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Direct detection of superoxide anion generated in C_{60}-photosensitized oxidation of NADH and an analogue by molecular oxygen

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Visible-light irradiation of poly(vinylpyrrolidone) (PVP)-solubilized C_{60} in water in the presence of NADH (dihydronicotinamide adenine dinucleotide) and molecular oxygen (O_2) results in formation of superoxide anion ($O_2^{\bullet -}$). Formation of $O_2^{\bullet -}$ having a characteristic $g_{\parallel}$ value of 2.18 was evidenced by the direct observation with use of a low-temperature EPR technique at 77 K. Photoinduced $O_2^{\bullet -}$ formation was also observed for an *N*-methyl-2-pyrrolidone (NMP) solution of C_{60} and 1-benzyl-1,4-dihydronicotinamide (BNAH) in the presence of O_2, whereas C_{60} radical anion ($C_{60}^{\bullet -}$) was formed in the absence of O_2 under otherwise the same experimental conditions. These results suggest that C_{60}^{2-} formed in the photoinduced electron-transfer reduction of C_{60} by BNAH acts as an electron donor to O_2 to give $O_2^{\bullet -}$ in NMP.

Introduction

Fullerenes are known to act as very strong photosensitizing agents and to exhibit bioactivity against biomolecules under photoirradiation, which could lead to their wider application as pharmaceuticals.[1–11] Yamakoshi *et al.* have previously reported photoinduced DNA cleavage in the presence of NADH (dihydronicotinamide adenine dinucleotide) in water,[4a] when C_{60} or C_{70} used as a sensitizer was dissolved with use of poly(vinylpyrrolidone) (PVP).[12] The singlet excited state of C_{60} ($^1C_{60}$*) produced initially upon irradiation is efficiently converted to the triplet excited state $^3C_{60}$* by the fast intersystem crossing.[13] $^3C_{60}$* thus formed is known to undergo energy transfer to molecular oxygen (O_2) to give singlet oxygen ($^1O_2$)[14–19] or electron transfer from reductants to produce the radical anion $C_{60}^{\bullet -}$.[20–31] $^1O_2$ has been reported to oxidize NADH *via* an electron transfer from NADH to $^1O_2$ to yield the two-electron oxidized form, *i.e.*, NAD^+.[32,33] Photoinduced electron transfer from the singlet excited state of NADH and analogues to O_2 can also initiate the reduction of O_2 in the protic media to hydrogen peroxide.[34,35] In any case, no direct observation of superoxide anion ($O_2^{\bullet -}$) produced in the electron-transfer reactions involving NADH has so far been reported. As such, the active oxygen species such as $O_2^{\bullet -}$ and $^{\bullet}OH$ involved in the DNA cleavage in the aqueous C_{60}–PVP–NADH–O_2 system under irradiation have yet to be identified clearly.

We report herein the direct detection of $O_2^{\bullet -}$ generated in the aqueous C_{60}–PVP–NADH–O_2 system under irradiation with use of low-temperature EPR spectroscopy. Photoinduced $O_2^{\bullet -}$ formation was also observed for the *N*-methyl-2-pyrrolidone (NMP) solution of C_{60} and 1-benzyl-1,4-dihydronicotinamide (BNAH) in the presence of O_2, whereas $C_{60}^{\bullet -}$ was formed in the absence of O_2 under otherwise the same experimental conditions. The mechanism of $O_2^{\bullet -}$ formation in NMP is discussed based on the spectroscopic and electrochemical data obtained in this study, providing a valuable insight into the photoinduced DNA cleavage in the aqueous C_{60}–PVP–NADH–O_2 system.

Experimental

Materials

C_{60} (>99.99% pure) was purchased from MER Co., Tucson, AZ, USA. Poly(vinylpyrrolidone) K30 (PVP K-30, MW 40 000) was obtained commercially from Wako Pure Chemical Ind. Ltd., Japan. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was obtained commercially from Oxis International Inc. Dihydronicotinamide adenine dinucleotide (NADH) and 1-benzyl-1,4-dihydronicotinamide (BNAH) were purchased from Sigma Chemical Co. and Tokyo Chemical Industry Co., Ltd., Japan, respectively. Tetra-*n*-butylammonium perchlorate (TBAP) used as a supporting electrolyte was recrystallized from ethanol and dried under vacuum at 313 K. *N*-methyl-2-pyrrolidone (NMP) was purchased from Wako Pure Chemical Ind. Ltd., Japan, and used as received. Benzonitrile (PhCN) was distilled over P_2O_5 prior to use.[36]

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Preparation of PVP-solubilized C_{60} in water

PVP-solubilized C_{60} in water was prepared by the literature procedure.[12] C_{60} (0.4 mg) dissolved in toluene (1.0 mL) was added to 2.0 mL of $CHCl_3$ containing 100 mg of PVP (K-30). The solution was well mixed and the solvent was thoroughly evaporated under vacuum. The residue was redissolved in 2.0 mL of water and the aqueous solution thus obtained was sonicated to produce a clear brown solution.

EPR measurements

A quartz EPR tube (4.5 mm id) containing an oxygen-saturated aqueous solution (50 mM phosphate buffer, pH 7.4) containing C_{60} (1.5×10^{-4} M), NADH (1.5×10^{-4} M), PVP (0.4%) was irradiated with the focused light of a 1000 W high-pressure Hg lamp (USH–1005D) through an aqueous filter for 60 s, and then O_2 bubbling for 60 s at 298 K. The EPR spectrum of $O_2^{\bullet -}$ in frozen aqueous solution at 77 K was measured with a JEOL X-band spectrometer (JES-RE1XE) apparatus under non-saturating microwave power conditions. The *g* values were calibrated precisely with a Mn^{2+} marker which was used as a reference.

For the detection of DEPMPO–OOH, an NMP solution containing C_{60} (1.4×10^{-4} M), BNAH (1.4×10^{-3} M), and DEPMPO (1.4×10^{-2} M) in a LABOTEC LLC-04B EPR sample tube was irradiated in the presence of O_2 with a 300 W reflector lamp for 30 s.

Cyclic voltammetry

Cyclic voltammetry measurements were performed at 298 K on a BAS 100W electrochemical analyzer in deaerated NMP containing TBAP as a supporting electrolyte. A conventional three-electrode cell with a platinum working electrode (surface area of 0.3 mm^2) and a platinum wire as the counter electrode were utilized. The Pt working electrode (BAS) was polished with a BAS polishing alumina suspension and rinsed with acetone before use. The measured potentials were recorded with respect to the Ag/AgNO$_3$ (0.01 M) reference electrode. All potentials (*vs.* Ag/Ag$^+$) were converted to values *vs.* SCE by adding 0.29 V.[37] The electrochemical measurements of C_{60} in NMP were carried out under an atmospheric pressure of argon, while the reduction potential of O_2 was measured in O_2-saturated NMP.

Laser flash photolysis

The procedure and the equipment for the measurements of the triplet–triplet absorption spectrum of $^3C_{60}{}^*$ were described previously.[31]

Spectroscopic measurements

Typically, to a deaerated NMP solution (3 mL) of C_{60} (1.4×10^{-4} M) in a quartz cuvette (10 mm id) was added BNAH (1.4×10^{-4} M) under an atmospheric pressure of argon, and the solution was irradiated at 532 nm with a Shimadzu RF-5300PC spectrofluorophotometer at 298 K. The Vis–NIR spectra were measured on a Shimadzu UV-1600PC UV–visible spectrophotometer which was thermostated at 298 K.

Near-IR luminescence emission spectra were measured on a Hamamatsu Photonics R5509-72 photomultiplier under irradiation at 560 nm with use of a Cosmo System LVU-200S monochromator.

Results and discussion

Photosensitized formation of singlet oxygen in an aqueous PVP system

Poly(vinylpyrrolidone) (PVP), a detergent, was used to dissolve C_{60} into water.[12] A PVP-solubilized aqueous solution of C_{60} is brown, although C_{60} in non-polar media, such as toluene, benzene and *n*-hexane, is purple. This may be due to a strong interaction between C_{60} and PVP in water.[12,38] C_{60} is known to act as a strong photosensitizer, which produces singlet oxygen ($^1O_2$) by photoirradiation in the presence of molecular oxygen (O_2) *via* energy transfer from the triplet state $^3C_{60}{}^*$ to O_2.[14–19] Photoinduced $^1O_2$ formation in the aqueous C_{60}–PVP–O_2 system was examined with use of near-IR luminescence emission spectroscopy.[39] The characteristic luminescence emission band of $^1O_2$ at 1270 nm was observed for a C_6D_6 solution of C_{60} as shown in Fig. 1a. On the other hand, no luminescence emission band of $^1O_2$ was observed for the aqueous solution of C_{60}-PVP (Fig. 1b). The absence of the luminescence emission band of $^1O_2$ in the aqueous C_{60}–PVP–O_2 system suggests that the energy transfer from $^3C_{60}{}^*$ to O_2 is prohibited due to a strong interaction between C_{60} and PVP in water. The strong coordination of PVP to C_{60} has recently been indicated by the large formation constant ($K = 1331$ M^{-1}) for a strong charge transfer complex formed between PVP and C_{60}.[38] Alternatively $^1O_2$ formed under the present experimental conditions is too short-lived to be detected. Thus, even if $^1O_2$ is formed in the aqueous C_{60}–PVP–O_2 system, it cannot be involved in the oxidation of NADH.

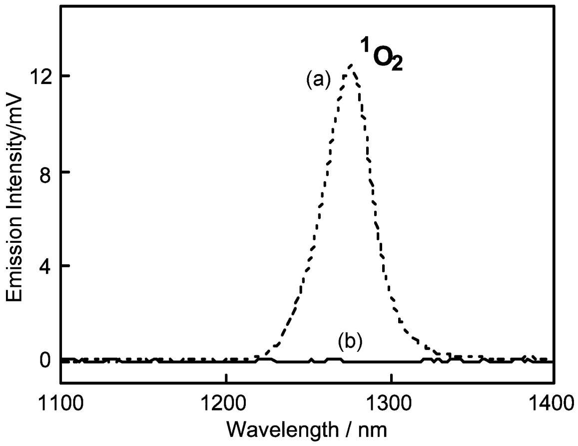


Fig. 1 Near-IR luminescence emission spectra for (a) C_6D_6 solution of C_{60} (2.0×10^{-3} M) (dashed line) and (b) the 0.4% PVP-solubilized phosphate buffer solution (50 mM; pH 7.4) of C_{60} (1.5×10^{-4} M) (solid line) in the presence of O_2 at 298 K.

Photoinduced $O_2^{\bullet -}$ generation in an aqueous C_{60}–PVP–NADH–O_2 system

Visible-light irradiation of the aqueous C_{60}–PVP–O_2 system in the presence of a reductant, such as NADH, results in formation of superoxide anion ($O_2^{\bullet -}$).[4a] Yamakoshi *et al.* have reported that the characteristic EPR signals of the $O_2^{\bullet -}$-adduct of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), DMPO–OOH, are observed under visible-light irradiation of the aqueous solution containing C_{60}–PVP, NADH, and DMPO in the presence of O_2.[4a] Formation of $O_2^{\bullet -}$ was confirmed directly by a low-temperature EPR method. The EPR spectrum of $O_2^{\bullet -}$ generated by visible-light irradiation of the aqueous C_{60}–PVP–NADH–O_2 system is shown in Fig. 2, where the characteristic EPR signal due to $O_2^{\bullet -}$ which has the $g_{\parallel}$ value of 2.18 is clearly observed at 77 K.[40]

Electrochemical behavior of C_{60} in NMP

N-Methyl-2-pyrrolidone (NMP), a monomeric component of PVP, was employed as a non-aqueous polar solvent to investigate the photoinduced $O_2^{\bullet -}$ formation in the presence of C_{60} and reductants. The solubility of C_{60} in NMP is significantly high (0.89 mg mL^{-1}).[41] First, the electrochemical behavior of

Table 1 Reduction potentials (*vs.* SCE) of C_{60} in non-aqueous polar media

Solvent	$E^0_{\text{red}}{}^a$/V (ΔE^b/mV)			
	1st	2nd	3rd	Fc^+/Fc
NMP	−0.35 (104)	−0.86 (119)	—	0.40 (116)
PhCN	−0.43 (72)	−0.87 (72)	−1.34 (76)	0.50 (69)

[a] Determined by cyclic voltammetry. Supporting electrolyte: 0.1 M TBAP; Working electrode: Pt; Scan rate: 0.1 V s^{-1}. [b] $\Delta E = |E_{pc} - E_{pa}|$.

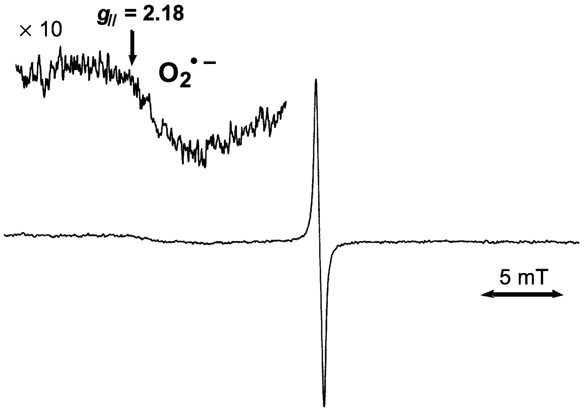


Fig. 2 X-Band EPR spectrum of $O_2^{\bullet -}$ generated by visible-light irradiation of a phosphate buffer solution (50 mM; pH 7.4) of C_{60} (1.5×10^{-4} M), NADH (1.5×10^{-4} M), PVP (0.4%) and O_2 at 77 K.

C_{60} in NMP was examined using cyclic voltammetry (CV). Two reversible redox couples were observed for an NMP solution of C_{60} containing 0.1 M TBAP as shown in Fig. 3, although the third reduction process could not be observed because of the reduction of NMP itself. The reduction potentials thus obtained are listed in Table 1 together with those in benzonitrile (PhCN).[42] Table 1 also shows the redox potentials of ferrocene (Fc) as an internal standard. The first reduction potential of C_{60} to produce $C_{60}^{\bullet -}$ in NMP is shifted slightly in the positive direction as compared to that in PhCN. Such a positive shift may be ascribed to the stronger solvation of NMP to $C_{60}^{\bullet -}$ as compared to that of PhCN.[38] On the other hand, the second reduction occurs at about the same potential. From the reversibility of the cyclic voltamogram in Fig. 3, the singly and doubly reduced C_{60} are stable under the CV time scale in deaerated NMP.

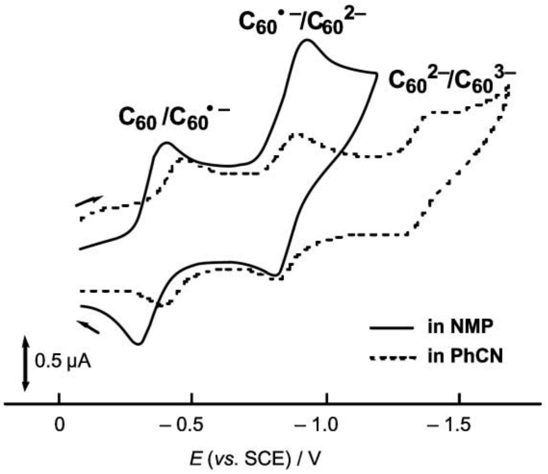


Fig. 3 Cyclic voltammograms of C_{60} in NMP (solid line) and in PhCN (dashed line) containing 0.1 M TBAP at 298 K. Working electrode: Pt; scan rate 0.1 V s^{-1}.

Photoinduced $O_2^{\bullet -}$ generation in a C_{60}–BNAH–O_2 system in NMP

The singlet excited state of C_{60} produced initially upon irradiation is known to be efficiently converted to the triplet excited state by the fast intersystem crossing.[13] The transient absorption spectra in the visible and near-IR region are observed by the laser flash photolysis of a deaerated NMP solution of C_{60} with 532 nm laser light as shown in Fig. 4a. The triplet–triplet absorption band at 740 nm appears immediately after nanosecond laser exposure (Fig. 4).[30,31] The $^3C_{60}$* thus formed may undergo the energy-transfer reaction to molecular oxygen (O_2) to produce $^1O_2$[14–19] or the electron-transfer reduction by reductants to produce $C_{60}^{\bullet -}$.[20–31] As in the case of the aqueous system described above, $^1O_2$ could not be detected for an NMP solution of C_{60} in the presence of O_2 by near-IR luminescence emission spectroscopy. In the presence of an NADH model compound, 1-benzyl-1,4-dihydronicotinamide (BNAH), $O_2^{\bullet -}$ was detected with the use of 5-diethoxy-phosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) used as an $O_2^{\bullet -}$-trapping agent.[43] DEPMPO reacts with $O_2^{\bullet -}$ to form the $O_2^{\bullet -}$-adduct, DEPMPO–OOH, which shows a characteristic EPR signal different from that of $^\bullet$OH-adduct, DEPMPO–OH.[43] Fig. 5 shows the EPR spectrum of DEPMPO–OOH observed after visible-light irradiation of an NMP solution of C_{60}, BNAH, and DEPMPO in the presence of O_2 at 298 K. Thus, a charge transfer interaction between NMP and C_{60} prohibits energy transfer from $^3C_{60}$* to O_2 but

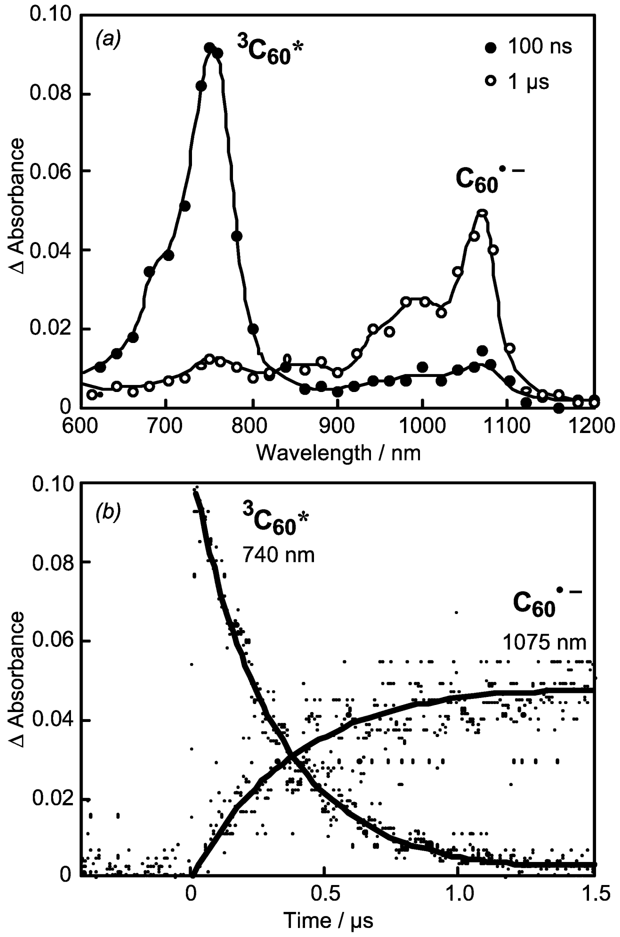


Fig. 4 (a) Transient absorption spectra observed in the photoreduction of C_{60} (1.0×10^{-4} M) by BNAH (2.0×10^{-3} M) after laser excitation in deaerated NMP at 295 K. (b) Time course of the absorbance changes at 740 nm for the decay of $^3C_{60}$* and 1075 nm for the formation of $C_{60}^{\bullet -}$ in the photoreduction of C_{60} (1.0×10^{-4} M) by BNAH (2.0×10^{-3} M) after laser excitation in deaerated NMP at 295 K.

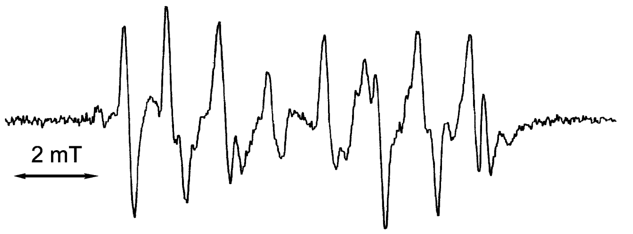
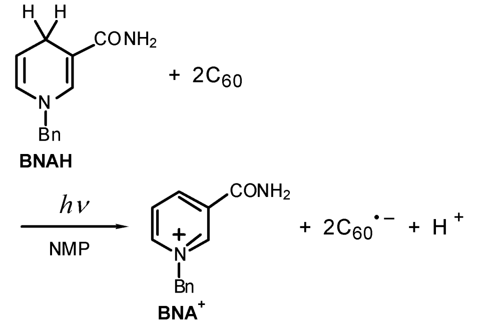


Fig. 5 X-Band EPR spectrum of DEPMPO–OOH formed by visible-light irradiation (30 s) of an NMP solution of C_{60} (1.4×10^{-4} M), BNAH (1.4×10^{-3} M), and DEPMPO (1.4×10^{-2} M) in the presence of O_2 using a 300 W reflector lamp at 298 K.

electron transfer from BNAH to $^3C_{60}$* is not retarded by the interaction with NMP.

In the absence of O_2, the photoinduced electron-transfer reduction of C_{60} by BNAH indeed occurs to produce $C_{60}^{\bullet-}$ [eqn. (1)]. The characteristic near-IR band at 1075 nm[44] due to $C_{60}^{\bullet-}$ was observed under visible-light irradiation at 532 nm of a deaerated NMP solution of C_{60} and BNAH.[31]



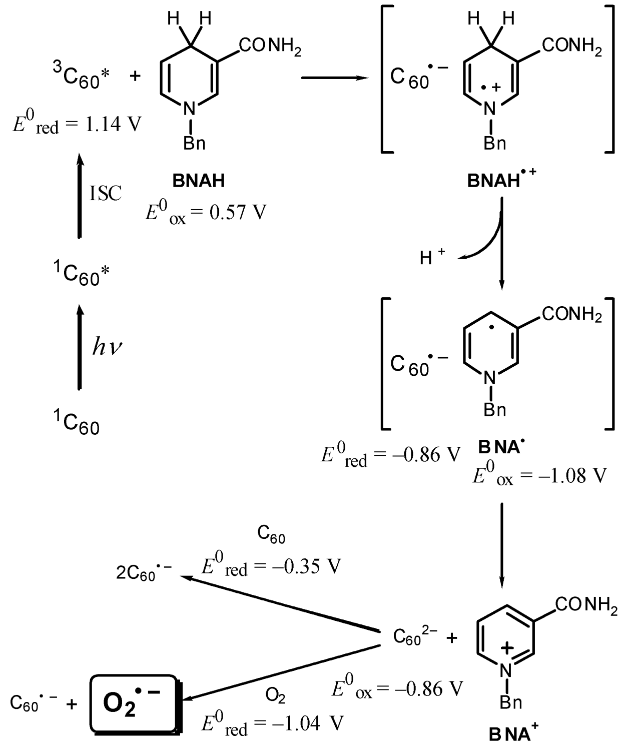
(1)

The triplet excited state ($^3C_{60}$*) is responsible for the photoinduced electron transfer from BNAH, since the decay of the transient absorption band at 740 nm due to $^3C_{60}$* appearing immediately after nanosecond laser pulse is accompanied by appearance of a new absorption band at 1075 nm due to $C_{60}^{\bullet-}$ as shown in Fig. 4a. The decay of the absorbance at 740 nm due to $^3C_{60}$* obeys pseudo-first-order kinetics, coinciding with the rise of the absorbance at 1075 nm due to $C_{60}^{\bullet-}$ (Fig. 4b). The rate constant of photoinduced electron transfer from BNAH to $^3C_{60}$* is determined as 1.3×10^9 M^{-1} s^{-1}.

Mechanism of $O_2^{\bullet-}$ generation

From the results described above, the mechanism for photo-induced $O_2^{\bullet-}$ formation in NMP is proposed as shown in Scheme 1, where the redox potential for each compound is presented. Visible-light irradiation of C_{60} gives the singlet excited state $^1C_{60}$*, which is followed by the intersystem crossing (ISC) to produce the triplet excited state of $^3C_{60}$*.[13] Then, the electron-transfer reduction of $^3C_{60}$* having a high reduction potential of $E^0_{red} = 1.14$ V *vs.* SCE by BNAH ($E^0_{ox} = 0.57$ V *vs.* SCE)[45] occurs to give a radical ion pair of $C_{60}^{\bullet-}$ and the BNA radical cation ($BNAH^{\bullet+}$).[13,31] $BNAH^{\bullet+}$ undergoes deprotonation to give the BNA radical ($BNA^{\bullet}$), which is a very strong electron donor having an oxidation potential of $E^0{}_{ox} = -1.08$ V *vs.* SCE.[31,45] The $BNA^{\bullet}$ can reduce $C_{60}^{\bullet-}$ to C_{60}^{2-}, since the oxidation potential of $BNA^{\bullet}$ is more negative than the reduction potential of $C_{60}^{\bullet-}$ ($E^0_{red} = -0.86$ V *vs.* SCE). In the absence of O_2, electron transfer from C_{60}^{2-} to C_{60} yields two equivalents of $C_{60}^{\bullet-}$. In the presence of O_2, however, electron transfer from C_{60}^{2-} ($E^0_{ox} = -0.86$ V *vs.* SCE) to O_2 having a reduction potential at -1.04 V *vs.* SCE in NMP occurs to give $C_{60}^{\bullet-}$ and $O_2^{\bullet-}$.

In conclusion, the primary active oxygen species involved in the C_{60}–PVP–NADH–O_2 system has been shown to be $O_2^{\bullet-}$ rather than $^1O_2$. The results obtained above with use of NMP as a solvent suggest that $O_2^{\bullet-}$ is formed *via* the electron transfer reduction of O_2 by C_{60}^{2-} which is produced in the photoreduction of C_{60}–PVP by NADH in an aqueous solution.



Scheme 1

Acknowledgements

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