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Sterically shielded spin labels for in-cell EPR spectroscopy: Analysis of stability in reducing environment

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Abstract
Electron paramagnetic resonance (EPR) spectroscopy is a powerful and widely used technique for studying structure and dynamics of biomolecules under bio-orthogonal conditions. In-cell EPR is an emerging area in this field; however, it is hampered by the reducing environment present in cells, which reduces most nitroxide spin labels to their corresponding diamagnetic *N*-hydroxyl derivatives. To determine which radicals are best suited for in-cell EPR studies, we systematically studied the effects of substitution on radical stability using five different classes of radicals, specifically piperidine-, imidazolidine-, pyrrolidine-, and isoindoline-based nitroxides as well as the Finland trityl radical. Thermodynamic parameters of nitroxide reduction were determined by cyclic voltammetry; the rate of reduction in the presence of ascorbate, cellular extracts, and after injection into oocytes was measured by continuous-wave EPR spectroscopy. Our study revealed that tetraethyl-substituted nitroxides are good candidates for in-cell EPR studies, in particular pyrrolidine derivatives, which are slightly more stable than the trityl radical.

Keywords: nitroxide reduction, aminoxyl radical, radical stability, spin labeling, trityl radical

Introduction

RNA molecules have a central role in cellular processes and gene regulation. Their three-dimensional structures and conformational dynamics are essential for their functions as biological catalysts, structural scaffolds, and regulators of gene expression [1]. Thus, information about structure and motion can give insights into RNA function and how it might be modulated. Besides X-ray crystallography [2], nuclear magnetic resonance (NMR) [3], and fluorescence spectroscopy [4], pulsed electron–electron double resonance (PELDOR or DEER) spectroscopy [5] has, over the past few years, demonstrated its applicability to map the global structure of nucleic acids [6,7] and other macromolecules [8,9] through distance measurements in the range of 1.5–8 nm, utilizing distance-dependent magnetic dipole–dipole interaction between two covalently attached aminoxyl (nitroxide) radicals. EPR spectroscopy also holds promise for in-cell measurements because there is no background interference, as can be the case with fluorescence spectroscopy. In fact, PELDOR was recently used to study structural aspects of nucleic acids [10,11] and proteins [12] inside intact *Xenopus laevis* oocytes. However, the short lifetimes of nitroxide spin labels under cellular conditions is a severe limitation to the general applicability of PELDOR for in-cell measurements [13].

In addition to the use as labels for EPR spectroscopy, cyclic nitroxide radicals are an important class of compounds for biological and medical applications [14–16], such as contrast agents for magnetic resonance imaging (MRI) [17], as antioxidants, and as superoxide dismutase mimics [18], where the nitroxide is involved in redox reactions by a one-electron exchange between its reduced and oxidized state [19]. Additionally, nitroxide radicals have been used to determine the partial pressure of oxygen [20] and pH [21] values in living tissues via EPR spectroscopy. The aforementioned applications of nitroxides in biological fluids are adversely affected when the paramagnetic center is readily reduced to the EPR-silent hydroxylamine [22].

Since the application of nitroxides inside living cells is of growing interest, extensive efforts have been taken for the design and synthesis of nitroxide radicals that are more resistant toward reduction [23,24]. Several factors affect the stability of nitroxides, such as ring size (piperidine, pyrrolidine, and isoindoline), the presence of heteroatoms within the ring (imidazolidine), substituents (neutral or charged), and the identity of the alkyl substituents in the positions adjacent to the nitroxide functional group. Although the stability of a variety of nitroxides in the presence of ascorbate has been reported [22,25–36], the biostability of radicals has invariably been investigated under different conditions [26–30,37–41]. Furthermore, each of these biostability studies has focused on a small number of radicals. The study of Kinoshita et al. is similar to the work described here in terms of the techniques and

conditions used for evaluation of radical stability. They also compared tetramethyl and tetraethyl piperidine nitroxides; however, our work additionally includes imidazolidine and isoindoline nitroxides as well as a triphenylmethyl (trityl) radical [37].

In this paper, we compare redox properties of a series of nitroxides varying with ring type, substituents, and charge, using several approaches with the aim of identifying radicals that are suitable for in-cell studies. A trityl radical, which has been shown to be relatively stable under reductive conditions [42], was chosen for comparison. We also determined the hyperfine coupling constants (A_{iso}) of all the nitroxides that are sensitive to the polarity around the nitroxide moiety, by continuous-wave (CW) EPR. With regard to the nitroxides, we focused on tetraethyl-substituted radicals with pyrrolidine-, piperidine-, isoindoline-, and imidazolidine-based structures and compared them with the more reactive tetramethyl-substituted analogs. The effect of electronegative and charged substituents on the nitroxide rings was also evaluated. In addition to determination of the kinetic and thermodynamic stability of the radicals in the presence of ascorbic acid, their stability was tested in a cytosolic extract from *X. laevis* oocytes and inside living oocyte cells.

Material and methods

Preparation of radicals

Chemicals were purchased primarily from Sigma-Aldrich Chemical Company and Acros, Belgium, and were used without further purification. Thin-layer chromatography (TLC) was performed on glass-backed TLC plates with extra hard layer (Kieselgel 60 F$_{254}$, 250 µm, Silicycle) and compounds were visualized using UV light at 254 nm. Silica gel (230–400 mesh, 60 Å) was purchased from Silicycle, and used for flash chromatography. Molecular masses of organic compounds were determined by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (Bruker, MicroTof-Q).

Tetramethyl-derived spin labels **1**, **2**, **3** and pyrrolidine spin label **12** were purchased, while tetraethyl piperidine derivative **4** was prepared according to the reported procedure [37] and used for the synthesis of compounds **5** and **6** [37,43]. Compounds **11** and **13**–**15** were synthesized as previously described [44–47].

Compounds **8** and **10** were prepared using a modification of previously reported method [48]. In the reported procedure for the synthesis of **8**, compound **16a** was oxidized and then nitrated to obtain **18a**. However, nitration gave multiple spots in our hands; therefore, compound **16a** was first nitrated to obtain compound **17a** (Scheme 1) [49]. Subsequent oxidation with meta-chloroperoxybenzoic acid (m-CPBA) yielded compound **18a** in moderate yields, which was converted to compound **8** using the previously published protocol [48]. This modified procedure was also used for the synthesis of the corresponding tetraethyl derivative **10**. Preparation of compound **8** using
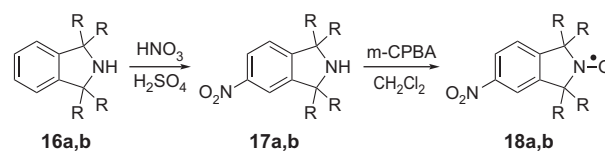
Scheme 1. Syntheses of isoindoline-derived radicals **18a** and **b**, used for synthesis of **8** and **10**; R is either methyl (**a**) or ethyl (**b**).

this strategy has recently been reported by Mileo et al. [50].

1,1,3,3-Tetramethyl-5-nitroisoindoline 18a

To a solution of compound **17a** (0.9 g, 4 mmol) in CH$_2$Cl$_2$ (5 mL) at 0°C, a solution of *m*-CPBA (1.4 g, 8.17 mmol) in CH$_2$Cl$_2$ (6 mL) was added. The resulting solution was stirred at 0°C for 1 h and then at 24°C for 2 h. The reaction mixture was diluted with CH$_2$Cl$_2$ (10 mL) and the organic layer was washed successively with an aqueous NaOH solution (2.5 N, 2×10 mL) and brine (10 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, and then concentrated *in vacuo*. The crude reaction mixture was purified by column chromatography (5% MeOH in CH$_2$Cl$_2$) to yield **18a** as a yellow solid (0.9 g, 94% yield). HR-ESI-MS: 258.0975 (M + Na), calcd. 235.0953 for C$_{12}$H$_{15}$N$_2$O$_3$.

Tetraethyl-5-nitroisoindoline 18b

To a solution of compound **17b** (0.120 g, 0.43 mmol) in CH$_2$Cl$_2$ (2 mL) at 0°C, a solution of *m*-CPBA (0.089 g, 0.5 mmol) in CH$_2$Cl$_2$ (1 mL) was added. The resulting solution was stirred at 0°C for 1 h and then at 24°C for 3 h. The reaction mixture was diluted with CH$_2$Cl$_2$ (5 mL) and the organic layer was washed successively with an aqueous NaOH solution (2.5 N, 2×10 mL) and brine (10 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, and then concentrated *in vacuo*. The crude reaction mixture was purified by column chromatography (30% ethyl acetate in petroleum ether) to yield **18b** as a yellow solid (0.1 g, 79% yield). HR-ESI-MS: 314.1602 (M + Na), calcd. 291.1601 for C$_{16}$H$_{23}$N$_2$O$_3$.

Cyclic voltammetry

Cyclic voltammetry experiments were performed in a 4 mM phosphate buffer solution (PBS; pH: 7.2) containing 1 mM radical concentration. A three-electrode cell arrangement was used encompassing glassy carbon working electrode, which was polished before each measurement, a reference electrode Ag/AgCl/KCl(3 M), and a double-wire platinum counter electrode. The following parameters were used: scan rate, 0.1 V/s; starting potential, 0.0 V; upper vertex potential, 1.0 V; lower vertex potential, -1.5; cathodic step potential, -0.00244 V; and anodic step potential, 0.00244 V. In case of compound **6**, the starting potential was -0.2 V, because of similar anodic and cathodic peak potentials.

Monitoring reduction of radicals in ascorbic acid and in cell extract using CW-EPR

Preparation of the crude cytoplasmic extracts from *X. laevis* oocytes was as previously described [10]; 19 μL of 5 mM ascorbic acid solution in 4 mM PBS (pH = 7.4), or 19 μL of cell extract were mixed with 1 μL of a 4 mM radical solution (final radical concentration, 200 μM). The solution was transferred into an EPR tube (1 mm inner diameter, Wilmad, USA) and the EPR signal intensity was measured as a function of time using a Bruker E500 CW-EPR spectrometer at X-band (9.43 GHz), using the following settings: modulation frequency, 9.43 GHz; time constant, 40.96 ms; microwave power, 1.0 mW; conversion time, 40.96 ms; modulation amplitude, 1.0 G; number of points, 1024; field sweep, 70 G; and sweeps, 160–170.

Monitoring reduction of radicals inside cells by CW-EPR

Oocytes from *X. laevis* trapped in stage VI, characterized by ca.1 mm diameter and ca.1 μL volume were used [10]. Samples were prepared by microinjection of ca. 40 nL of 4 mM spin label stock solution into 50 oocytes and subsequent incubation at room temperature for a variable time (0, 15, 30, 60, and 120 min) before freezing the cells in liquid nitrogen, until the radical concentration was determined by CW-EPR: microwave power, 0.2 mW; modulation amplitude, 2.0 G; number of scans, 10; and temperature, 125 K.

Results and discussion

Selection of radicals

Five classes of spin labels were investigated, each of which was based on a specific cyclic nitroxide, that is, piperidine (Figure 1A), isoindoline (Figure 1B), imidazolidine (Figure 1C), and pyrrolidine derivatives (Figure 1C) or a trityl radical (Figure 1C). The piperidine series served to investigate the effects of the substituents on the ring, all of which were electron withdrawing, except for the alkyl groups flanking the nitroxide. The isoindolines (Figure 1B) had ionizable groups to facilitate solubility in aqueous solutions—a sulfonate in compounds **7** [51,52] and **9** [51,52], and a tetraalkylammonium ion in compounds **8** and **10**. Each type of nitroxide was prepared as tetramethyl and tetraethyl derivatives. The trityl radical **15** was the only carbon-based radical in this study.

Thermodynamic parameters for reduction in the presence of ascorbic acid

Ascorbic acid, a reducing agent present to some extent in biological systems [53–55], is commonly used for evaluation of radical stability [29] and was used to investigate the thermodynamic parameters of the radicals. To determine the Gibbs free energy (ΔG) and the reaction equilibrium con-
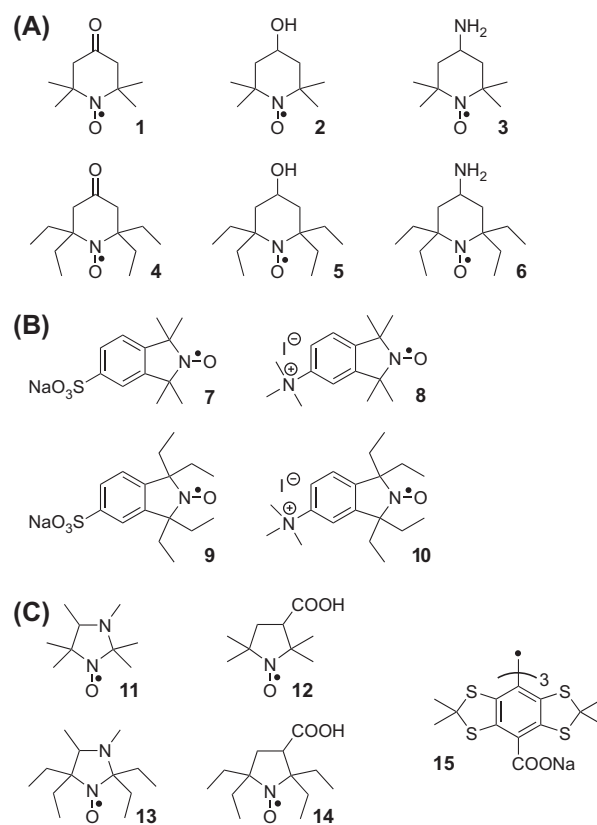

Figure 1. Radicals for evaluation of reductive stability. (A) Piperidine-based nitroxides. (B) Isoindoline-based nitroxides. (C) Imidazolidine- and pyrrolidine-based nitroxides and a trityl radical.

stant (K) for the reduction, the redox potential of each radical was measured by cyclic voltammetry. The half-wave potentials correspond to standard redox potentials as long as the diffusion coefficients of the reduced and oxidized state are equal, which is generally the case.

A typical voltammogram of a nitroxide, recorded by sweeping the potential of a glassy carbon electrode between −1.5 and 1 V (vs Ag/AgCl/KCl) in both the anodic and the cathodic direction, respectively, contains two peak couples (Supplementary Figure 4A to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.979409). The first peak couple appears at the anodic potential of the voltammogram and originates from the reversible oxidation of the nitroxide to an oxoammonium cation (Supplementary Figure 4B to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.979409). The peak separation is close to the theoretical value of 59 mV for a one-electron transfer. This reaction is not important in a biological context, where nitroxide radicals are reduced. The second peak couple represents the reduction of the nitroxide (Supplementary Figure 4C to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.979409) and the corresponding oxidation of hydroxylamine. In this case, the electron transfer to the nitroxide is coupled to a chemical reaction, making the peak separation larger. Since the redox behavior of trityl radicals differs significantly from

nitroxides, compound **15** was not studied by cyclic volta-mmetry.

The half-wave potentials ($E_{1/2}$) were determined from the cyclic voltammograms as the midpoint between the oxidation and the reduction peak potentials for the second peak couple: $E_{1/2} = (E_p^{ox} + E_p^{red})/2$ (Table I). The tetram-ethyl-substituted piperidines had the highest $E_{1/2}$ value (*ca.* -0.07 V), while the tetraethyl-substituted isoindo-line- and pyrrolidine-derived nitroxides displayed the low-est redox potential (-0.32 V), making them the most resistant to reduction. The differences between the stan-dard redox potential of ascorbic acid ($E_0 = -0.15$ V vs Ag/AgCl/KCl) and the measured $E_{1/2}$ of each nitroxide were used to determine ΔG and K for the reduction of the nitroxide with ascorbate [56] (Table I). The ΔG values for the reduction were negative for the tetramethyl-derived piperidines, thus making the nitroxide reduction energeti-cally favorable. In contrast, the tetraethyl-substituted nitroxides yielded ΔG values of about 6–16 kJ/mol and small equilibrium constants, showing that their equilib-rium lies toward the educts.

Using the equilibrium constants determined from the cyclic voltammetry measurements and known starting concentrations of educts, the equilibrium concentrations of the nitroxide radicals were calculated, normalized to the starting concentration ($[N]_{eq}/[N]_0$), and expressed in Table I as percentages. The equilibrium concentrations of the nitroxides in reaction with ascorbic acid were also deter-mined experimentally by following the intensity of the low-field nitroxide line of the EPR spectra as a function of time (Figure 2A). The decaying EPR signal was fitted with the pseudo-first-order reaction kinetic implicitly including the equilibrium signal as a fit parameter (see Kinetics of the reduction of radicals in ascorbic acid solution). This signal originates from radicals present at equilibrium and was compared with the equilibrium
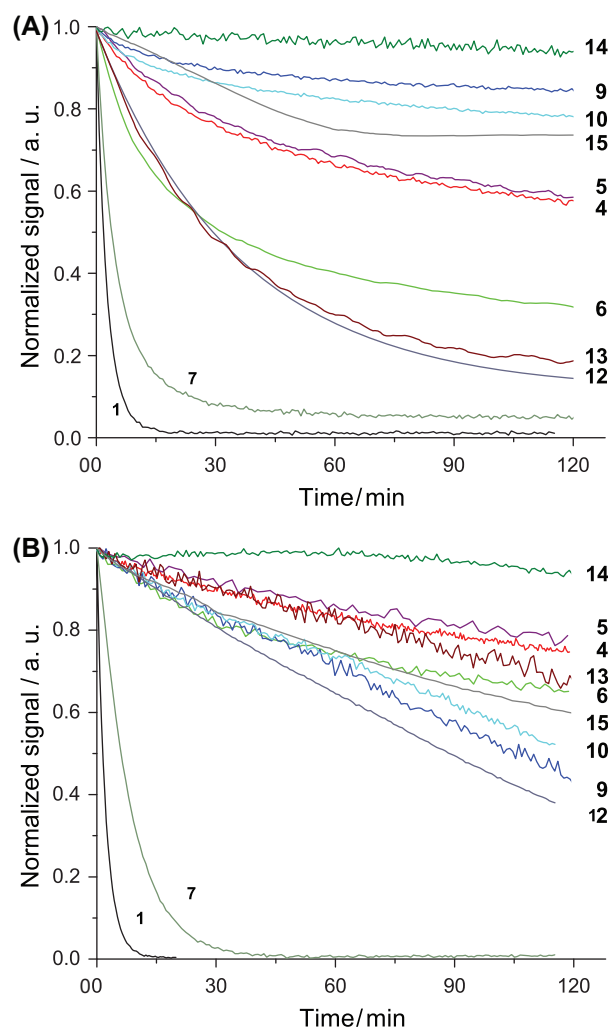

Figure 2. (A) Reduction of selected radicals with ascorbic acid (200 µM conc. of radical and 5 mM ascorbic acid in PBS buffer, pH: 7.2, except for compounds **1–3**, when ascorbate conc. was 1 mM). The EPR signal intensity is plotted as a function of time. (B) Decay curves for the radicals in a cytosolic extract.

Table I. Half-wave potentials ($E_{1/2}$) of nitroxide radicals, Gibbs free energy (ΔG) and equilibrium constants (K) for reduction of nitroxides with ascorbic acid, and equilibrium concentrations of the nitroxides (calculated and measured).

Rad.*	$E_{1/2}$[†] [V]	ΔG[‡] [kJ/mol]	$K = e^{-(\Delta G/RT)}$	$[N]_{eq}/[N]_0$ (%)	
				Calculated	Measured
1	-0.08	-7 ± 2	16 ± 5	1.4 ± 0.5	1.2 ± 0.1
4	-0.27	12 ± 1	0.009 ± 0.003	59 ± 6	53 ± 3
2	-0.08	-7 ± 2	16 ± 5	1.4 ± 0.5	1.4 ± 0.1
5	-0.27	12 ± 1	0.009 ± 0.003	59 ± 6	55 ± 2
3	-0.07	-7 ± 2	16 ± 5	0.9 ± 0.5	1.5 ± 0.3
6	-0.23	8 ± 1	0.04 ± 0.02	33 ± 6	31 ± 2
7	-0.16	1 ± 1	0.7 ± 0.3	4.5 ± 2.0	5.0 ± 0.5
9	-0.32	16 ± 1	0.0012 ± 0.0005	82 ± 4	84 ± 2
8	-0.16	0.5 ± 1	0.8 ± 0.3	3.8 ± 1.2	4.8 ± 0.5
10	-0.32	16 ± 1	0.0012 ± 0.0005	82 ± 4	76 ± 2
11	-0.16	1 ± 1	0.7 ± 0.3	4.5 ± 2.0	5 ± 2
13	-0.21	6 ± 1	0.09 ± 0.04	22 ± 5	16 ± 1
12	-0.18	3 ± 1	0.3 ± 0.1	9 ± 3	11 ± 1
14	-0.32	16 ± 1	0.0012 ± 0.0005	86 ± 4	91 ± 7

*Radicals.
[†]For the half-wave potential ($E_{1/2}$) the error is ± 0.01.
[‡]$\Delta G = -nF \Delta E° = -RT \ln(K)$.

concentrations of nitroxides that were calculated from the redox potentials. Comparison of the last two columns in Table I shows an excellent agreement between the equilibrium concentrations of the nitroxide radicals as measured by EPR spectroscopy and those calculated from the thermodynamic parameters. Thus, knowledge of the redox properties of radicals can be used to predict the equilibrium concentration with high precision, for their reduction with ascorbate anion.

Kinetics of the reduction of radicals in ascorbic acid solution

The large excess of ascorbate that was used for reduction of radicals ensured (pseudo) first-order kinetics and allowed fitting of the decay curve with a first-order exponential function: $[N] = [N]_{eq} + [N]_0 \times e^{-k't}$, where $[N]$ is the concentration of the radical at time t, $[N]_0$ is the initial concentration, and k' is the pseudo-first-order rate constant. $[N]_{eq}$ is a constant offset given by the thermodynamic equilibrium of nitroxide radicals under steady-state conditions. Dividing k' by the concentration of ascorbic acid yielded the bimolecular rate constants (Table II).

The calculated bimolecular rate constants confirmed that the rate of reduction depends on several factors: the size of the nitroxide ring system, the nature of the substituents, and shielding of the nitroxide moiety (Figure 2A and Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.979409). Replacing the methyl groups adjacent to the nitroxides with ethyl groups had the largest impact on radical stability, presumably by sterically restricting access

of reductants to the nitroxide [22,25]. The hyperfine coupling constants (A_{iso}) of the radicals were determined from their CW-EPR spectra (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.979409) and confirmed that the ethyl-derivatized nitroxides had a lower A_{iso} value than their corresponding methyl derivatives, as expected for a more hydrophobic environment. The steric effect was most pronounced in the case of the piperidine derivatives, where the tetraethyl-substituted nitroxides **4**, **5**, and **6** were found to be ca. 100 times more stable against reduction than the tetramethyl derivatives **1**, **2**, and **3**. These data are in agreement with the recently published study of Kajer et al. [57]. It has been shown that cyclohexyl or pyran groups do not further increase radical stability [22,58], making tetraethyl-derived nitroxide radicals good candidates for *in vivo* EPR.

Other substituents also affected stability of the radicals, although to a lesser extent than the alkyl groups adjacent to the nitroxides. Positively charged substituents decreased the stability of the radicals, presumably by attracting the ascorbate anion. For example, compound **3**, where the amino group is protonated at pH: 7, is threefold more reactive than compound **2**. The imidazolidine **13** was considerably less stable than the other tetraethyl derivatives, presumably due to protonation [44]. For the isoindoline derivatives, the effect of charge was not as pronounced, presumably because the charge is farther from the nitroxide functional group; the positively charged compound **8** is less than twofold more reactive than the negatively charged **7**, while no difference was observed for the tetraethyl-substituted compounds **9** and **10**.

Table II. Half-life of the radicals and their corresponding second-order reaction rate constant for reduction with ascorbic acid, and EPR signal intensities after incubation (2 h) with ascorbic acid in the cell extracts and inside oocyte cells.

Rad.*	Lifetime [s][†]	2nd order rate const [(M·s)$^{-1}$]	Extent of reduction[‡]		
			Asc.[§]	Cell extract	Oocyte cells
1	158 ± 1	5.75 ± 0.04	1	0	−
4	2600 ± 50	0.066 ± 0.001	57	75	34
2	165 ± 2	5.51 ± 0.005	1	0	−
5	2640 ± 60	0.058 ± 0.002	58	78	15
3	54 ± 2	16.8 ± 0.7	1	0	−
6	1480 ± 40	0.115 ± 0.003	31	65	66
7	365 ± 5	0.44 ± 0.01	5	0	−
9	1980 ± 50	0.079 ± 0.003	84	45	51
8	220 ± 3	0.74 ± 0.01	5	0	−
10	2350 ± 60	0.081 ± 0.001	78	52	55
11	100 ± 2	1.62 ± 0.04	5	0	−
13	1940 ± 30	0.084 ± 0.002	19	66	25
12	2160 ± 20	0.079 ± 0.001	14	38	−
14	85000 ± 30000	0.002 ± 0.001	94	94	88
15	10500 ± 1000	0.016 ± 0.002	74	60	70

*Radicals. The starting concentration of radicals was 200 mM and concentration of ascorbic acid was 5 mM, except for spin label **1–3** where it was 1 mM.
[†]Calculated as $t_1 = 1/k'$.
[‡]listed as a percentage, after reaction time of 2 h.
[§]Ascorbic acid.

The largest effect on the nitroxide stability of the tetramethyl-substituted nitroxides was caused by the structure of the nitroxide-bearing ring. The five-membered pyrrolidine, imidazolidine, and isoindoline derivatives were three- to ten-fold more stable toward reduction than the six-membered piperidine derivatives [32]. Similar rates of reduction for compounds **7** and **12** indicate that the pyrrolidine and isoindoline rings have similar stability. The slowest reduction rate was found to be that for the negatively charged tetraethyl pyrrolidine derivative **14**.

Radical stability in cell extract

The rate of radical reduction was also investigated in cytoplasmic extracts of oocytes and as in the case of ascorbate reduction, all tetramethyl-substituted radicals were rapidly reduced in the cellular extract (Figure 2B and Supplementary Figure 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.979409). The EPR time traces (Figure 2B) did not follow a single exponential decay, presumably due to the presence of various reducing agents (ascorbate, glutathione, cysteine, nicotinamide adenine dinucleotide [NAD] hydrogen, and NAD phosphate hydrogen) and a significant amount of dissolved oxygen in the cell extract. Therefore, signal intensities after reaction time of 2 h were used for comparing the stability of the radicals in the cytosolic fluid. As seen from Figure 2B and Table II, the most resistant radical is the pyrrolidine derivative **14**, which was 95% intact after 2 h, followed by the tetraethyl piperidines **4** and **5** (75%), tetraethyl imidazolidine and tetraethyl piperidine **6** (65%), trityl (60%), and tetraethyl-isoindoline (50%). The different relative stabilities of isoindoline *versus* piperidine nitroxides in the cell extract, compared with ascorbate reduction, are presumably due to the presence of different reducing agents in the cell. In addition, different rates of re-oxidation of the corresponding hydroxylamines to nitroxides by oxidants in the cell extract, including molecular oxygen, could also be a contributing factor [59].

Radical stability inside cells

As stated above, the primary aim of this study was to compare, under identical experimental conditions, the stability of potential spin labels for investigation of biomolecules in living cells by pulsed EPR spectroscopy. Therefore, the ultimate test was their persistence in oocyte cells. Charged radicals **6**, **9**, **10**, **14**, and **15** gave reduction profiles that are very similar to those recorded in cell extracts (Table II), presumably because they were distributed in the cytosol which should yield reduction kinetics similar to that of cell extracts (see Supplementary Figures 2 and 3 to be found online at http://informa healthcare.com/doi/abs/10.3109/10715762.2014.979409 for decay curves for all radicals in cytosolic extract and inside cells). In contrast, the reduction kinetics for non-charged radicals **4**, **5**, and **13** inside cells were much faster than in the cell extracts, e.g., only about 15% of **5** remained after 2 h. Radical **6**, containing the same ring size but carrying a protonated amino group, retained 65% of the signal. A possible explanation for this discrepancy is that the non-charged radicals could have entered intracellular membranes and been reduced rapidly by the electron transport chain components in the mitochondrial membrane [60]. Pyrrolidine **14** was the most persistent radical, retaining more than 85% of the signal, with the trityl radical **15** following closely behind (70%).

Conclusions

In conclusion, introduction of bulky ethyl groups next to a nitroxide group leads to significant stabilization against reduction by both ascorbic acid and the reductants present in living cells, yielding radicals that are more stable in cells than trityl radical **15**. The tetraethyl-substituted pyrrolidine-based nitroxide carrying a carboxylic group (**14**) demonstrated superior stability against reduction due to combination of sterical shielding, ring size, and charge, all of which are factors that should be taken into account for the design of spin labels for *in vivo* studies. Charged and neutral radicals showed different relative stabilities in cell extracts than in cells, indicating that conjugation to biomolecules could have a large effect on the stability of these radicals. The trityl radical exhibited considerable stability toward reduction, especially in cells.

Acknowledgements

Declaration of interest

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Supplementary material available online

Supplementary Figures 1–4 and Table I.