Synergistic antioxidant effect of green tea polyphenols with α-tocopherol on free radical initiated peroxidation of linoleic acid in micelles

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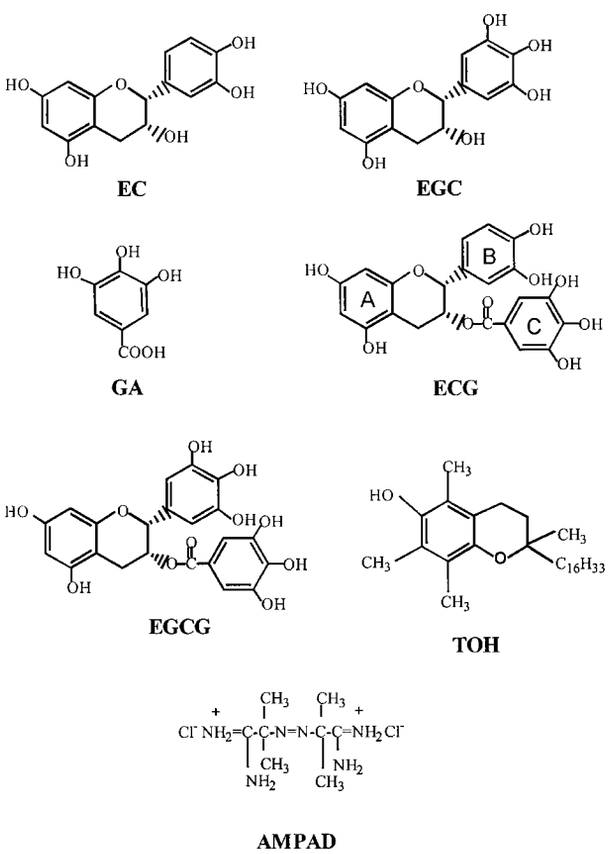
The antioxidant effect of the main polyphenolic components extracted from green tea leaves, *i.e.*, (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG) and gallic acid (GA), against peroxidation of linoleic acid has been studied in sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) micelles. The peroxidation was initiated thermally by a water-soluble azo initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPAD) and the reaction kinetics were followed by formation of linoleic acid hydroperoxides and consumption of the antioxidant. Kinetic analysis of the antioxidation process demonstrates that these green tea polyphenols are effective antioxidants in micelles used either alone or in combination with α-tocopherol (vitamin E). The antioxidative action may involve trapping the initiating radicals in the bulk water phase, trapping the propagating lipid peroxyl radicals on the surface of the micelle and regenerating α-tocopherol by reducing α-tocopheroxyl radical. The antioxidant activity of these green tea polyphenols depends significantly on the microenvironment of the reaction medium, the oxidation potential and the size of the molecule.

Introduction

A large body of clinical and experimental evidence has accumulated in the past decade suggesting that free radical mediated membrane lipid peroxidation is associated with a variety of chronic health problems, such as cancer, atherosclerosis and ageing, and that natural antioxidants, such as α-tocopherol (vitamin E), L-ascorbic acid (vitamin C) and β-carotene may have beneficial effects in protecting against these diseases.[1–4] A number of polyphenolic compounds extracted from green tea leaves, especially (−)-epigallocatechin gallate (EGCG), have been found to be good antioxidants against lipid peroxidation in phospholipid bilayers,[5,6] in low density lipoprotein,[7] in epidermal microsomes,[8] in synaptosomes[9] and in animal model systems,[10] and against tumourigenesis.[11,12] We[13] found recently that some polyphenolic components of green tea exhibited strong synergistic antioxidative activity with α-tocopherol in homogeneous solutions. It has been recognized that the antioxidant activity in homogeneous solutions may not parallel that in heterogeneous media, let alone the activity *in vivo*.[14,15] In order to bridge the gap between chemical and biological activities, it is essential to understand and evaluate the dependence of the antioxidant activity upon the microenvironment of the reaction media. A general methodology is to carry out the reaction in membrane mimetic systems, such as micelles and artificial bilayers.[16] Indeed, we have found that the activity sequence of lipophilic vitamin C derivatives obtained from micellar systems[14] coincides well with those in artificial bilayers,[17,18] in erythrocytes[19] and in low density lipoprotein.[20] Therefore, we present herein a kinetic and mechanistic study on the antioxidative effect of the main polyphenolic components extracted from green tea leaves, *i.e.*, (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG) and gallic acid (GA), against peroxidation of linoleic acid in sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) micelles.



The peroxidation was initiated thermally by a water soluble azo initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPAD). The interaction of these green tea polyphenols with α-tocopherol (TOH, vitamin E) was also investigated.

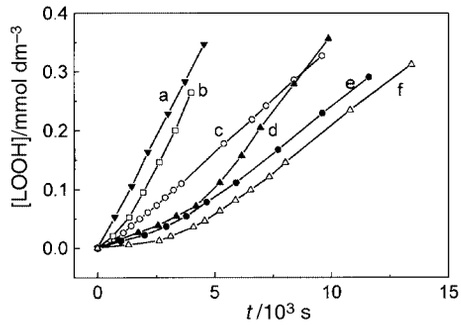


Fig. 1 Formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in 0.1 mol dm^{-3} SDS micelles at pH 7.4 and 37 °C, initiated with AMPAD and inhibited by green tea polyphenols (GOH). $[LH]_0 = 15.2$ mmol dm^{-3}; $[AMPAD]_0 = 6.3$ mmol dm^{-3}; $[GOH]_0 = 10$ μmol dm^{-3}. (a) uninhibited peroxidation; (b) inhibited with GA; (c) inhibited with EC; (d) inhibited with ECG; (e) inhibited with EGC; (f) inhibited with EGCG.

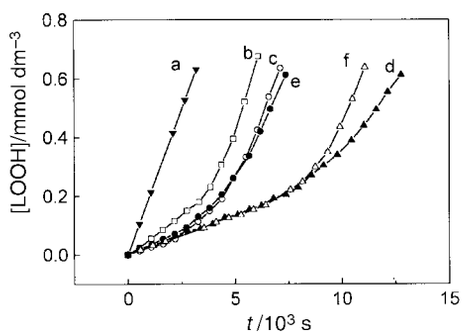


Fig. 2 Formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in 0.015 mol dm^{-3} CTAB micelles at pH 7.4 and 37 °C, initiated with AMPAD and inhibited by green tea polyphenols (GOH). $[LH]_0 = 15.2$ mmol dm^{-3}; $[AMPAD]_0 = 6.3$ mmol dm^{-3}; $[GOH]_0 = 10$ μmol dm^{-3}. (a) uninhibited peroxidation; (b) inhibited with GA; (c) inhibited with EC; (d) inhibited with ECG; (e) inhibited with EGC; (f) inhibited with EGCG.

Results and discussion

Inhibition of formation of linoleic acid peroxides by green tea polyphenols

The primary peroxidation products of linoleic acid are hydroperoxides formed by oxygen addition at C-9 or C-13 positions with either *trans*,*cis*- or *trans*,*trans*-diene stereochemistry.[21] They showed characteristic ultra-violet absorption at 235 nm[22] which was used to monitor the formation of the total hydroperoxides formed during the peroxidation after separation of the reaction mixture by high performance liquid chromatography (HPLC). A set of representative kinetic curves of the total hydroperoxides formation in SDS micelles is shown in Fig. 1. It is seen from Fig. 1 that the concentration of the hydroperoxides increased linearly with time in the absence of antioxidants upon AMPAD-initiation, demonstrating fast peroxidation of linoleic acid. The slope of this line corresponds to the rate of propagation, R_p (*vide infra*). The peroxide formation was inhibited remarkably by addition of ECG, EGC or EGCG in the so-called 'inhibition period' (t_{inh}) or induction period, then turned faster when the green tea polyphenol was exhausted. GA and EC showed less effect, but the inhibition period was still appreciable. This behaviour is distinctly different from that in homogeneous solutions reported previously.[13] In homogeneous solutions the green tea polyphenols only decrease the rate of peroxidation, but no inhibition period could be observed (compare Fig. 1 with Figs. 1 and 2 in our previous paper[13]). A similar antioxidative effect was observed in CTAB micelles, but the effect was much more pronounced than that in SDS micelles (Fig. 2). The same amount of the green tea polyphenol produced approximately double the inhibition period in CTAB micelles in comparison with that in SDS micelles except in the case of EGC. On the basis of the inhibition period the antioxidant activity follows the sequence ECG ~ EGCG ~ EGC > GA ~ EC in SDS micelles, while EGCG ~ ECG > EC ~ EGC > GA in CTAB micelles. It is also seen that after the inhibition period the rate of peroxide formation recovered approximately to the original rate in CTAB micelles except ECG, while in SDS micelles the rate was significantly lower than the original for most of the green tea polyphenols except GA.

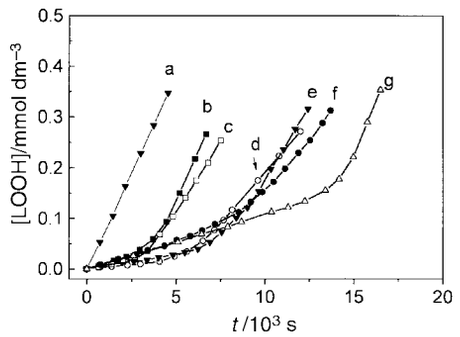


Fig. 3 Formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in 0.1 mol dm^{-3} SDS micelles at pH 7.4 and 37 °C, initiated with AMPAD and inhibited by green tea polyphenols (GOH) and/or α-tocopherol (TOH). $[LH]_0 = 15.2$ mmol dm^{-3}; $[AMPAD]_0 = 6.3$ mmol dm^{-3}; $[GOH]_0 = 10$ μmol dm^{-3}; $[TOH]_0 = 7.5$ μmol dm^{-3}. (a) uninhibited peroxidation; (b) inhibited with TOH; (c) inhibited with GA and TOH; (d) inhibited with EC and TOH; (e) inhibited with ECG and TOH; (f) inhibited with EGC and TOH; (g) inhibited with EGCG and TOH.

Antioxidant effect of green tea polyphenols in the presence of α-tocopherol

α-Tocopherol is a well-known chain-breaking antioxidant.[23] It shows typical antioxidative kinetics upon linoleic acid peroxidation in both SDS and CTAB micelles (line b in Figs. 3 and 4), in accordance with previous reports.[13–17,24] It is seen that the inhibition period of α-tocopherol is substantially shorter in CTAB than in SDS micelles, *i.e.*, the same amount of α-tocopherol produced only half the inhibition period in CTAB micelles in comparison with that in SDS micelles, being distinctly different to the behaviour of the green tea polyphenols (*vide supra*). Addition of the green tea polyphenol together with α-tocopherol prolonged the inhibition period of the latter except GA in SDS micelles, but the effectiveness depends significantly on the reaction medium and the polyphenol. In SDS micelles EGCG shows a remarkable synergistic antioxidant effect, *i.e.*, the inhibition period when the two antioxidants were used in combination was much longer than the sum when they were used individually. EGC and EC show an additive effect, *i.e.*, the inhibition period when the two antioxidants were used in combination was the sum of when they were used individually. The effect of ECG and GA is less than additive (Fig. 3). It is worth noting that in CTAB micelles the peroxidation is completely inhibited in the inhibition period and the cooperative effect is basically additive (Fig. 4).

Consumption of α-tocopherol and EGCG

In order to rationalize the mechanism of the antioxidant synergism of α-tocopherol and EGCG their decay kinetics were studied by HPLC separation of the reaction mixture followed by electrochemical determination of the antioxidants. It was found that both α-tocopherol and EGCG decayed approximately linearly with time within two half-lives when they were used individually in SDS and CTAB micelles (lines a and b in Figs. 5 and 6), in accordance with the kinetic demand for antioxidation reactions (eqn. (14), *vide infra*). The decay rate of

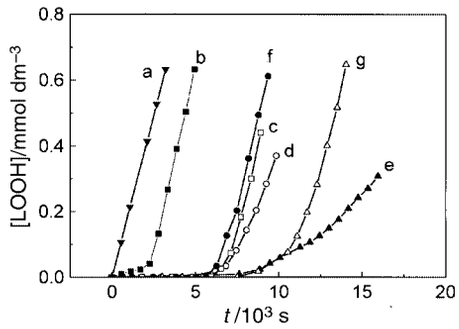


Fig. 4 Formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in 0.015 mol dm^{-3} CTAB micelles at pH 7.4 and 37 °C, initiated with AMPAD and inhibited by green tea polyphenols (GOH) and/or α-tocopherol (TOH). $[LH]_0 = 15.2$ mmol dm^{-3}; $[AMPAD]_0 = 6.3$ mmol dm^{-3}; $[GOH]_0 = 10$ μmol dm^{-3}; $[TOH]_0 = 9.5$ μmol dm^{-3}. (a) uninhibited peroxidation; (b) inhibited with TOH; (c) inhibited with GA and TOH; (d) inhibited with EC and TOH; (e) inhibited with ECG and TOH; (f) inhibited with EGC and TOH; (g) inhibited with EGCG and TOH.

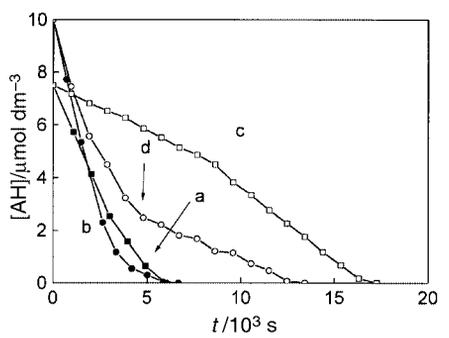


Fig. 5 Consumption of antioxidants (AH) during the antioxidation of linoleic acid in SDS micelles. The reaction conditions are the same as described in the legend of Fig. 3. (a) decay of TOH in the absence of EGCG; (b) decay of EGCG in the absence of TOH; (c) decay of TOH in the presence of EGCG; (d) decay of EGCG in the presence of TOH.

α-tocopherol was 1.6 and 4.3 nmol dm^{-3} s^{-1} in SDS and CTAB micelles respectively due to the different rate of initiation in the micelles (*vide infra*). Interestingly, the decay of α-tocopherol in the presence of EGCG behaved somewhat differently in SDS and CTAB micelles. In SDS micelles the decay decreased remarkably to 0.33 nmol dm^{-3} s^{-1} before EGCG was completely consumed, then the decay increased to 0.55 nmol dm^{-3} s^{-1}. On the other hand, although the decay of α-tocopherol also decreased in the presence of EGCG in CTAB micelles, no appreciable turning point could be observed besides that in the very beginning of the reaction (Figs. 5 and 6).

Kinetics and mechanism

It has been proved that the reaction kinetics of the peroxidation in micelles and biomembranes follow the same rate law as that in homogenous solutions.[16,25] Therefore, the peroxidation of linoleic acid (LH) initiated by azo-compounds (R–N=N–R) can be described by the following simplified scheme [eqns. (1)–(6)],

Initiation:

$$\text{R–N=N–R} \xrightarrow{k_d} 2\,e\,\text{R}^{\bullet} + \text{N}_2 + (1 - e)\,\text{R–R} \qquad (1)$$

$$\text{R}^{\bullet} + \text{O}_2 \longrightarrow \text{ROO}^{\bullet} \qquad (2)$$

$$\text{ROO}^{\bullet} + \text{LH} \longrightarrow \text{ROOH} + \text{L}^{\bullet} \qquad (3)$$

Propagation:

$$\text{L}^{\bullet} + \text{O}_2 \longrightarrow \text{LOO}^{\bullet} \qquad (4)$$

$$\text{LOO}^{\bullet} + \text{LH} \xrightarrow{k_p} \text{L}^{\bullet} + \text{LOOH} \qquad (5)$$

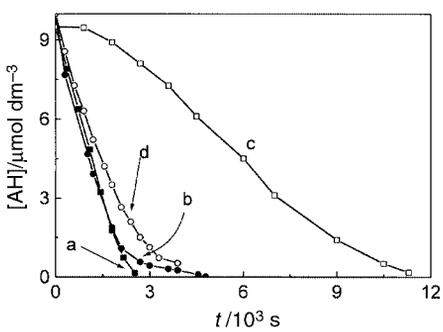


Fig. 6 Consumption of antioxidants (AH) during the antioxidation of linoleic acid in CTAB micelles. The reaction conditions are the same as described in the legend of Fig. 4. (a) decay of TOH in the absence of EGCG; (b) decay of EGCG in the absence of TOH; (c) decay of TOH in the presence of EGCG; (d) decay of EGCG in the presence of TOH.

Termination:

$$2\,\text{LOO}^{\bullet} \xrightarrow{2k_t} \text{Molecular products} \qquad (6)$$

where k_d, k_p and k_t are rate constants for the decomposition of the initiator, for the chain propagation and termination respectively, and e is the efficiency of the initiator which denotes the fraction of the initiator effective in initiating the peroxidation due to the cage effect.

Based on the steady state kinetic treatment, the rate of peroxide formation can be expressed as eqn. (7). The apparent rate of the chain initiation is given by eqn. (8).

$$\text{d[LOOH]/d}t = R_p = \{k_p/(2\,k_t)^{1/2}\}\; R_i^{1/2}\,[\text{LH}] \qquad (7)$$

$$R_i = 2\,k_d\,e\,[\text{R–N=N–R}] \qquad (8)$$

Although the radical generation rate of AMPAD is known to be 1.3×10^{-6} [AMPAD] s^{-1} at 37 °C for liposomal dispersions,[26] the cage effect parameter e depends appreciably on the medium and the concentration of the antioxidants and the initiator.[26] Therefore, the R_i value is generally determined from the inhibition time and/or decay rate of α-tocopherol [eqns. (12) and (13), *vide infra*].

In the presence of a chain-breaking antioxidant molecule (AH) the peroxyl radical can be trapped and a new antioxidant radical (A$^{\bullet}$) produced [eqn. (9)]. If the A$^{\bullet}$ is a stabilized radical

$$\text{LOO}^{\bullet} + \text{AH} \xrightarrow{k_{inh}} \text{LOOH} + \text{A}^{\bullet} \qquad (9)$$

(*e.g.* α-tocopheroxyl radical or ascorbate radical anion) which can promote the rate-limiting hydrogen abstraction reaction [eqn. (9)] and undergo a fast termination reaction [eqn. (10)], the peroxidation would be inhibited.

$$\text{LOO}^{\bullet} + \text{A}^{\bullet} \xrightarrow{\text{fast}} \text{LOOA} \qquad (10)$$

During the inhibition period the rate of formation of peroxyl radicals by initiation equals the rate at which peroxyl radicals are trapped, therefore eqn. (11) holds, where n is the stoichiometric factor that denotes the number of peroxyl radicals trapped by each antioxidant molecule and is given by eqn. (12).

$$R_{inh} = k_{inh}\,n\,[\text{AH}][\text{LOO}^{\bullet}] \qquad (11)$$

$$n = R_i\,t_{inh}/[\text{AH}]_0 \qquad (12)$$

The k_{inh} in eqn. (11) represents the activity of the antioxidants. From eqns. (9)–(12) we have eqn. (13).

$$-\text{d[AH]/d}t = R_i/n \qquad (13)$$

Table 1 Inhibition of AMPAD-initiated peroxidation of linoleic acid by green tea polyphenols (GOH) in micelles[a,b]

Micelle	GOH	$R_p/10^{-8}$ mol dm^{-3} s^{-1}	$R_{inh}/10^{-8}$ mol dm^{-3} s^{-1}	$t_{inh}/10^3$ s	$k_{inh}/10^4$ dm^3 mol^{-1} s^{-1}	n	kcl$_p$	kcl$_{inh}$
SDS	None	7.1					22	
SDS	EGCG	2.4	0.58	4.0	3.6	1.7	7.5	1.8
SDS	ECG	6.4	1.5	4.5	1.3	1.9	20	4.7
SDS	EGC	5.2	1.3	4.0	1.7	1.7	16	4.2
SDS	EC	3.4	2.1	1.2	3.7	0.52	11	6.6
SDS	GA	8.9	3.6	1.7	1.5	0.73	28	11
CTAB	None	18					22	
CTAB	EGCG	18	2.6	8.8	0.35	7.7	22	3.1
CTAB	ECG	7.9	2.7	8.3	0.37	7.3	9.5	3.2
CTAB	EGC	12	3.2	3.8	0.80	3.3	15	3.8
CTAB	EC	18	2.5	4.1	0.90	3.6	22	3.0
CTAB	GA	24	5.4	2.3	0.38	2.0	29	6.4

[a] The reaction conditions and the initial concentration of the substrates are the same as described in the legends of Figs. 1 and 2 for reactions conducted in SDS and CTAB micelles respectively. Data are the average of three determinations which were reproducible with deviation less than ±10%. [b] Taking R_i as 3.1 and 8.3 nmol dm^{-3} s^{-1} in SDS and CTAB micelles respectively, see text.

Table 2 Inhibition of AMPAD-initiated peroxidation of linoleic acid by green tea polyphenols (GOH) and α-tocopherol (TOH) in micelles[a,b]

Micelle	Antioxidant	$R_p/10^{-8}$ mol dm^{-3} s^{-1}	$R_{inh}/10^{-8}$ mol dm^{-3} s^{-1}	$t_{inh}/10^3$ s	$k_{inh}/10^4$ dm^3 mol^{-1} s^{-1}	n'[c]	kcl$_p$	kcl$_{inh}$	SE (%)
SDS	TOH	7.8	6.1	4.7	3.6	2.0	24	1.9	
SDS	EGCG + TOH	5.4	10	14.9	0.67	2.7	17	3.1	75
SDS	ECG + TOH	5.6	3.8	7.4	3.7	1.3	18	1.2	−18
SDS	EGC + TOH	5.1	12	9.2	0.77	1.6	16	3.8	~0
SDS	EC + TOH	7.4	3.2	6.4	4.1	1.1	23	1.0	12
SDS	GA + TOH	5.5	7.1	3.3	4.0	0.6	17	2.2	−47
CTAB	TOH	22	1.8	2.3	2.0	2.0	27	2.1	
CTAB	TOH + EGCG	3.8	~0	11.2	[d]	4.8	4.6	[d]	~0
CTAB	TOH + ECG	5.6	~0	10.3	[d]	4.4	6.7	[d]	~0
CTAB	TOH + EGC	21	~0	6.4	[d]	2.7	25	[d]	~0
CTAB	TOH + EC	14	~0	7.2	[d]	3.1	17	[d]	~0
CTAB	TOH + GA	36	~0	7.3	[d]	3.1	43	[d]	~0

[a] The reaction conditions and the initial concentration of the substrates are the same as described in the legends of Figs. 3 and 4 for reactions conducted in SDS and CTAB micelles respectively. Data are the average of three determinations which were reproducible with deviation less than ±10%. [b] Taking R_i as 3.1 and 8.3 nmol dm^{-3} s^{-1} in SDS and CTAB micelles respectively, see text. [c] $n' = R_i\, t_{inh}/([GOH]_0 + [TOH]_0)$. [d] Could not be calculated because R_{inh} is approximately zero.

The n value of α-tocopherol is generally assumed to be 2,[16,25] thus the R_i value can be determined from the inhibition period or from the decay rate of α-tocopherol. The rate of peroxide formation during the inhibition period, R_{inh}, can be derived from the steady-state treatment of the above equations as eqn. (14).

$$R_{inh} = d[LOOH]/dt = k_p\, R_i\, [LH]/(n\, k_{inh}\, [AH]) \quad (14)$$

The kinetic chain length (kcl) defines the number of chain propagations initiated by each initiating radical and is given by eqns. (15) and (16) for inhibited and uninhibited peroxidation

$$kcl_{inh} = R_{inh}/R_i \quad (15)$$

$$kcl_p = R_p/R_i \quad (16)$$

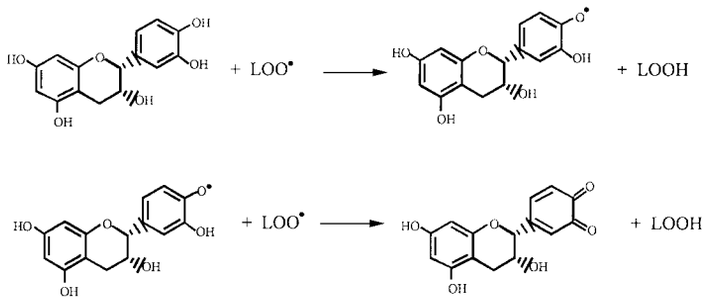
respectively. The kinetic parameters deduced from Figs. 1–4 are listed in Tables 1 and 2.

It can be seen from Figs. 1–4 and Tables 1 and 2 that the reaction medium exerts significant effects on the rate of initiation, and on the antioxidant activity of α-tocopherol (TOH) and green tea polyphenols (GOH).

The R_i values calculated from the inhibition period [eqn. (12)] are 3.1 and 8.3 nmol dm^{-3} s^{-1} in SDS and CTAB micelles respectively, which are in good agreement with the values of 3.0 and 8.2 nmol dm^{-3} s^{-1} respectively, obtained from the decay of TOH [eqn. (13)]. Taking the concentration of AMPAD as 6.3 mmol dm^{-3} the R_i value in CTAB micelles corresponds to 1.3×10^{-6} [AMPAD] s^{-1}, in good agreement with the previously reported value in liposomal dispersions.[26] However, the R_i value of AMPAD in SDS micelles is substantially smaller than that in CTAB micelles. This is understood because AMPAD is positively charged, hence it is prone to adsorb on the surface of SDS micelles which, in turn, would reduce the effective initiation due to the cage effect [eqn. (1)]. On the other hand, the inhibition rate constant, k_{inh}, of α-tocopherol (TOH) is higher in SDS than in CTAB micelles. This is due to the fact that lipid peroxyl radicals are polar (dipole moment of *ca*. 2.6 Debye) and electrophilic,[25] hence they should move to the surface of micelles more quickly in SDS than in CTAB micelles to react with the antioxidant whose reactive phenoxyl functional group resides on the surface of the micelle,[23] according to the floating peroxyl hypothesis proposed by Barclay.[16]

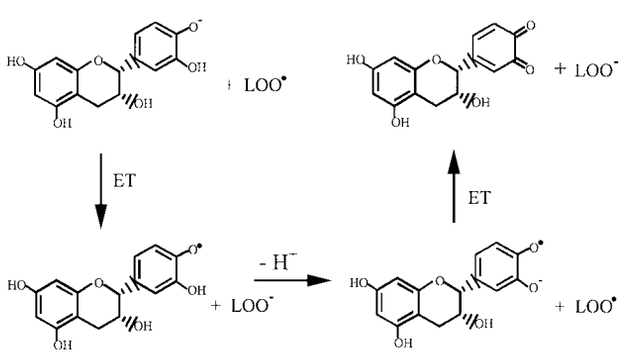
It can be seen from Figs. 1–2 and Table 1 that the green tea polyphenols (GOHs) show clear inhibition periods and behave well as chain-breaking antioxidants in both SDS and CTAB micelles. The antioxidant activity of GOHs towards peroxyl radicals is obviously due to their *ortho*-dihydroxy structure in the B and/or C rings that makes the oxidation intermediate, *ortho*-semiquinone, fairly stable and easily further oxidized to form the final product *ortho*-quinone (*e.g.*, Scheme 1).

It is worth noting that the antioxidation behaviour of GOH in micelles is different from the behaviour in homogeneous



Scheme 1 Antioxidation reactions of GOH.

solutions where no inhibition period could be observed.[13] Jovanovic *et al.*[27] have studied in detail the oxidation of GOHs in aqueous solutions by pulse radiolysis and laser photolysis. It was concluded that subsequent to the one electron oxidation of the polyphenols the resorcinol phenoxyl (at the A ring) transforms to the gallate phenoxyl (at the B and/or C rings) or the catechol phenoxyl (at the B ring) *via* fast inter- and/or intramolecular electron transfer. Since the phenoxyl radicals are considerably acidic (the pK_a of the phenoxyl radicals of ECG, EGCG, EC, GA and EGC are 4.3, 4.4, 4.6, 5.0 and 5.5 respectively),[27] the neutral radicals are prone to lose a proton to form the corresponding phenoxyl radical anions as relatively stable intermediates. In addition, GOH may partially dissociate in neutral media to form the corresponding phenoxide (*e.g.*, the pK_{a1}s of EGCG, EC and GA are 7.75, 8.64 and 8.73 respectively).[27] It is well-known that the phenoxide of GOH more easily undergoes electron transfer oxidation producing the radical anion of GOH which is electron paramagnetic resonance (EPR) detectable in alkaline solutions.[28] Therefore, the electron transfer antioxidation of GOH (Scheme 2) might take



Scheme 2 Antioxidation reactions of GOH *via* electron transfer.

place simultaneously with the direct hydrogen abstraction (Scheme 1). Obviously, the relative contributions of these two processes (hydrogen abstraction and electron transfer) depend on the reaction medium, the pH of the solution, and the oxidation potential and the acid dissociation constant (pK_a) of GOH. Our previous results showed that GOH could only trap the initiating radicals in homogeneous solutions,[13] while the present results reveal that GOH could also effectively trap the lipid peroxyl radicals in micelles. This suggests that GOH may bind to the Stern layer of micelles by hydrogen bonding interactions, hence facilitating their dissociation and electron transfer antioxidation towards the lipid peroxyl radical which also resides in the surface of the micelle.

The antioxidant potential of GOH can be expressed by comparing either the inhibition rate constant, k_{inh}, or the inhibition time, t_{inh}. It is interesting to note from Table 1 that although the inhibition rate constant, k_{inh}, of GOH in CTAB micelles is smaller than in SDS micelles, the inhibition time, t_{inh}, and the stoichiometric factor, n, are larger in CTAB than in SDS micelles. Assuming each α-tocopherol traps two peroxyl radicals[16,25] [eqns. (9) and (10)], the n values of GOH are calculated and listed in Table 1. It can be seen that each GOH molecule can trap less than two peroxyl radicals in SDS micelles as expected, while it can trap up to seven peroxyl radicals in CTAB micelles. This implies that in CTAB micelles the phenoxyl groups at both B and C rings are involved in the antioxidative action, because the positively charged micellar surface of CTAB can tightly hold the phenoxide of GOH allowing repeated attacks of the lipid peroxyl radicals migrating from the inner core of the micelle. Jovanovic *et al.*[27] have reported that EGCG exhibited a second pK_a of 5.5, revealing the presence of the two radicals, probably at both the gallate moieties. Therefore, EGCG and ECG are the most effective GOHs. In addition, it has been reported that the oxidation products of GOH, *e.g.*, theaflavins, are also antioxidative,[29,30] hence after the inhibition period the rate of peroxide formation is smaller than the intrinsic propagation rate, and the n value of EGCG and ECG is even larger than four in CTAB micelles. Indeed, after complete consumption of EGCG in CTAB micelles (*ca.* 5×10^3 s, Fig. 6) the peroxidation was still inhibited ($t_{inh} = 8.8 \times 10^3$ s, Fig. 2 and Table 1). The lower inhibition rate in CTAB micelles is understood, since the positively charged micellar surface would retard the electrophilic peroxyl radicals migrating from the inner core to react with the GOH located at the surface of the micelle.[16]

It is hard to predict precisely the relative antioxidative activity sequence of GOHs in micelles because the micellar microenvironment would appreciably influence the oxidation potential, acid dissociation constant and the diffusion rate of the molecule which bound to the micellar surface. In addition, the stability of the phenoxyl radical anion intermediate and the antioxidative activity of the primary oxidation products of GOH are also influenced by the micelle and play an important role in the activity. Generally, GOHs with two gallate moieties, *i.e.*, EGCG and ECG which possess the lowest oxidation peak potentials (0.23 and 0.27 V *vs.* SCE respectively) and two active moieties, are most active, while GA and EC, which possess the highest oxidation peak potential (0.42 and 0.33 V *vs.* SCE respectively), are least active on the basis of the inhibition period. This activity sequence is generally in accordance with the activity sequence of GOHs with lipid peroxyl radicals in homogeneous solutions[13] and in low density lipoprotein,[5] and with superoxide radical anion in homogeneous solutions.[27] On the other hand, if one compares the activity on the basis of rate of inhibition the GOH with the smaller size possesses the higher activity, especially in CTAB micelles, implying that diffusion of GOH on the micellar surface and diffusion of peroxyl radicals from the interior to the surface of the micelle might be the predominant factor that controls the rate, as reported previously.[31]

Synergistic and/or additive antioxidant effect of green tea polyphenols with α-tocopherol

It can be seen from Figs. 3 and 4 and Table 2 that addition of the green tea polyphenol (GOH) together with α-tocopherol

(TOH) significantly increases the inhibition period of the latter, especially EGCG in SDS micelles. The t_{inh} of TOH and EGCG when they were used together in SDS micelles was 75% longer than the sum of the t_{inh}s when the two antioxidants were used individually, as expressed by the synergistic efficiency SE% [eqn. (17)],[32] demonstrating clearly the antioxidant synergism of EGCG with TOH.

$$SE = \{t_{inh}(TOH + GOH) - [t_{inh}(TOH) + t_{inh}(GOH)]\}/[t_{inh}(TOH) + t_{inh}(GOH)] \times 100 \quad (17)$$

It can been seen from Fig. 5 that TOH decayed linearly with time in SDS micelles within two half-life times in the absence of EGCG, indicating a typical antioxidant decay as defined by eqn. (13). However, the decay was remarkably suppressed by the coexistent EGCG until most of the EGCG was consumed. These results are similar to the kinetic behaviour observed in their homogeneous solutions,[13] and in the regeneration of TOH by L-ascorbic acid,[33] and hence strongly suggest that EGCG can reduce α-tocopheroxyl radical (TO$^{\bullet}$) to regenerate the parent molecule [eqn. (18)]. The oxidation potentials of EGCG

$$TO^{\bullet} + GOH \longrightarrow TOH + GO^{\bullet} \quad (18)$$

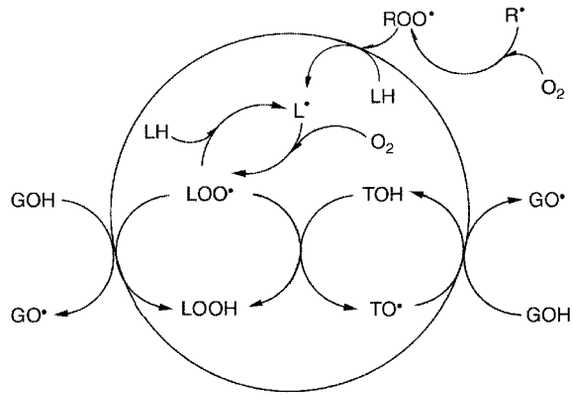
and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water soluble analogue of TOH) are 0.43 and 0.48 V *vs*. NHE respectively,[27] making this TOH regeneration reaction a preferable reaction.

This TOH regeneration reaction could be expressed alternatively as an electron transfer reaction [eqn. (19)] as discussed

$$TO^{\bullet} + GOH \rightleftharpoons TO^{-} + GOH^{\bullet +} \quad (19)$$

previously,[13,28] hence the relative contribution of the forward and backward reactions should depend on the oxidation potential of TOH and GOH. As a matter of fact, GA exhibits a negative synergistic effect since its oxidation potential is *ca*. 0.15 V higher than that of TOH which makes its phenoxyl radical prefer to oxidize TOH rather than to reduce TO$^{\bullet}$. The synergistic effects of ECG, EGC and EC are quite small since their oxidation potential is close to that of TOH. The reason for the very small synergistic effect in CTAB micelles is not clear, probably the reaction of GOH with peroxyl radicals (Scheme 1) competes with the TOH regeneration reaction [eqns. (18) and (19)], hence obscures the latter if the former reaction is fast enough. Indeed, the peroxidation was totally inhibited in CTAB micelles when GOH and TOH were used together (Fig. 4), implying an infinitely large k_{inh}. In addition, EGCG decays much faster in CTAB than in SDS micelles, and after its complete consumption the decay of α-tocopherol is still significantly suppressed (Fig. 6), suggesting that the oxidation products of EGCG are much more antioxidative in CTAB than in SDS micelles. Therefore, although a short period of completely suppressed decay of TOH seems appreciable in the very beginning of the reaction (Fig. 6), the synergistic effect is not obvious.

In conclusion, this work demonstrates that the principal components of green tea polyphenols (GOH), *i.e*., EGCG, EGC, ECG, EC and GA, are effective antioxidants against linoleic acid peroxidation in SDS and CTAB micelles, especially in the latter medium. The antioxidative action may involve trapping the initiating radicals (ROO$^{\bullet}$) in the bulk water phase, trapping the propagating peroxyl radicals (LOO$^{\bullet}$) on the surface of the micelle, and regenerating α-tocopherol (TOH) by reducing α-tocopheroxyl radical (TO$^{\bullet}$), as illustrated in Scheme 3. The relative contributions of these processes depend significantly on the microenvironment of the reaction medium, as well as the oxidation potential and the size of the green tea polyphenols.



Scheme 3 The antioxidation mechanism of GOH in micelles.

Experimental

Materials

(−)-Epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG) and gallic acid (GA) were isolated from green tea leaves by extraction with methanol, water and ethyl acetate consecutively and chromatographic separation on a Sephadex LH-20 column, with reference to procedures reported previously.[34,35] Their structures and purity were confirmed by ^{1}H and ^{13}C NMR spectra and HPLC, respectively, as reported previously.[36] Linoleic acid (Sigma, Chromatographic pure), *dl*-α-tocopherol (Merck, Biochemical reagent, >99.9%) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPAD) were used as received and kept under nitrogen in a refrigerator before use. The surfactants SDS and CTAB were recrystallized from ethyl alcohol and acetone–water (9 : 1 v/v) respectively.

Determination of linoleic acid hydroperoxides

Aliquots of the reaction mixture in an open vessel were taken out at appropriate time intervals and subjected to high performance liquid chromatography (HPLC) analysis using a Gilson liquid chromatograph with a ZORBAX ODS reversed phase column (6 × 250 mm, Du Pont Instruments) and eluted with methanol–propan-2-ol (3 : 1 v/v). The flow rate was set at 1.0 ml min^{-1}. A Gilson model 116 UV detector was used to monitor the total linoleic acid hydroperoxides at 234 nm.[22] Every experiment was repeated at least three times to ensure the experimental deviation within ±10%.

Determination of α-tocopherol and EGCG

The procedure was the same as described above for analysis of linoleic acid hydroperoxides, except that a Gilson model 142 electrochemical detector set at +700 mV *vs*. SCE was used for simultaneous monitoring of both TOH and EGCG. The column was eluted with methanol–propan-2-ol–formic acid (80 : 20 : 1, v/v/v) containing 50 mmol dm^{-3} of sodium perchlorate as a supporting electrolyte.

Determination of oxidation potential

The oxidation potentials of GOH were determined with a PAR model 173 potentiostat using a glassy carbon electrode in phosphate buffered solution at pH 6.8 as described previously.[37] The potential was recorded relative to a saturated calomel electrode (SCE) reference electrode.

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