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Effect of complexation with randomly methylated β-cyclodextrin on the aqueous solubility, photostability and antioxidant activity of an indolinonic nitroxide radical

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Abstract

The interaction between the hydrophobic indolinonic nitroxide radical, 1,2-dihydro-2-methyl-2-phenyl-3H-indole-3-one-1-oxyl and hydrophilic α-, β- and γ-cyclodextrin derivatives was investigated in water by phase-solubility analysis. Among the studied cyclodextrins, random methyl-β-cyclodextrin (RM-β-CD) had the greatest solubilizing activity (1312-fold increase in the intrinsic aqueous solubility). Solid complexes were prepared by the freeze-drying method and characterized by powder X-ray diffractometry and thermal analysis. Complexation of the nitroxide with RM-β-CD was also confirmed in solution by electron paramagnetic resonance (EPR) spectroscopy. Photodegradation of the nitroxide was reduced by complexation with RM-β-CD, this effect being more pronounced in the solid-state (the extent of degradation was 28.0% for the complex vs. 78.8% for uncomplexed nitroxide) than in solution (41.2 vs. 69.1% for uncomplexed nitroxide). The antioxidant activity of the complex was also investigated on the peroxidation of methyl linoleate micelles and on protein oxidation induced by free radical generators, and in both systems the free form of the nitroxide as well as its complex with RM-β-CD, showed essentially the same degree of protection. Moreover, EPR experiments showed a time-dependent decrease in the EPR signal of both the complexed and uncomplexed nitroxides with the free-radical generators. Therefore, RM-β-CD complexation of the nitroxide represents an effective strategy to improve its aqueous solubility and photostability, which is essential for certain biological applications, while it does not interfere with its radical scavenging efficiency.

Keywords: *Indolinonic nitroxide, cyclodextrin, complexation, aqueous solubility, photodegradation, sntioxidant activity*

Introduction

Nitroxides are among the most stable free radicals known that are often experimentally attached covalently to membrane or proteins so that the behavior of macromolecules can be followed by changes in the electron paramagnetic resonance (EPR) spectrum, a well-developed technique known as spin-labeling.[1] Besides this popular use of nitroxides, a wealth of literature now exists and is continuously increasing which demonstrates the potential use of nitroxide radicals as antioxidant compounds. By acting as electron donors, electron acceptors and radical scavengers, they either reduce or inhibit the generation or the effects of reactive nitrogen oxygen species (RNOS) in diverse biological systems.[2–4] The majority of these compounds are, however, hydrophobic, as is the case of aromatic indolinonic nitroxides that have been studied extensively by our group. In fact, the chemical reactivity of indolinonic nitroxides towards several biologically relevant radical species has now been well characterized[5–11] and

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this has been followed up by many antioxidant studies on lipids, proteins, DNA, intact cells, which strongly demonstrate the inherent antioxidant abilities of these compounds.[12–18] However, to explore further the potential applications of these aromatic nitroxides, especially in an aqueous environment, one is limited by their poor water solubility and for certain studies, also by their photosensitivity. A higher hydrosolubility could improve bioavailability in addition to the important fact that biological assays would be easier to carry out.

On the other hand, cyclodextrins are amply employed in the pharmaceutical, cosmetics and food industries primarily as additive and complexing agents for various compounds such as drugs, vitamins and food colorants.[19–22] These are cyclic oligosaccharides shaped as a truncated cone with a hydrophilic outer surface and a non-polar cavity interior. They are able to encapsulate appropriately sized hydrophobic compounds or some lipophilic portion of a molecule into their apolar cavity via non-covalent bonds.[21] This complexation process can affect some of the physico-chemical properties of the included substance and can lead to enhanced stability to air and light and increased apparent aqueous solubility.[23,24]

In the present work, we describe the preparation and characterization of an inclusion complex between an indolinonic nitroxide, namely 1,2-dihydro-2-methyl-2-phenyl-3H-indole-3-one-1-oxyl and random methyl-β-cyclodextrin (RM-β-CD) and investigate the influence of complexation on the aqueous solubility, photochemical stability and antioxidant activity of the above nitroxide.

Materials and methods

Materials

The indolinonic nitroxide, 1,2-dihydro-2-methyl-2-phenyl-3H-indole-3-one-1-oxyl (Figure 1) was synthesized according to the literature.[25] The identity and purity of the compound was checked by thin layer chromatography and by mass spectrometry on a Carlo Erba QMD 1000 spectrometer in EI^+ mode (Milan, Italy). The cyclodextrins used in this study included: hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-α-cyclodextrin (HP-α-CD), hydroxypropyl-γ-cyclodextrin (HP-γ-CD) and RM-β-CD. These were purchased from Sigma–Aldrich (Milan, Italy). Methanol, acetonitrile and water were high performance liquid chromatography (HPLC)-grade from Merck (Darmstadt, Germany). All other chemicals were analytical-grade reagents purchased from Sigma–Aldrich (Milan, Italy). The lipid soluble, free-radical generator, AMVN [2,2′-azo-bis(2,4-dimethylvaleronitrile)], was kindly prepared and donated by Prof. R. Leardini from the University of Bologna (Italy) according to the method described in the literature.[26]

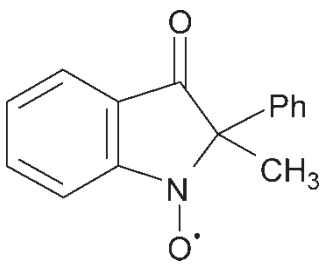

Figure 1. Chemical structure of 1,2-dihydro-2-methyl-2-phenyl-3H-indole-3-one-1-oxyl.

High performance liquid chromatography

The HPLC apparatus comprised of a Model LabFlow 3000 pump (LabService Analytica, Bologna, Italy), a Model 7125 injection valve with a 20 μl sample loop (Rheodyne, Cotati, CA, USA) and a Model 975-UV variable wavelength UV–Vis detector (Jasco, Tokyo, Japan) set at 350 nm. Data acquisition and processing were accomplished with a personal computer using Borwin software (JBMS Developments, Le Fontanil, France). Sample injections were done with a Model 701 syringe (10 μl; Hamilton, Bonaduz, Switzerland). Separations were performed on a 5 μm Zorbax SB-C18 column (150 × 4.6 mm i.d.; Agilent Technologies, USA) fitted with a guard column (5 μm particles, 4 × 2 mm i.d.) and eluted isocratically, at a flow-rate of 1.0 ml/min, with methanol–acetonitrile–water (55:25:20, v/v/v). The identity of the nitroxide peak was assigned by co-chromatography with the authentic standard. Quantification was carried out by integration of the peak areas using the external standardization method.

Phase-solubility studies

Solubility analyses were carried out according to Higuchi and Connors.[27] An excess amount of nitroxide was added to aqueous solutions (5 ml) in vials containing the different cyclodextrins examined (0–90 mM). The vials were sealed to avoid changes due to evaporation and the samples were shaken on a horizontal rotary shaker at 25 ± 1°C and shielded from light. After equilibrium had been reached (3 days, as demonstrated by a constant content of solubilized nitroxide in three successive samples at 3, 5 and 7 days), the content of each vial was filtered through 0.45-μm membrane filters (Whatman, Clifton, NJ) and analyzed for the nitroxide by HPLC as outlined above. Data were determined from the average of at least three determinations. Solubility diagrams were constructed by plotting the molar concentration of the indolinonic nitroxide in solution against the molar concentration of cyclodextrin. The stability constant

values were calculated using the following equation:

$$K = \frac{\text{slope}}{S_0(1 - \text{slope})}$$

where S_0 represents the solubility of the nitroxide radical in the absence of cyclodextrins and slope is the slope of the obtained phase solubility diagram.

Inclusion complex preparation

The inclusion complex was prepared at a 1:1 molar ratio of nitroxide to RM-β-CD by the freeze-drying method, as reported below. The calculated amount of nitroxide was added to an aqueous solution (5 ml) containing an equimolar quantity of cyclodextrin. The suspension was stirred for 3 days at room temperature and shielded from light. Following sample filtration, the resulting solution was subjected to lyophilization and the residue stored in a desiccator. The amount of nitroxide incorporated into the complex was determined by HPLC after proper dilution. A physical mixture was prepared by mixing in a mortar the nitroxide and RM-β-CD and subjecting the blend to freeze-drying.

X-ray diffractometry

The powder X-ray diffraction patterns were recorded on a D 5000 powder diffractometer (Siemens, Munich, Germany) using a voltage of 45 kV and a current of 25 mA for the generator, with Cu anode material. The wavelength of the graphite-monochromated radiation was 1.5406 Å. The diffractograms were recorded from 3° (2θ) to 50° (2θ) at an angular speed of 1° (2θ) per minute using 1–1–1–0.15° slits.

Thermal analysis

Differential thermal analysis (DTA) was carried out on a Netzsch STA 409 simultaneous thermal analyzer (Netzsch Italiana, Verona, Italy). The samples (6–7 mg) were accurately weighed in platinum pans (Netzsch) and heated from 20 to 200°C, at a scanning rate of 20°C/min.

EPR spectroscopy

EPR measurements were performed on a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany) equipped with an XL Microwave frequency counter, Model 3120 for the determination of the *g*-factors. Temperature was controlled by a Stelar VTC87 temperature controller. Spectra were recorded with the following instrumental settings: 5 mW microwave power, 0.5 G modulation amplitude and 100 kHz field modulation.

For determination of coupling constants and *g*-factors, buffer solutions (50 mM phosphate, pH 7.4) of the complex and ethanol solutions of the nitroxide were prepared at a concentration of 2 mM. Aliquots (100 μl) were then transferred into an inverted U cell containing the buffer to give a final concentration of 200 μM. Samples were thoroughly purged with argon and spectra were recorded. For the EPR study on the scavenging ability of the nitroxide or its complex toward radicals generated from the water-soluble azo-initiator, AAPH [2,2-azobis(2-methylpropionamidine)dihydrochloride], appropriate stock solutions of AAPH in buffer, complex in buffer and nitroxide in ethanol were prepared and aliquots were transferred to glass capillary tubes (1 mm i.d.) containing buffer, to give final concentrations of 20 mM AAPH and 200 μM nitroxide in free or complexed form. After thorough degassing under argon, spectra were recorded at 40°C for 75 min until complete disappearance of the EPR signal.

Peroxidation studies of methyl linoleate

Methyl linoleate micelles were prepared by mixing appropriate amounts of methyl linoleate, free-radical generators (AMVN in acetonitrile or AAPH in buffer), complex (in buffer) or nitroxide (in acetonitrile), with a buffer solution (5 mM phosphate, 0.1 mM EDTA, 0.9% NaCl, pH 7.4) of 0.5 M SDS (sodium dodecyl sulphate) followed by vigorous mixing for 1 min, according to a modified method of Foti *et al.* [28] Samples were then transferred to quartz cuvettes and placed in the sample compartment of a UV Kontron 941 spectrophotometer thermostated at 40°C. The progress of peroxidation was then monitored by recording the absorbance of conjugated diene formation at 234 nm for 1 h against an appropriate blank which lacked methyl linoleate.

Protein oxidation

Protein samples were prepared by dissolving 3 mg/ml of bovine serum albumin (BSA) in 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.4. The samples were then incubated at 50°C for 1 h in the presence or absence of 5 mM AAPH and 100 μM of nitroxide or its complex. The nitroxide or its complex were added to the protein as acetonitrile (2.5% v/v) or buffer solutions, respectively, and the mixture was vortexed prior to addition of AAPH for thorough incorporation. The extent of protein oxidation was monitored by the method of Levine *et al.* which uses the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl groups of oxidized proteins. [29] Briefly, 0.5 ml of 20 mM DNPH in 2.5 M HCl was added to 0.5 ml of each sample; blank samples lacked DNPH. Following 1 h of incubation at room temperature with continuous shaking, the protein

was precipitated by addition of 2 ml, 20% trichloroacetic acid (TCA) and centrifuged at 3000*g* for 10 min. The protein was washed twice with ethanol/ethylacetate (1:1) and dissolved in 1 ml of 6 M guanidine HCl, pH 6.5. The absorbance was then read at 370 nm and protein carbonyls were evaluated using a molar absorption coefficient of 22,000/M/cm.

Photodegradation studies

Photodecomposition experiments were performed in the solid-state or in 30% (v/v) acetonitrile in water solutions containing 2 mM nitroxide. Solid samples (ca. 10 mg) were weighed and spread onto the bottom of a beaker. The solutions (0.5 ml) were transferred into quartz cells (2 mm path length) closed with screw caps and inserted horizontally in the test chamber. The preparations containing free or complexed nitroxide were irradiated for 2 h with a solar simulator (Suntest CPS +; Atlas, Linsengericht, Germany) equipped with a Xenon lamp, an optical filter to cut off wavelengths shorter than 290 nm and an IR-block filter to avoid thermal effects. The solar simulator emission was maintained at 250 W/m^2. After the exposure interval (2 h), the samples were quantitatively transferred into 10 ml calibrated flasks with acetonitrile, diluted to volume and the remaining nitroxide concentration was quantified by HPLC, as outlined above. The product deriving from the photodegradation of the nitroxide has not yet been isolated and identified. All samples were protected from light both before and after irradiation. The degree of photodegradation was evaluated by comparing the peak areas of nitroxide from the irradiated samples, with those obtained by analysis of an equivalent amount of the non-irradiated preparations.

Results and discussion

Complex characterization

Solubility analysis was used initially for studying the interaction of the nitroxide with cyclodextrins in water. Because of the limited aqueous solubility of unmodified cyclodextrins,[21] the highly water-soluble hydroxypropylated- (i.e. HP-α-CD, HP-β-CD, HP-γ-CD) and randomly methylated- (i.e. RM-β-CD) derivatives were selected for this investigation. Figure 2 shows the influence of the examined cyclodextrins on the aqueous solubility of the nitroxide radical. According to the classification of Higuchi and Connors,[27] two different types of phase solubility curves were observed, namely A_L plots with a linear relationship and an A_n type solubility curve with a negative deviation from linearity. The diagrams obtained (Figure 2) demonstrate that the major solubility enhancement (1312-fold increase) was produced by RM-β-CD and hence this cyclodextrin

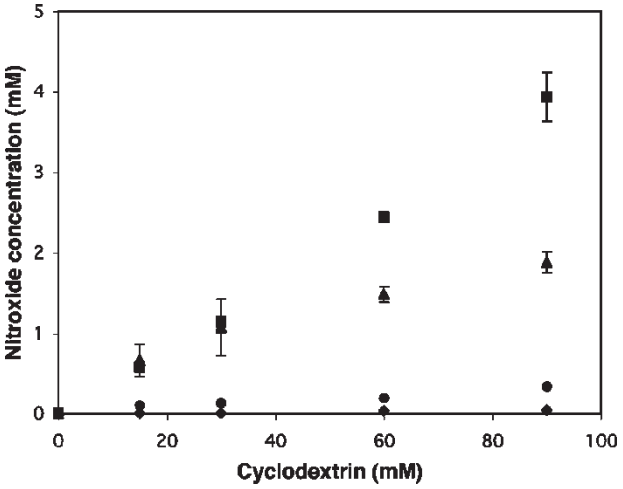

Figure 2. Phase-solubility diagrams for nitroxide with different cyclodextrins in purified water at 25°C. Key: (◆) HP-α-CD; (●) HP-γ-CD; (■) RM-β-CD; (▲) HP-β-CD. Each point represents the mean ± SD of at least three experiments.

interacts more strongly with the indolinonic nitroxide than HP-α-CD, HP-β-CD and HP-γ-CD. This difference may be traced to the extended hydrophobic surface of the RM-β-CD inner cavity.[30] Over the concentration range used in this study, the apparent solubility of the nitroxide increased linearly ($r > 0.99$) with increasing RM-β-CD concentrations (A_L-type) suggesting the formation of a complex with a 1:1 stoichiometry. The stability constant ($K_{1:1}$) for the inclusion complex calculated according to the method of Higuchi and Connors[26] was found to be 11431 ± 879/M.

Powder X-ray diffraction analysis was performed to examine the interaction of the nitroxide with RM-β-CD in the solid-state. The preparation of the complex was carried out at a 1:1 (guest:host) molar ratio using the freeze-drying method. The diffractogram of the physical mixture (Figure 3B) shows peaks corresponding to the nitroxide (Figure 3A), indicating that the radical retained its crystalline structure in this system. On the contrary, the diffraction pattern of the freeze-dried nitroxide/RM-β-CD complex was completely diffuse thus demonstrating the formation of an amorphous solid dispersion which could be attributed to the inclusion of the radical into the cyclodextrin cavity.[31]

Additional solid-state characterization of the nitroxide/RM-β-CD system was carried out by thermal analysis (thermograms not shown). The DTA profile of the nitroxide exhibited an endothermic peak at 165°C corresponding to its melting point. RM-β-CD gave a broad endotherm around 45°C which can be referred to the loss of water. The nitroxide melting peak was present in the physical mixture thermogram, although it was shifted to a lower temperature (136°C) suggesting a weak interaction between the components of the physical mixture. The complete

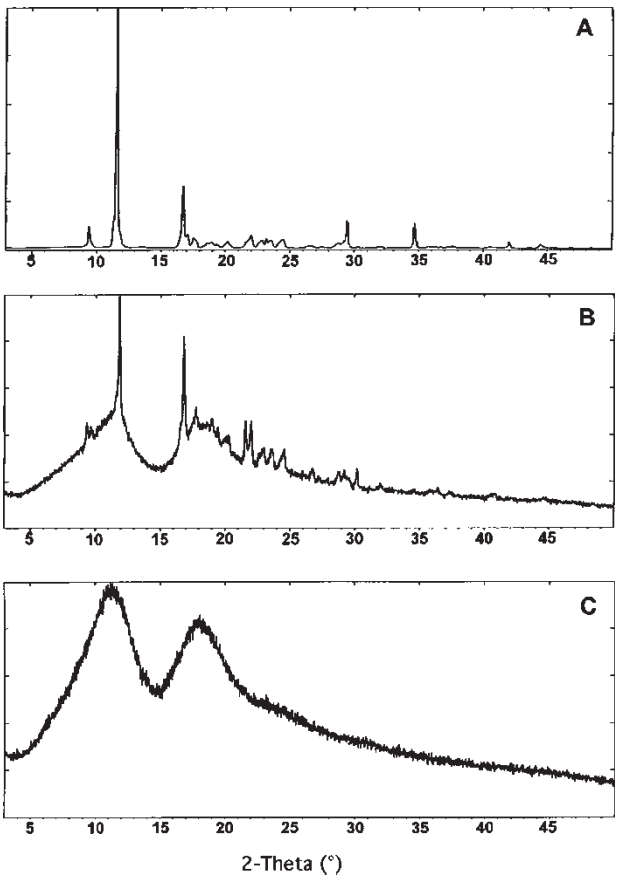

Figure 3. Powder X-ray diffraction patterns of nitroxide (A), nitroxide/RM-β-CD physical mixture (B), nitroxide/RM-β-CD complex (C).

disappearance of the characteristic nitroxide endothermal transition was instead observed in the DTA curve of the complex. These results provide further evidence of inclusion complex formation between the nitroxide radical and RM-β-CD,[32] corroborating the X-ray diffractrometry data.

EPR studies

The nitroxide radical/RM-β-CD interaction was further confirmed in solution by EPR spectroscopic studies as this technique is a valid tool for distinguishing the signals between complexed and uncomplexed species. Table I shows the hyperfine splitting constants and the *g*-factors of the nitroxide in free or complexed form in aqueous solution. The decrease in the nitroxide's nitrogen and hydrogen hyperfine splittings (hfs) as well as it is *g*-value as compared to the free nitroxide, reveals that the nitroxide is experiencing a slightly more hydrophobic environment of the RM-β-CD host cavity.

Table I. Hyperfine splitting constants and *g*-factor of RM-β-CD inclusion complex and of the free nitroxide, in 50 mM phosphate buffer, pH 7.4.

	Hyperfine coupling constants (hfcs) in Gauss			
Sample	a_N	$a_{H\text{-}5,7}$	$a_{H\text{-}4,6}$	*g*-Factor
Complex	8.92	2.77	0.95	2.00238
Nitroxide (10% ethanol v/v)	10.58	3.31	1.08	2.00546

In addition, on comparing the spectra of the free nitroxide (Figure 4A) and the nitroxide/RM-β-CD (Figure 4B) complex in aqueous solution, clear differences can be observed. In particular, the higher field wing of the EPR spectrum of the nitroxide/RM-β-CD complex has a considerably weaker amplitude (broader line width) than the other manifold, indicating that the probe is under motional constraint. These observations are in total agreement with the few literature reports on the EPR spectra of nitroxides complexed with cyclodextrins.[33,34]

Antioxidant studies

These studies were performed to investigate whether the reactivity of the nitroxide may decrease if the nitroxide moiety or the benzene ring which both contribute to the scavenging of radicals, are embedded in the CD cavity. For the *in vitro* evaluation of the antioxidant activity of the nitroxide and of its complex with RM-β-CD, inhibition of lipid peroxidation in aqueous micelles of SDS containing methyl linoleate was monitored spectrophotometrically at 234 nm. At this wavelength, a strong absorption is produced by the conjugated dienes formed upon methyl linoleate peroxidation induced in the micellar phase by radical initiators which, on thermolysis, provide peroxyl radicals at constant rate.[28] Two radical sources were employed in this study: AMVN which is a lipophilic azo-initiator that locates in the lipid region of micelles or membranes generating radicals initially within the lipid region, and AAPH which is hydrophilic and generates radicals in the aqueous region.[35] These were chosen as it was of interest to examine if the antioxidant activity of the water-soluble complexed nitroxide and of the lipid-soluble nitroxide could be affected by the different hydro/-lipophilicity of the radicals generated in the micellar system. Figure 5A,B show the kinetic traces monitoring the formation of methyl linoleate conjugated dienes during peroxidation induced by AMVN and AAPH, respectively, in the presence and absence of nitroxide or its complex. As can be observed, peroxidation proceeds at a steady rate in both cases, and the presence of 1 μM nitroxide or 1 μM complexed nitroxide inhibits peroxidation to very similar extents (~60%) in both cases. On an attentive examination of Figure 5, a clear cut lag phase can be observed during which peroxidation is fully blocked during the first 20 min after which it slowly resumes and roughly reaches its rate before inhibition. In fact,

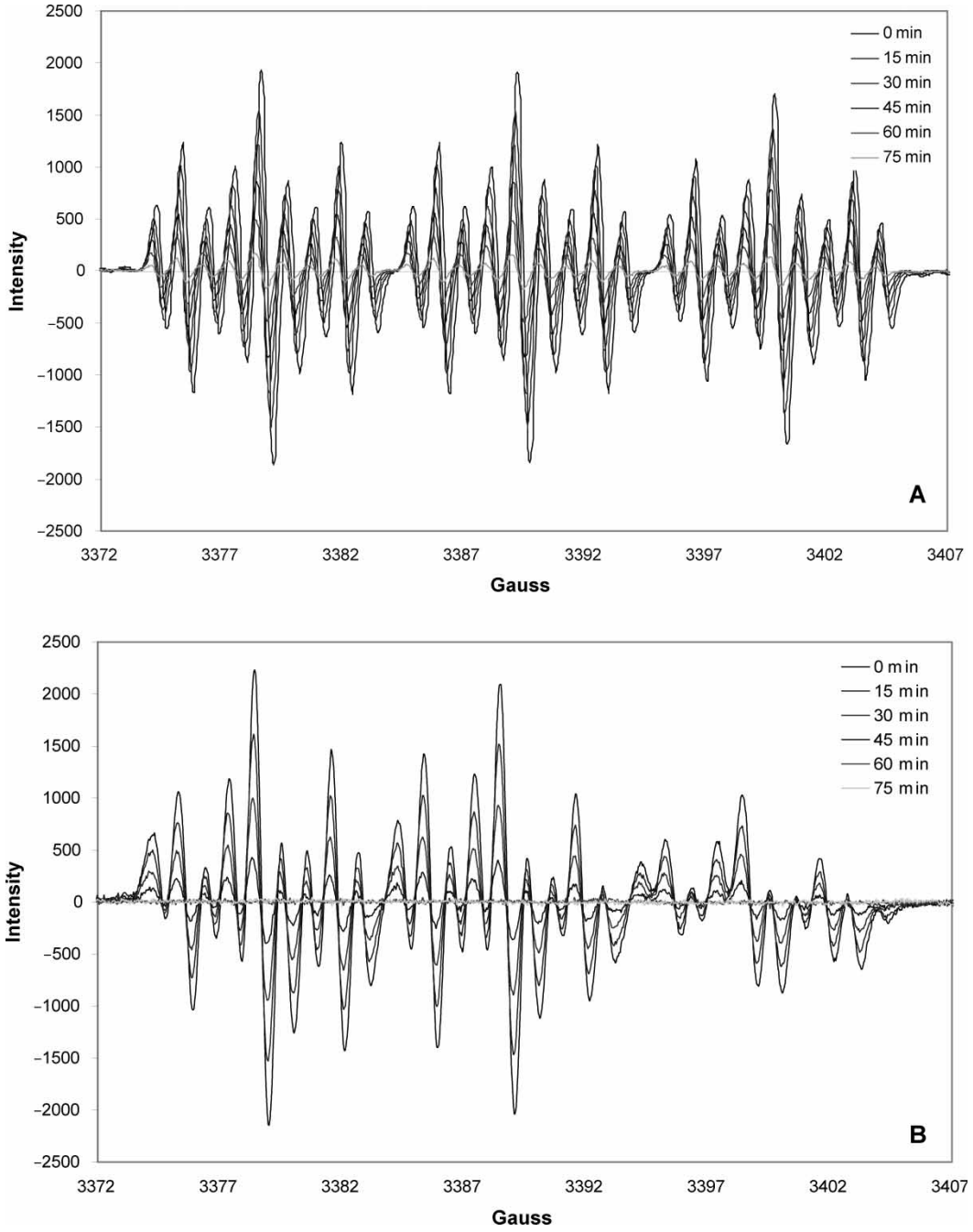

Figure 4. EPR spectra of 200 μM nitroxide (A) and 200 μM nitroxide/RM-β-CD complex (B), incubated with 20 mM AAPH in 5 mM phosphate buffer, 0.1 mM EDTA, 0.9% NaCl, pH 7.4 at 40°C.

during the first 20 min, ~0.16% of AAPH has decomposed which corresponds to 1.6 μM of radicals formed.[35] Now considering that the efficiency of radical release from the decomposition of AAPH is affected by the medium (rates of half-life are referred to in water only) and that AAPH derived carbon-centered radicals are known to recombine in the solvent cage to give dimer and/or disproportionation products, it is reasonable to expect that 1 μM nitroxide fully blocks methyl linoleate oxidation for the first 20 min. At 5 μM of nitroxide or its complex with RM-β-CD, there was total inhibition in both cases for the duration of the experiment (results not shown). In fact, at 60 min radical release from AAPH corresponds to ~5 μM which justifies this result. It is interesting to observe that the complexed nitroxide compared to its free form still retains the same antioxidant efficiency and that no differences are observed as to whether the free radicals generated are lipophilic or hydrophilic, indicating the versatility of this compound as antioxidant both in an aqueous and in a lipid environment.

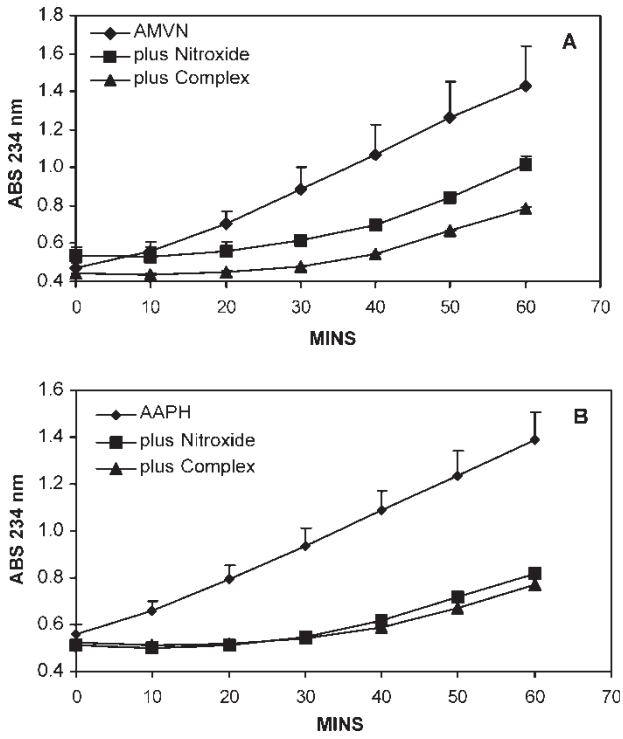

Figure 5. Effect of 1 μM nitroxide and 1 μM nitroxide/RM-β-CD complex on the formation of methyl linoleate conjugated dienes during peroxidation induced by AMVN (A) or AAPH (B), in a pH 7.4 micellar solution of 0.5 M SDS at 40°C. Methyl linoleate: 1.5 mM; AMVN, AAPH: 1 mM. The results are the mean ± SD of at least three experiments.

To corroborate further the antioxidant activity data, studies were also performed on a protein model system consisting of BSA that was oxidized with AAPH in the presence and absence of the complexed and free form of the nitroxide. The extent of oxidation was determined using DNPH that forms a hydrazone with the carbonyl groups produced during protein oxidation, quantifiable at 370 nm.[29] Figure 6 shows that the increase in oxidative modification of BSA during incubation with AAPH is reduced by almost 50% in the presence of 100 μM nitroxide or its complex with RM-β-CD, confirming the results previously reported on lipid peroxidation. The differences between the concentrations of the nitroxide which provide almost 50% protection in the two antioxidant assays (Figures 5 and 6) can probably be ascribed to the different mechanisms involved in fatty acid and protein oxidation.

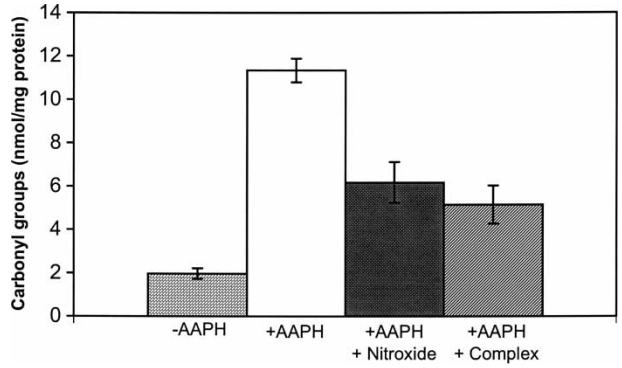

Figure 6. Effect of 100 μM nitroxide and 100 μM nitroxide/RM-β-CD complex on protein carbonyl formation in BSA (3 mg/ml) exposed to 5 mM AAPH for 1 h at 50°C in 50 mM phosphate buffer, 0.1 mM EDTA, pH 7.4.

To verify that the results obtained on the antioxidant activity of the water-soluble nitroxide/RM-β-CD complex were actually due to its intrinsic capacity to react with AAPH-derived radicals and not to the free nitroxide in equilibrium with the complexed form (no covalent bonds are involved in complex formation), EPR experiments were performed in the presence of AAPH only. The EPR profiles reported in Figure 4A,B show a time-dependent decrease in the EPR signal of both the complexed and uncomplexed nitroxide with AAPH. No decrease was observed in the control reactions when AAPH was omitted. Furthermore the differences in the EPR spectrum features between the nitroxide (Figure 4A) and its complex with RM-β-CD (Figure 4B) were maintained during the trapping of the radicals produced by the azoinitiator. This indicates that the indolinonic nitroxide acts as radical scavenger even when included into the cyclodextrin cavity. It is assumed that the decrease in the EPR signal is due to the formation of the alkylated hydroxylamine arising from radical–radical coupling between the nitroxide moiety and the carbon-centered radicals generated upon thermal decomposition of the azo-initiator.[5] The rate of EPR signal disappearance was roughly similar for both complexed and uncomplexed nitroxide even if upon repetitive experiments, a slightly faster rate was always observed with the complex.

The enhancement of water solubility of lipophilic antioxidants, such as α-tocopherol,[36,37] carotenoids[38] and 3-hydroxyflavones[39] using cyclodextrins, and in particular β-cyclodextrin, has recently been observed by others and in all cases, the antioxidant activity was always retained. The results presented in this paper further confirm that complexation with cyclodextrins does not affect the antioxidant character of a compound whether it is diamagnetic or paramagnetic as in the case of the indolinonic nitroxide. Interestingly, it appears that complexation with cyclodextrins significantly stabilizes nitroxide spin adducts obtained during spin-trapping of superoxide anion with nitrones and protects them from ascorbate reduction,[40] a particularly important finding if one is to consider using inclusion complexes of nitrones or nitroxides for biological applications.

Photostability Studies

In order to investigate the effect of RM-β-CD on the photochemical behavior of the nitroxide radical, photolysis studies were carried out initially in the solid-state. Uncomplexed or complexed nitroxide was exposed for 2 h to the solar simulator and the extent of photodegradation was measured by HPLC.[41] In the sample containing the nitroxide alone, the degree of photodecomposition was 78.8% which decreased to 28.0% for the nitroxide/RM-β-CD complex (Table II).

These data demonstrate that complexation with RM-β-CD remarkably enhances the photostability of the solid indolinonic radical. Subsequent experiments were performed on solutions (30% acetonitrile in water), using the same irradiation conditions reported above. In the preparation containing uncomplexed nitroxide, the loss of the radical reached 69.1%, whereas a 52.7% decrease in the nitroxide level was measured in the solution containing its complex with RM-β-CD. Although, the observed difference was statistically significant, the reduction in the light-induced degradation of the indolinonic radical attained by complexation with RM-β-CD was not as marked as that measured in the solid-state (Table II). This effect can be probably ascribed to a decrease in the fraction of substance included in the cyclodextrin cavity upon complex dilution.[42] Consequently, additional studies were carried out on solutions containing the nitroxide radical/RM-β-CD complex with a 4-fold molar excess of cyclodextrin to reduce the percentage of the free guest in equilibrium with the included form. Under these conditions, the extent of the nitroxide photolytic decomposition was 41.2% (Table II). These data demonstrated a further increase in the photostabilization activity achieved by the RM-β-CD complex in solution.

In conclusion, the combined use of different analytical techniques (X-ray diffractometry, thermal analysis, EPR spectroscopy) has enabled us to fully characterize the inclusion compound of a nitroxide with cyclodextrins, which to our knowledge is the first study of its kind. In addition, the study illustrates that the inclusion complex of 1,2-dihydro-2-methyl-2-phenyl-3H-indole-3-one-1-oxyl with RM-β-CD retains the antioxidant activity of the indolinonic nitroxide while enhancing its aqueous solubility and photostability. This is an important finding which leaves room for expansion on the biological application in a hydrophilic environment of lipophilic nitroxides, especially aromatic indolinonic ones which possess a more variegated reactivity towards radical species[5–11] compared to other nitroxides commonly used like the piperidines, TEMPO and TEMPOL.[43–45] For example, they could be routinely employed in the aqueous medium of fluids for organ preservation during transplantation and for diminishing graft rejection or in perfusates utilized during ischemia/reperfusion of several organs.

Table II. Comparative photodegradation data for RM-β-CD inclusion complex and for the free nitroxide after 2 h irradiation with the solar simulator.

	Nitroxide radical loss⋆ (%)		
Sample	Nitroxide	Complex	$P^{\dagger}$
Solid product	78.8 ± 5.1	28.0 ± 6.4	<0.001
Solution	69.1 ± 7.0	52.7 ± 4.0	<0.001
Solution + excess RM-β-CD	72.5 ± 4.2	41.2 ± 4.4	<0.001

⋆Each value is the mean ± SD of eight determinations.$^{\dagger}P$ values (unpaired *t*-test) vs. uncomplexed nitroxide.

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