Electrochemical behaviour of hypocrellin A in acetonitrile and aqueous micellar media

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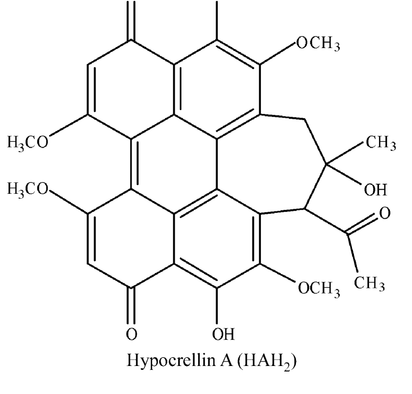
The electrochemical behaviour of hypocrellin A (HAH_2) has been studied in acetonitrile and in aqueous micellar media. In acetonitrile (CH_3CN) the two successive one-electron transfers *are* reversible on the cyclic voltammetry time scale and allow different reduced forms of HAH_2 to be produced by exhaustive electrolysis. In addition, the one-electron reduction of HAH_2 has been studied by UV-visible and EPR spectroscopy, showing that the one-electron transfer is associated with a protonation phenomenon leading to the formation of $HAH_2^{\bullet -}$ and $HAH_3^{\bullet}$. In an aqueous micellar medium the first reduction step behaves similarly to that in an organic medium and has allowed us to establish the influence of the pH value on $E_{1/2}$ and to determine the two pK_a values of HAH_2, while pK_a values of the one-electron reduced forms of HAH_2 have been evaluated.

Hypocrellin A (4,9-dihydroxyperylene-3,10-quinone), which can be extracted from *Hypocrella bambuase*, a parasitic fungus of *Siramudinaria*,[1,2] is structurally related to perihydroxylated polycyclic quinones, such as hypericin, and displays antitumoral and antiviral activities against several types of viruses, including human immunodeficiency virus.[3,4] This natural photosensitising agent has been traditionally used in China in a variety of medicinal treatments.[2] Moreover, Cheng and Wang[5] have demonstrated that, as for hypericin,[3] these biological properties are activated by photochemical irradiation but, unlike hypericin, hypocrellin A absolutely requires molecular oxygen for its antiviral activity.[6] The mechanism is not well understood.[7,8] Meanwhile, it has been shown that photoexcitation of HAH_2 can lead, in aerobic conditions, to the formation of radical species such as superoxide anion $O_2^{\bullet -}$ as shown by EPR titration.[9] Because of the very low solubility of HAH_2 in aqueous media, these studies have been performed in hydroorganic solutions: either H_2O–DMF[10] or H_2O–DMSO mixtures containing microsomes in order to reach suitable photosensitiser concentrations.[9] Hu *et al.*[10] have demonstrated that the production of the superoxide radical anion, through irradiation of HAH_2 solution, was strongly dependent on the pH of the solution. This is not surprising since at pH = 6 no reduction of molecular oxygen is displayed whereas this reduction process is detected at pH = 10.



Hypocrellin A (HAH_2)

In this photoredox context, an important point deals with the determination of the redox potential of HAH_2 in the ground state and the influence of the pH on its electrochemical behaviour. Although several studies have reported the formation of the one-electron reduced form of HAH_2 by photochemical means,[7–10] no electrochemical study has yet been described.

In this paper, we present the first electrochemical study of HAH_2, combined with spectrophotometry, in both organic and aqueous media. In an organic medium, its electrochemical behaviour has been investigated in order to estimate the stability of the reduced forms of HAH_2. In an aqueous medium, because of the very low solubility of HAH_2, Brij 35 miscelles have been used as a solubilising agent to obtain 0.1 mmol HAH_2 solutions and to reproduce *in vivo* conditions. Under these conditions, we have examined the influence of the pH value on the first one-electron reduction of HAH_2 in order to estimate the protonic behaviour of the reduced species of HAH_2. This study was also concerned with the characterisation of these reduced forms, obtained in both organic and aqueous media, by EPR and UV-visible spectroscopy. These results have been compared to those of hypericin[11] in order to explain the different efficiency of these two photosensitising agents in the production of superoxide anion.

Experimental

Cyclic voltammograms (CV) were recorded using a potentiostat EGG 273 or a polarographic analyser EGG 264 A for experiments performed in CH_3CN or aqueous electrolytes, respectively. For studies in aqueous media adsorption phenomena, which are favoured on a mercury drop electrode, are likely to be involved in the better resolution of the CV compared to results obtained on solid electrodes. In CH_3CN electrolyte, CV were performed using a platinum or vitreous carbon disc (5 mm diameter) as the working electrode. An Ag/10 mM $Ag^+ + CH_3CN + 0.1$ M tetra-*n*-butylammonium perchlorate (TBAP) electrode and an Ag/AgCl aqueous satu-

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rated KCl electrode were used as reference electrodes in CH_3CN and aqueous electrolyte, respectively. In order to compare the results in organic and aqueous media, potentials have been converted to the NHE scale using Ag/Ag^+ 10 mM + CH_3CN + 0.1 M TBAP = 0.55 V *vs.* NHE and Ag/AgCl = 0.20 V *vs.* NHE.[12] Dry TBAP, used as the supporting electrolyte in CH_3CN medium, was purchased from Aldrich, recrystallised from ethyl acetate–cyclohexane and dried under vacuum at 80 °C for 3 days. Commercial phosphate salts (Aldrich) were used without any further purification for buffered aqueous solutions between pH = 3 and 12. CH_3CN (Rathburn) was used as received, nitrogen flushed and the experiments were carried out under an argon atmosphere in a dry glove box.

UV-visible spectra were recorded on a HP 8456 spectrophotometer for experiments performed in CH_3CN solution and a Perkin Elmer apparatus in aqueous media. EPR spectra were recorded with a Brucker ESP 300 E spectrometer at room temperature (22–24 °C) with the following parameters: microwave 1 mW, modulation amplitude 0.197 G, time constant 327.68 ms, scan rate 1342 s, scan width 8 G, X-band, modulation frequency 100 kHz for experiments carried out in acetonitrile. In aqueous media the same parameters were applied except for the scan rate and the scan width, which were equal to 671 s and 13 G, respectively.

Brij 35 (Merck) micellar solutions were prepared following the injection method previously described.[13] An aqueous solution of Brij 35 (0.1 M) containing hypocrellin A (0.2 mM) was injected in a buffered aqueous phosphate solution at different pH values (concentration of the phosphate salt = 0.2 M). The final Brij 35 concentration in aqueous solution was 50 mM. The hypocrellin A concentration in aqueous micellar solution was determined by UV-visible titration. By means of the absorption at $\lambda = 464$ nm ($\varepsilon = 23\,000$ M^{-1} cm^{-1} at pH = 7), one can establish that 0.1 mmol HAH_2 concentration has been obtained. The electrolyses in the micellar medium have been performed using a vitreous carbon plate as the working electrode. It should be noted that, in these conditions, the strong background current prevents a precise determination of the coulombic yield for the one-electron reduction of hypocrellin A.

Results and discussion

Electrochemical behaviour in acetonitrile medium

As for hypericin,[11] HAH_2 is reduced following two successive one-electron transfer steps. The CV on carbon vitreous electrode in CH_3CN + 0.1 M TBAP exhibits in the cathodic region two well-defined reversible systems at −0.37 and −0.63 V/NHE ($\Delta E_p = 60$ and 65 mV, respectively), whereas in the anodic region an irreversible system ($E_{p_a} = 1.66$ V) is observed (Fig. 1, scan A). The one-electron reduction of HAH_2 is confirmed by exhaustive electrolysis at −0.44 V on a vitreous carbon plate electrode, which consumes close to 1 F mol^{-1}. During the reduction the intense red colour of HAH_2 is displaced by the formation of a green reduced species ($\lambda = 638$ nm, $\varepsilon = 13\,300$ M^{-1} cm^{-1}) (Fig. 2). The CV of the resulting reduced solution shows, beside the expected oxidation of $HAH_2^{\cdot -}$ ($E_{p_a} = -0.34$ V), a second irreversible one at more positive potential ($E_{p_a} = 0.59$ V, scan B in Fig. 1). This indicates that the electrochemical reduction of HAH_2 is associated to a chemical reaction leading to the formation of two one-electron reduced forms of HAH_2. It should be noted that this coupling reaction is reversible as demonstrated by the total restitution of the starting HAH_2 compound by an exhaustive reoxidation at 0.78 V of the reduced solution. One can suggest that proton transfer (by the residual water present in the electrolyte) could correspond to this coupling reaction.

The EPR spectrum (Fig. 3) of this reduced solution confirms the presence of two radical species since a careful analysis demonstrated that this spectrum results from the overlap of two sets of signals whose intensity ratio is close to unity and which correspond to $HAH_2^{\cdot -}$ and $HAH_3^{\cdot}$. This molar ratio can be modified by addition of perchloric acid to the medium. In the presence of one molar equivalent of acid, this molar ratio becomes 1 : 4 to give $HAH_3^{\cdot}$ as the major product. However, attempts to obtain $HAH_2^{\cdot -}$ as the sole product by the electrochemical reduction of HAH_2 solution in the presence of base (*i.e.*, by addition of collidine or butyllithium–THF solution) the electrolyte were unsuccessful and led to EPR-silent reduced solutions. The comparison of EPR spectra obtained in the acidic and neutral conditions allows us to *deduce* the shape of the EPR spectra of $HAH_2^{\cdot -}$ and

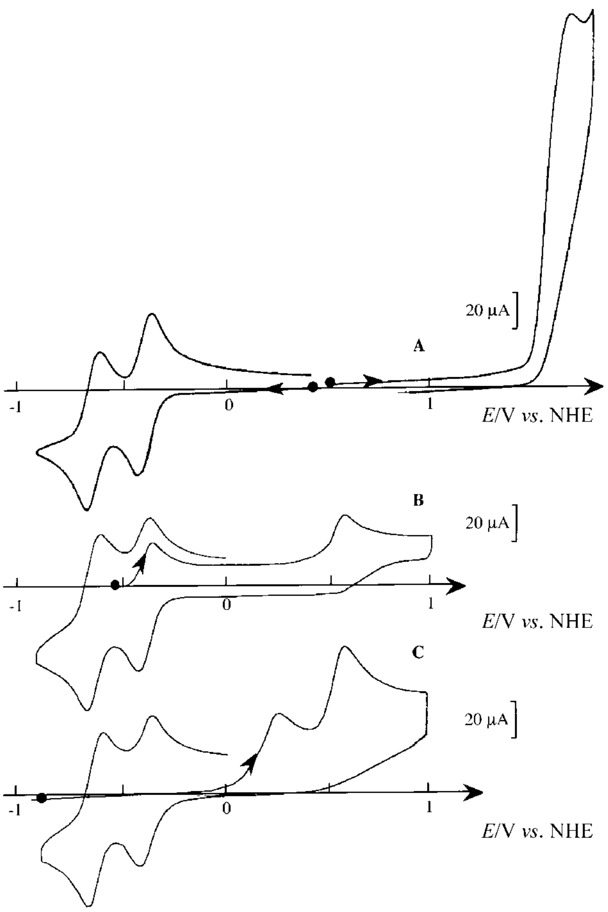

Fig. 1 (A) Cyclic voltammogram on a carbon vitreous electrode (diameter 5 mm) of hypocrellin A (10^{-4} M) in CH_3CN + 0.1 M TBAP, scan rate 100 mV s^{-1}. (B) After exhaustive electrolysis at −0.44 V/NHE. (C) After exhaustive electrolysis at −0.70 V/NHE.

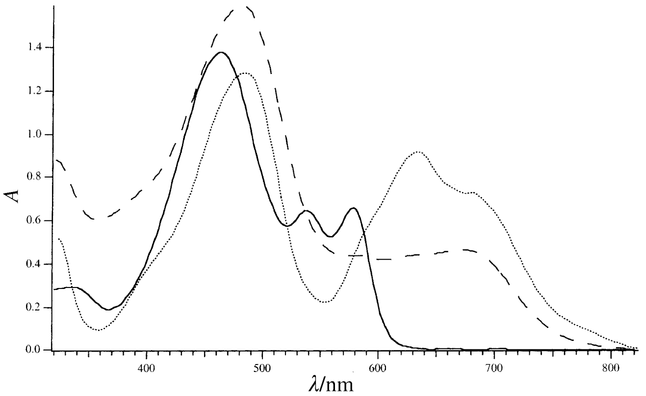

Fig. 2 Absorption spectrum before reduction (——) and after one- (···) and two-electron (– – –) reduction at −0.44 and −0.70 V/NHE, respectively.

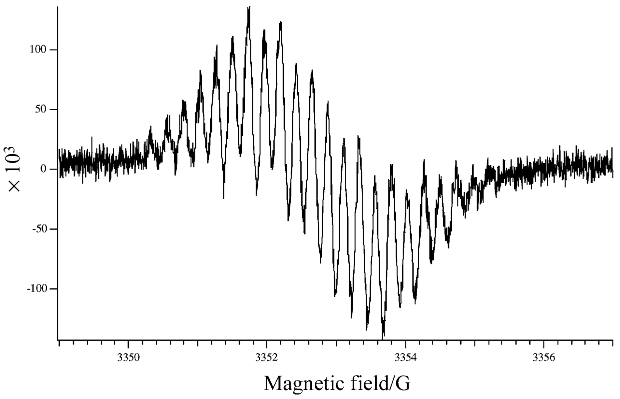

Fig. 3 EPR spectrum of the one-electron reduced species of hypocrellin A in CH_3CN + 0.1 M TBAP.

$HAH_3^{\cdot}$. It is expected from the structure of HAH_2 that the hyperfine EPR spectra of its reduced species will result from the coupling interaction of the unpaired electron with four (or less) different kinds of proton spins: aromatic, phenolic hydroxyl, methylene and methoxy group protons. The protons of the side chains should be less involved in the splitting of the EPR spectra to a significant extent as already reported.[14] Spectral simulations were conducted in order to produce the best fit to the experimental spectra according to the parameters listed in Table 1; the simulated spectra are represented in Fig. 4. The larger splitting constants were attributed to the phenolic hydroxyl protons and are of the same order magnitude of these determined for a similar compound, hypocrellin B.[15]

This study has been extended to the second one-electron reduction process. A potentiostatic electrolysis at −0.70 V consumes 2 electrons per mol of HAH_2 and leads to a green solution (λ = 672 nm, ε = 6800 M^{-1} cm^{-1}) (Fig. 2). The CV of the resulting solution now shows two irreversible anodic peaks at 0.26 and 0.60 V whereas, on a further cathodic scan the typical $HAH_2/HAH_2^{\cdot -}$ and $HAH_2^{\cdot -}/HAH_2^{2-}$ systems are almost entirely restored (Fig. 1, scan C). The initial HAH_2 solution can be retrieved by exhaustive electrolysis at 0.80 V, also consuming two electrons per mol. As for the one-electron reduction, one can suggest that protonation/deprotonation steps should be taken into account in the overall electrochemical process, considering that the two-electron reduced HAH_2^{2-} is a strongly basic species.

Although protonation steps are coupled to the electron transfer, leading to protonated forms of both $HAH_2^{\cdot -}$ and HAH_2^{2-}, the reversibility of the overall reduction processes allows the quasi total restitution of the starting solution by reoxidation, indicating that the one- and two-electron reduced forms of HAH_2 are stable on the time scale of the electrolyses.

Chemical behaviour in aqueous micellar solution

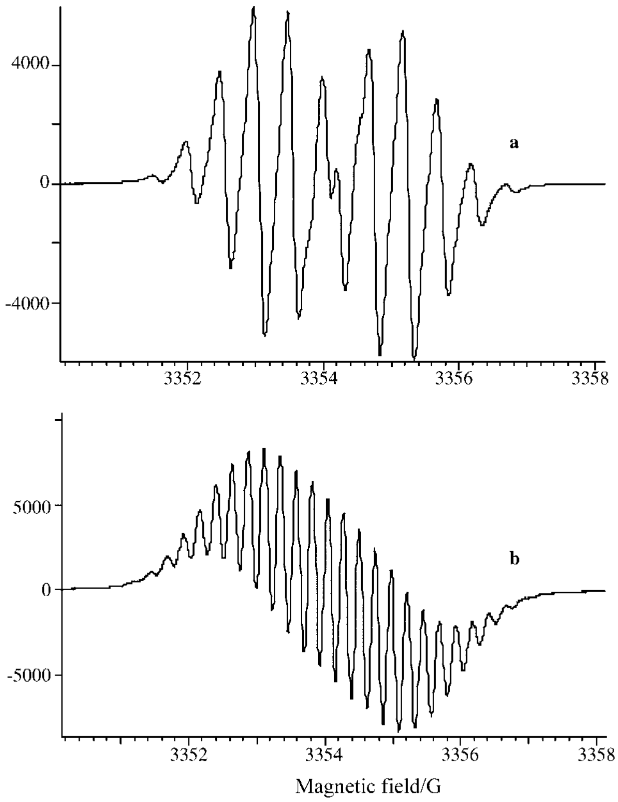

Fig. 4 Simulated EPR spectra of (a) $HAH_2^{\cdot -}$ and (b) $HAH_3^{\cdot}$.

Determination of pK_a values of HAH_2. Since HAH_2 carries two phenolic hydroxy groups, two dissociation processes should be observable through changes in the UV-visible absorption spectrum as the pH of the solution is varied. As expected, two dramatic changes are observed in two pH ranges. For pH values between 7 and 9, the first pK_a value can be determined by the shift of the absorption band from $\lambda = 464$ nm ($\varepsilon = 23\,000$ M^{-1} cm^{-1}) up to $\lambda = 474$ nm ($\varepsilon = 20\,000$ M^{-1} cm^{-1}) concomitantly with the appearance of a new absorption band at λ = 631 nm (ε = 7000 M^{-1} cm^{-1}). As the pH is further increased, the second pK_a value can be estimated from the bathochromic shift of the absorption band at λ = 631 to 637 nm (Fig. 5). Thus, the pK_a values have been determined for eqn. (1) and (2), respectively.

$$HAH_2 \rightleftarrows HAH^- + H^+ \quad pK_{a_1} = 8.2 \qquad (1)$$

$$HAH^- \rightleftarrows HAH^{2-} + H^+ \quad pK_{a_2} = 11.4 \qquad (2)$$

This protonic behaviour can also be confirmed by the evolution of the fluorescence spectra *vs.* pH. Indeed, HAH_2 is well known for its fluorescence properties[8,16] and we demonstrated that this phenomenon is correlated with pH. By photoexcitation of aqueous solutions of HAH_2 at λ = 464 or 542 nm, in acidic conditions up to pH = 4 an intense fluorescence band is

Table 1 Experimental[a] and simulated[b] EPR spectral parameters of one-electron reduced HAH_2 and of the reduced form of HAH_2

		Splitting constant/G			
Species	g factor[a]	Phenolic hydroxy[b]	Aromatic[b]	Methoxy[b]	Linewidth[a] /G
$HAH_2^{\cdot -}$	2.004 73	1.7 (2H)	0.5 (2H)	0.5 (12H)	0.3
$HAH_3^{\cdot}$	2.004 84	1.38 (1H) 1.7 (2H)	0.47 (2H)	0.69 (12H)	0.25

[a] Experimental. [b] Simulated.

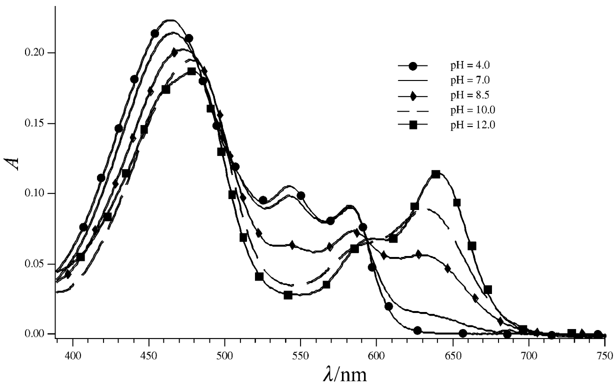

Fig. 5 Absorption spectrum *vs.* pH of hypocrellin A in buffered aqueous micellar solution ($[HAH_2] \approx 10^{-4}$ M).

observed at $\lambda = 598$ nm, which strongly decreases with increasing pH to disappear completely at pH = 10. In more basic conditions, a new emission band is detected at $\lambda = 661$ nm whose intensity increases with pH from 10 to 13 (Fig. 6). Using this method, pK_{a_1} and pK_{a_2} have been determined to be equal to 8.2 and 11.3, respectively. Although a good correlation between these two methods was obtained, the comparison with pK_a values evaluated by Hu *et al.*[10] ($pK_{a_1} = 7.84$ and $pK_{a_2} = 10.29$) shows that the nature of the solvent might have an important influence on the determination of these thermal constants since these authors performed their experiments in aqueous DMF solutions. In addition, we will see below that these two pK_a values are in good accord with those determined by electrochemical experiments.

Electrochemical study in aqueous micellar buffered solutions. In buffered aqueous micellar Brij 35 solutions of HAH_2 (0.1 mM), the shape of the CV depends on the acidic character of the analysed solution (Fig. 7). At low pH, only one reversible system ($\Delta E_p = 0.04$ V) is observed with an $E_{1/2}$ value dependent on the pH. With increasing pH the reduction peak shifts to more negative values from 0.03 V at pH = 4.5 up to −0.11 V at pH = 7.0 (*i.e.*, 56 mV per pH unit). The value at pH = 7.0 is similar to that in the literature[10] but very different from that determined in CH_3CN solution.[14] This specific electrochemical behaviour could be due to the influence of the following parameters.

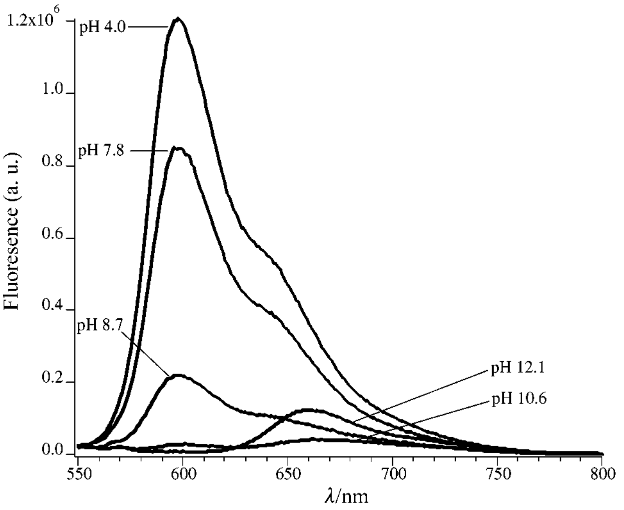

Fig. 6 Emission spectrum *vs.* pH of hypocrellin A in buffered aqueous micellar solution ($[HAH_2] \approx 10^{-4}$ M).

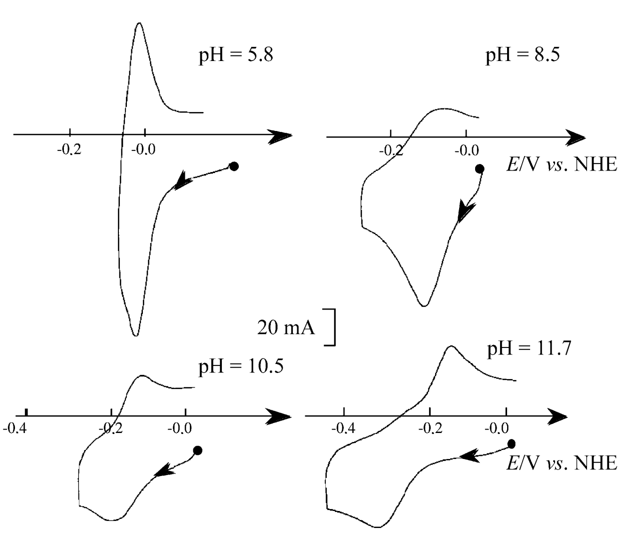

Fig. 7 Cyclic voltammograms on a mercury electrode of 0.1 mM hypocrellin A in buffered aqueous micellar solution + phosphate (0.1 M) at different pH.

(i) The ΔE_p value depends on the nature of the electrode material. Using a vitreous carbon electrode this value increases up to 80 mV but leads to a poorly resolved CV. As for hypericin,[11] some adsorption phenomena are likely to be involved for a better resolution of the CV on the mercury drop electrode. Moreover, the small ΔE_p value, lower than the theoretical value of 60 mV for a one-electron transfer, cannot be attributed to a mechanism involving a disproportionation process as previously invoked in acidic media.[17] Indeed, we will see later that, for pH between 4.5 and 9.5, the electrolyses lead to EPR spectra that attest to the one-electron character of this radical species and its stability. The expected catalysis of the disproportionation process by the proton can be invoked for pH lower than 4.5 since in our conditions the electrolysed solutions are EPR-silent. Special effects of the micellar medium could contribute to an improved stability of the reduced species of HAH_2.

(ii) The discrepancy between these two $E_{1/2}$ values determined in aqueous micellar electrolyte and in acetonitrile medium (−0.11 V/NHE at pH = 7.0 and −0.37 V/NHE, respectively) could be due to a different extent of solvation of HAH_2. One can suggest that the aqueous medium makes homoassociation phenomena easier, leading to the formation of dimers that are, as proposed by Diwu *et al.*,[16] more easily reducible. However, we have not observed such homoassociation phenomena for hypericin, a related compound.[18]

In the pH range between 4.5 and 7.0, the $E_{1/2}$ *vs.* pH variation allows us to establish that a protonation step is coupled to the one-electron transfer, leading to the formation of $HAH_3^{\bullet}$ since the deprotonation of HAH_2 occurs at high pH values:

$$HAH_2 + H^+ + e^- \rightleftarrows HAH_3^{\bullet} \qquad (3)$$

In basic conditions (pH 7.0 to 10.0) the $E_{1/2}$ value remains almost constant with an ΔE_p value close to 55 mV and the deprotonation of HAH_2 is observed. For pH up to 8.2 (the pK_{a_1} of HAH_2) the independence of $E_{1/2}$ *vs.* pH suggests that the redox couple $HAH_2/HAH_2^{\bullet -}$ is involved:

$$HAH_2 + e^- \rightleftarrows HAH_2^{\bullet -} \qquad (4)$$

For pH between 8.2 and 10.0 hypocrellin A is in its deprotonated form HAH^-. In these conditions the absence of $E_{1/2}$ *vs.* pH variation could be due to a redox equilibrium, precluding a proton transfer. Thus, for this pH range, the redox couple $HAH^-/HAH^{\bullet 2-}$ has to be considered:

$$HAH^- + e^- \rightleftarrows HAH^{\bullet 2-} \qquad (5)$$

The electrochemical behaviour of HAH_2 in aqueous micellar medium relative to its one-electron reduction can be illustrated by the $E_{1/2}$ *vs.* pH diagram (Fig. 8), allowing us to assign to the pH values equal to 7.0 and 8.5, the pK_a values for the two successive protonation steps of the reduced form of HAH_2:

$$HAH_3^{\bullet} \rightleftarrows HAH_2^{\bullet -} + H^+ \quad pK_{a_3} = 7.0 \qquad (6)$$

$$HAH_2^{\bullet -} \rightleftarrows HAH^{\bullet 2-} + H^+ \quad pK_{a_4} = 8.5 \qquad (7)$$

For pH values above 10.0, the CV become less resolved leading to an increase of the ΔE_p value up to 200 mV at pH = 11.5 (Fig. 7). This poor resolution does not allow a good determination of the $E_{1/2}$ value of the redox couple involved, consequently the determination of the pK_a values of the acid–base couples HAH^-/HA^{2-} and $HAH^{\bullet 2-}/HA^{\bullet 3-}$ (estimated as 10.4 and 11.7, respectively) becomes less precise.

This $E_{1/2}$ *vs.* pH diagram allows a better understanding of the production of superoxide anion radical through irradia-

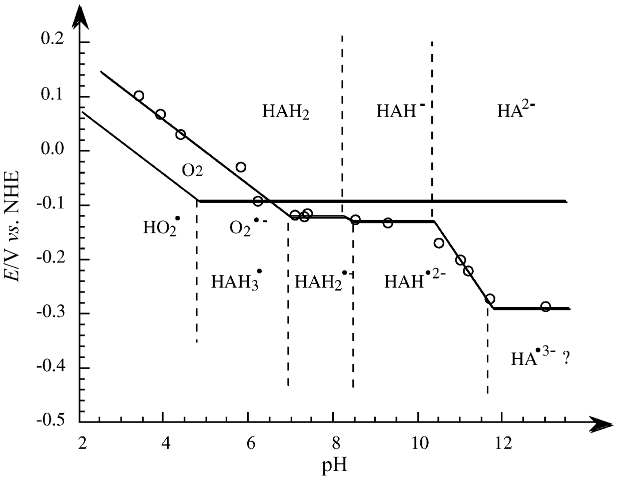

Fig. 8 $E_{1/2}$ *vs.* pH diagram of hypocrellin A in buffered aqueous micellar solution.

tion of HAH_2. The production of $O_2^{\bullet-}$ could be obtained through different mechanisms, involving as a first step the reactivity of the excited state of the photosensitiser (HAH_2^*). For this excited species the triplet state, which has a longer lifetime (14 μs in the micellar media and 4–6 μs in organic solvent[19,20]) than the singlet state (1 ns in micellar media and organic solvent[8]), should be considered. In addition, the quantum yield of formation of the triplet state is considerable (0.83 in micellar media and 0.78 in cyclohexane[19]). Thus, the redox potential of the excited state can be estimated using the Rehm–Weller relation.[21] From the energy of the triplet state (1.77 eV[8]), the $E°$ value of the redox couple $HAH_2^*/HAH_2^{\bullet-}$ has been determined to be 1.65 V/NHE. In this context, four mechanisms can be proposed to explain the photogeneration of superoxide anion.

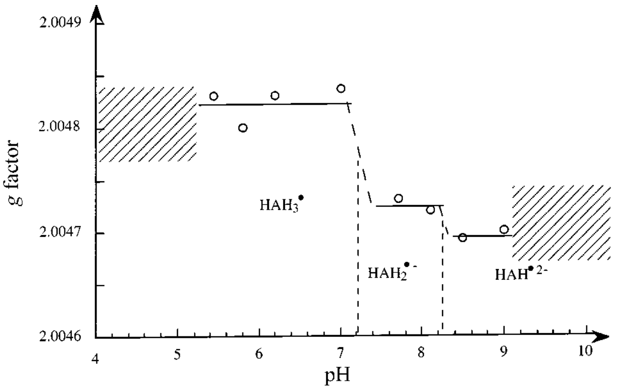

Fig. 9 Variation of the *g* factor of one-electron reduced species of hypocrellin A in buffered aqueous micellar solution.

A (*a*) $HAH_2^* + HAH_2 \rightleftarrows HAH_2^{\bullet-} + HAH_2^{\bullet+}$

(*b*) $HAH_2^{\bullet-} + O_2 \rightleftarrows HAH_2 + O_2^{\bullet-}$

B (*a*) $HAH_2^* + HAH_2^* \rightleftarrows HAH_2^{\bullet-} + HAH_2^{\bullet+}$

(*b*) $HAH_2^{\bullet-} + O_2 \rightleftarrows HAH_2 + O_2^{\bullet-}$

C (*a*) $HAH_2^* + D \rightleftarrows HAH_2^{\bullet-} + D^{\bullet+}$

(*b*) $HAH_2^{\bullet-} + O_2 \rightleftarrows HAH_2 + O_2^{\bullet-}$

D (*a*) $HAH_2^* + O_2 \rightleftarrows HAH_2^{\bullet+} + O_2^{\bullet-}$

Mechanism **A** and particularly reaction *a* is thermodynamically forbidden, whereas for mechanism **B**, reaction *a* requires the meeting of two excited species with a short lifetime. Thus, this reaction is unlikely. This process could be involved in particular conditions such as a high local concentration of sensitiser or for a high photon density.

Mechanism **C** is the most probable. In the presence of a good electron donor with a redox potential lower than 1.65 V/NHE, such as a protein or an amino acid, reaction *a* can be considered. Therefore, reaction *b*, which implies the reduction of the molecular oxygen by the reduced form of HAH_2 can be thermodynamically discussed. Using $E°$ ($O_2/O_2^{\bullet-}$ = −0.09 V and pK_a ($O_2^{\bullet-}/HO_2^{\bullet}$) = 4.8[22] we are able to confirm that the formation of $O_2^{\bullet-}$, by reduction of molecular oxygen by photogenerated reduced HAH_2, is inhibited for pH below 6.4. On the other hand, as described previously[9] in more basic solutions, the production of $O_2^{\bullet-}$ should be very efficient at pH = 10.0.

Concerning mechanism **D**, this process is more complex and seems to be in competition with the formation of singlet oxygen by energy transfer to molecular oxygen.[23]

Table 2 Spectrophotometric data of the different protonated forms of HAH_2 and their one-electron reduced species

pH	Redox couple species	λ_1/nm ε_1/M^{-1} cm^{-1}	λ_2/nm ε_2/M^{-1} cm^{-1}	λ_3/nm ε_3/M^{-1} cm^{-1}	λ(isosbestic)/nm
5.44	HAH_2	464 23 000	543 11 000	584 10 200	428
	$HAH_3^{\bullet}$	426 16 000	494 10 800	581 3260	
7.87	HAH_2	469 21 600	543 10 200	584 9600	582
	$HAH_2^{\bullet-}$	410 5300	628 7700	698 3200	
9.43	HAH^-	474 20 000	588 8200	631 8400	632, 662
	$HAH^{\bullet 2-}$	398 5200	492 7900	698 2600	
12.1	HA^{2-}	479 19 900	589 8160	637 10 800	568
	$HA^{\bullet 3-}$	487 18 900	591 7600	638 9500	

The exhaustive electrolyses of buffered HAH_2 aqueous micellar solutions allowed us to obtain EPR and UV-visible spectra characteristic of the different reduced forms of HAH_2. EPR spectra have been obtained in the pH range between 4.5 and 9.3 and, although the poorly resolved structure of these spectra prevents a successful simulation (*i.e.* the determination of the splitting constants), the evaluation of the g factor is significant (Fig. 9). The EPR-silent solution in basic conditions ($pH > 9.5$), as already observed in CH_3CN medium containing base, could be due to a diamagnetic coupling between two radicals, as previously observed in a basic solvent such as DMSO for the one-electron reduction of hypericin.[11] As expected, between pH 4.5 and 7.0 the g factor remains constant, indicating that in this pH range the electrochemical reduction of HAH_2 leads to the formation of a single species, HAH_3^{-}. In the same manner, an absence of g factor variation is observed for two other pH ranges (pH between 7.5 and 8.0 and between pH 8.4 and 9.3), showing that $HAH_2^{\bullet -}$ and $HAH^{\bullet 2-}$ are respectively produced in more basic conditions. In narrow pH ranges (from 7.1 to 7.3 and from 8.1 to 8.4) the poor resolution of the spectra seems to indicate that two one-electron reduced forms of HAH_2 are present in solution. Indeed, in these pH ranges the two acid/base couples, $HAH_2^{\bullet -}/HAH_3^{\bullet}$ or $HAH^{\bullet 2-}/HAH_2^{\bullet -}$, are concomitantly present in the reduced solutions, leading to a convolution of the spectra of each component of the mixture. It should be noted that g factor values for $HAH_2^{\bullet -}$ and $HAH_3^{\bullet}$, (*i.e.*, 2.004 83 and 2.004 71, respectively) are very close to those determined in acetonitrile medium, confirming that in both organic and aqueous micellar media the one-electron reduction of HAH_2 is associated with proton transfer.

These reduced species have also been characterised by UV-visible spectroscopy (Table 2). The spectra of the reduced species depend on the acidic character of the solution and some drastic changes are observed according to the pH value. In acidic media, HAH_3^{-} is characterised by two absorption bands at 494 and 426 nm. These spectrophotometric data are consistent with a one-electron process since the two-electron reduced form of HAH_2 does not present an intense band at 426 nm for a similar pH value.[17] In less acidic conditions, taking into account that hypocrellin A is in its mono-deprotonated form, the reduction leads to less important absorption changes as previously observed.[17] In basic media ($pH = 12.1$), due to the strong anionic character of HA^{2-} and its reduced form $HA^{\bullet 3-}$, the weak spectral changes do not allow a very significant observation of the electrochemical process.

Conclusion

The analysis of the cathodic electrochemical behaviour of hypocrellin A shows that, in both organic and aqueous media, the one-electron reduction is reversible on the time scale of the CV. Moreover, electrolyses carried out in an organic medium have shown that the electrogenerated radical species $HAH_2^{\bullet -}$ is basic enough to be protonated by the residual water. In an aqueous micellar medium the variation of $E_{1/2}$ *vs.* pH for the first electrochemical process allowed the determination of the pK_as of the HAH_2/HAH^{-}, HAH^{-}/HA^{2-} and $HAH_3^{\bullet}/HAH_2^{\bullet -}$, $HAH_2^{\bullet -}/HAH^{\bullet 2-}$ couples. This electrochemical feature has allowed us to demonstrate that the influence of the pH has significant implications, particularly for the formation of superoxide radical anion through irradiation of hypocrellin A.[9] Indeed, these electrochemical data are consistent with the preferred reduction of molecular oxygen by the one-electron reduced form of HAH_2 in basic conditions. This study has been completed by the characterisation of these reduced forms of HAH_2 by both EPR and UV-visible spectroscopy, showing that, in micellar media, the disproportionation of the electrogenerated radical does not occur, on the time scale of the electrolyses, in the pH range between 4.5 and 9.5.

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