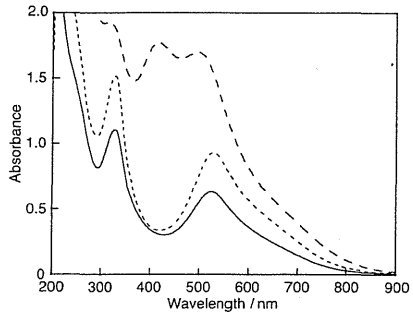

Fig. 1 UV-vis spectra of extracts of the mixture of DPPH• (0.23 mmol) and β-CD (0.45 mmol) in 15 mL of boiling phosphate buffer (0.1 M, pH 7.4) (—), acetate buffer (50 mM, pH 4.4) (- - -) and borate buffer (14 mM, pH 9.1) (– – –).

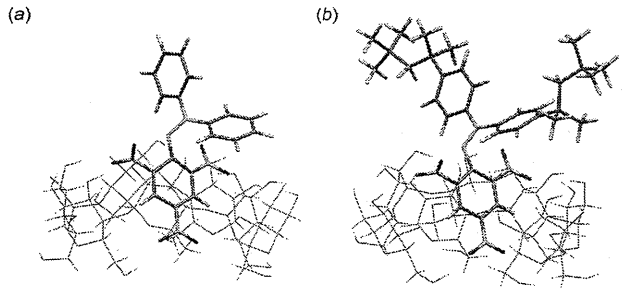

Fig. 2 Optimised structures of the inclusion complex of (a) DPPH• with β-CD and (b) DOPPH• with β-CD calculated by DFT (UB3LYP/3-21G:C-PCM solvation model parameterised for water).

DPPH• was not solubilised in water. The 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl radical (DOPPH•) could not be solubilised in water by β-CD in the same manner, either. Fig. 2a shows an optimised structure of the inclusion complex of DPPH• with β-CD calculated by the density functional theory (DFT) (UB3LYP/3-21G:C-PCM solvation model parameterised for water) (see the ESI†). The picryl moiety of DPPH• is incorporated into the hydrophobic cavity of β-CD. DOPPH• is also incorporated into β-CD as shown in Fig. 2b. The calculated association energy between DPPH• and β-CD (−31 kcal mol^{-1}) by DFT is significantly less negative than that between DOPPH• and β-CD (−44 kcal mol^{-1}). These theoretical results suggest that DOPPH• may not be solubilised by β-CD due to the hydrophobic *tert*-octyl groups. DPPH• solubilised by β-CD in water or the phosphate buffer solution (0.1 M, pH 7.4) is stable at least for several days at room temperature. When a boiling acetate buffer solution (50 mM, pH 4.4) was used instead of the phosphate buffer, DPPH• could also be solubilised by β-CD (Fig. 1). On the other hand, a brown solution with absorption bands at 416 and 505 nm was obtained using the boiling borate buffer solution (14 mM, pH 9.1) as shown in Fig. 1. This suggests that DPPH• is unstable under basic conditions as reported previously.[9] In fact, addition of 0.75 mL of a borate buffer solution (0.1 M, pH 9.1) to DPPH•/β-CD in water (Milli-Q) (2.3 mL) resulted in a gradual decrease in the absorption band at 527 nm, accompanied by an increase in the band at 412 nm with clear isosbestic points at 252, 304, 346 and 491 nm as shown in Fig. 3. The one-electron reduced DPPH• ($DPPH^-$) is reported to have an

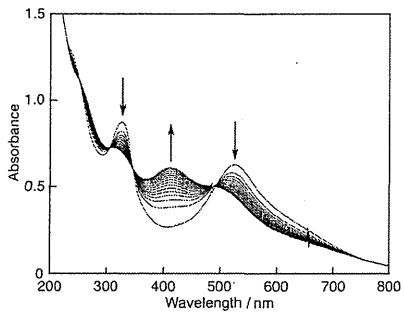

Fig. 3 Spectral change observed for DPPH•/β-CD (5.8 × 10^{-5} M) in borate buffer (25 mM, pH 9.1) at 298 K. Interval: 20 min.

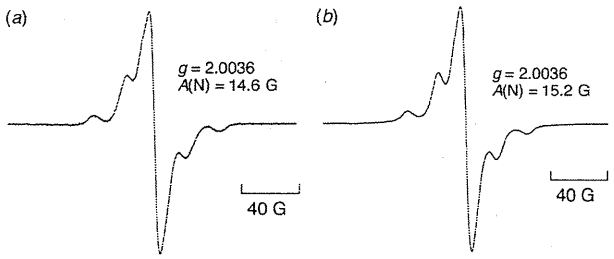

Fig. 4 EPR spectra of (a) DPPH•/β-CD (1.0 × 10^{-5} M) in distilled water at 77 K and (b) DPPH• (1.0 × 10^{-5} M) in MeOH at 77 K.

absorption band at 426 nm in a 1:1 ethanol–buffer solution,[9] suggesting that $DPPH^-$ may be included in the products. However, the detailed reaction mechanism of DPPH• under basic conditions is under investigation and will be reported elsewhere.

The EPR spectrum of DPPH•/β-CD observed in water at room temperature has the same *g* value (2.0036) and hyperfine coupling constant (7.8 G) as those of DPPH• in MeOH (2.0036 and 7.9 G, respectively) (see the Fig. S1, ESI†). A slightly small hyperfine coupling constant (14.6 G) was observed in water as compared to that in MeOH (15.2 G) for the EPR spectra recorded at 77 K (Fig. 4).

When ascorbic acid ($AscH_2$) was added to the phosphate buffer solution (0.1 M, pH 7.4) of DPPH•/β-CD, the band at 527 nm disappeared immediately with clear isosbestic points at 320, 338 and 431 nm as shown in Fig. 5a. Since the pK_a value of $AscH_2$ is reported to be 4.1,[12] $AscH_2$ undergoes deprotonation and exists in its anionic form, $AscH^-$, in phosphate buffer solution (0.1 M, pH 7.4). Thus, this spectral change indicates that $AscH^-$ efficiently scavenged DPPH• in phosphate buffer [eqn (1)]. When $AscH^-$ was replaced by Trolox, a water-soluble analogue of α-tocopherol (vitamin E), a similar spectral change was observed due to the scavenging reaction of DPPH• by Trolox [Fig. 5b and eqn (2)].[13]

$$DPPH^•/\beta\text{-CD} + AscH^- \rightarrow DPPH_2/\beta\text{-CD} + Asc^{•-} \quad (1)$$

$$DPPH^•/\beta\text{-CD} + \text{Trolox} \rightarrow DPPH_2/\beta\text{-CD} + \text{Trolox}(-H)^• \quad (2)$$

Spectral titrations (insets of Fig. 3a and b) show the same stoichiometry with both antioxidants, the DPPH•/antioxidant molar ratio being 2:1.[7] The decay of the absorbance at 527 nm monitored by a stopped-flow technique obeyed pseudo-first-order kinetics,

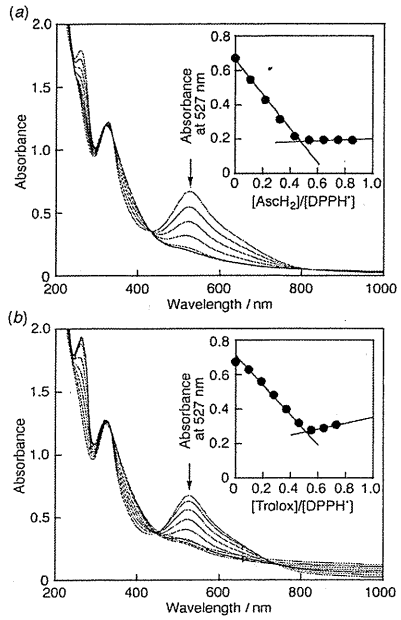

Fig. 5 Spectral changes observed upon addition of (a) $AscH_2$ ($0–5.4 \times 10^{-5}$ M) or (b) Trolox ($0–4.6 \times 10^{-5}$ M) to DPPH•/β-CD (6.3×10^{-5} M) in phosphate buffer (0.1 M, pH 7.4). Insets: plots of the absorbance at 527 nm *vs.* (a) $[AscH_2]/[DPPH^•]$ and (b) $[Trolox]/[DPPH^•]$.

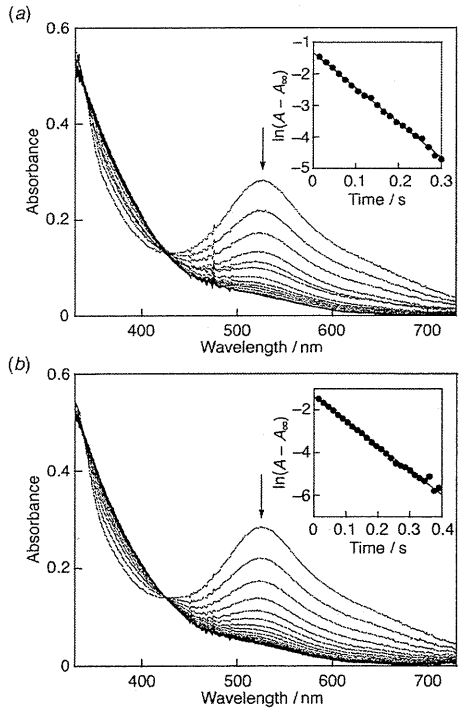

Fig. 6 Spectral changes (interval: 25 ms) observed during reactions of (a) $AscH_2$ (1.4×10^{-3} M) with DPPH•/β-CD (2.6×10^{-5} M) and (b) Trolox (6.3×10^{-4} M) with DPPH•/β-CD (3.0×10^{-5} M) in phosphate buffer (0.1 M, pH 7.4) at 298 K. Insets: the first-order plots of the absorbance at 527 nm.

when the concentration of $AscH_2$ ($[AscH_2]$) was maintained at more than a 10-fold excess of DPPH•/β-CD concentration (Fig. 6a).

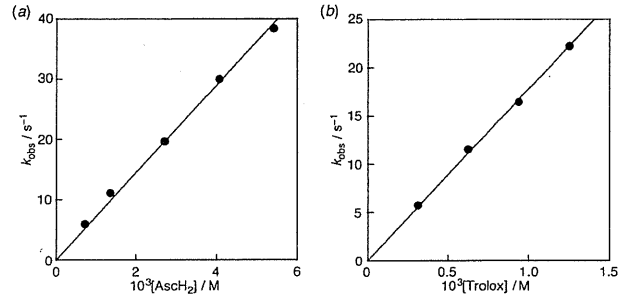

Fig. 7 Plots of k_{obs} *vs.* (a) $[AscH_2]$ and (b) [Trolox].

The pseudo-first-order rate constant (k_{obs}) linearly increased with increasing $[AscH_2]$ (Fig. 7a). From the slope of the linear plot the second-order rate constant (k) for the scavenging of DPPH•/β-CD by $AscH_2$ was determined in a phosphate buffer (0.1 M, pH 7.4) to be 7.2×10^3 M^{-1} s^{-1}. The k value for Trolox was also determined in the same manner to be 1.8×10^4 M^{-1} s^{-1} (Fig. 6b and 7b), which is exactly the same as that measured in a 1:1 ethanol–buffer (pH 7.4) solution.[9,14] Thus, β-CD does not inhibit the reaction of DPPH• with the antioxidants, while the stability and reactivity of nitroxyl radicals were reported to be significantly changed by the complexation with cyclodextrins.[15]

In summary, β-CD-solubilised DPPH• in water has been demonstrated to be a powerful tool to evaluate the antioxidative activity of antioxidants in aqueous media, especially in highly concentrated buffer solutions without precipitation of buffer salts.

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