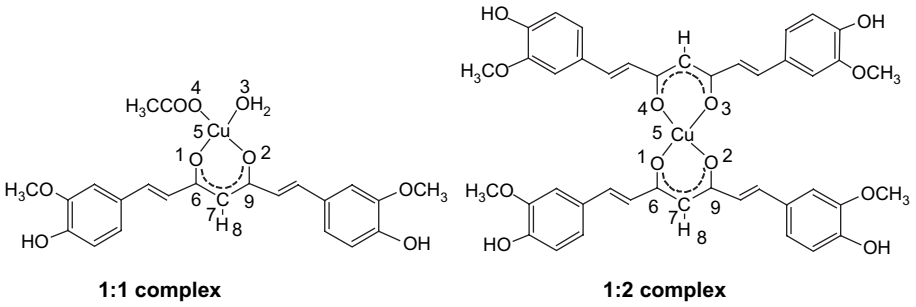

Fig. 1. Molecular structures of Cu(II)–curcumin complexes (1:1 and 1:2).

of $-\log K_f/0.059$. For the estimation of K_f for 1:1 Cu(II)–curcumin complex, 500 μM copper acetate in DMSO was titrated with 1 μM to 10 mM curcumin and by fitting the data to a linear plot (Fig. 3a) according to Eq. (2), K_f was determined to be 3.7×10^{14}. Similar exercise was carried out in the case of 1:2 complex, where 500 μM copper chloride solution was titrated with 1 μM to 10 mM curcumin and by fitting the data to a linear plot (Fig. 3b) according to Eq. (2) K_f was determined to be 3.9×10^{15}. These results therefore indicate that both 1:1 and 1:2 Cu(II)–curcumin complexes are considerably stable in the medium studied and 1:2 complex was shown to have higher stability as compared to that of 1:1 complex.

The EPR spectra for both 1:1 and 1:2 Cu(II)–curcumin complexes were recorded in DMSO at 77 K as given in Fig. 4a and b, respectively. In the same figure, the EPR spectra, simulated by using Bruker Simfonia software are superimposed with the dotted line. The EPR spectrum of 1:2 Cu(II)–curcumin complex showed axial symmetry ($g_{||} = 2.295$, $g_{\perp} = 2.0852$, $A_{||} = 170$ G) which is associated with square planar coordination of four equivalent oxygen atoms around copper(II) ions. Earlier we reported that the EPR spectrum of 1:1 complex shows slightly orthorhombic g tensor, which could be due to asymmetric coordination around Cu^{2+} ion, as Cu^{+2} is coordinated to four inequivalent oxygen atoms [23]. To recognize this asymmetry in the orthorhombic g tensor more distinctly, we performed careful EPR simulations. The results confirm that the 1:1 complex has distorted square planar structure around Cu^{2+} ion and the values of orthorhombic g tensor have been estimated to be $g_1 = 2.325$, $g_2 = 2.071$, $g_3 = 2.062$, $A_1 = 155$ G. The values of A_2 and A_3 were unresolved as they are within the line widths. Diaz et al. have reported a good correlation between f factor ($g_{||}/A_{||}$, where $A_{||}$ is expressed in cm^{-1}) and SOD like activity of copper(II) complexes [27]. A f factor value smaller than 135 cm is obtained for square planar Cu(II) complexes, and this value increases with increasing tetrahedral distortion. The f value for Cu, Zn SOD is 160 cm, indicating a tetrahedral distortion from square planar geometry and is one of the features that enhance the catalytic activity of the enzyme. From the above EPR data the f values for 1:1 and 1:2 complexes were determined to be 150 (g_1/A_1), and 135 cm ($g_{||}/A_{||}$), respectively. Therefore 1:1 Cu(II)–curcumin complex exhibiting appreciable square planar distortion is expected to show high SOD-like activity. These EPR results have been further supported by quantum chemical calculation.

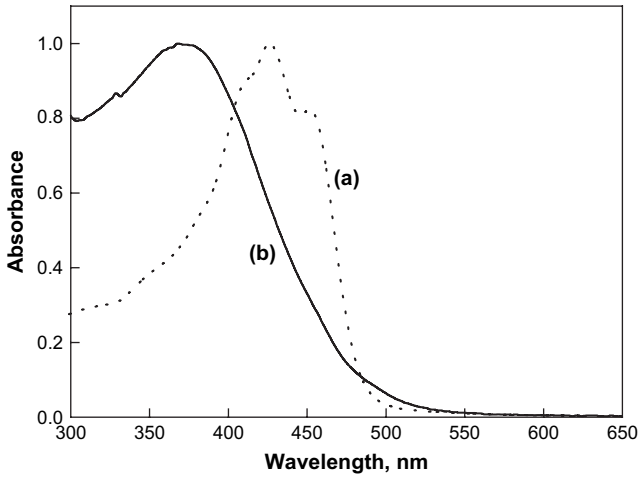

Fig. 2. Normalized absorption spectrum (a) and (b) of 1:1 and 1:2 Cu(II)–curcumin complex, respectively in DMSO.

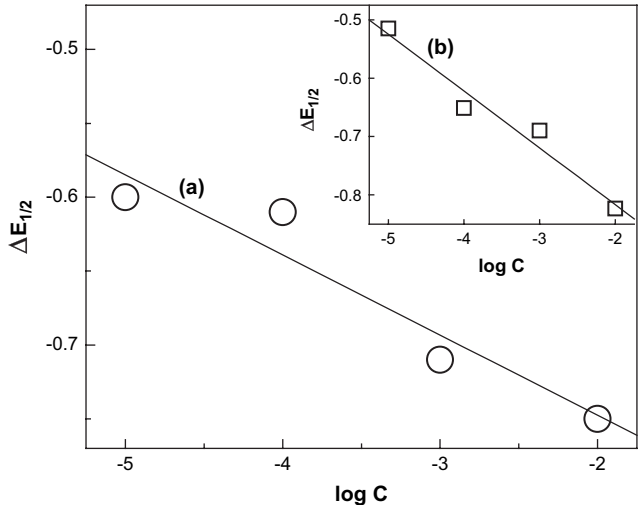

Fig. 3. Variation in the half wave potential of Cu^{+2} ion in presence of different concentrations of curcumin, (a) 500 μM $Cu(OCOCH_3)_2$ and (b) 500 μM $CuCl_2$. Solid lines represent linear fit for the data according to Eq. (2). Experimental conditions: copper salts dissolved in DMSO with 0.1 M TEAP as supporting electrolyte, saturated with nitrogen, scan rate 100 mV/s.

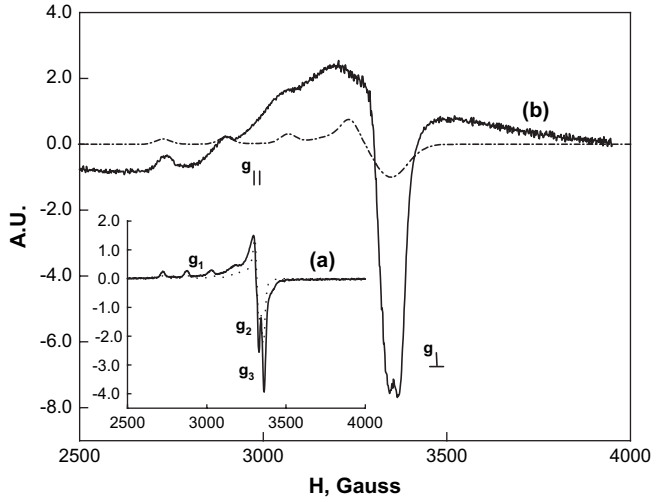

Fig. 4. EPR spectra of (a) 1:1 and (b) 1:2 Cu(II)–curcumin complexes in DMSO at 77 K. Solid lines represent experimental spectra and dashed lines represent the simulated spectra.

2.2. Superoxide scavenging reactions of the complexes

Earlier we reported the superoxide scavenging ability of 1:1 complex using NBT^{+2} as a standard, monitoring NBT^{+} formed by the reduction of NBT^{+2} with superoxide radical generated by xanthine/xanthine oxidase system and the IC_{50} value for 1:1 complex was reported to be 0.17 μM [23], under the same conditions, the 1:2 complex gave an IC_{50} value of ~2 μM. After extensive literature search, it was found that NBT^{+2} is not very selective to superoxide radicals and may get reduced by xanthine oxidase even under anaerobic conditions and thereby introduces errors in the estimations of SOD activity. Therefore we employed a more sensitive method using cytochrome *c* as standard to estimate their comparative SOD activity [28].

When 1:1 and 1:2 complexes were added to the reaction medium comprising of xanthine, xanthine oxidase, EDTA, cytochrome *c* (Fe^{3+}), in Tris buffer, there is a competition between cytochrome *c* (Fe^{3+}) and the complexes to react with superoxide radical as given in Eqs. (3) and (4).

$$\text{cytochrome } c\left(Fe^{3+}\right) + O_2^{\cdot -} \xrightarrow{k_1} \text{cytochrome } c\left(Fe^{2+}\right) \quad (3)$$

$$\text{Complex} + O_2^{\cdot -} \xrightarrow{k_2} \text{Product} \quad (4)$$

In presence of the complex, the Δ*A*/min at 550 nm due to cytochrome *c* (Fe^{2+}) was found to be increased, and the final absorbance at 550 nm decreased with the increasing concentration of the complex. The percentage inhibition (*%I*) of the Cu(II)–curcumin complex was calculated according to Eq. (5).

$$\%I = \frac{\Delta A/\min(\text{Uninhibited}) - \Delta A/\min(\text{Inhibited})}{\Delta A/\min(\text{Uninhibited}) - \Delta A/\min(\text{Blank})} \times 100 \quad (5)$$

Here, "Uninhibited" denotes the system consisting of xanthine, xanthine oxidase, EDTA and cytochrome *c*, "Inhibited" denotes the same system with the addition of Cu(II)–complex and "Blank" denotes the system without the enzyme xanthine oxidase. This *%I* when plotted against the concentration of the complex as shown in Fig. 5 indicates linearity in the concentration range studied, from which the IC_{50} value (the concentration at which the absorbance is half its initial value) was determined to be 6.7 μM and 68 μM for 1:1 and 1:2 complexes, respectively. The IC_{50} value for curcumin under the same condition was found to be 86.8 μM, which is slightly higher than that of the 1:2 complex, but 1:1 complex shows 10 times more SOD activity as compared to both 1:2 complex and curcumin. The IC_{50} values for SOD activity for the two complexes estimated by NBT and cytochrome *c* methods, although differ significantly indicate same trend that 1:1 complex is more active than 1:2 complex.

The rate constants for the scavenging of superoxide radical with Cu(II)–curcumin complexes were calculated according to the competition kinetics method for the above competing reactions given in Eqs. (3) and (4), using the Eq. (6) given below:

$$\left(\frac{A_0}{A} - 1\right) = \frac{k_2}{k_1}\left(\frac{[\text{complex}]}{[\text{cytochrome } c]}\right) \quad (6)$$

where A_0 and A are the maximum absorbance values at 550 nm in the absence and presence of the complex, respectively. Slope of the linear plot for $[(A_0/A)-1]$ vs. [complex]/[cytochrome *c*], gives k_2/k_1, using the value of k_1 as $5.8 \times 10^5\ M^{-1}\ s^{-1}$ for the reaction of superoxide radical with cytochrome *c* [29], k_2 was estimated and values for 1:1 and 1:2 complexes are listed in Table 1. The rate constant determined

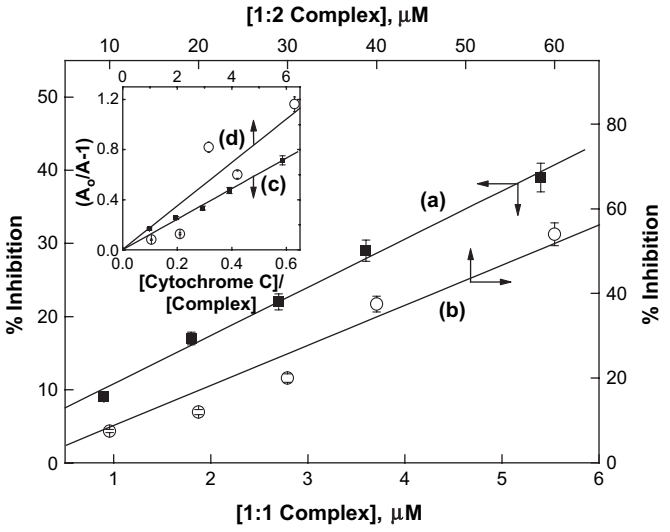

Fig. 5. Percentage inhibition (*%I*) of superoxide radicals formed by xanthine/xanthine oxidase enzyme by different concentrations of the complexes assayed by cytochrome *c* (Fe^{2+}) absorption at 550 nm, (a) in presence of 1:1 Cu(II)–curcumin complex and (b) in presence of 1:2 Cu(II) –curcumin complex. Inset shows competition kinetic plots for the reaction of superoxide radical with cytochrome *c* in presence of (c) 1:1 Cu(II)–curcumin complex and (d) 1:2 Cu(II)–curcumin complex. (Experimental conditions: 50 μM xanthine, 10 mU/ml of xanthine oxidase, 9.52 μM cytochrome *c*, and 600 μM EDTA, 30% DMSO).

Table 1
Comparative properties of Cu(II)–curcumin complexes (1:1 and 1:2)

Property	Complex (1:1)	Complex (1:2)
Absorption maximum (DMSO)	426 nm[a], shoulders at 410 nm and 450 nm	370 nm
Absorption maximum (2% Tx-100)	426 nm	380 nm
Extinction coefficient at absorption maximum (DMSO) ($M^{-1}\,cm^{-1}$)	97000 ± 200[a]	22125 ± 100
Cu(II)/Cu(I) potential vs. NHE	0.38 ± 0.02 V	0.42 ± 0.03 V
Formation constant (K_f)	3.7×10^{14}	3.9×10^{15}
EPR spectra in DMSO at 77 K	$g_1 = 2.325$, $A_1 = 155$ G, $g_2 = 2.071$, $g_3 = 2.0620$	$g_{\|\|} = 2.295$, $A_{\|\|} = 170$ G, $g_\perp = 2.0852$
k ($O_2^{\bullet-}$) + compound, competition kinetics with cytochrome c ($M^{-1}\,s^{-1}$)	$7.1 \pm 0.1 \times 10^5$	$1.04 \pm 0.17 \times 10^5$
IC_{50} value (xanthine/xanthine oxidase assay)	6.7 μM	68.0 μM
Inhibition of lipid peroxidation in liposomes, 10 μM, dose 210 Gy	98%	15%
k (DPPH) + compound, DMSO ($M^{-1}\,s^{-1}$)	156 ± 12[a]	$(1.1 \pm 0.1) \times 10^3$
k ($N_3^{\bullet}$) + compound, Tx-100 ($M^{-1}\,s^{-1}$)	$(2.1 \pm 0.4) \times 10^9$[a]	$(4.4 \pm 1.1) \times 10^8$
Absorption maximum of phenoxyl radical	510–520 nm[a]	520–530 nm
Electron affinity (kcal/mol)	−73.61	−40.67
O–H bond dissociation energy (kcal/mol)	88.51[a]	87.83
O–H proton dissociation enthalpy (kcal/mol)	322.28	327.81
Gas phase ionization potential (kcal/mol)	149.35[a]	142.65

[a] From Ref. [23].

for curcumin by this method ($4.06 \pm 0.6 \times 10^4\ M^{-1}\,s^{-1}$) agrees well with that estimated earlier by pulse radiolysis [30]. The 1:1 complex shows nearly seven times higher rate constant for superoxide radical as compared to the 1:2 complex.

2.3. Inhibition of lipid peroxidation by the complexes

In order to test the antioxidant activity of the complexes, we studied their ability to inhibit lipid peroxidation in liposomal solutions. Peroxidation of lipid is a measure of damage to the membrane lipids caused by the attack of reactive oxygen species. Inhibition of lipid peroxidation by any external agent is used to evaluate its antioxidant capacity [24].

Fig. 6 shows bar graph for the comparative TBARS formation in liposomes in absence and presence of 10 μM of 1:1 and 1:2 Cu(II)–curcumin complexes on irradiation with γ-radiation at absorbed doses of 210, 420 and 630 Gy. The results confirm that both the complexes inhibit lipid peroxidation at all the doses. However, 1:1 Cu(II)–curcumin complex inhibits peroxidation to a greater extent than either the 1:2 Cu(II)–curcumin complex or curcumin, therefore it is expected to be a better antioxidant.

2.4. Free radical-scavenging ability of the complexes

Earlier [23] we reported the free radical kinetics for the reactions of 1:1 complex with azide radicals ($N_3^{\bullet}$ radicals) and DPPH radicals using pulse radiolysis and stopped-flow spectrometer. While $N_3^{\bullet}$ radicals react by electron transfer, the reaction with DPPH occurs by hydrogen atom abstraction [31,32]. Generally hydrogen atom transfer is slower than that of an electron transfer process. The same experiments were carried out with 1:2 complex and compared with 1:1 complex.

As the 1:2 complex is insoluble in water, it was dissolved in aqueous Tx-100 solutions. The transient spectrum obtained on reaction of $N_3^{\bullet}$ with 1:2 complex recorded 40 μs after the pulse, is given in Fig. 7. The spectrum matches qualitatively with that obtained on reaction of $N_3^{\bullet}$ with 1:1 complex and curcumin, indicating that the reaction produces similar phenoxyl radicals which are generated by one-electron oxidation followed by proton loss from curcumin phenolic OH group [31]. The rate constants for the reaction of $N_3^{\bullet}$ radicals with 1:2 Cu(II)–curcumin complex was estimated to be $4.4 \pm 1.1 \times 10^8\ M^{-1}\,s^{-1}$ by following the observed pseudo first order rate constant for the formation of the transient at 500 nm as a function

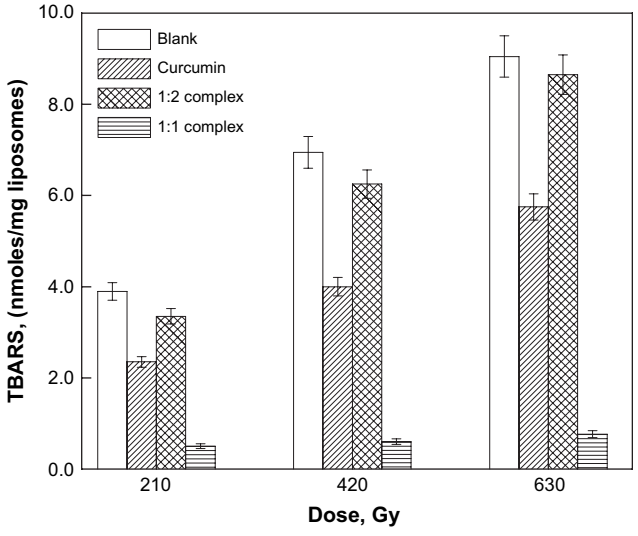

Fig. 6. γ-Radiation induced lipid peroxidation in liposomes (1 mg/ml) in the absence and presence of curcumin, 1:2 and 1:1 Cu(II)–curcumin complex [substrate = 10 μM] at different absorbed doses. Lipid peroxidation was assayed by reduction in formation of TBARS.

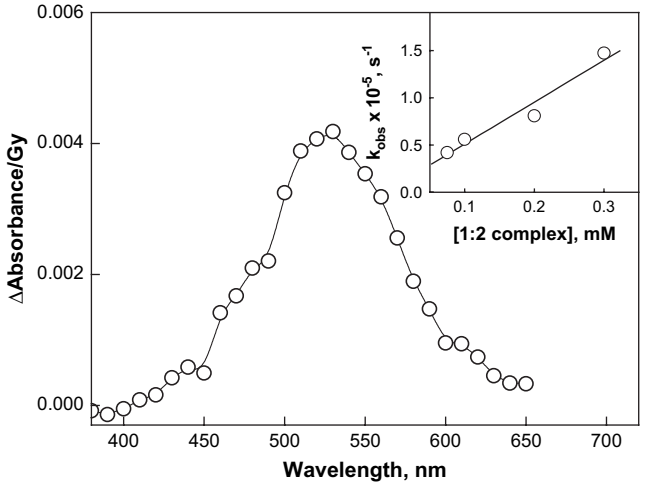

Fig. 7. Difference absorption spectrum of transient obtained by reaction of 0.1 mM 1:2 Cu(II)–curcumin complex with $N_3^{\bullet}$ radical at pH 7; (experimental conditions, N_2O saturated aqueous solutions of 1:2 complex containing 30 mM Tx-100 and 0.1 M sodium azide were pulse radiolysed, dose/pulse: 13 Gy). Inset shows variation in the observed rate constant as a function of concentration of 1:2 Cu(II)–curcumin complex.

of concentration as given as inset of Fig. 7, the value reported for 1:1 complex under similar conditions is given in Table 1. As observed earlier, the transients in all the cases do not react with oxygen and therefore not converted into any other new radicals [23].

The reaction of DPPH with 1:2 complex was studied in DMSO. The absorption–time plots for the decay of DPPH (10 μM) in presence of the complex (250 μM to 1 mM) at 517 nm could be fitted to single exponential function to obtain the observed rate constant. In presence of different concentrations of the complex, the observed rate constant was plotted as a function of the concentration of the complex and from the slope, the bimolecular rate constants for the reaction of 1:2 Cu(II)–curcumin complexes with DPPH was determined to be $1.1 \times 10^3\ M^{-1}\ s^{-1}$. The bimolecular rate constant for the reaction of DPPH with 1:1 complex was earlier reported to be $1.6 \times 10^2\ M^{-1}\ s^{-1}$. The reaction of these complexes with $N_3^{\bullet}$ radicals and DPPH radicals indicate that the two complexes are able to participate in both electron transfer and hydrogen atom transfer reactions. This may be mainly from the phenolic OH groups of curcumin which are intact and unaffected by the chelation with copper. All these above estimated parameters for the two complexes are listed in Table 1.

2.5. Quantum chemical calculations

The physicochemical properties of the complexes (1:1 and 1:2 Cu(II)–curcumin) have been studied employing the DFT calculations, which have been successfully used in determining the metal–chelating modes [15c,33] and in exploring the radical-scavenging mechanisms of antioxidants as well [34].

Table 2 lists selected structural parameters of curcumin and the complexes (1:1 and 1:2 Cu(II)–curcumin). It can be seen that the four Cu(II)–O bond lengths of the 1:1 Cu(II)–curcumin complex are different (Table 2), while those of the 1:2 Cu(II)–curcumin complex are identical (1.94 Å, Table 2), which is consistent with the EPR-derived results where orthorhombic symmetry was observed due to coordination to four non-equivalent oxygens in the 1:1 Cu(II)–curcumin complex, while square planar coordination is observed around the copper in the 1:2 Cu(II)–curcumin complex. In addition, the dihedral angle of O1–O2–O3–O4 of the 1:2 Cu(II)–curcumin complex (22.39°) is much larger than that of the 1:1 Cu(II)–curcumin complex (5.36°), which likely results from the steric repulsion between the methoxy groups in both curcumins of the 1:2 Cu(II)–curcumin complex (Fig. 8). Moreover, as shown in Table 2, copper coordination has little influence on the geometry of parent curcumin, except for the binding site. As the B3LYP/LANL2DZ method can provide quite accurate structural parameters of Cu(II) complexes, such as bond length, with a error of less than 0.01 Å [15c], the theoretical geometries of the complexes (1:1 and 1:2 Cu(II)–curcumin) are of significance in comparing with other SOD mimics.

Table 2
Selected structural parameters (bond length in Å and dihedral angle in degree) of curcumin and the complexes (1:1 and 1:2)

	Complex (1:1)	Complex (1:2)	Curcumin
O1–Cu5 (H5)	1.89[a]	1.94	1.04
O2–Cu5 (H5)	1.92[a]	1.94	1.55
O3–Cu5	2.01[a]	1.94	–
O4–Cu5	1.95[a]	1.94	–
O1–C6	1.32	1.32	1.36
C6–C7	1.42	1.42	1.40
C7–H8	1.09	1.09	1.08
C7–C9	1.41	1.42	1.44
C9–C2	1.33	1.32	1.30
∠O1–O2–O3–O4	5.36	22.39	–

The numbering of the atoms corresponds to that shown in Fig. 1.
[a] From Ref. [23].

According to the calculated results (Table 1), the EA of 1:1 Cu(II)–curcumin complex is ∼30 kcal/mol lower than that of 1:2 complex, which agrees well with the lower reduction potential of the former than the latter. The O–H PDE of the 1:1 Cu(II)–curcumin complex is 5.53 kcal/mol lower than that of the 1:2 counterpart, while the O–H BDE of the 1:1 Cu(II)–curcumin complex is about 0.68 kcal/mol higher than that of the 1:2 Cu(II)–curcumin complex, in line with the experimental observation that the former is more active in scavenging superoxide anion radical (through proton transfer or electron transfer), but less active in scavenging DPPH radical (through H-atom transfer) than the later. In addition,

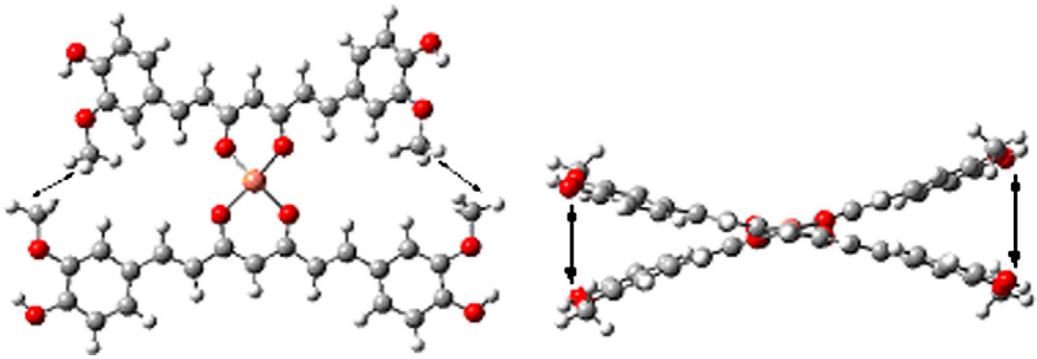

Fig. 8. Stereo views of optimized structures of Cu(II)–curcumin complex (1:2). The arrows indicate the steric repulsion between the methoxy groups of both curcumins.

as known to us, the azide radical-scavenging ability can be characterized by their IP and the lower the IP higher the activity. However, the different azide radical-scavenging activity of the complexes (1:1 and 1:2 Cu(II)–curcumin) (Table 1) cannot be elucidated by their difference in IP (Table 1), which may arise from the fact that solvent effect is not considered during the calculation.

In brief, a large part of physicochemical properties of Cu(II)–curcumin complexes (1:1 and 1:2) can be elucidated by DFT calculations, which not only provide deeper insights into the experimental findings but also display the potential of theoretical methods in antioxidant study.

3. Conclusions

In the recent years, metal-chelating properties of curcumin and its implications in use of curcumin as a multipotent agent to combat oxidative stress and Alzheimer's disease and also as a probable SOD mimic is gaining a lot of significance. Curcumin forms both 1:1 and 1:2 complexes with copper(II). We were able to synthesize and characterize these two complexes. EPR spectra confirm the structures of the complexes as distorted orthorhombic and symmetric square planar, respectively. Reduction potential of the reversible Cu^{+2}/Cu^{+} couple was found to be ~0.4 V which is in within the range expected for SOD mimics [25]. However, the SOD activity of the complexes studied by xanthine and xanthine oxidase assay indicated that the 1:1 Cu(II)–curcumin complex is nearly ten times more potent as SOD mimic than the 1:2 Cu(II)–curcumin complex. Similarly the rate constant for the scavenging of superoxide radical of the 1:1 complex has been found to be seven times higher than that of the 1:2 Cu(II)–curcumin complex. The complexes were also tested for their ability to inhibit radiation induced lipid peroxidation in liposomes, where the 1:1 Cu(II)–curcumin complex was found to exhibit inhibition of lipid peroxidation to a greater extent than the 1:2 Cu(II)–curcumin complex. The two complexes show similar electron and hydrogen atom transfer reactions with free radicals and produce the phenoxyl radicals similar to those produced from curcumin. Factors like steric hindrance, O–H bond dissociation energies and ionization potential are responsible for this. Theoretical calculations support these observations.

From these studies it appears that the increasing flexibility of the complex to accommodate the reduced cuprous species in tetrahedral or linear environments during superoxide radical reaction plays a crucial role in the overall antioxidant and SOD activity. The EPR spectral results indicate appreciable distortion from the square planar geometry for 1:1 complex. From this it can be concluded that the 1:1 complex would be able to undergo and sustain the distortion from square planar geometry to the distorted tetrahedral one during its reaction with superoxide radical. This allows for the compound to remain intact and undergo many redox cycles and hence as an efficient antioxidant. The 1:2 complex on the other hand is planar but rigid and hence cannot undergo the distortions and therefore is less powerful antioxidant. Thus the 1:1 Cu(II)–curcumin complex having distortion from square planar geometry is expected to exhibit a better SOD activity and is also a good free radical scavenger.

4. Experimental

4.1. Chemicals

Curcumin, xanthine, xanthine oxidase, cytochrome *c*, SOD, DPPH, Tx-100 (Sigma/Aldrich) were purchased from the local market. AR grade, cupric chloride ($CuCl_2 \cdot 2H_2O$) and cupric acetate ($Cu(OOCCH_3)_2 \cdot 2H_2O$) and spectrograde dimethyl sulfoxide (DMSO) from Spectro Chem. India, Mumbai were used as received. All the other reagents were of the best purity available. Absorption spectrometric studies were carried out using JASCO V–530 spectrophotometer.

The synthesis of 1:1 and 1:2 complexes between copper(II) ion and curcumin has been reported in our earlier papers and described briefly here [15a,23]. The 1:1 Cu(II)–curcumin complex was synthesized by mixing equimolar ratio of copper acetate and curcumin in dry ethanol and refluxed for 3 h under nitrogen atmosphere. 1:2 Cu(II)–curcumin complex was synthesized by mixing methanolic solution of curcumin and aqueous solution of $CuCl_2 \cdot H_2O$ under reflux for 3 h in the stoichiometry of 1:2 (copper:curcumin). The precipitated complexes were filtered, washed with cold water and ethanol and dried in vacuum.

4.2. Analytical experiments

EPR spectra were recorded on Bruker ESP 300 spectrometer, operated at X-band frequency (9–10 GHz) using 100 kHz field modulation. DPPH sample was used as a field marker. The spectra were recorded at 77 K using a liquid nitrogen Dewar insert.

Cyclic voltammetry was carried out using Eco-Chemie potentiostat AUTOLAB-20 cyclic voltameter attached with VA 663 stand. For the electrochemical estimation of Cu^{+2}/Cu^{+} couple, solutions of the above complexes in DMSO were scanned employing three-electrode system of Ag/AgCl electrode as the reference electrode, glassy carbon electrode as the working electrode, and platinum rod as the counter electrode. The electrochemical setup has been calibrated using Cd^{+2} solution. For the estimation of formation constant of the complexes, electrochemical scanning was carried out taking the Cu(II) salt solution, both in the presence and absence of varying concentration of curcumin in an electrochemical cell comprising of a hanging mercury drop electrode, a Ag/AgCl electrode as reference and a platinum rod as the counter electrode. The voltammograms were recorded at room temperature in DMSO solvent using 0.1 M tetraethyl ammonium perchlorate (TEAP) as the supporting electrolyte. The solution was kept under nitrogen purging to avoid interference from oxygen.

Superoxide radical was generated by enzymatic reaction of xanthine (50 μM) with xanthine oxidase (10 mU/ml) in presence of Tris buffer (pH 7.4) and 600 μM EDTA according to the procedure given in reference [35]. The superoxide radical generated by this method was allowed to react with cytochrome *c* (Fe^{3+}) (9.5 μM) to produce reduced cytochrome *c* (Fe^{2+}), absorbing at 550 nm. The change in absorbance per unit time ΔA/min was monitored up to 300 s, where ΔA is the difference in absorbance at 550 nm. The concentration of xanthine oxidase is adjusted such that ΔA/min is $\sim$0.025.

Lipid peroxidation studies were also carried out in phosphatidylcholine liposomes (2:1 mixture of phosphatidylcholine and cholesterol). Liposomes were prepared according to the procedure given in Ref. [36]. Liposomes suspended in pH 7.4 phosphate buffers with and without substrate were exposed for different doses of γ-radiation from ^{60}Co γ-source with a dose rate of 42 Gy min^{-1} as measured by standard Fricke dosimeter [37], and the lipid peroxidation was estimated by TBARS at 532 nm ($\varepsilon_{532} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$) as given in earlier Refs. [38,39]. All the above experiments were repeated twice to confirm their reproducibility, wherever the applicable standard deviations of different experimental data have been given as errors.

The kinetics of DPPH reactions with the complexes was studied using SX-18 MV stopped-flow spectrometer from Applied Photophysics Ltd., UK in the single mixing mode. Here the two mixing syringes contain DMSO solutions of DPPH and Cu(II)–curcumin complexes separately and the time-dependent absorption changes at 517 nm due to DPPH after mixing were monitored in presence of different concentrations of the complex [31,32]. Three independent runs were analysed to obtain average observed rate constant.

Pulse radiolysis studies were carried out using 7 MeV electron pulses (50 ns) from a linear electron accelerator coupled with absorption detection [40]. The dose per pulse was close to 12–14 Gy. One-electron specific oxidants and azide radicals ($E(N_3^{\bullet}/N_3^{-}) = 1.33$ V vs. NHE) were generated by the radiolysis of N_2O purged aqueous solutions containing 0.1 M sodium azide according to the procedure reported in Ref. [41].

4.3. Theoretical

The theoretical calculation procedures are as follows. The full geometry optimization for each molecule was performed *in vacuo* by employing hybrid density functional theory (DFT) [42] and B3LYP functional [43]. The standard double-ζ basis set was used for all light elements, while for metals, nonrelativistic effective core potential (ECP) was employed. The valence basis set used in connection with the ECP is essentially of double-ζ quality (the LANL2DZ basis set). As the molecules are rather large, the B3LYP/LANL2DZ method failed to give zero point vibrational energy and thermal correction to energy. However, according to previous studies [15c,44], the physicochemical parameters derived from total electronic energy (TE) are fairly accurate in a relative sense. All the calculations were accomplished by Gaussian 03 program package [45].

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