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Design of liposome-based pH sensitive nanoSPIN probes: nano-sized particles with incorporated nitroxides

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Abstract

Liposome-based nanoSized Particles with Incorporated Nitroxides, or nanoSPINs, were designed for EPR applications as pH probes in biological systems. Phospholipid membrane of the liposomes with incorporated gramicidin A showed selective permeability to a small analyte, H⁺, while protecting entrapped sensing nitroxide from biological reductants. An application of the pH-sensitive nanoSPIN in an ischemia model in rat heart homogenate allows for monitoring ischemia-induced acidosis while protecting encapsulated nitroxide against bioreduction.

Introduction

Nitroxyl radicals (NRs) are very useful functional EPR probes allowing measurement of redox state,¹ oxygen,² pH,^{3–5} NO,^{6–8} thiols,^{9–11} and structural studies of biological macromolecules.¹² However, comparatively fast reduction of the NRs to EPR-silent hydroxylamines limits their application. NRs encapsulation in the inner aqueous phase of inert nanospheres, such as lipid vesicles, may result in the development of stable paramagnetic probes. The semipermeable membrane of these nanospheres prevents chemical interactions of the probe with the reducing microenvironment, while retaining the probe's ability to monitor small analytes (*e.g.* NO or H⁺). We term these paramagnetic sensors nanoSPINs (nanoSized Particles with Incorporated Nitroxides). Swartz *et al.* were the first to demonstrate that NRs incorporated in liposomes¹³ or proteinaceous microspheres¹⁴ have higher resistance to bioreduction and might be useful probes for *in vivo* EPR oximetry. Later our labs^{8,15} and others¹⁶ used encapsulation of NO-sensitive NRs in phospholipid liposomes to protect extremely unstable nitronyl nitroxides against bioreduction. In this paper we describe the pH-sensitive nanoSPINs based on liposomal encapsulation of the imidazoline NRs.

Imidazoline NRs were found to be the most effective spin pH probes due to the presence of protonatable N-3 atom in the vicinity to the N–O• radical fragment.¹⁷ While a wide variety of pH-sensitive imidazoline NRs have been synthesized, the development of more stable probes remains a critical step for their successful applications, particularly *in vivo*.^{18,19}

Materials and methods

Chemicals

pH-sensitive membrane-impermeable NRs were synthesized as described below. Egg α -phosphatidylcholine and L-glutathione were purchased from Sigma-Aldrich. Gramicidin A was purchased from CALBIOCHEM. Triarylmethyl free radical, TAM Oxo63, was a gift from Nycomed Innovations Co (Malmö, Sweden).

Synthesis of trimethylammonium methyl sulfate, nitroxide NR1

The nitroxide **NR1** was prepared through alkylation of 4-(2-aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazole, **a2**, according to the Scheme 1.

4-(2-Aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazole, **a2**, was synthesized through hydrolysis of the iminophosphorane derivative **a1** described previously.²⁰

4-[(2-Dimethylaminoethyl)amino]-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazole **a3**. Formic acid (90%, 12.5 mmol) was added portion-wise to an ice-cooled solution of amine **a2** (0.5 g, 2.5 mmol) in a 35% solution of formaldehyde (5.5 mmol). The resulting mixture was heated in an oil bath at 60 °C for 30 min, diluted with water, basified (NaOH) to pH 12, and extracted with ether (7 × 5 mL). An organic extract was dried over K₂CO₃, the solvent was removed under reduced pressure, and **a3** was obtained as yellow crystals (0.4 g, 1.8 mmol, 70%); mp 89–90 °C dec (hexane–EtOAc, 1 : 1); IR (KBr, cm⁻¹) 3325 (N(CH₃)₂), 1627 (C=N), 1548 (NH). Anal. calcd. for C₁₁H₂₃N₄O × 1/6H₂O: C, 57.39; H, 10.14; N, 24.35. Found: C, 57.37; H, 9.94; N, 24.10. Mass spectrum (M⁺): calcd. for C₁₁H₂₃N₄O 227.18718, found 227.18862.

[2-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-4-yl-amino)ethyl] trimethylammonium methyl sulfate, NR1. (CH₃O)₂SO₂ (0.034 mL, 0.33 mmol) was added to a solution of **a3** (0.075 g, 0.33 mmol) in dry ether (5 mL) and the resulting mixture was allowed to stay at ambient temperature for 6 h. The residue, obtained after evaporation of solvent, solidified after triturating with an ether–CH₃CN mixture (20 : 1) to give **NR1** as pale yellow crystals (0.071 g, 0.2 mmol, 61%); mp 115–117 °C dec (ether–CH₃CN, 2 : 1); IR (KBr, cm⁻¹) 3353 (NH), 1628 (C=N), 1543 (NH). Anal. Calcd for C₁₃H₂₉N₄O₅·0.25H₂O: C, 43.63; H, 8.25; N, 15.66. Found: C, 43.53; H, 8.24; N, 15.39.

Synthesis of the nitroxide NR2

2-(4-(Chloromethyl)phenyl)-4-(dimethylamino)-2,5,5-triethyl-2,5-dihydro-1*H*-imidazol-1-oxyl, **b9**, was prepared according to Scheme 2.

The nitroxide **b7** was prepared from 3-hydroxyamino-3-ethylpentan-2-one hydrochloride **b1**¹⁸ using a modified general procedure.^{21,22} The nitroxide **b7** was then treated with methane sulfonyl chloride to give methanesulfonate **b8**, a highly reactive nitroxide. The latter undergoes nucleophilic substitution of methanesulfonate group with chloride anion upon acidification with hydrochloric acid to give **b9**. The nitroxide **b9** is stable in the form of hydrochloride and can be used as alkylating pH-sensitive spin label.

(4-(4,4-Diethyl-5-methyl-3-oxy-4*H*-imidazol-2-yl)phenyl)methanol, **b3**. 4-Hydroxymethyl-benzaldehyde was prepared from benzene-1,4-dicarbaldehyde (Aldrich) using the procedure described previously.²³ The crude 4-hydroxymethyl-benzaldehyde (2.5 g, *ca.* 17 mmol) was added to a solution of 3-hydroxyamino-3-ethylpentan-2-one hydrochloride **b1** (3 g, 17 mmol) and ammonium acetate (6 g, 78 mmol) in 50 mL of 50% aqueous ethanol. The solution was

stirred for 7 h. The ethanol was removed under reduced pressure and crude 5,5-diethyl-2-(4-(hydroxymethyl)phenyl)-4-methyl-2,5-dihydroimidazol-1-ol (**b2**) was extracted with chloroform. The chloroform extract was dried over MgSO_4 , filtered and stirred for 1 h with PbO_2 (5 g). The oxidant was filtered off, chloroform was removed under reduced pressure and the residue was separated on silica gel column, eluent chloroform, to give 3.8 g (87%) of **b3**, light-orange crystals, mp 109–111 °C (hexane–EtOAc 1 : 1), (Found, %: C, 69.37; H, 7.65; N, 10.79; Calcd for $\text{C}_{15}\text{H}_{20}\text{N}_2\text{O}_2$: C, 69.20; H, 7.74; N, 10.76); $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$ 3257 br, 2971, 2934, 2870, 2817, 1611, 1585, 1565, 1547, 1497, 1459, 1395, 1389, 1371, 1353, 1294, 1266, 1201, 1174, 1115, 1055, 1019, 842, 830, 766, 719, 681, 561; $\lambda_{\text{max}}(\text{EtOH})/\text{nm}$ 335 (log ϵ 3.88), 254 (4.38); $\delta_{\text{H}}(300 \text{ MHz}; \text{CDCl}_3)$ 0.46 (6 H, t, J 7.2 Hz, $2 \times \text{CH}_3$, Et), 1.68 and 2.00 (each 2H, AB q, J_{q} 7.2 Hz, J_{AB} 14.5 Hz, $2 \times \text{CH}_2$, Et), 2.16 (s, 3H, 5- CH_3), 4.25 (br, 1H, OH), 4.66 (s, 2H, CH_2), 7.35, 8.51 (AA'BB', 4H, J 8.0 Hz, Ar); $\delta_{\text{C}}(75 \text{ MHz}; \text{CDCl}_3)$ 7.00 and 7.12 (CH_3 , 4-Et), 17.07 (5- CH_3), 28.28 and 28.29 (CH_2 , 4-Et), 64.13 (CH_2), 90.04 (4-C), 147.82 (C=N–O), 179.79 (C=N), 144.55 (Ar, p-i), 125.15 (Ar, i), 127.72 (Ar, o), 126.42 (Ar, m).

2-(4-(Hydroxymethyl)phenyl)-4,4-diethyl-4*H*-imidazole-4-carbaldehyde oxime 3-oxide, **b4**. Na (1 g, 41 mmol) was dissolved in isopropanol (30 mL); after the reaction becomes slow the mixture was heated to 60 °C until Na was completely dissolved. The solution was allowed to cool to room temperature to form a suspension of *i*-PrONa. Isopropyl nitrite (3.5 mL, 39 mmol) and a solution of **b3** (3.8 g, 14.5 mmol) in 20 mL of isopropanol were added subsequently to the stirred suspension of *i*-PrONa in isopropanol and the mixture was allowed to stay for 2 h. After the reaction was complete (TLC, Silufol UV-254, eluent EtOAc) the mixture was acidified with AcOH to pH 6–7 and isopropanol was removed in vacuum. A saturated solution of NaCl (20 mL) was added to the residue and the precipitate of **b4** was filtered off and recrystallized from EtOAc. Yield 3.0 g (71%), brown powder, mp 207–210 °C, (Found, %: C, 62.43; H, 6.59; N, 14.22; Calcd for $\text{C}_{15}\text{H}_{19}\text{N}_3\text{O}_3$: C, 62.27; H, 6.62; N, 14.52); $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$ 1611, 1571, 1538, 1474, 1454, 1424, 1403, 1365, 1306, 1206, 1048, 1018, 820, 720; $\lambda_{\text{max}}(\text{EtOH})/\text{nm}$ 380 (log ϵ 3.49), 274 (4.37); $\delta_{\text{C}}(75 \text{ MHz}; (\text{CD}_3)_2\text{SO})$ 0.56 (6 H, t, J 7.2 Hz, $2 \times \text{CH}_3$, Et), 2.12 (2H) and 2.24 (2H, AB q, J_{q} 7.2 Hz, J_{AB} 14.5 Hz, $2 \times \text{CH}_2$, Et), 4.71 (s, 2H, CH_2), 4.60 (br, 1H, C–OH), 8.04 (c, 1H, N=CH), 7.52, 8.63 (AA'BB', 4H, J 8.0 Hz, Ar); $\delta_{\text{C}}(75 \text{ MHz}; (\text{CD}_3)_2\text{SO})$ 7.60 (CH_3 , Et), 31.09 (CH_2 , Et), 64.26 (CH_2), 91.52 (4-C), 144.44 (C=N–OH), 150.51 (C=N–O), 173.17 (C=N), 145.64 (Ar, i), 125.56 (Ar, p-i), 128.57 (Ar, o), 127.16 (Ar, m).

2-(4-(Hydroxymethyl)phenyl)-4,4-diethyl-4*H*-imidazole-5-carbonitrile 3-oxide, **b5**. TsCl (1.68 g, 8.8 mmol) was added portion-wise to a stirred solution of oxime **b4** (2.55 g, 8.8 mmol) in a mixture of CHCl_3 (35 mL) and triethylamine (2.5 mL, 17.6 mmol). The resulting solution was stirred for 1 h, washed with water and dried over MgSO_4 . CHCl_3 was removed in vacuum and the residue was separated by column chromatography (Kieselgel 60, Merck, eluent chloroform) to give **b5**. Yield 2.14 g (90%), yellow crystals, mp 112–116 °C (hexane–*tert*-butyl methyl ether–isopropanol 1 : 1 : 1). (Found, %: C, 66.36; H, 6.29; N, 15.33; Calcd for $\text{C}_{15}\text{H}_{17}\text{N}_3\text{O}_2$: C, 66.40; H, 6.32; N, 15.49); $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$ 3464, 2974, 2926, 2894, 2858, 2222, 1609, 1536, 1520, 1474, 1458, 1424, 1387, 1349, 1306, 1204, 1180, 1059, 1015, 989, 963, 836, 819; $\lambda_{\text{max}}(\text{EtOH})/\text{nm}$ 400 (log ϵ 3.62), 283 (4.33); $\delta_{\text{H}}(300 \text{ MHz}; \text{CDCl}_3)$ 0.70 (6H, t, J 7.2 Hz, $2 \times \text{CH}_3$, Et), 2.12 (4H q, J_{q} 7.2 Hz, $2 \times \text{CH}_2$, Et), 4.76 (s, 2H, CH_2), 7.51, 8.56 (AA'BB', 4H, J 8.0 Hz, Ar); $\delta_{\text{C}}(75 \text{ MHz}; \text{CDCl}_3)$ 7.18 (CH_3 , Et), 29.05 (CH_2 , Et), 64.50 (CH_2), 92.72 (4-C), 111.55 (C $\equiv$ N), 149.19 (C=N–O), 148.75 (C=N), 144.76 (Ar, p-i), 124.56 (Ar, i), 127.28 (Ar, o), 126.76 (Ar, m).

[4-(5-Dimethylamino-4,4-diethyl-3-oxy-4*H*-imidazol-2-yl)-phenyl]-methanol, **b6**. Liquid cold (0 °C) dimethylamine (5 mL, 75 mmol) was added to a solution of **b5** (2.0 g, 7.4 mmol) in CHCl_3 (30 mL). The reaction mixture was allowed to stay for 5 h, diluted with ethanol (5 mL), washed with brine, dried over Na_2CO_3 . The solvent was removed in vacuum and the

residue was recrystallized from CHCl_3 – CCl_4 3 : 1 to yield **b6** (1.7 g, 80%), yellow crystals, mp 208–210 °C, (Found, %: C, 66.19; H, 8.07; N, 14.42; Calcd for $\text{C}_{16}\text{H}_{23}\text{N}_3\text{O}_2$: C, 66.41; H, 8.01; N 14.52); $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$ 2968, 2931, 2875, 1610, 1541, 1440, 1406, 1380, 1265, 1136, 1120, 1066, 1034, 1020, 961, 943, 887, 855, 826, 764; $\lambda_{\text{max}}(\text{EtOH})/\text{nm}$ 368 (log ϵ 3.76), 268 (4.45); $\delta_{\text{H}}(300 \text{ MHz}; \text{CDCl}_3)$ 0.66 (6 H, t, J 7.2 Hz, $2 \times \text{CH}_3$, Et), 1.85 and 2.24 (each 2H, AB q, J_{q} 7.2 Hz, J_{AB} 14.5 Hz, $2 \times \text{CH}_2$, Et), 3.16 (s, 6H, N– CH_3), 4.62 (s, 2H, CH_2), 4.93 (br, 1H, OH), 7.31 and 8.51 (AA'BB', 4H, J 8.0 Hz, Ar); $\delta_{\text{C}}(75 \text{ MHz}; \text{CDCl}_3)$ 7.85 (CH_3 , Et), 27.44 (CH_2 , Et), 37.90 (N– CH_3), 64.03 (CH_2), 84.06 (4-C), 150.19 (C=N–O), 170.47 (C=N), 145.08 (Ar, p-i), 125.49 (Ar, i), 128.63 (Ar, o), 125.95 (Ar, m).

2-(4-(Hydroxymethyl)phenyl)-4-dimethylamino-2,5,5-triethyl-2,5-dihydro-1*H*-imidazole-1-oxyl, **b7**. A 1 M solution of EtMgBr in THF (10 mL) was added dropwise to a stirred solution of **b6** (1.3 g, 4.5 mmol) in THF (20 mL). The reaction mixture was allowed to stay for 0.5 h. Then water (3 mL) was added dropwise under vigorous stirring followed by MnO_2 (3 g, 34.5 mmol) addition. The mixture was stirred vigorously for 2 h, diluted with *t*-BuOMe (40 mL), the oxidant was filtered off and the filtrate was dried over Na_2CO_3 . The solvent was removed in vacuum and the nitroxide **b7** was isolated from the residue by column chromatography on Al_2O_3 , eluent CHCl_3 . Yield 0.7 g (50%), orange crystals, mp 89–91 °C (hexane), (Found, %: C, 68.13; H, 9.15; N, 13.16; Calcd for $\text{C}_{18}\text{H}_{28}\text{N}_3\text{O}_2$: C, 67.89; H, 8.86; N, 13.20); $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$ 3224 br, 2972, 2938, 2875, 1595, 1573, 1458, 1412, 1307, 1269, 1219, 1103, 1063, 1020, 968, 949, 935, 910, 883, 814, 784; $\lambda_{\text{max}}(\text{EtOH})/\text{nm}$ 218 (log ϵ 4.30).

4-(Dimethylamino)-2-(4-(chloromethyl)phenyl)-2,5,5-triethyl-2,5-dihydro-1*H*-imidazol-1-oxyl hydrochloride, **b9**. A solution of nitroxide **b7** (630 mg, 2 mmol) and triethylamine (0.3 mL, 4 mmol) in dry chloroform (10 mL) was cooled to –10 °C and methanesulfonyl chloride (0.156 mL) was added dropwise upon stirring. The mixture was stirred for 1 h, then the solution was washed with water and dried over Na_2SO_4 . The solvent was removed in vacuum and the nitroxide **b8** was isolated from the residue by column chromatography on silica gel, eluent CHCl_3 , as an orange oil, yield 530 mg (67%). The nitroxide **b8** was immediately dissolved in diethyl ether (15 mL) and 7% aqueous hydrochloric acid was added dropwise to the solution upon stirring until the pH of the water phase reached 1. The aqueous solution was separated and water was removed in vacuum. The crystalline residue was dissolved in acetonitrile (*ca.* 3 mL), filtered and dry diethyl ether (20 mL) was added. The mixture was allowed to stay for 10 h at –10 °C, the crystalline precipitate of nitroxide **b9** was filtered off and washed with diethyl ether, overall yield 390 mg (50%), mp 167–172 °C, (Found, %: C, 57.62; H, 7.41; N, 11.05; Cl, 18.79; Calcd for $\text{C}_{18}\text{H}_{28}\text{N}_3\text{OCl}_2$: C, 57.91; H, 7.59; N, 11.26; Cl, 18.99); $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$ 3432 br, 2971, 2934, 2878, 2604 br, 1674, 1494, 1458, 1413, 1387, 1307, 1281, 943, 907, 845, 826, 812.

Synthesis of the nitroxide NR2 from b9 precursor—NR2 was synthesized through the alkylation of glutathione with 2-(4-(chloromethyl)phenyl)-4-dimethylamino-1-oxyl-2,5,5-triethyl-2,5-dihydro-1*H*-imidazol **b9**. Nitroxide **b9** (6.8 mg, 20 μmol) was added to a solution of glutathione (10 mmol, 1 mL) and pH was brought to 11 by adding NaOH. The reaction mixture was allowed to stay for 5 h. The unreacted nitroxide **b9** was extracted with chloroform ($3 \times 0.7 \text{ mL}$). Yield of the nitroxide **NR2** (3.1 mM, 1 mL), determined by EPR, was 31%. The **NR2** has distinguishable EPR spectrum from NR **b9** reflecting longer rotation correlation time in agreement with its larger molecular weight ($M_{\text{w}} = 607$ and the ratio of amplitudes of central- and high-field spectral components, $I_{\text{c}}/I_{\text{h}} = 1.6$ for the **NR2**; $M_{\text{w}} = 336$ and $I_{\text{c}}/I_{\text{h}} = 1.2$ for the NR **b9**). Mass spectrum of the **NR2-H**⁺ (M^+): calcd. for $\text{C}_{28}\text{H}_{44}\text{N}_6\text{O}_7\text{S}$ 608.29, found 608.3.

Liposomes preparation

Liposomes preparation by extrusion—Large (200 nm diameter) unilamellar liposomes from egg phosphatidylcholine (PC) were prepared by slightly modified extrusion method described previously²⁴ using LiposoFast extruder (Avestine, Inc., Ottawa, Canada). A mixture of the ethanol solutions of PC (50 mg, 0.5 mL) and gramicidin A (25 μ M, 40 μ L) was dried on the walls of rotating cylinder under the nitrogen flow and then kept under the vacuum for 0.5 h. For EPR experiments, the lipid film was hydrated by moderate shaking for 1 h in 1 mL of 70 mM phosphate buffer, pH 7.2, containing 1 mM NR. The resulting suspension was passed through the extruder. In order to remove spin label from outer liposomal volume, the liposome suspension was passed through the gel-filtration column (Sephacrose CL2B, 10 $\times$ 0.8 cm) equilibrated with the same buffer and liposome fraction was collected.

Preparation of liposomes by reverse-phase evaporation—Large unilamellar liposomes were prepared by slightly modified reverse-phase evaporation approach described previously.²⁵ PC (45 mg) was dissolved in chloroform (3 mL). A solution of gramicidin A (25 μ M, 40 μ L) in chloroform was added. The mixture was placed to a 50 mL round-bottom flask with a long extension neck, and the solvent is removed under reduced pressure by a rotary evaporator. Then lipid was redissolved in the diethyl ether, and 1 mL of 1–2 mM solution of the NR in 50 mM phosphate buffer, pH 7.4, was added. The resulting two-phase system is sonicated for 5 min in a bath-type sonicator (Branson 3510) until the mixture becomes a homogeneous opalescent dispersion that does not separate for at least 30 min after sonication. The sonication temperature is 0–5 $^{\circ}$ C. The mixture is then placed on the rotary evaporator and the organic solvent is removed under reduced pressure. The liposomes were purified from the nonencapsulated NR by passing through a Sephadex G25 column.

The prepared liposome-based nanoSPINs were characterized and used on the day of preparation. The leakage of the probe from inner space of the liposomes was monitored by addition of membrane-impermeable paramagnetic broadening agent, potassium ferricyanide, and was less than 10% after 24 h incubation at room temperature.

EPR studies of oxygen consumption and ischemia-induced acidosis in rat heart homogenate

A heart was excised from a Sprague-Dawley rat (about 300 g) and kept in liquid nitrogen before homogenization. The heart was homogenized in the presence of an equal volume of NaCl isotonic solution. The homogenate was centrifugated at 4 $^{\circ}$ C, 8000 g and supernatant was collected for further experiments.

The oxygen-sensitive EPR probe, triarylmethyl radical Oxo63, was used to measure oxygen consumption in the rat heart homogenate after addition of succinate. The dependence of the EPR linewidth of Oxo63 probe on oxygen concentration was calibrated in 0.1 M phosphate buffer, pH 7.0 and 37 $^{\circ}$ C, saturated by various nitrogen–oxygen gas mixtures using a Temperature and Gas Controller (Noxygen, Germany).

The EPR spectra of the nitroxide **NR2** and encapsulated **NR2** were measured in separate experiments in the presence of homogenate after addition of 10 mM succinate at 37 $^{\circ}$ C. The decay of the integral intensity of the EPR signal and the nitrogen hyperfine splitting, a_N , measured as the distance between low- and high-field spectral components, were used to calculate NR concentration and pH of the sample, respectively. The temperature of the sample during the experiment was controlled by a Temperature and Gas Controller (Noxygen, Germany).

EPR measurements

EPR measurements were performed in 50 μ L capillary tubes using a Bruker X-band EMX spectrometer. Parameters of the acquisition were as follows: microwave power, 20 mW; scan time, 20.97 s; modulation amplitude, 1.0 G for the **NR1** and 1.5 G for the **NR2**. For the Oxo 63 TAM probe the spectra acquisition parameters were as follows: microwave power, 0.6 mW; modulation amplitude, 0.1 G; scan time, 20.48 s.

Characterization of the synthesized compounds

The IR spectra were recorded on a Bruker Vector 22 FT-IR spectrometer in KBr pellets (the concentration was 0.25%; the pellet thickness was 1 mm).

The UV spectra were measured on a HP Agilent 8453 spectrometer in EtOH.

The NMR spectra were recorded on a Bruker AV 300 (300.132 MHz for ^1H and 75.476 MHz for ^{13}C) spectrometer for 5–10% solutions at 300 K using the signal of the solvent as the standard. The assignment of the signals in the ^{13}C NMR spectra was made based on analysis of intensities, on the spectra measured in *J*-modulation mode.

Results and discussion

Design of liposome-based pH-sensitive nanoSPINs requires the following: (i) synthesis of membrane impermeable pH probe to avoid probe leakage from the inner liposomal space; and (ii) incorporation of membrane pores to ensure free movement of analyte proton. Fig. 1 illustrates the realization of this concept using phospholipid liposomes with incorporated gramicidin A channels.²⁶ To prevent diffusion of NRs across phospholipid membranes, imidazoline nitroxides **NR1** and **NR2** bearing a positively charged trimethylammonium group and a highly hydrophilic membrane-impermeable tripeptide glutathione moiety, correspondingly, were synthesized (Schemes 1 and 2).

Both **NR1** and **NR2** were found to be localized in the inner aqueous space of the liposome as confirmed by addition of membrane-impermeable paramagnetic broadening agent, potassium ferricyanide, to the bulk solution of the liposomes (see Fig. 2 for the **NR1**). No significant leakage of the NRs was observed during several hours of exposure.

Fig. 3 displays the EPR spectra of the **NR1** at various pH. The EPR spectrum measured at pH close to pK_a of the NR represents superposition of the spectra of protonated, NRH^+ , and nonprotonated, NR, forms of the radicals as clearly seen from the high-field spectral components in Fig. 3B. The ratio $[\text{NRH}^+]/[\text{NR}]$ reversibly varies with pH according to the Henderson–Hasselbalch equation providing an experimental tool for pH determination by EPR. In general, spectral simulation is required for accurate $[\text{NRH}^+]/[\text{NR}]$ determination. In practice, nitrogen hyperfine splitting measured as a distance between unresolved spectral components can be used as a highly sensitive pH marker as shown in Fig. 4.

Liposomes prepared without the gramicidin pore sustained a transmembrane pH gradient of several units of pH.^{27,28} Incorporation of gramicidin A channels resulted in dissipation of the transmembrane proton gradient allowing for the monitoring of extraliposomal pH by the encapsulated spin pH probe. Indeed, similar pH dependences of a_N were obtained for the NR free in solution and encapsulated in liposomes (see Fig. 4).

Fig. 5 illustrates the protective effect of the liposomal encapsulation of the **NR2** against a physiologically relevant reducing agent, ascorbate. Insignificant, about 5%, loss of EPR signal, apparently attributed to slow transmembrane diffusion of ascorbic acid,²⁹ was observed in the presence of 10 mM ascorbate (100 fold excess of the ascorbate over the NR) after 25 min of

incubation. Note that EPR signal of the **NR2** free in solution was practically undetectable after 25 min incubation in the presence of significantly lower, 1.5 mM, concentration of ascorbate (see Fig. 5).

We applied **NR2** encapsulated in gramicidine-containing liposomes to monitor ischemia-induced acidosis in the rat heart homogenates. The oxygen consumption by homogenate oxidative metabolism was measured by EPR using oxygen-sensitive probe Oxo63. As shown in Fig. 6C oxygen level dropped down below detectable level 10 min after succinate addition. During this time the encapsulated **NR2** kept 85% of its signal intensity. On the other side, EPR signal of the **NR2** added to homogenate free in solution was practically undetectable after 10 min of incubation (see Fig. 6A). Therefore, use of **NR2** containing nanoSPINs allowed us for accurate measurement of the a_N and calculation of corresponding pH values shown in Fig. 6B.

Previously we applied pH-sensitive imidazoline NR with similar range of pH sensitivity (pK 6.1) to monitor ischemia-induced acidosis in isolated rat heart using L-band EPR-spectroscopy.⁵ However, the loss of about 90% of the EPR signal intensity of the NR incubated in the heart during 30 min of global ischemia limits its application. The pilot experiment using the encapsulated **NR2** in the ischemic rat heart shows four times higher stability of the EPR signal (data not shown), therefore supposing pH-sensitive nanoSPINs to be a useful tool for the studies of myocardial acidosis.

Conclusion

In this work we formulated the concept of a stable paramagnetic nano-sized analytical probe, nanoSPIN, and designed the first liposome-based nanoSPINs for pH monitoring in biological systems using EPR spectroscopy. NanoSPINs significantly enhance the stability of the nitroxide probes, and may become useful analytical tools, particularly taking into account the importance of pH status in various physiological and patho-physiological processes, *e.g.* extracellular pH in tumors^{30,31} and ischemia-induced myocardial acidosis. Further improvements in stability of the liposomes may be achieved using archaeobacterial lipids^{32,33} or UV-induced cross-linking polymerization of lipid monomers.^{34–36} An alternative strategy to nanoSPIN design may be based on preparation of capsules with a polyamide membrane³⁷ used for encapsulation of enzymes or preparation of artificial cells.³⁸ Recent developments of *PEBBLEs* (nanosized Photonic Explorers for Bioanalysis with Biologically Localized Embedding)³⁹ and polymer nanocapsules⁴⁰ demonstrate alternative strategies for encapsulation of molecular probes within an inert matrix which can be explored for the design of the nanoSPINs.

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Fig. 1. Schematic design of pH-sensitive nanoSPINs. The phospholipid membrane of the liposomes with incorporated gramicidin A showed selective permeability to a small analyte, H^+ , while protecting the entrapped sensing nitroxide from biological reductants.

Fig. 2.

EPR spectra of aqueous solution of **NR1** before (red line) and after (blue line) addition of broadening agent, 10 mM potassium ferricyanide: (a) 1 mM **NR1** in 70 mM sodium phosphate buffer, pH 7.2; (b) 1 mM **NR1** in inner aqueous space of large (200 nm diameter) unilamellar liposomes prepared from egg phosphatidylcholine, 40 mg mL⁻¹, by extrusion method. The spectrometer settings were the same except for the gain: 2×10^3 (a) and 2×10^4 (b).

Fig. 3.
EPR spectra of 0.1 mM solution of the **NR1** in 50 mM sodium phosphate buffer measured at various pH: (A) 7.06; (B) 4.21; (C) 2.56.

Fig. 4. pH dependences of nitrogen hyperfine splitting, a_{N_i} , for the **NR1** and **NR2** in aqueous solution (○) and in gramicidin-containing liposomes (●). Solid lines correspond to the best fit of experimental data to standard titration equation yielding pK_a values for the **NR1** and **NR2**, 4.15 ± 0.15 and 6.2 ± 0.1 , respectively (mean values of three different sample preparation).

Fig. 5. Kinetics of the **NR2** reduction measured from its EPR signal intensity decay in aqueous solution of various concentrations of sodium ascorbate: 0.4 mM (Δ), 0.75 mM ($\circ$) and 1.5 mM ($\blacksquare$). Symbols ($\bullet$) denote the time evolution of the concentration of the **NR2** encapsulated into liposomes after addition of 10 mM sodium ascorbate.

Fig. 6.

Oxygen depletion and ischemia-induced acidosis in the rat heart homogenate measured using **NR2** containing nanoSPIN. (A) Kinetics of the **NR2** reduction measured from its EPR signal intensity decay in the rat heart homogenate. **NR2** encapsulated into gramicidin-containing liposomes (●) or free in solution (○) was added to homogenate simultaneously with addition of 10 mM succinate. (B) Ischemia-induced pH decrease calculated from the EPR spectra of the encapsulated **NR2** using calibration shown in the Fig. 5. (C) Time evolution of the linewidth of the Oxo63 probe during the incubation in rat heart homogenate. The narrowing of the linewidth from about 205 mG to 175 mG corresponds to decrease in oxygen content from 15% to that below detectable level.

Scheme 1.
Synthetic route to the **NR1** nitroxide.

Scheme 2.
Synthetic route to the nitroxide **NR2**.