Iron complexes of gallocatechins. Antioxidant action or iron regulation?

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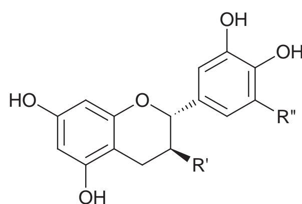
Iron(III) complexes of gallocatechins were studied in aqueous solutions by UV–VIS spectrometry, HPLC, cyclic voltammetry, laser photolysis and pulse radiolysis techniques. The blue–violet colored complexes are readily formed in water. The Job plots indicate 1 : 2 stoichiometry for the reaction of iron(III) with gallocatechins and methyl gallate, and 1 : 3 for that of iron(III) and catechin. This suggests that the three phenol groups of the gallate moiety play a role in complex formation. The formation constants of the complexes are found to be pH dependent, as expected for polyhydroxybenzene derivatives. $pK_{a1} = 4.3$ and $pK_{a2} = 7.4$ for the polyphenols with the gallate ester moiety (epigallocatechin gallate and epicatechin gallate) are lower than those of epigallocatechin (EGC) and catechin ($pK_{a1} = 4.9$ and $pK_{a2} = 8.4$), very probably because of the electron-withdrawing effect of the ester. Apparent stability constants of iron(III) gallocatechin complexes are high at pH 7, $\log K \approx 27$, comparable to those of the catechol derivatives. Photoionization of the iron complexes by the 248 nm laser is more efficient at higher pH, $\varphi = 0.13$ at pH 7 *vs.* $\varphi = 0.26$ at pH 11.5. The absorption spectra, which resemble those of ligand phenoxyl radicals, indicate that photoionization yields unstable phenoxyls, $t_{1/2}$ ~1 ms. Similar spectra are recorded when one-electron oxidation by the azide radical, $N_3^{\bullet}$, is used to generate the ligand radicals. The reduction potential of Fe(III)gallocatechins is −0.325 V *vs.* NHE, which is ~0.45 V less negative than the reduction potential of the Fe(II)/Fe(III) couple. In the case of the catechins with the gallate ester moiety, namely EGCG and ECG, the high pH cyclic voltammograms exhibit a quasi-reversible oxidation–reduction not seen in the other derivatives. The relevance of these findings for the physiological function and antioxidant and chemopreventive action of gallocatechins is discussed.

Introduction

Gallocatechins are the major constituents of green tea. These polyhydroxybenzene derivatives are shown to be extraordinary antioxidants *in vivo* and *in vitro*.[1–10] The antioxidant action of tea catechins originates from favorable one-electron donation properties. Of particular importance is the ability to repair the vitamin E radical, which is a unique characteristic of the gallocatechins.[5] In addition, these tea phenols are very efficient scavengers of biologically damaging oxyl species, such as the superoxide radical, $O_2^{\bullet -}$, and singlet oxygen, $^1O_2$.[1,5]



R' = OH; R" = H - Epicatechin
R' = OH; R" = OH - Epigallocatechin
R' = gallate ester; R" = H - Epicatechin gallate
R' = gallate ester; R" = OH - Epigallocatechin gallate

Favorable antioxidant properties of the gallocatechins are the basis for efficient chemoprevention of oxyl radical mediated degenerative diseases, such as cancer.[6,9,10–14]

The ability of plant phenolics to form transition metal complexes is obvious from the development of intense color in aqueous solutions of iron and gallocatechins. This has been used to determine the gallocatechin content of beverages, like beer and tea.[15] Because of the extraordinary stability of the complexes, it has been argued that the consumption of tea would interfere with the absorption from food and bioavailability of iron and alumina.[16,17]

Oxidative stress in general and oxygen free radicals in particular have been implicated in degenerative changes leading to Alzheimer's disease.[18–21] It has been shown that lipid peroxidation is a contributing factor to the formation of senile plaques and to neuronal cell death. More recently, increased levels of the biomarker of oxidative stress, 8-hydroxy-2′-deoxyguanosine, were found in paretial cortex of Alzheimer patients.[20] Of particular importance are iron accumulation in the pre-frontal cortex and alterations in the levels of the distribution of ferritin, which were found to contribute to increased free radical formation, possibly through Fenton-type chemistry. If there is a way to further decrease the concentration of free iron by substitute chelators, such as gallocatechins, then the Fenton chemistry contribution to Alzheimer's disease would be substantially reduced.

Interestingly, neither structure and stability constants of any metal–gallocatechin complexes nor the antioxidant properties of these complexes have been reported yet. It is obvious that these properties are of crucial importance in any meaningful assessment of the chemopreventive action of gallocatechins.

We have investigated the iron(III) complexes of the gallocatechins in aqueous solutions by various physicochemical techniques in a manner similar to studies of various catechol

complexes.[22–30] The ability of the catechin complexes to accommodate and stabilize ligand phenoxyl radicals has been studied in the present work by laser flash photolysis and pulse radiolysis. These measurements offered clues for the structure of the complexes, since various attempts to crystallize complexes have been unsuccessful. Our results have indicated that the antioxidant ability of the gallocatechins is practically lost in the complexes. Thus, bioregulation of free iron could be an essential contribution of these nutrients to the prevention of free radical mediated degenerative diseases.

Materials and methods

All chemicals used in this study were of the highest purity available. (−)-Epicatechin, EC, (−)-epicatechin gallate, ECG, (−)-epigallocatechin, EGC, and (−)-epigallocatechin gallate, EGCG, were produced from green tea by Mitsui Norin, Inc., as detailed elsewhere.[31] Methyl gallate, 2-chloroethanol, sodium azide, phosphate and borate buffers, NaOH, $HClO_4$ and potassium thiocyanate (Merck), and ferric chloride and ferrous sulfate (Aldrich), were used as received. Water was purified through a Millipore Milli-Q system. All solutions were freshly prepared before each experiment. High purity (>99.99%) N_2O and N_2 were purged through solutions either to enable conversion of e^-_{aq} to the hydroxyl radical,[32] or to exclude oxygen and prevent its interference with radical reactions.

UV–VIS spectra were measured with a Shimadzu UV–VIS and an HP 8450A diode array spectrophotometer. 1 and 10 mm Supra-sil quartz cuvettes were used.

The 3 MeV Van-de-Graaff pulse radiolysis equipment with optical detection at the Max-Planck-Institut für Strahlenchemie[33] was used for the pulse radiolysis studies. A 2 cm supra-sil quartz cell with temperature variation through a thermostatically controlled liquid jacket was used for sample irradiation. The spectra of the radicals were measured at 5–10 Gy/pulse, whereas the rate constants were determined at considerably lower 1–2 Gy/pulse to minimize interference from radical–radical reactions. Thiocyanate dosimetry was used in dose determinations, assuming $G[(SCN)_2^{\cdot -}] = 6.0$ in N_2O-saturated 10 mM KSCN aqueous solutions.

Fully computerized laser photolysis (λ = 248 nm) at the Max-Planck-Institut[34] was used for photochemical investigations of the complexes. The photoionization yield was measured using $\varphi(e^-_{aq}) = 0.67$ for photoionization of aqueous $K_4[Fe(CN)_6]$[35] as a reference. 0.1 M 2-chloroethanol was used to scavenge the photogenerated electron.

An EG&G potentiostat driven by the Research Electrochemistry Software Version 4.11 was used for cyclic voltammetry. The working electrode was glassy carbon, with a platinum auxiliary, and saturated calomel as a reference. The complexes were prepared in deaerated water containing 0.1 M KCl and 50 mM phosphate buffer (pH 7.0 and 11.5).

An HP1100 series liquid chromatograph with an autosampler and a variable wavelength (VWD) and an HP1049 electrochemical detector (ECD) connected in series was used for the HPLC of the complexes. ECD operated in the amperometric mode with a glassy carbon working electrode, platinum auxiliary and a saturated calomel reference.

Results and discussion

Stoichiometry of Fe(III) complex formation at pH 7

Mixing solutions of the gallocatechins with $FeSO_4$ or $FeCl_3$ at pH 7.0 results in the development of a blue–violet color, which is indicative of the formation of the complex. The UV–VIS spectrum reveals the appearance of the broad ligand-to-metal charge transfer band in the visible region, with a maximum around 540 nm. Assuming that only one complex between the metal and the catechin derivative is formed, the Job's method of continuous variations may be used to determine the composition of the complex.

Table 1 Stoichiometric composition of metal complexes of gallocatechins determined by the Job's method at pH 7, 20 °C

Complex	n
Fe(III)(catechin)	3
Fe(III)(epigallocatechin gallate)	2
Fe(III)(epigallocatechin)	2
Fe(III)(epicatechin gallate)	2
Fe(III)(methyl gallate)	2
Fe(III)(catechol)	3

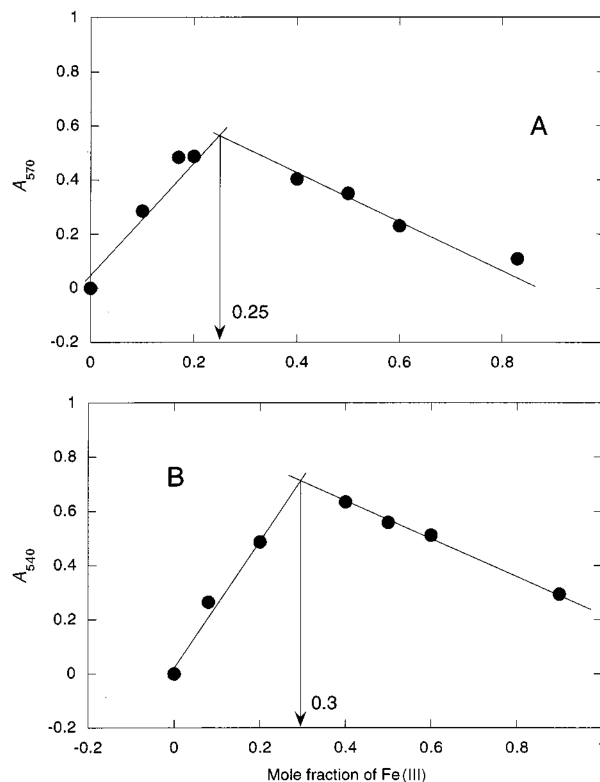


Fig. 1 The Job plots for A) $Fe(catechin)_3$ and B) $Fe(epigallocatechin)_2$.

The Job's plots for the Fe(III) complexes of catechin (or epicatechin) and epigallocatechin gallate are presented in Fig. 1. The maximum in the Job's plot corresponds to a condition where [molar fraction of metal] = $1/(n + 1)$, where n = number of ligands in the complex. From Fig. 1, in the case of the Fe(III) complex with catechin, $1/(n + 1) = 0.25 = 1/4$; which results in $n = 3$. In the case of the Fe(III) complex with epigallocatechin gallate, $1/(n + 1) = 0.3 = 1/3$. Consequently, the number of ligands is 2. All gallocatechins were investigated by the Job's method, and the results are summarized in Table 1.

The results clearly show that iron(III) forms a complex with two molecules of gallocatechin and three molecules of catechin. The comparison with the simple phenolic models, methyl gallate and catechol (see Table 1), indicates that this difference in the number of ligands probably originates from the availability of three phenolic oxygens in the gallate moiety for the coordination bonds with the metal.

Acid–base equilibria of Fe(III)bis(gallocatechins)

UV–VIS spectra of Fe(III)bis(gallocatechins) feature three charge-transfer bands, which are clearly pH dependent. Similarly to other iron–phenolate complexes[22,24–26,29,30,36–40] iron(III)-bis(gallocatechin) complexes exhibit the acid–base equilibria (1)–(3) in the pH range from 2 to 12. The UV–VIS spectra of

Table 2 Acid–base equilibria of Fe(III)bis(gallocatechin)

Gallocatechin	pK_{a1} [a]	pK_{a2} [a]
Epicatechin	5.40	8.35
Epigallocatechin	4.90	8.60
Epigallocatechin gallate	4.40	7.40
Epicatechin gallate	4.30	7.30

[a] Estimated to be accurate to ±0.1 pH unit.

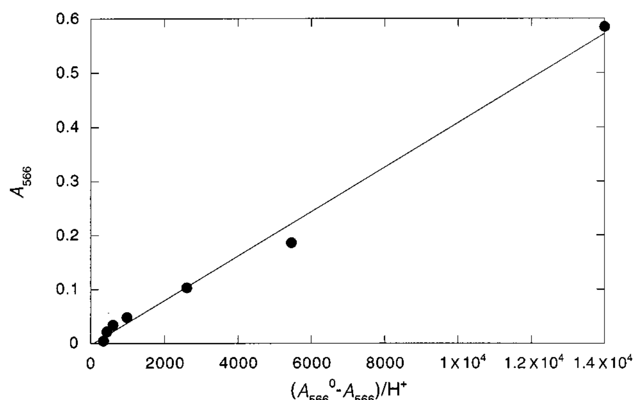


Fig. 2 Schwarzenbach's plot of Fe(III)bisEGCG in the pH range from 4 to 7.

$$Fe(III) + LH_3 \rightleftharpoons Fe(III)LH_2^- + H^+, K_1 \quad (1)$$

$$Fe(III)LH_2^- + LH_2 \rightleftharpoons Fe(III)LH^{2-} + LH_2^+, K_2 \quad (2)$$

$$Fe(III)LH^{2-} + LH_2 \rightleftharpoons Fe(III)L^{3-} + LH_3^+, K_3 \quad (3)$$

the Fe(III)bis(gallocatechins) were studied in the pH range from 2 to 10 (gallocatechins are extremely unstable in alkaline media, which precludes accurate determination of the third equilibrium constant), and the results are summarized in Table 2.

The stoichiometry of the protonation equilibria of the Fe(III)-bis(gallocatechin) complexes was verified by the Schwartzenbach plots[37] of the measured absorbances in the pH ranges where the most prominent absorbance changes were observed. The plots were linear only if one proton exchange was taken into account (Fig. 2).

The gallocatechins with the gallate ester moiety apparently have lower protonation equilibrium constants, presumably because of the electron-withdrawing effect of the ester group. Similar shifts in the protonation equilibrium constants were reported for model phenols, *e.g.* $pK_a = 8.03$ for methyl gallate *vs.* $pK_a = 8.73$ for gallic acid.[5]

It is noteworthy that the pK_a values of the Fe(III) complexes are considerably lower than the pK_a values of the free ligands (see ref. 5), which is explained by the participation of the phenol groups in the formation of the complexes.

Apparent stability constants of Fe(III)bis(gallocatechins)

Apparent stability constants of the Fe(III)bis(gallocatechins) were determined from competition equilibria with EDTA, in a manner similar to the approach by Harris *et al.*[25]

The complexes were prepared in a nitrogen-purged aqueous solution at pH 7, containing 0.1 mM Fe(III), 15 mM phosphate buffer pH 7, and 0.5 mM gallocatechin. The solution was allowed to equilibrate for at least 30 minutes prior to the addition of EDTA. Again equilibration with EDTA was allowed for at least another 30 minutes. All measurements were done at 20 °C.

The absorption of the Fe(III)gallocatechin complexes was then recorded. The competition constant, *K*, was determined from the plots $A_{540}{}^m/(A_{540}{}^0 - A_{540}{}^m)$ *vs.* [gallocatechin]^2/[EDTA], where A^m and A^0 correspond to the absorbance of the solution with EDTA added and prior to the EDTA addition, respectively. The slope of the linear plot is K/K_{EDTA}, which is summarized in Table 3.

Table 3 Competition constants at pH 7, 20 °C

Complex	K/K_{EDTA} ± 10%	K [a]
$Fe[EGCG]_2$	400	4×10^{27}
$Fe[ECG]_2$	200	2×10^{27}
$Fe[EGC]_2$	250	2.5×10^{27}

[a] Taking log $\beta = 25.0$ for Fe[EDTA].

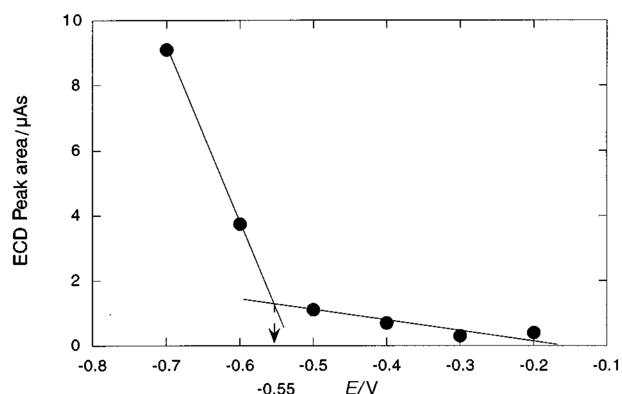


Fig. 3 Plot of area of the Fe(III)bisECG peak *vs.* voltage of ECD.

The apparent stability constants of Fe(III)bis(gallocatechins) are high, $K \sim 10^{27}$ M^{-2}, similar to those observed for the catechol complexes.[30] Such high stability constants suggest that the gallocatechins are extremely efficient chelators of free iron.

Reduction of Fe(III)bis(gallocatechins)

The reduction of Fe(III)bis(gallocatechins) was investigated in nitrogen-purged aqueous solutions at pH 7 using HPLC with VWD and ECD connected in series. An Fe-complex was prepared from 1 mM $FeSO_4$ and 5 mM gallocatechin in nitrogen-purged aqueous solution containing 10 mM phosphate buffer pH 7. This solution was then run through the reverse phase column (Genesis, C18, 4.6 × 150 mm, 4 µ particles) using the following gradient program: from 0–15 min 0–50% methanol starting with 100% 50 mM phosphate buffer pH 7 (containing 1 mM KCl), then an equilibration program from 50–0% methanol in 5 min prior to the next injection. The VWD was set at the maximum absorption of the complexes (484 nm), whereas the amperometric detector was operated in the reduction mode, with a stepwise increase in voltage from −0.7 to 0.0 V (0.1 V per injection) *vs.* SCE. A peak which appeared both in VWD and ECD chromatograms was taken to correspond to a complex. In all cases, only one major peak was found, which indicates that a single complex predominates at pH 7.

The plot of peak area *vs.* voltage for $Fe[ECG]_2$ is presented in Fig. 3. Similar plots were obtained for $Fe[EGCG]_2$, $Fe[EGC]_2$, and $Fe[C]_3$.

Obviously, the reduction potential of the complexes can be derived as −0.55 V (*vs.* SCE) = −0.325 V (*vs.* NHE), which is ~0.45 V less negative than the reduction potential of Fe(III)/Fe(II) couple ($E_0 = -0.77$ V *vs.* NHE).

The reduction of the complex was also studied by pulse radiolysis. In an N_2O-saturated aqueous solution of 1 M propan-2-ol at pH 10.0 and at varying concentrations of $Fe[EGCG]_2$, the 2-propyl radical, $(CH_3)_2$–$C^•$–OH, is exclusively generated. This highly reducing radical, $E \sim -1.8$ V *vs.* NHE, readily reduces the complex, as is visible from the bleaching of the absorbance at 540 nm (dose/pulse = 2 Gy) [reaction (4)].

Table 4 Irreversible oxidation potentials of Fe(III) complexes of gallocatechins determined by cyclic voltammetry in aqueous solutions

Substrate	*E*/V (*vs.* NHE) at pH 11.5	*E*/V (*vs.* NHE) at pH 7
Fe(Epigallocatechin)$_2$	1.241	
Epigallocatechin	0.141	
Fe(Epicatechin)$_3$	0.55	
Epicatechin	0.33	
Fe(Epigallocatechin gallate)$_2$	−0.17[a]	0.69
Epigallocatechin gallate	0.31	
Fe(Epicatechin gallate)$_2$	−0.17[a]	0.69
Epicatechin gallate	0.19	

[a] Another peak appears in the reverse (reduction sweep) cycle at −0.68 V *vs.* SCE (−0.44 V *vs.* NHE).

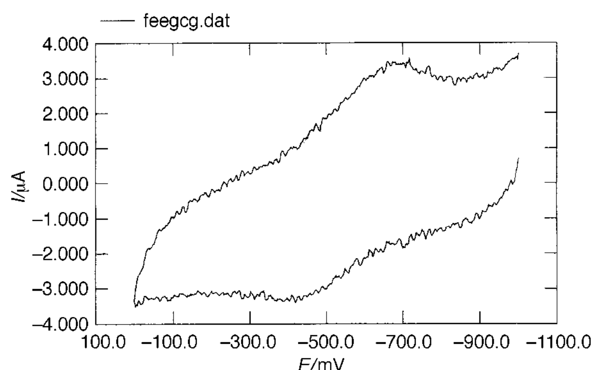
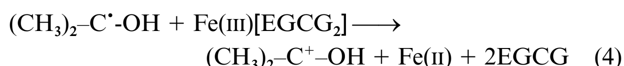


Fig. 4 Cyclic voltammogram of Fe(III)[EGCG$_2$] at pH 11.5, recorded in Ar-saturated aqueous solution of 0.5 mM FeCl$_3$, 1.0 mM EGCG, 5 mM phosphate buffer pH 11.5, and 0.1 M KCl, sweep rate 0.1 V min^{-1}.



$$(CH_3)_2–C^{\bullet}\text{-}OH + Fe(III)[EGCG_2] \longrightarrow (CH_3)_2–C^{+}–OH + Fe(II) + 2EGCG \quad (4)$$

The second order rate constant of $k = (6.7 \pm 1) \times 10^4\ M^{-1}\ s^{-1}$ can be derived from the pseudo first order rate constants obtained for the three concentrations of the complex.

Oxidation of Fe(III)bis(gallocatechins)

Gallocatechins are potent antioxidants–electron donors.[1,5] To investigate how their antioxidant action is affected by complexation with Fe(III), cyclic voltammetry at pH 7 and 11.5 was used to determine the oxidation potentials. None of the complexes exhibited a reversible one-electron redox reaction. Such electrochemical behavior suggests that only the ligands participate in electron transfer processes. The irreversible potentials are read at the peak of voltammograms recorded at 0.1 V min^{-1}. The results are summarized in Table 4.

At pH 7, Fe(III)bis(gallocatechins) have high oxidation potentials (Table 4), which means that complexation with Fe(III) almost completely removes their antioxidant properties. The oxidation potentials are lower at pH 11.5, as expected from the deprotonation of the complex.

Interestingly, at pH 11.5, the Fe(III) complexes of gallocatechins which have the gallate ester moiety, Fe[ECG$_2$] and Fe[EGCG$_2$], exhibit quasi-reversible voltammograms (since the voltammograms were repeatable and independent of the sweep rate), as illustrated in Fig. 4. The voltammograms would indicate a four-electron process, which is difficult to explain considering the structure of the complexes. We hypothesize that the oxidation process takes place at the phenol ring, generating the phenoxyl radical, whereas the gallate ester group is reduced in the reduction process (possibly with the expulsion of CO_2).

Oxidation of the complexes results in the formation of the potentially harmful radical species. Stability of the ligand phenoxyl radical is very important for the assessment of the biological function of Fe(III)bis(gallocatechins).

Table 5 Photoionization ($\lambda_{exc} = 248$ nm) yields of Fe(III)[gallocatechin]$_2$ complexes

Complex	pH	$\varphi(e^-_{aq})$[a]
Fe(Epicatechin)$_3$	11.5	0.26
Fe(Epicatechin gallate)$_2$	11.5	0.26
Fe(Epigallocatechin)$_2$	11.5	0.26
Fe(Epigallocatechin gallate)$_2$	11.5	0.26
	7.5	0.13
Fe(Methyl gallate)$_2$	7.5	0.13
	13.4	[b]

[a] Photoionization is monophotonic. [b] Biphotonic process.

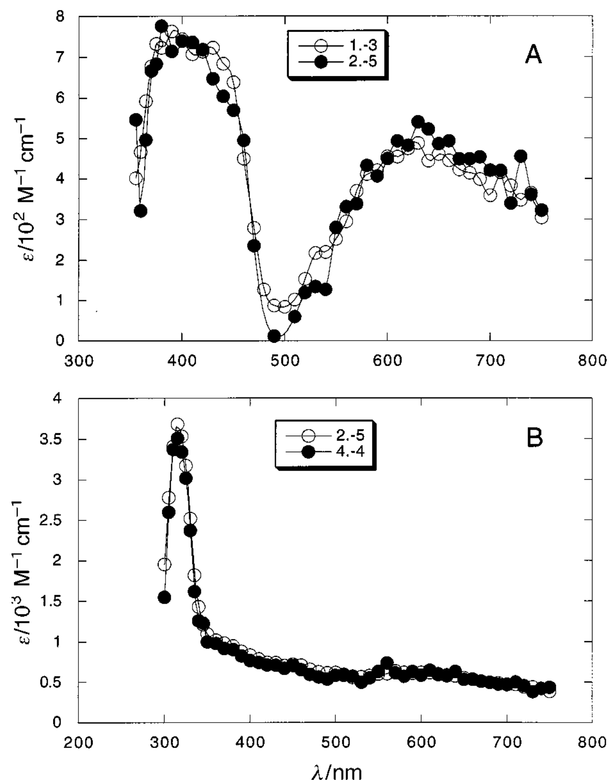


Fig. 5 Transient spectra of A) Fe(III)[(EGCG)(EGCG$^{\bullet}$)] and B) Fe(III)[(EGC)(EGC$^{\bullet}$)] generated by chemionization with $N_3^{\bullet}$, in N_2O-saturated aqueous solutions of 0.1 M NaN_3, 0.1 mM Fe(III)-[(gallocatechin)$_2$], pH 11.5.

The photoionization of the metal complexes with a 248 nm laser results in the ejection of an electron from a ligand, as previously observed for the Fe(III) complexes of phenols.[39] The photochemical yield of photoionization is considerably higher at pH 11.5 than in a neutral medium (see Table 5). The higher photoionization yield is very probably due to the deprotonation of the complexes, which makes them easier to oxidize.

The absorption spectra of one-electron oxidized complexes at pH 11.5 are also recorded upon reaction with the azide radical, and are similar to those obtained by photoionization. The representative spectra of the one-electron oxidized Fe(III) complexes of gallocatechins are presented in Fig. 5.

The complexes are readily oxidized (within 20 μs) by the azide radical to give relatively unstable, $t_{1/2} \sim 1$ ms, phenoxyl radicals. Clearly, the absorption spectra of the radicals in the complex with Fe(III) resemble those of the uncomplexed phenoxyls,[5] with an additional broad band at ~600 nm. This band can be attributed to the charge transfer band of the complexed phenoxyl radical.

Conclusions

Fe(III)bis(gallocatechin) complexes are very stable at biologically significant pH 7. Log $K \sim 27$ is derived from the

competition with EDTA. Based on the bioavailability of tea catechins in plasma 1 h after consumption of 1.2 g of decaffeinated green tea (a total of 176–554 mg l^{-1} or on average ~1 mM catechins),[41] the gallocatechins may take up to ~0.5 mM of free iron from the blood. Even with a very low concentration of the gallocatechins in the brain[41] (0.003–0.01% of ingested ^{3}H-EGCG is found in the brain of F344 rats), they can still aid in iron regulation by taking ~1 μM free iron.

Iron(III) appears to form coordinate bonds with the phenolic oxygens from the gallate moiety. It is conceivable that the ester group in ECG and EGCG also participates in the complex formation, especially at lower pH, where the gallate phenol groups are protonated. The unusual quasi-reversible cyclic voltammograms of ECG and EGCG at pH 11.5 could be explained by the participation of the ester group. The oxidation results in the gallate phenoxyl radicals, as in Fe(III)[EGC], whereas the reduction might occur at the ester moiety with the expulsion of CO_2.

Fe(III)bis(gallocatechins) can be oxidized by very strong oxidants. As seen in other Fe(III)phenol complexes, the ligand is oxidized to a corresponding phenoxyl radical, which can still participate in the coordination with the metal. The Fe(III) complexes with the phenoxyl radicals are not stable and disappear within several ms to generate stable products.

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