Novel pH-Sensitive Nitroxide Di- and Tri-radical Spin Labels

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Stable nitroxide di- and tri-radicals **2–4**, synthesized from diethylenetriamine or tris(aminoethyl)amine, display pH-sensitive EPR spectra owing to changes in through-space exchange upon protonation of the central amine nitrogen atom.

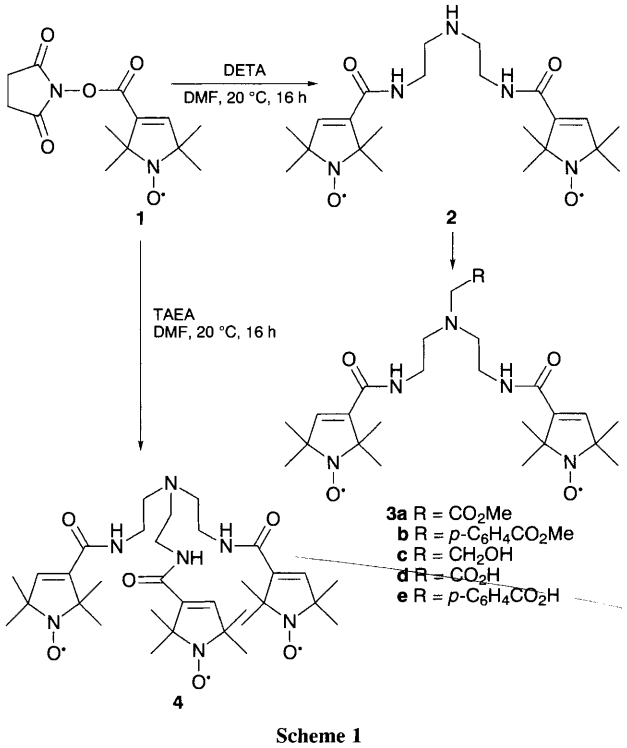
Stable nitroxide free radicals continue to constitute a widely used class of biophysical spin labels.[1] Electron paramagnetic resonance (EPR) spectroscopy provides information concerning the local environment and motional characteristics of nitroxide radicals without the requirement of optical transparency of the sample. The synthesis and chemistry of stable nitroxide free radicals and their use as spin labels in biophysical studies has been well reviewed.[2] Less attention has been paid to nitroxide diradicals,[3] which in some cases have advantages as spin labels over monoradicals.[4] Diradical EPR spectra are markedly influenced by the intramolecular interactions between the nitroxide centres and are characterized by the value of the spin–spin exchange integral J. Two mechanisms[3a] (see ref. 5, however) are considered to contribute to J: direct exchange by through-space overlap of the orbitals of the unpaired electrons, and indirect exchange interaction *via* bonds connecting the two radical centres. Direct exchange interactions are especially sensitive to structural factors and can result in EPR spectra that respond to changes in the environment of the radical centres.[3] Diradical spin labels have been successfully employed in the study of micelle formation,[4a] cyclodextrin complexation,[4b] enzyme mechanisms,[4c–f] and the structure and function of biomembranes.[4g–i]

Changes in EPR spectra caused by pH changes have been observed for diradicals containing two ionizable carboxylic acid or amino groups.[6] Spectral changes were attributed to an electrostatic repulsion between similarly charged moieties.[6a] Later, pH-sensitive mononitroxide spin labels[7] having an ionizable group in close proximity to the nitroxide radical centre were introduced.[8] Their use has been especially rewarding in studies of membranes biodynamics,[9] and has stimulated further development of nitroxides with pH-sensitive EPR spectra.[10]

In a continuation of our research program in the area of nitroxide polyradicals for spin labelling and MRI applications,[11] we report herein the synthesis and pH sensitivity of a new series of di- and tri-nitroxide spin labels containing a protonatable amino group. Diradical **2** and triradical **4** were prepared by acylation of diethylenetriamine (DETA) and tris(aminoethyl)amine (TAEA), respectively, with *N*-hydroxysuccinimide ester **1** in DMF (16 h, 20 °C). Functionalized derivatives **3a–c** were synthesized from **2** in high yields using standard alkylation or hydroxyethylation procedures† (Scheme 1). Diradical carboxylic acids **3d** and **e** were prepared by basic hydrolysis of the corresponding esters.‡

EPR spectra§ of diradicals **2** and **3a–c** in dilute solutions consisted of a five line pattern which was solvent and temperature dependent (direct exchange[3a]). Strong spin–spin exchange was observed for **2** and **3a–c** in toluene ($J \gg a_N$) and medium-to-strong ($J > a_N$) exchange was observed in water.

The EPR spectra are also pH dependent. Spin exchange increases as the pH is lowered. Apparently protonation of the central nitrogen atom decreases the angles between substituents, thus favouring conformations with closer nitroxide centres. The EPR spectra also show the expected increase in the relative



Scheme 1

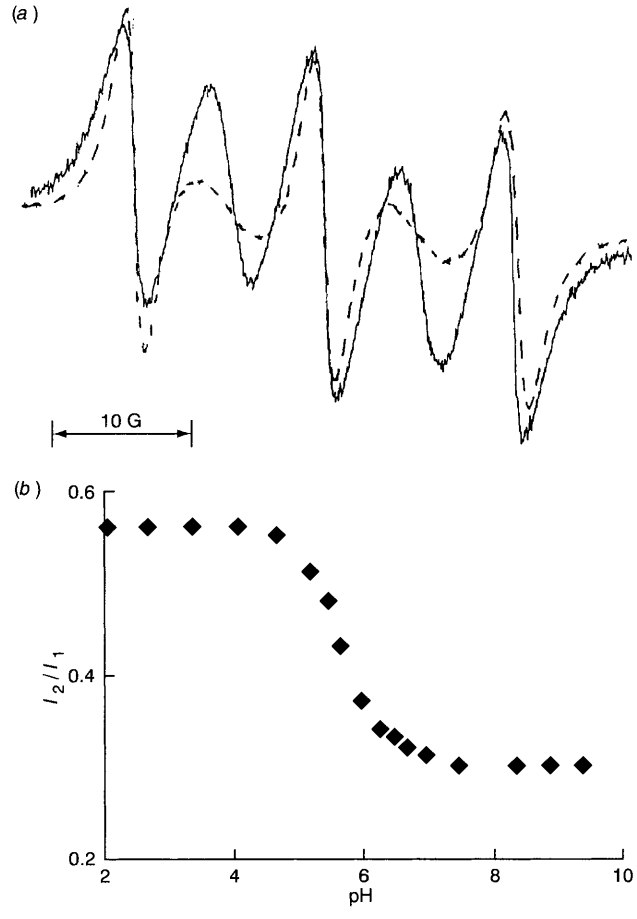


Fig. 1 (*a*) EPR spectra of diradical **3b** at pH = 10 (dashed) and pH = 2 (solid). (*b*) Titration curve for diradical **3c**.

Table 1 pH-Induced changes in EPR spectra of the di- and tri-nitroxides

	pH = 10		pH = 2.6		
Compound	a_N	I_2/I_2	a_N	I_2/I_1	S^a
2	15.8	0.27	15.6	0.38	41
3a	15.7	0.31	15.6	0.40	29
3b	15.9	0.20	15.5	0.73	265
3c	15.6	0.30	15.5	0.56	87
4	15.9	0.69	15.5	1.05	52

[a] $S = 100\cdot[(I_2/I_1)_{pH\ 2.6} - (I_2/I_1)_{pH\ 10}]/(I_2/I_1)_{pH\ 10}$ (see text).

intensity of the second and fourth 'diradical' lines upon protonation (Fig. 1). The ratio of intensity of the second line in the quintet relative to that of the first line (I_2/I_1) was measured at pH 2.6 and at pH 10 for nitroxides **2**, **3a–c** and **4**. The ratios were then used to calculate the pH sensitivity parameter S, where $S = 100\cdot[(I_2/I_1)_{pH\ 2.6} - (I_2/I_1)_{pH\ 10}]/(I_2/I_1)_{pH\ 10}$ for each nitroxide (Table 1). Whereas monoradical pH-sensitive spin labels show relatively small differences in the hyperfine splitting constant for protonated and non-protonated forms (Δa_N = *ca.* 1 G[7–10]; $S = 100\cdot[\Delta a_N/a_N]$ = *ca.* 100·1 G/15 G = *ca.* 7), these new diradicals as well as the triradical **4** show an increased range of measurement with respect to I_2/I_1 (*e.g.*, S = 265 for **3b**). A plot of I_2/I_1 *vs.* pH (inflection point, pH = *ca.* 5.8) is illustrated for **3c** [Fig. 1(*b*)].

The working range of these pH-sensitive spin labels is determined by the pK_a of the central amino group. The synthetic route is flexible and should accommodate a variety of available nitroxide units differing in their lipophilicity and/or redox properties.¶ The carboxyl groups in **3d** and **e** permit attachment of these pH-sensitive spin labels to biomolecular targets using conventional spin labelling techniques.[2c]

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Footnotes

† *Selected spectroscopic data* for **2**: Yield 86%; mp 134–135 °C·(from EtOAc); IR (KBr) ν/cm^{-1} 1658, 1649, 1619, 1610, 1555. For **4**: Yield 71%; mp 93–95 °C (from EtOAc); IR (KBr) ν/cm^{-1} 1653, 1611, 1545. For **3a**: By alkylation of **2** with methyl bromoacetate–K_2CO_3 in MeCN (4 h, 40 °C); yield 91%; mp 52–54 °C (hexane–EtOAc 1 : 1); IR (KBr) ν/cm^{-1} 1744, 1654, 1646, 1619, 1605, 1543. For **3b**: By alkylation of **2** with methyl 4-bromomethylbenzoate–K_2CO_3 in MeCN (16 h, 60 °C); yield 93%; mp 162–164 °C (hexane from diethyl ether); IR (KBr) ν/cm^{-1} 1722, 1661, 1539. For **3c**: By treatment of **2** with ethylene oxide (20x excess) in MeOH (3 d, 20 °C); yield 79%; mp 122–123 °C (hexane–EtOAc 1 : 2); IR (KBr) ν/cm^{-1} 1658, 1645, 1615, 1604, 1538. Satisfactory C, H, N elemental analyses were obtained for **2**, **3a–e**, and **4**.

‡ *Selected spectroscopic data* for **3d**: Yield 70%; mp 124–126 °C (EtOAc); IR (KBr) ν/cm^{-1} 1661, 1619, 1537. For **3e**: Yield 77%; mp 157–159 °C (diethyl ether from MeOH); IR (KBr) ν/cm^{-1} 1715, 1662, 1611, 1539.

§ EPR spectra were recorded on a Bruker ESP-300 spectrometer using 10^{-4} mol dm^{-3} solutions in a Wilmad WG-812 flat cell at 20 °C. Aqueous glycine buffers (0.1 mol dm^{-3})[6] were used as solvents; however, no significant changes were detected in plain water solutions or while using 5–10% of methanol as a co-solvent.[6] All pH-induced changes in spectra were reversible; hydrolysis products **3d–e** were not detected in the solutions of **3a–b** over the period of investigation (5–10 min).

¶ Bioreduction, should it occur to a significant extent, may be detected by double integration[12] of the EPR spectra.

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