Supporting Information

Nitroxide-fluorophore double probes: a potential tool for studying membrane heterogeneity by ESR and fluorescence

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1. Absorption and emission spectra

1.1 Procedures

All absorption spectra were recorded with 5·10⁻⁵ M solutions of probe in methanol. Fluorescent spectra were recorded with 10⁻⁷ M solutions of probe in methanol. Reduction of nitroxide of double probe: to the 10⁻⁴ M solution of the probe was added solution of ascorbic acid (20 equiv) in methanol, the mixture was allowed to stand for 20 min and then diluted to the final concentration with methanol.

Labelling of liposomes with probes: solvent was removed under reduced pressure from 100 µL of 10⁻⁴ M stock solution of the probe in methanol. Alternatively the nitroxide was first reduced as described above with solution of ascorbic acid in methanol and than the solvent was removed under reduced pressure. To the resuting film of probe 10 µL of 10⁻³ M solution of TRITON X-100 in PBS buffer (pH = 7.4) and 990 µL of PBS buffer were added. To the 40 µL of the obtained solution 13,3 µL of liposomes (lipid concentration was 3·10⁻³ M, preparation is described below) were added and the mixture was diluted to the final volume of 4 mL with PBS buffer. Concentrations of components in the final mixture were 10⁻⁷ M for tested probe, 10⁻⁷ M for TRITON X-100 and 4·10⁻⁵ M for lipids.

The liposomes were prepared by a reverse phase evaporation method originally described by Moscho et al. (Moscho, Orwar, Chiu, Modi, & Zare, 1996) and later modified by Kulin et al. (Kulin, Kishore, Helmerson, & Locascio, 2003). The lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was dissolved in chloroform at a concentration of 30 mM. From this solution, 50 µL was added to a 50 mL round-bottom flask. Then, 3 mL of aqueous phase (10 mM Hepes buffer with 11 mM MgCl₂, pH = 7.4) was carefully added along the side of the flask wall using a pipet. The organic solvent, chloroform, was then removed in a rotary evaporator under reduced pressure. The final pressure was 40 mm of Hg at 40 °C while rotating at 40 rpm.

1.2 Spectra

![Figure S1](image1.png)

Figure S1. Left: Normalised absorption and fluorescence emission spectra shown together with fluorescence emission spectrum after reduction of the nitroxide of double probe 16a in methanol (λ<sub>ex</sub> = 450 nm). Right: Fluorescence emission spectra of double probe 16a incorporated into the liposomes before and after reduction of the nitroxide (λ<sub>ex</sub> = 470 nm).
Figure S2. Left: Normalised absorption and fluorescence emission spectra of probe 16b in methanol ($\lambda_{\text{ex}} = 450$ nm). Right: Fluorescence emission spectra of probe 16b incorporated into the liposomes ($\lambda_{\text{ex}} = 470$ nm).

Figure S3. Left: Normalised absorption and fluorescence emission spectra shown together with fluorescence emission spectrum after reduction of the nitroxide of double probe 19a in methanol ($\lambda_{\text{ex}} = 450$ nm). Right: Fluorescence emission spectra of double probe 19a incorporated into the liposomes before and after reduction of the nitroxide ($\lambda_{\text{ex}} = 470$ nm).

Figure S4. Left: Normalised absorption and fluorescence emission spectra of probe 19b in methanol ($\lambda_{\text{ex}} = 450$ nm). Right: Fluorescence emission spectra of probe 19b incorporated into the liposomes ($\lambda_{\text{ex}} = 470$ nm).
2. ESR spectra

2.1 Procedures

Liposomes were prepared from 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (60%), cholesterol (40%) and PBS buffer (50 mg/mL) by lipid film method.

Labelling: a stock solution of probes in methanol (10⁻³ M) was added into a test tube. Ethanol was removed by a stream of nitrogen to obtain a film of compounds 1, 16a and 19a on the test tube wall. Liposomes were added to the film and the test tube was shaken for 30 min.

2.2 Spectra

Figure S5. Normalised ESR spectra of liposomes labelled with 1, 16a and 19a, spectra were recorded at 308 K.
3. $^1$H and $^{13}$C NMR spectra

Figure S6. $^1$H-NMR spectrum of compound 5 (300 MHz, CDCl$_3$).

Figure S7. $^{13}$C-NMR spectrum of compound 5 (75 MHz, CDCl$_3$).
Figure S8. $^1$H-NMR spectrum of compound 7 (300 MHz, CDCl$_3$).

Figure S9. $^{13}$C-NMR spectrum of compound 7 (75 MHz, CDCl$_3$).
Figure S10. $^1$H-NMR spectrum of compound 9 (300 MHz, CDCl$_3$).

Figure S11. $^{13}$C-NMR spectrum of compound 9 (75 MHz, CDCl$_3$).
Figure S12. $^1$H-NMR spectrum of compound 12 (300 MHz, CDCl$_3$).

Figure S13. $^{13}$C-NMR spectrum of compound 12 (75 MHz, CDCl$_3$).
Figure S14. $^1$H-NMR spectrum of compound 13 (300 MHz, CDCl$_3$).

Figure S15. $^{13}$C-NMR spectrum of compound 13 (75 MHz, CDCl$_3$).
Figure S16. $^1$H-NMR spectrum of compound 14b (300 MHz, CDCl$_3$).

Figure S17. $^{13}$C-NMR spectrum of compound 14b (75 MHz, CDCl$_3$).
Figure S18. $^1$H-NMR spectrum of compound $15b$ (300 MHz, Pyr-d$_5$).

Figure S19. $^{13}$C-NMR spectrum of compound $15b$ (75 MHz, CD$_3$OD).
Figure S20. $^1$H-NMR spectrum of compound 15b (75 MHz, DMSO-d$_6$, recorded at 358K).
Figure S21. \( ^1H \)-NMR spectrum of compound 16b (300 MHz, Acetone-d\(_6\)).

Figure S22. \( ^13C \)-NMR spectrum of compound 16b (75 MHz, Acetone-d\(_6\)).
Figure S23. $^1$H-NMR spectrum of compound 17b (300 MHz, CDCl$_3$).

Figure S24. $^{13}$C-NMR spectrum of compound 17b (75 MHz, CDCl$_3$).
Figure S25. $^1$H-NMR spectrum of compound 18b (300 MHz, Pyr-d$_5$).

Figure S26. $^{13}$C-NMR spectrum of compound 18b (75 MHz, Pyr-d$_5$).
Figure S27. $^1$H-NMR spectrum of compound 19b (300 MHz, Acetone-$d_6$).

Figure S28. $^{13}$C-NMR spectrum of compound 19b (75 MHz, Acetone-$d_6$).
Figure S29. $^{13}$C-NMR spectrum of compound 19b (75 MHz, DMSO-d$_6$, recorded at 353K).