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Designing Molecular Probes to Prolong Intracellular Retention: Application to Nitroxide Spin Probes

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Abstract

Targeted delivery of molecular probes into cells enables cellular imaging through optical and magnetic modalities. Probe molecules that are well retained by cells can accumulate to higher intracellular concentrations, and thus increase the signal-to-noise ratio of, and widen the temporal window for, imaging. Here we synthesize a paramagnetic spin probe bearing six ionic functional groups and show that it has long intracellular half-life (> 12 hours) and exceptional biostability in living cells. We demonstrate that judicious incorporation of ionic substituents on probe molecules systematically increases intracellular retention time, and should therefore be beneficial to imaging experiments.

Introduction

Locating and tracking specific cell types *in situ* is a long-term goal in medicine, which should enhance clinical diagnostics (e.g., for evaluating tumor growth and metastasis) and offer insights into human physiology and disease that could lead to more effective therapies.

Currently, magnetic resonance imaging (MRI) is the foremost technique for imaging anatomical features with high spatial resolution. However, because MRI images ubiquitous endogenous protons, it has limited ability to provide physiological information. In contrast, electron paramagnetic resonance imaging (EPRI) is an emergent modality that images exogenous, paramagnetic molecular probes (so-called “spin-probes”). The use of spin probes confers unique advantages to EPRI. First, because spin probes can be targeted to specific cell types, they can highlight the target cells against the rest of the body. Second, spin probes can be chemically “tailored” to have flexible functionality. For example, spin probes can be

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tailored for desired pharmacokinetics or designed to report specific physiological information (e.g., oxygen tension, pH, redox status).^{2–10}

As for all imaging applications, signal-to-noise ratio (SNR) is crucial for *in vivo* EPRI. Upon delivery into cells, a spin probe that has a short intracellular lifetime limits the maximal attainable signal, and consequently shortens the time window for imaging. Conversely, a spin probe that has a long intracellular lifetime lengthens the temporal window for imaging, which enables greater signal averaging, and thus improves SNR. Therefore, development of spin probes with long intracellular lifetimes is desirable. Key determinants of a spin probe's intracellular lifetime are biostability and resistance to extrusion by cellular transporters. Owing to their excellent stability under physiological conditions,¹¹ pyrrolidinyloxyls (Chart 1) are well suited for *in vivo* cellular imaging applications.

We have shown that rational modification of the basic pyrrolidinyloxyl structure can increase resistance to extrusion by cellular organic anion transporters, and thus improve intracellular retention.¹ Specifically, when loaded into cells in culture, at 37 °C, nitroxides **1**, **2**, and **3** have intracellular half-lives of 6.8, 21, and 79 min, respectively (see Fig. 1). This comparison shows that nitroxides bearing more ionic groups are retained intracellularly for longer times. Furthermore, having both cationic and anionic functional groups may also be beneficial.^{1, 12}

Recently, we have used immunoliposomes to target delivery of nitroxide **3** (Fig. 1) to solid tumors in mice, where the tumor cells overexpress the human epidermal growth factor receptor 2 (HER2).^{13–14} However, because tumor cells did not retain nitroxide **3** long enough to permit imaging, our attempts to perform *in vivo* EPRI on the tumors were unsuccessful. Here, we report the chemical synthesis and biological testing of nitroxide **4** (Chart 2), which has a total of six ionically charged functional groups at physiological pH — four negative and two positive. Using cultured cells, we demonstrate that nitroxide **4** has superior intracellular retention — $t_{1/2}$ of ~13 hours, and exceptional biostability. Highly-charged nitroxides such as **4** should make *in vivo* EPRI of nitroxide-loaded tumors feasible.

Results and Discussion

Nitroxide **4** was prepared as its tetraethyl ester derivative **12**, which was assembled from the ethylenediamine-*N,N*-diacetate derivative **7** and *trans*-3,4-dicarboxypyrrolidinyloxyl **11**, as summarized in Scheme 1. Compound **7** was prepared as follows: ethylenediamine was monoprotected with Boc (**5**), then dialkylated with ethyl bromoacetate (**6**), followed by removal of Boc with trifluoroacetic acid (**7**). To prepare *trans*-3,4-dicarboxypyrrolidinyloxyl (**11**), 3-carbamoylpyrrolinyloxyl (**8**) was dehydrated with tosyl chloride to yield 3-cyanopyrrolinyloxyl (**9**), conjugate addition of cyanide to which gave the dicyanopyrrolidinyloxyl as a mixture of *cis* and *trans* isomers, which could be separated chromatographically on the basis of polarity difference. Once assembled, the tetraethyl ester **12** was hydrolyzed with tetrabutylammonium hydroxide and then alkylated with bromomethyl acetate to afford the tetrakis(acetoxymethyl) (AM) ester **13**.

To examine how well the highly charged nitroxide **4** is retained in cells, we loaded the nitroxide into cultured human T-lymphocytes by incubation with the AM ester **13**. With all carboxyl groups masked as AM esters, **13** can permeate into cells (Fig. 2). Once inside cells, abundant cellular esterases cleave the AM esters to liberate nitroxide **4** which, being multiply charged, is trapped and accumulated in the cell. Thus, the tetra-AM ester **13** may be viewed as the “prodrug” form of nitroxide **4**.

After incubation with the tetra-AM ester, we quantified intracellular and extracellular nitroxide **4** as a function of time. The time course for transfer of nitroxide **4** from the intracellular compartment to the extracellular medium is shown in Fig. 3A. The average of the half-lives from nonlinear least-squares curve fits to the two time courses was $t_{1/2} = 12.5 \pm 1.1$ h (or exponential lifetime of $\tau = 18.0 \pm 1.6$ h) at 37 °C. Such a long intracellular retention time should be ample for any *in vivo* imaging applications.

Because the environment inside the cell is reducing, intracellularly trapped nitroxide could be reduced and thus lose its EPR signal, which would be undesirable. To determine whether nitroxide **4** was being reduced, we measured the total amount of nitroxide (i.e., intracellular plus extracellular) at each time point. As shown in Fig. 3B, the total nitroxide content remained essentially constant over the 6-hour course of the experiment, at 4.69 ± 0.15 nmol. Thus nitroxide **4** is remarkably resistant to bio-reduction—a desirable characteristic for a spin probe designed for EPR imaging. This bodes well for the use of highly-charged nitroxides *in vivo*, where tissue O₂ levels are typically ~10%, compared with ~18% in cell culture *in vitro* (water-saturated air containing 5% CO₂ at 37 °C).

Knowing the total number of Jurkat lymphocytes (1.325×10^8), the mean cell volume (7.65×10^{-13} L),¹⁵ and the total amount of nitroxide initially loaded into the cells (4.69 ± 0.15 nmol) leads to the estimate that the intracellular concentration of nitroxide **4** was initially ~50 μM. This level of intracellular loading is comparable to what has been achieved when cells are incubated with the AM esters of common fluorescent polycarboxylate ion indicators,^{16–18} but is much lower than what we have achieved by incubation with the AM esters of nitroxides **1**, **2**, and **3**.^{1, 19} The simplest rationale for the difference in loading is the aqueous solubility of the AM esters. The tetra-AM ester **13** has MW = 834, approaching that of the AM esters of the common polycarboxylate indicators (e.g., fura-2 AM and fluo-4 AM, MW ~1,000), which are known to be sparingly soluble (< 1 μM) in aqueous media.¹⁸ In contrast, the AM esters of nitroxides **1** – **3** range in MW from 258 to 431. Since only dissolved AM ester molecules can permeate the cell membrane, the flux of AM esters into the cell is much less when the solubility is very low. Importantly, the K_m of the intracellular esterases has been estimated to be in the several-μM range.^{20–23} Therefore, hydrolysis of AM esters whose solubility is less than K_m would be expected to proceed with low velocity. Low AM ester solubility thus severely limits the intracellular accumulation of the hydrolyzed form of the probe molecules. Finally, it is important to stress that limitations of the AM ester do not affect the utility of the nitroxide in immunoliposome applications, where the nitroxides are encapsulated as their *salts* at high concentrations in the liposomes and delivered into cells through an entirely different mechanism—namely receptor-mediated endocytosis.

To put the present findings in perspective, we examined the relationship between intracellular half-life ($t_{1/2}$) to the total number of charged groups (Z_{tot}) in the probe molecule, as shown in Fig. 4. It can be seen that increasing the total number of ionic functional groups in the molecule resulted in systematic lengthening of the intracellular half-life. This validates the strategy of increasing the number of charged functional groups to deter extrusion, and thus enhance retention in the cytosol.

A final important issue to consider is the dependence of the EPR spectral linewidth on molecular structure. Linewidth (LW) is a determinant of signal-to-noise ratio because a narrower line has higher spectral amplitude. Molecular structure affects the tumbling correlation time, τ_c ; a larger molecule is expected to tumble more slowly and thus have a longer τ_c . Under typical physiological conditions, nitroxide spin probes are in the fast-tumbling regime, and one expects $LW \propto \tau_c$, i.e., a longer correlation time means a larger linewidth. The correlation time can be modeled by a modified form of the Stokes-Einstein

relation:²⁴ $\tau_c = \frac{C_{\text{slip}} \eta V}{kT}$, where η is the solvent viscosity, V is the volume of the molecule, k is the Boltzmann constant, T is the absolute temperature, and C_{slip} is an empirical parameter that accounts for deviation of the spin probe from spherical symmetry and for its interactions with the solvent ($0 < C_{\text{slip}} < 1$). Fig. 5 shows the X-band EPR spectra of nitroxides **1** and **4**; simulation of the spectra in EasySpin permits one to extract τ_c . Table 1 presents the values of τ_c and C_{slip} for nitroxides **1** and **4** determined in this study, as well as for nitroxide **8** and OX063, whose properties have been published.^{25–28}

Generally, the values in Table 1 show that, not surprisingly, bulkier molecules tend to have longer τ_c . Moreover, charged or highly polar substituents that are expected to interact strongly with water hinder tumbling. For example, nitroxide ²H₁₂-**8**, which is 3 – 5% larger than nitroxide **1**, has a τ_c that is actually 35% *shorter*. This is consistent with ²H₁₂-**8** having an amide substituent where **1** has a carboxylate; the latter is expected to interact more strongly with water molecules. Similarly, if nitroxides **1** and **4** strictly obey the Stokes-Einstein relation, then the ratio of the correlation times, $\tau_{c,4}/\tau_{c,1}$, should equal the ratio of the molecular volumes, $V_4/V_1 = 1400/576 = 2.43$, whereas the observed ratio is $135/20 = 6.75$. This deviation is reflected in nitroxide **4** having a larger C_{slip} , which takes into account two effects—six charged functional groups that can interact strongly with water and an extended structure that precludes isotropic tumbling (see Fig. 6). In this context it is interesting to compare nitroxide **4** with the highly symmetrical trityl radical, OX063 (Fig. 6): not quite doubling the molecular volume nearly quadruples τ_c . OX063 has three CO₂[−] and 12 OH groups, all of which are expected to interact with water thus hinder tumbling.

The above considerations suggest two ways to reduce τ_c : 1) reducing the number of charged functional groups, and 2) reducing the size and asymmetry of the molecule. In view of the finding that charged groups improve retention, we must reject option 1. In contrast, the structure of nitroxide **4** can be made much more compact while retaining the six essential charged functional groups. To further narrow the EPR lines and thereby increase signal amplitude, the pyrrolidinyloxyl ring can be isotopically substituted with ¹⁵N and deuterium.³¹ Synthesis of such nitroxides is under way.

Conclusions

Targeted delivery of molecular probes into cells enables cellular imaging through optical and magnetic modalities. Probe molecules that resist clearance from the intracellular space are retained for longer periods of time and can be accumulated to higher intracellular concentrations, and thus enhance the ease and the signal-to-noise ratio of imaging measurements. We show that judicious incorporation of ionic substituents on probe molecules can systematically increase intracellular retention time and should therefore be beneficial to imaging experiments.

Experimental Procedures

General

For organic synthesis, chemical reagents and solvents of ACS or higher grade from commercial vendors were used without further purification. TLC plates (Silica gel 60, EMD Millipore) were used for analytical chromatography, and silica gel (Silica Gel 60, 230 – 400 mesh, EMD Millipore) was used for flash chromatography. NMR spectra were recorded at 400 MHz (400 MR, Varian Instruments) in CDCl₃ and referenced to tetramethylsilane. Abbreviations denoting the multiplicity of NMR resonances are: s = singlet, d = doublet, t = triplet, m = multiplet. IR spectra were recorded in CHCl₃ on an FT-IR spectrometer (model 6100, JASCO, Inc.). High-resolution mass spectrometry (electrospray ionization) was performed at the mass spectrometry facility in the Chemistry and Biochemistry Department at the University of Maryland, College Park. Procedures and instrumentation for determination of rotational correlations times are detailed in a separate section below. For biological experiments, EPR spectra were acquired on a benchtop X-band spectrometer (microESR, Active Spectrum, Inc.). All cell culture media, reagents, and supplements were purchased from Gibco (Invitrogen).

1-(*tert*-Butoxycarbonyl)ethylenediamine (5)—The procedure reported by Yavin and colleagues was used.³² To a vigorously stirred solution of ethylenediamine (8.4 g, 9.3 mL, 140 mmol) in CH₂Cl₂ (50 mL) at room temperature, a solution of di-*tert*-butyl dicarbonate (3.05 g, 14 mmol) in CH₂Cl₂ (200 mL) was added dropwise over 6 h. Thereafter, the reaction mixture was stirred for 24 h, filtered, and washed with aqueous 2 M Na₂CO₃ (50 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered, and evaporated to dryness to give a thick oil (1.80 g, 80% yield). The crude product was used in the subsequent reaction without further purification. ¹H-NMR (CDCl₃): 1.40 (s, 2H, NH₂), 1.46 (s, 9H, *t*-butyl), 2.80 (t, 2H, CH₂CH₂NH), 3.18 (d, 2H, CH₂CH₂NH), 4.91 (br, 1H, NH).

***N*-*tert*-butoxycarbonyl-*N*',*N*'-bis(ethoxycarbonylmethyl)ethylenediamine (6)**—The synthesis was adapted from that reported by Hamachi *et al.*³³ Anhydrous K₂CO₃ (2.6 g, 18.8 mmol) was added to a solution of the Boc-protected ethylenediamine (5) (1 g, 6.3 mmol) in DMSO (7 mL), and the mixture was stirred at room temperature for 5 min. Ethyl bromoacetate (2.3 g, 1.53 mL, 13.8 mmol) was then added, and stirring was continued for 5 h. Thereafter, water (50 mL) was added, and the mixture was extracted with CH₂Cl₂ (4 × 50 mL). The combined organic phase was dried over anhydrous Na₂SO₄, filtered, and CH₂Cl₂ was removed by rotary evaporation. Residual DMSO was removed under high vacuum.

Chromatography on silica gel with petroleum ether:ethyl acetate (10:3) as eluent afforded compound **6** as a thick oil (1.5 g, 70% yield). ¹H-NMR (CDCl₃): 1.26 (t, 6H, -CH₂CH₃), 1.43 (s, 9H, *t*-butyl), 2.85 (m, 2H, NHCH₂CH₂N=), 3.15 (m, 2H, NHCH₂CH₂N=), 3.50 (s, 4H, -NCH₂CO-), 4.20 (q, 4H, -CH₂CH₃).

***N,N*-bis(ethoxycarbonylmethyl)ethylenediamine (7)**—The synthesis was adapted from Hamachi, *et al.*³³ A solution of trifluoroacetic acid (2.38 mL, 30.9 mmol) in CH₂Cl₂ (15 mL) was added dropwise over 30 min to a solution of the dialkylated, Boc-protected ethylenediamine (**6**) (0.7 g, 2.1 mmol) in CH₂Cl₂ (5 mL) maintained at 0 °C. The reaction mixture was stirred at room temperature for 3 h more; thereafter, rotary evaporation gave an oil. The oil was dissolved ethyl acetate (50 mL) and carefully washed with saturated aqueous NaHCO₃. The organic solution was dried over anhydrous Na₂SO₄, filtered, and evaporated to dryness on a rotary evaporator. The crude product **7** (0.33 g, 66% yield) was used in the subsequent step without further purification. ¹H-NMR (CDCl₃): 1.27 (t, 6H, -CH₂CH₃), 3.15 (m, 4H, NHCH₂CH₂N-), 3.62 (s, 4H, -NCH₂CO-), 4.19 (q, 4H, -CH₂CH₃), 7.55, (s, 2H, NH₂).

3-Cyano-2,2,5,5-tetramethyl-1-pyrrolinyloxyl (9)—To a stirred solution of 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrrolinyloxyl (**8**); 7.6 g, 41.5 mmol; prepared by the method of Rozantsev³⁴) in pyridine (50 mL) was added *p*-toluenesulfonyl chloride (14.4 g, 75.8 mmol), whereupon the solution became warm. The reaction mixture was kept at room temperature for 2 d. An aqueous solution of KOH (5 g in 125 mL water) was then added, and the mixture was heated to 80 °C. Upon reaching 80 °C the mixture was immediately cooled in an ice bath, and extracted with ether (4 × 100 mL). The ether solution was washed first with ice-cold dilute HCl (10%) until the pH of the aqueous wash solution was ~2–3, and then with saturated aqueous Na₂CO₃ until the pH of the aqueous wash solution was 10. After drying over anhydrous Na₂SO₄ and filtration, the ether solution was reduced to dryness by rotary evaporation. The resulting solid was purified on silica gel (4:1 hexane:ethyl ether) to yield compound **9** as a yellow solid (3 g, 45%) A small sample recrystallized from hexane had mp = 63 °C.³⁴

***trans*-3,4-Dicyano-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (10)**—To a solution of pyrrolinyloxyl **9** (3.2 gm, 14.4 mmol) in absolute ethanol (100 mL) was added an aqueous solution (100 mL) of KCN (4 g, 61.5 mmol) and NH₄Cl (3.4 g, 64 mmol); the reaction mixture was heated to 70 °C for 5 h. The reaction mixture was then cooled in an ice bath, and extracted with ether (4 × 100 mL). The combined organic phase was reduced to dryness by rotary evaporation. The residue was dissolved in ether; the solution was dried over anhydrous Na₂SO₄, filtered, and again reduced to dryness on a rotary evaporator. The crude solid was purified on silica gel: a small amount of the starting material was eluted with 3:1 hexane:ethyl ether; elution with 1:1 hexane: ethyl ether afforded *trans* 3,4-dicyano-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (**10**) as a yellow solid (1.7 g, 46%). A suspension of the solid in hexane was maintained at reflux and ethyl ether was added to achieve complete dissolution. Cooling the solution at –10 °C yielded yellow crystals; mp = 141 – 142 °C.³⁵ IR (CHCl₃): 2229 (CN) cm⁻¹.

***trans*-3,4-Dicarboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (11)**—Hydrolysis of *trans* 3,4-dicyano-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl by sodium hydroxide was performed, as described by Chatani and colleagues.³⁶ Aqueous NaOH (2 M, 60 mL) was added to the dicyano compound **10** (1 g, 5.2 mmol). The mixture was refluxed for several days until evolution of NH₃ ceased (judged by dampened pH paper). After cooling, the alkaline solution was extracted with ethyl ether (2 × 100 mL) to remove any starting material. The solution was then acidified to pH ~1 – 2 with cold HCl (10%), and extracted exhaustively with ethyl ether. The combined organic extract was dried over anhydrous Na₂SO₄, filtered, and dried by rotary evaporation to yield *trans* 3,4-dicarboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (**11**) as a light yellow powder (0.84 g, 70%), mp = 222 – 224 °C.³⁵

***trans*-3,4-bis[2-bis(Ethoxycarbonylmethyl)aminoethylaminocarbonyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (12).**^{35–38}—Diisopropylethylamine (0.36 g, 0.48 mL, 2.38 mmol) was added to a solution of *N,N*-bis(ethoxycarbonylmethyl)ethylenediamine (**7**) (0.33 g, 1.4 mmol) and *trans*-3,4-dicarboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (**11**) (0.15 g, 0.7 mmol) in 10 mL DMF and the mixture was stirred for 30 min. Thereafter, (benzotriazol-1-yloxy)tris(dimethyl-amino)phosphonium hexafluorophosphate (BOP) (0.62 g, 1.4 mmol) was added, and the mixture was stirred for 5 h at room temperature. Saturated NaCl solution (50 mL), saturated NaHCO₃ (5 mL) and CH₂Cl₂ (100 mL) were added to the reaction mixture. The organic layer was separated, dried over anhydrous Na₂SO₄, filtered, and dried by rotary evaporation; residual DMF was removed under high vacuum. The residue was purified on silica gel with CHCl₃:acetone (4:1) to yield compound **12** as a thick yellow oil, which eventually solidified at room temperature. Recrystallization by dropwise addition of ethyl ether to a boiling hexane solution afforded compound **12** as a light yellow powder (0.2 g, 44% yield); mp = 84 – 85 °C; IR (CHCl₃): 3335 (N-H), 1668 and 1735 (C=O) cm⁻¹; HRMS(ESI): [M+H]⁺ calcd 659.3736, found 659.3737.

***trans*-3,4-bis[2-bis(acetoxymethoxycarbonylmethyl)aminoethylaminocarbonyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (13)**—Methanolic tetrabutylammonium hydroxide (43.2% solution, 323 µL, 489 µmol) was added to the tetraethyl ester **12** (40.3 mg, 61.2 µmol), which rapidly dissolved to give a golden yellow solution. The solution remained homogeneous after addition of water (100 µL). The sealed flask containing the solution was maintained at 45 – 50 °C for 24 h. The solvent was removed by rotary evaporation. The residue was dissolved in water (1 mL) and aqueous 1 M HCl (172 µL) was added to adjust the pH to ~9; the solution was frozen and lyophilized to yield a fine powder. With magnetic stirring, the powder was dissolved in DMSO (dried over CaH₂; 300 µL). After sequential addition of diisopropylethylamine (63.2 mg, 85.2 µL, 489 µmol) and bromomethyl acetate (74.7 mg, 47.9 µL, 489 µmol), the reaction mixture was stirred under dry argon at 65 – 70 °C for 40 h. The residue remaining after of all volatile components have been removed under high vacuum was purified by flash chromatography on silica gel (1:9 hexane:ethyl acetate containing 1% v/v Et₃N and 0.5% v/v EtOH) to yield the tetraacetoxymethyl ester **13** as a light yellow solid (15.1 mg, 29.6%). HRMS(ESI): [M+H]⁺ calcd 835.3335, found 835.3353.

Cell culture

Jurkat lymphocytes (gift of Dr. Alfredo Garzino-Demo) were grown at 37 °C in suspension culture, in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% (v/v) fetal bovine serum, and equilibrated with humidified air containing 5% CO₂. Culture density was maintained at 1.5×10^6 cells/mL.

Loading cells with AM ester 13 for studying intracellular retention of nitroxide 4

Tetra-AM ester **13** (14.7 mg, 17.6 µmol, in 600 µL DMSO) was mixed with 330 µL of Pluronic F-127 (20% w/v in DMSO). The mixture was dispersed into 200 mL of serum-free RPMI 1640 medium (final concentration of tetra-AM ester was 88 µM). The medium was used to suspend 6.63×10^8 Jurkat lymphocytes. The cell suspension was divided equally between two T-75 culture flasks, which were charged with carbogen (5% CO₂, 95% O₂), and gently rocked at room temperature for 3 h. Thereafter, the cells were sedimented by centrifugation at 400 *g*, resuspended in fresh serum-free RPMI medium equilibrated with carbogen, and gently rocked for a further 50 min at room temperature to ensure complete de-esterification of the intracellular nitroxides. The cells were then centrifuged, washed with fresh serum-free RPMI medium by resuspension, sedimented again, and finally resuspended in 200 mL serum-free RPMI.

The final cell suspension was equipartitioned into 5 15-cm plastic Petri dishes, with each dish receiving 40 mL of suspension containing 1.325×10^8 cells. The dishes were maintained in an incubator at 37 °C under a humidified air containing 5% CO₂. At 0, 45, 90, 180 and 360 min, a dish was harvested by transferring the cell suspension to a 50-mL culture tube. The cells were spun down at 400 *g*; 38 mL of supernatant medium were withdrawn; any remaining supernatant was rapidly removed by suction to leave the cell pellet. The 38-mL supernatant aliquots were rapidly frozen in liquid N₂, lyophilized to dryness, and reconstituted with 0.75 mL deionized water (18.2 MΩ·cm).

To each cell pellet was added 250 µL of 300 µM digitonin solution (15 µL 20 mM digitonin in DMSO per mL water containing 0.5 mM Na₄EGTA). The pellet was dispersed by repeated aspiration through a 1-mL plastic pipette tip. The lysate was sonicated for ~12 sec in a sonicator bath (Branson 2510), frozen with liquid N₂ and immediately thawed. The sonication/freeze-thaw cycle was performed 3 times. The final thawed lysate was centrifuged at 21,920 *g* for 10 min at 10 °C to sediment cell debris and produce a clear lysate.

A separate suspension of lymphocytes *not* incubated with the AM ester was processed identically to produce nitroxide-free supernatant medium and clear lysate for preparing standard samples of nitroxide **4**.

Analysis of cell lysates

The EPR spectra of the lysates and the reconstituted supernatant media from the experimental group were recorded. The spectra of standard samples of nitroxide **4** at known concentrations in nitroxide-free lysate and supernatant media were also recorded. Because the corresponding experimental and standard samples are in the same solution medium,

matrix effects are controlled for, which enables quantitative comparison of the spectra. Thus, the concentration of nitroxide **4** in each experimental sample could be determined.

The time course of the fraction of intracellular and extracellular nitroxide **4** (y vs t data) was fit to the linearized exponential function, $\ln(y - y_0) = A - t/\tau$ to determine the exponential time constant, τ . Total nitroxide at each time point is the sum of the moles of intra- and extracellular nitroxide **4**. The intracellular concentration of nitroxide **4** was calculated with the knowledge that the total amount of nitroxide **4** was originally in 1.33×10^8 Jurkat lymphocytes, whose mean volume is 7.65×10^{-13} L.¹⁵

Determination of tumbling correlation times

EPR spectroscopy was performed on an X-band spectrometer (EMX, Bruker). Measurements were conducted at room temperature (20 – 22 °C). Nitroxides (K^+ salts) were dissolved at 100 μ M in 0.9% (wt/vol; 0.154 M) NaCl containing 10 mM HEPES buffer at pH 7.4. Samples were placed in thin Teflon tubing, which were contained in quartz EPR tubes. As required, samples were de-oxygenated by passing N_2 through the space surrounding the Teflon tubing. Spectra of air-saturated and de-oxygenated samples were recorded. Oxygen removal did not significantly change the spectral envelope, which suggests that the line shape is dominated by hyperfine couplings of the multiple inequivalent protons. Spectral line shapes were simulated using EasySpin³⁹ software to permit calculation of the tumbling correlation time; anisotropic g and A values were assumed to be similar to values reported previously.²⁹

To estimate molecular volumes, the geometries of three molecules (nitroxides **1** and **4**, and the trityl radical, OX063) were optimized by the PM3 semiempirical method implemented in HyperChem⁴⁰ (vers. 7.52; HyperCube). For each molecule, 7 – 11 structures were optimized from different starting conformations, and the volumes of all optimized structure was computed and averaged. In all cases, standard deviations were less than 1% of the average volumes.

For calculations using the modified Stokes-Einstein relation, the viscosity of 0.154 M NaCl at 21 °C was taken to be $\eta = 0.995$ cP, calculated using tabulated values of temperature-and concentration-dependent density and viscosity of NaCl solutions.^{41–42}

Data analysis

Numerical and statistical analyses were performed through OriginPro software (OriginLabs). ANOVA was used to assess differences between means. Data are presented as mean $\pm$ standard deviation.

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Abbreviations

AM	acetoxymethyl
Boc	<i>tert</i> -butoxycarbonyl
BOP	(benzotriazol-1-yloxy)tris(dimethyl-amino)phosphonium hexafluorophosphate
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl-sulfoxide
EPR	electron paramagnetic resonance
EPRI	electron paramagnetic resonance imaging
MRI	magnetic resonance imaging
SNR	signal-to-noise ratio

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Figure 1.
Ionic pyrrolidinyloxyls and their intracellular retention half-lives at 37 °C.

Figure 2. Loading of nitroxide 4 into cells by incubation with the tetra-AM ester 13

The tetra-AM ester **13** permeates the plasma membrane to enter a cell. Cellular esterases cleave the AM esters to unmask the negatively charged carboxyl groups to yield nitroxide **4** which, being highly charged at physiologic pH, is retained intracellularly.

Figure 3. Fate of intracellular nitroxide 4

(A) Time course of intracellular and extracellular fractions of nitroxide **4** at 37 °C. Data at nominally 0, 0.75, 1.5, 3, and 6 hr (intracellular fraction: open blue circles; extracellular fraction: filled red circles). Curves are single-exponential time courses fit to the data (see Experimental Procedures for details). Half-life derived from the least-squares fit is $t_{1/2} = 12.5 \pm 1.1$ hr. (B) Total nitroxide (intracellular + extracellular) at various time points; average of the five time points = 4.69 ± 0.15 nmol.

Figure 4. Dependence of intracellular half-life ($t_{1/2}$) on total number of ionic groups (Z_{tot})

Numerals refer to nitroxide structures in Fig. 1 and Chart 2. Blue filled circles are experimentally determined $t_{1/2}$ in minutes. The red curve is the least-squares fit to a single-exponential function ($\log t_{1/2} = 4.426 - 4.281e^{-\frac{Z_{\text{tot}}}{5.891}}$). The exponential function was chosen because it has an asymptotic upper bound, but is not intended to have mechanistic implications. Data for nitroxides **1** – **3** are from our earlier study.¹

Blue traces are X-band EPR spectra recorded from deoxygenated aqueous solutions of 50 μM nitroxides **1** and **4**. Red traces are spectra simulated in EasySpin.

Figure 6. Structures of three radicals

Geometry-optimized structures of nitroxides **1** and **4** and the trityl OX063 (see Experimental Procedures for details). Color scheme: gray = C, white = H, blue = N, red = O, yellow = S.

Scheme 1.

a) (*t*-BuO₂C)₂O, CH₂Cl₂; *b)* BrCH₂CO₂Et, K₂CO₃, DMSO; *c)* F₃CCO₂H, CH₂Cl₂; *d)* Tosyl-Cl, pyridine; *e)* KCN, NH₄Cl, EtOH/H₂O; *f)* NaOH; *g)* BOP, *i*-Pr₂EtN, DMF; *h)* *i.* Bu₄NOH, *ii.* BrCH₂O₂CCH₃, *i*-Pr₂EtN DMSO.

2,2,5,5-tetramethyl-
1-pyrrolidinyloxy
or "proxyl"

Chart 1.
Pyrrolidinyloxy

Chart 2.
Pyrrolidinyloxyl derivative bearing six ionic functional groups

Table 1

Tumbling correlations times of radicals in water

	MW	V (Å ³) ^a	τ_c (ps)	C_{slip}
Nitroxide 1	185.2	576	20 ^{b,c}	0.14
Nitroxide 4	544.5	1,400	135 ^b	0.39
Nitroxide 8 (2,2,5,5- ² H ₁₂)	195.1	593	13 ^d	0.09
Trityl OX063	1357.8	2,730	500 ^e	0.75

^aVolume of structure optimized by semi-empirical modeling (see Experimental Procedures for details).^bAverage of values determined under aerated and deoxygenated conditions, which were not significantly different.^cCompare with 19 ps determined by pulse methods.²⁹^dReported in Biller et al. (2011)²⁵ for the dodecadeuterio analogue of **8** (structure shown in Scheme 1).^eLiterature value.^{27–28, 30}