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Phosphatidylserine content is a more important contributor than transmembrane potential to interactions of merocyanine 540 with lipid bilayers

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Abstract

Several factors, including the exposure of the negatively charged PL and transmembrane potential (TMP), may affect the binding of merocyanine 540 dye (MC540) to membrane lipids. Our aim was to quantify the significance of each of these two determinants in MC540 interactions with phosphatidylserine:phosphatidylcholine (PS/PC) vesicles. The effects of the altered PS content (PS/PC molar ratio: 5:95, 10:90 and 20:80) and TMP on MC540 binding were monitored using flow cytometry. Rapid [K^+] flux across the vesicle membrane lipid bilayer was generated using valinomycin.

We showed that the increased PS content leads to attenuated MC540 binding, while having no influence on the dynamic parameters of PS/PC vesicle membranes (electron spin resonance (ESR) spectrometry). Higher $[K^+]_{out}$ makes PS/PC liposomes bind more MC540, which implies that TMP—which becomes more positive inside the vesicles—favours the interactions of MC540 with the PL bilayer. Overall, the variability attributed to MC540–PL interactions is explained only to a minor extent by the generated TMP (7%) and largely by the variations in PS content (by up to 60%). In conclusion, the content of negatively charged PL is more important than TMP in determining the interactions of MC540 with PS/PC membranes.

Keywords: Merocyanine 540 (MC540); Phospholipid asymmetry; Transmembrane potential; Surface charge density; Lipid fluidity; Phosphatidylcholine/phosphatidylserine vesicle; Flow cytometry

1. Introduction

We have recently reported that merocyanine 540 (MC 540), the anionic fluorescent dye with a high affinity to membrane lipid components [19,21], may be successfully used for probing the changes in membrane phospholipid (PL) asymmetry that occur upon the activation of blood platelets [24]. Although we have shown that the redistribution of the negatively charged PS may relate to the suppressed interaction of MC540 with platelet and liposome PL bilayers [17,25], there are at least two other putative co-determinants (undergoing concomitant changes upon platelet activation) that might be responsible for such an attenuated MC540–bilayer interaction: transmembrane potential (TMP) and lipid packing [4,9–11,19,21]. For the last two decades MC540 has been successfully used in the studies on both biological and artificial membranes to show that shifted PL asymmetry (PS/PL) across a bilayer results in altered surface charge density and probably also in lipid packing (membrane lipid order parameter) [4,10,11,19,21,26]. How-

Abbreviations: BHT, butylated hydroxytoluene; $DiOC_5(3)$, 3,3′-dipentyloxacarbocyanine iodide; ESR, electron spin resonance; FF0, FF150 and FF300, FACS flow buffers containing 0, 150 or 300 mM KCl, respectively; *I*, ionic strength; $[K^+]_{out}$, outside concentration of potassium ions; MC540, merocyanine 540 dye; PL, phospholipids; PC, phosphatidylcholine; PS, phosphatidylserine; σ, surface charge density; Ψ_s, surface potential; TMP, transmembrane potential

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ever, none of those studies has established which of the above determinants is more important in defining MC540 binding. It still remains unclear whether the diminished staining with MC540 might be attributed merely to the enhanced exposure of negatively charged PL and the direct effects of electrostatic repulsion between anionic lipids and anionic MC540, or also—and to what extent—to alterations in TMP.

In the present study we employed the calculus of multiple regression to quantify the partial contributions of two important factors determining the interaction of MC540 with the PL bilayer: the content of PS in a lipid bilayer of PS/PC vesicles and the TMP. Our objective was to manipulate these two factors using model liposome membranes in order to relate the changes in MC540 binding to the PS/PC vesicles, which differed in PS content and were subjected to the increasing $[K^+]$ difference between the inner- and outer-vesicle mediums.

2. Materials and methods

2.1. Chemicals

All chemicals were from Sigma (St. Louis, MO, USA) unless stated otherwise. L-α-Phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphocholine) from fresh egg yolk (containing 48% unsaturated fatty acids) and L-α-phosphatidyl-L-serine (1,2-diacyl-*sn*-glycero-3-phospho-L-serine) (52% unsaturated fatty acids) from bovine brain were from Fluka (Switzerland). Chloroform was from Polish Chemicals (POCh, Gliwice). 3,3′-Dipentyloxacarbocyanine iodide ($DiOC_5(3)$) was from Molecular Probes, Oregon, USA. Water used for solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead, USA). Buffers and solutions used in a flow cytometry were filtered before use.

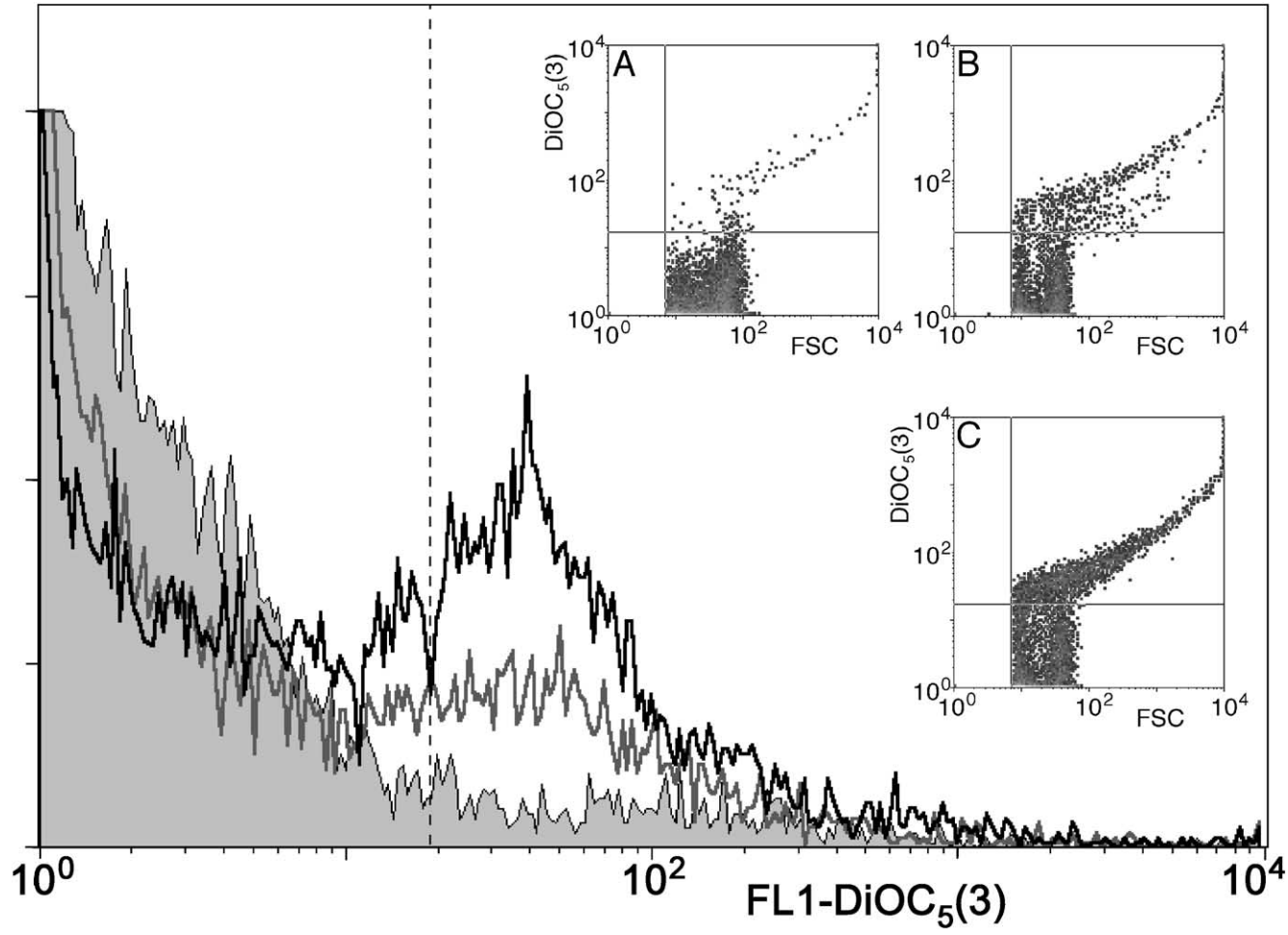

Fig. 1. Flow cytometric analysis of $DiOC_5(3)$ binding in PS/PC vesicles under conditions of increasing $[K^+]_{out}$. Exemplary histogram plots of $DiOC_5(3)$-positive vesicles containing 5 mol% PS suspended in an FF0 buffer (8 mM NaCl, 0.01 mM $CaCl_2$ and no K^+, with 1 μM valinomycin) (grey solid fill), an FF150 buffer (with 150 mM KCl) (grey outline) or an FF300 buffer (with 300 mM KCl) (black outline). The dashed line demarcates the marker of the specific $DiOC_5(3)$ binding in vesicles. Insert: Density plots of a forward light scatter vs. $DiOC_5(3)$-positive vesicles (FL1) for FF0 (A), FF150 (B) or FF300 (C). The lines demarcate the quadrants for which the estimations of $DiOC_5(3)$-positive vesicles were made. For further experimental details see Materials and methods.

2.2. Preparation of PS/PC vesicles

The phospholipids, L-α-phosphatidylcholine (phosphatidylcholine, PC) (estimated average mol. wt. 801.7) and L-α-phosphatidyl-L-serine (phosphatidylserine, PS) (estimated average mol. wt. 802.7) were used without further purification. The degree of lipid oxidation was estimated from UV absorbance [22], based on the ratio between the peak for dienes at 230 nm and the monodiene peak at 200 nm (<0.1).

For the preparation of liposomes by the injection method we used a protocol described by Disalvo et al. [5]. Phospholipid mixtures containing the increasing proportions of PS were dissolved in chloroform, supplemented with BHT and injected into 10 ml of FF0 buffer (8 mM NaCl, 0.01 mM $CaCl_2$) kept at 70 °C to a final concentration of 2 mg/ml PL and 0.01% BHT. Nitrogen was continuously bubbled through the solution during injection. Finally, the solution was annealed for 1 h at 70 °C and the vesicle suspension cooled down to room temperature (RT). Alternatively, lipid vesicles were made in PBS, pH 7.4, using the same protocol. It was shown earlier that samples thus prepared contained vesicles of an average diameter of about 500 nm. For the purpose of this study, liposomes with the increasing PS/PC molar ratio (5:95, 10:90 and 20:80 corresponding to the surface charge densities of 1/1200, 1/600 and 1/300 $Å^{-2}$, respectively) were prepared [6].

2.3. Flow cytometry analysis of liposomes labelled with MC540

Samples containing the increasing proportions of PS (2 mg/ml total PL in FF0 buffer) were incubated in the dark for 20 min at RT with MC540 prepared in an FF0 buffer to give a final dye concentration of 0.25 μM. Small volume of liposome suspension (50 μl) was supplemented with valinomycin (10-min incubation at RT, final concentration 1 μM), added to a 41-fold excess of either FF0, FF150 (8 mM NaCl, 150 mM KCl, 0.01 mM $CaCl_2$) or FF300 buffers (8 mM NaCl, 300 mM KCl, 0.01 mM $CaCl_2$) immediately before measurements, and monitored in the flow cytometer every 3 min for up to 15 min.

Flow cytometric analysis of MC540 bound to the liposome surface was performed with the forward light scatter (FSC) gate set on the liposome fraction (threshold 200). Laser excitation was at 488 nm and orange fluorescence was recorded for MC540 (FL2 channel). Fluorescence of at least 10000 vesicles stained with MC540 was measured with the photomultiplier (PMT) of the detector in cytometer channel 1 (FL1) set at 560 V and FL2 at 650 V. FL1–FL2 compensation was 1.0%, and that of FL2–FL1 was 21.4%. Percentage of the fluorescing liposomes with varying fractions of PS/PC was obtained after the subtraction of the nonspecific autofluorescence of liposomes not labelled with MC540.

2.4. Flow cytometry measurements of TMP

To monitor TMP in PS/PC vesicles the cationic molecule $DiOC_5(3)$ was used [7,15]. $DiOC_5(3)$ has been reported to accumulate in higher amounts in negatively charged inside of a cell/vesicle, and otherwise, the dye is released and escapes from the cell/vesicle when the inside becomes more positively charged [7,15]. When excited at 488 nm, the probe emits in the FL1 channel, and its fluorescence decreases with the increasing negative polarization of the cell inside since the internal dye concentration affects the $DiOC_5(3)$ fluorescence signal [16]. In our experiment, the PS/PC vesicles made of phospholipid mixtures in an FF0 buffer containing 50 nM $DiOC_5(3)$ were supplemented with 1 μM valinomycin, optionally labelled with MC540 (as described above), suspended in a 41-fold excess of either FF0, FF150 or FF300 buffer immediately before measurements, and monitored in the flow cytometer every 3 min for up to 15 min.

For the analysis of the minimum of 10000 of the $DiOC_5(3)$-positive vesicles, the PMT of the detector in FL1 was set at 570 V and FL2 at 524 V. FL1–FL2 compensation was 1.0%, and FL2–FL1 compensation was 21.4%.

2.5. Electron spin resonance (ESR) measurements of lipid fluidity in PS/PC vesicles

Ethanol solutions (10^{-3} mol/l) of [2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy] free radical

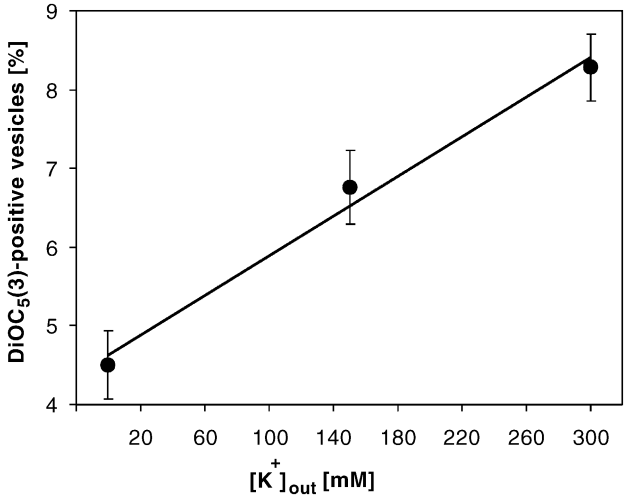

Fig. 2. Effect of $[K^+]_{out}$ on the fluorescence of $DiOC_5(3)$-positive PS/PC vesicles. The vesicles made of phospholipid mixtures containing 10% phosphatidylserine in an FF0 buffer solution of $DiOC_5(3)$ (8 mM NaCl, 0.01 mM $CaCl_2$, 50 nM $DiOC_5(3)$) were supplemented with 1 μM valinomycin, suspended in a 41-fold excess of either FF0 (no K^+), FF150 (with 150 mM KCl) or an FF300 buffer (with 300 mM KCl), and the fraction of $DiOC_5(3)$-positive vesicles was read in a flow cytometer after 15 min. For more details see Materials and methods. The estimated coefficients of the linear regression $y=ax+b$ were: $a=0.0252\pm0.0045$ ($P\ll0.0001$), $b=4.627\pm0.434$ ($P\ll0.0001$). The significance of the differences in $DiOC_5(3)$ binding, as estimated by two-way ANOVA, was $P\ll0.0001$ for both $[K^+]_{out}$ and PS content, with no significant interaction between the factors.

(5-doxylstearic acid, 5-DOXYL-Ste) or [2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy] free radical (16-doxylstearic acid, 16-DOXYL-Ste) were added to chloroform solutions of either PC or PS at a final concentration of 50 μmol/l and vortexed vigorously. Thus, spin-labelled PL mixtures containing the increasing proportions of PS were used for the preparation of liposome suspensions as described above, using either PBS pH 7.4 or FF0, FF150 and FF300 buffers as a suspending medium.

ESR scannings were routinely recorded as the first derivatives of absorption spectra. In all the ESR spectra, the ordinate was represented as the amplitude of an ESR signal and the estimated h_{+1}/h_0 ratios were calculated from the ESR spectra, taking into account the relevant amplitudes measured as the heights of the low-field and middle-field peaks, respectively (expressed in arbitrary units). For the purpose of this study, the estimated h_{+1}/h_0 parameter was further referred to as '(membrane) lipid fluidity' [23]. ESR measurements were performed at RT (22 ± 1 °C) in a Brüker SX-300E spectrometer.

2.6. Statistical analysis

Means ± S.E. are given for the normally distributed parameters. For data which showed the left- or right-skewed distributions and met the remaining criteria of normal distribution, we used either cubic or logarithmic transformation, respectively. Data were analysed with the Student *t*-test for independent or matched pair samples, or we used the ANOVA randomised complete block design depending on the number of groups compared. In order to verify how far the variations in PS content and $[K^+]_{out}$ explained the variability in the binding of MC540 to lipid vesicles, we employed the multiple regression analysis and partial correlation analysis [1].

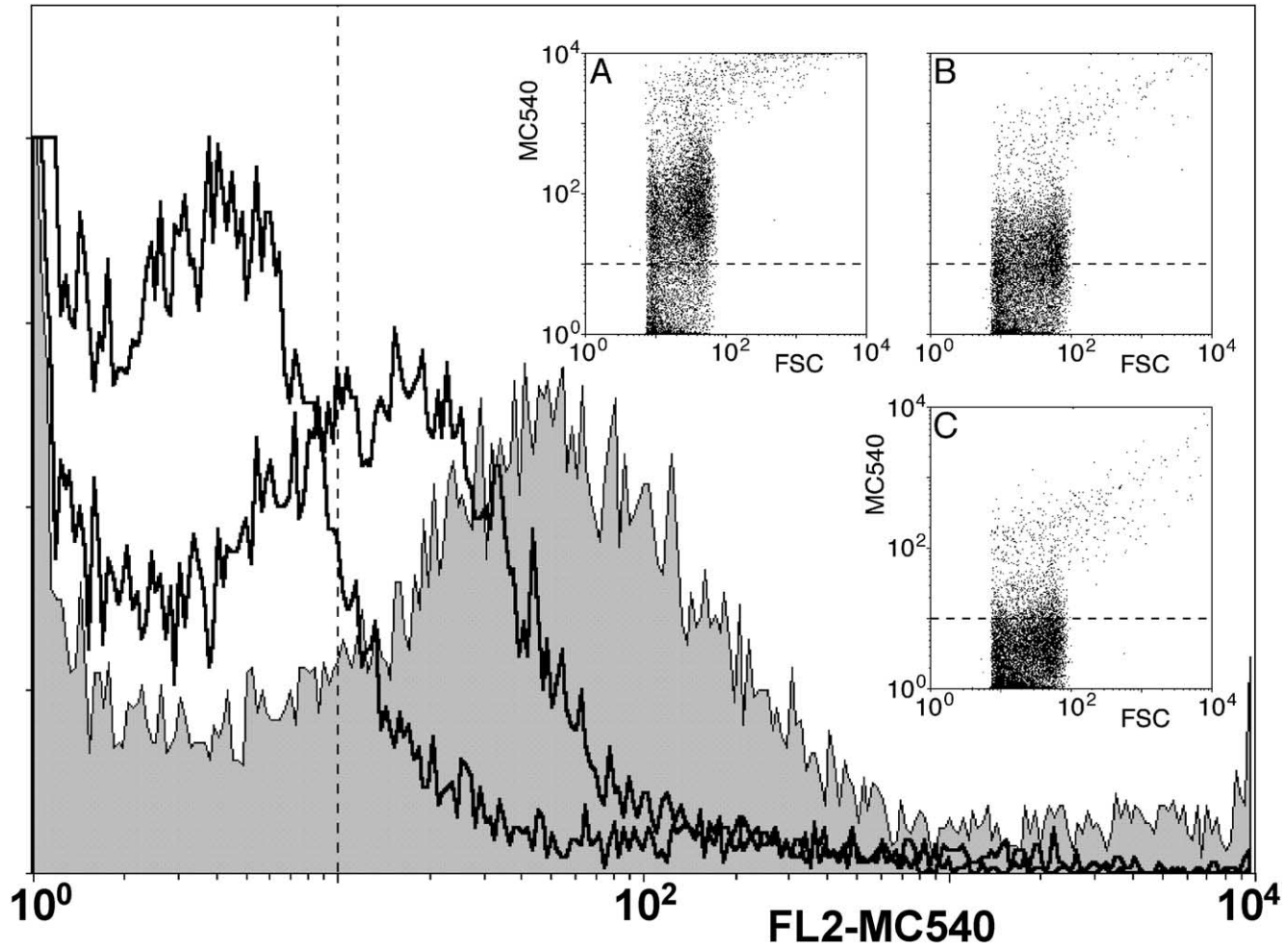

Fig. 3. Flow cytometric analysis of MC540 binding to PS/PC vesicles. Histogram plots of MC540-positive vesicles containing 5 mol% (grey solid fill), 10 or 20 mol% PS (outlines from right to left). Insert: Dot plots of a forward light scatter vs. MC540 binding (FL2) for PS/PC vesicles containing 5 mol% (A), 10 mol% (B) or 20 mol% PS (C). The dashed lines demarcate the markers of the specific binding of the dye to vesicles. For further experimental details see Materials and methods.

3. Results

3.1. Effect of $[K^+]_{out}$ on $DiOC_5(3)$ binding in PS/PC vesicles

In the presence of valinomycin, the PS/PC vesicles with a carbocyanine dye entrapped inside were able to generate TMP when placed in a hypertonic medium (0 min). Upon the addition of valinomycin, K^+ ions began to penetrate the vesicle membrane and accumulated inside, whereas the excess of the dye escaped from the vesicles. The higher the $[K^+]_{out}$ and the difference between the inside and outside $[K^+]$, the higher the fractions of PS/PC vesicles that became $DiOC_5(3)$-positive in the flow cytometer (Fig. 1). The fractions of $DiOC_5(3)$-positive vesicles observed under given conditions of $[K^+]_{out}$ remained statistically unchanged in the time course of the monitoring of the $DiOC_5(3)$-originated fluorescence in flow cytometer FL1. The vesicle size also remained unchanged, as reflected by the FSC. For further analyses we employed the recordings read at 15 min from the start of an experiment (time 0 min). The dependence between the increased $[K^+]_{out}$ and the elevated fraction of $DiOC_5(3)$-positive vesicles remained highly significant regardless of PS content ($r_{partial} = 0.487$, $P \ll 0.0001$). The difference in $[K^+]$ between the inside and the outside of the vesicles explained nearly 37% of the overall variability in $DiOC_5(3)$ fluorescence ($P < 0.002$). Such a positive association between the $[K^+]_{out}$ and the fraction of $DiOC_5(3)$-positive vesicles was observed for various contents of PS in vesicle membranes. However, the parameter of PS content was a highly significant independent determinant of the $DiOC_5(3)$-derived fluorescence ($r_{partial} = -0.426$, $P \ll 0.0001$) (Fig. 2).

3.2. Effects of PS content and $[K^+]_{out}$ on MC540 binding to PS/PC vesicles

We monitored the staining with MC540 of PL vesicles suspended in either PBS or FF0, FF150 and FF300 buffers and containing the increasing amounts of PS in the FL2 channel of a flow cytometer (Fig. 3). We observed a reciprocal linear dependence of MC540 binding to PS/PC vesicles on the molar content of PS: the fraction of MC540-positive vesicles became proportionally diminished along with the increasing PS/PC ratio (Fig. 4). Such a reciprocal dependence between PS content and MC540 binding occurred regardless of the suspension medium used for liposome preparation (PBS or FF0 buffer). Moreover, along with the increasing $[K^+]_{out}$, the interaction of MC540 with the vesicles became greater (Fig. 5).

To quantify the contribution of PS content and $[K^+]_{out}$ to the variability in MC540 binding to PS/PC vesicles, we employed the calculus of multiple regression and partial correlation analysis. PS content and $[K^+]_{out}$ taken together explained 62.5% of the overall variability in MC540-positive vesicles ($P \ll 0.0001$ with the tolerance over 0.998).

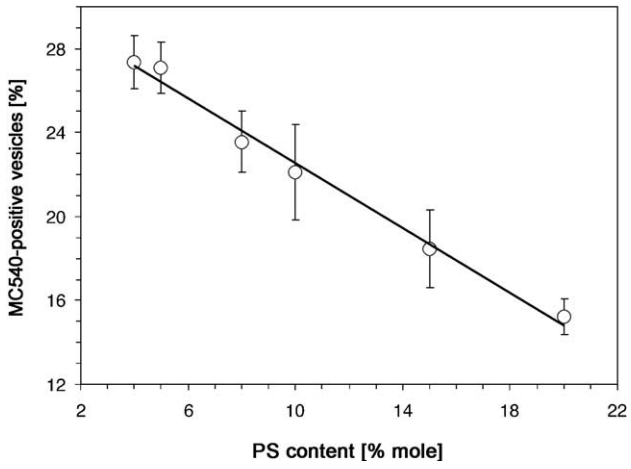

Fig. 4. Effect of PS content on MC540 binding to PS/PC vesicles. Data were presented as mean ± S.E., $n = 6$ experiments. The vesicles were made of phospholipid mixtures containing phosphatidylserine and phosphatidylcholine in PBS, pH 7.4; MC540 concentration was 3 μM. The dye binding to vesicles containing the increasing fractions of PS was expressed as MC540-positive vesicles. PS content expressed in mol% PS per 100 mol% PL. The estimated coefficients of the linear regression $y = ax + b$ were: $a = -0.78 \pm 0.12$ ($P \ll 0.0001$), $b = 30.3 \pm 1.4$ ($P \ll 0.0001$).

Whereas 56.3% of the total variability in MC540-positive vesicles could be attributed to the PS content alone ($P \ll 0.0001$, $r_{partial} = -0.774$), the respective contribution of $[K^+]_{out}$ (relevant to the generated TMP) was merely 7.2% ($P < 0.0001$, $r_{partial} = 0.387$). These outcomes clearly show that PS content is the major determinant, whereas TMP is the minor determinant of MC540 interaction(s) with vesicle membranes.

3.3. Effect of PS content on bilayer lipid fluidity in PS/PC liposomes

In order to ensure that the manipulation of increasing the PS content in the lipid bilayer of PL vesicles affects merely the surface charge density originating from the content of negative PS species and not the dynamic parameters of lipid bilayer, we used ESR spectrometry and two spin label probes, 5-Doxyl-Ste and 16-Doxyl-Ste. These lipophilic probes reflect the mobility of membrane phospholipid fatty acid chains and are commonly used to monitor the membrane lipid packing and 'lipid fluidity' at different depths of the hydrocarbon region in the lipid bilayer. The relevant increase or decrease in the h_{+1}/h_0 ratios of 5-Doxyl-Ste and 16-Doxyl-Ste may be regarded as the indicator of, respectively, enhanced or reduced membrane lipid fluidity in the 'near-the-surface' or deeper regions of the liposome lipid bilayer, whereas the values of $2T_{\parallel}$ correspond to the order parameter of a lipid bilayer [23]. In our study neither the h_{+1}/h_0 ratios of either spin label (relevant to membrane lipid fluidity at a given depth) nor the $2T_{\parallel}$ recorded for 16-Doxyl-Ste (relevant to membrane lipid order parameter) apparently changed with the increasing PS/PC ratio: PS content had no significant effect on the dynamic parameters

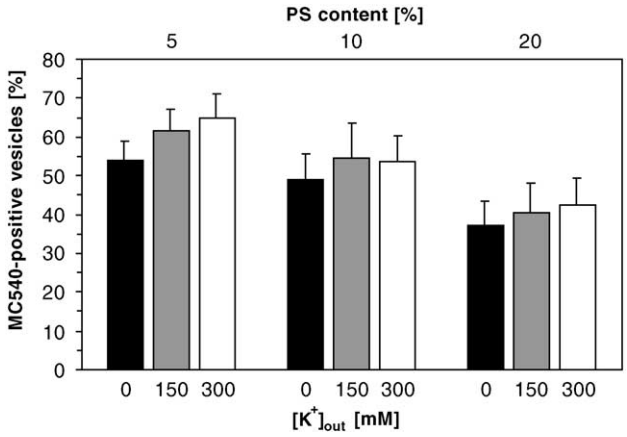

Fig. 5. Effects of PS content and $[K^+]_{out}$ on MC540 binding to PS/PC vesicles. The vesicles made of phospholipid mixtures containing phosphatidylserine and phosphatidylcholine in an FF0 buffer (8 mM NaCl, 0.01 mM $CaCl_2$) were supplemented with 1 μM valinomycin, labelled with MC540 (0.25 μM), suspended in a 41-fold excess of either an FF0 (no K^+), FF150 (with 150 mM KCl) or FF300 buffer (with 300 mM KCl), and the fraction of MC540-positive vesicles was read in a flow cytometer after 15 min. For more details see Materials and methods. The significance of the differences in MC540 binding, as estimated by two-way ANOVA, was $P \ll 0.0001$ for PS content and $P < 0.0002$ for $[K^+]_{out}$ with no significant interaction between the factors.

of a lipid bilayers in PS/PC vesicles, regardless of the buffer in which the suspension of liposomes was prepared (not shown).

4. Discussion

The preferential binding of MC540 to fluid, loosely packed, cholesterol-free PL domains implies that changes in a natural membrane PL distribution (relevant to PL asymmetry) result in the altered membrane staining with MC540: the tighter packing of the outer membrane leaflet leads to the decreased dye penetration into lipid membranes and vice versa [19,21]. In our recent study we have reported the reduced MC540 binding to the membrane PL of activated blood platelets and pointed out that such a weakened interaction is related to membrane PL symmetrization and increased PS exposure [24]. However, numerous reports published hitherto point out that certainly more than one parameter related to the chemico-physical properties of a lipid bilayer underlie the MC540 interaction(s) with membrane PL. Among them, phospholipid asymmetry (as well as the relevant lipid ordering) and TMP are believed to be the major candidates [4,9–11,18–21,26]. Nevertheless, the importance of each of those particular determinants has never been thoroughly explored.

In the present study, using the calculus of multivariate methods, we have shown that PS content in PS/PC vesicles is a more important determinant of MC540 binding than TMP. In particular, we have shown that with the increased content of PS in the lipid bilayer, the interaction(s) of PS/PC vesicles with MC540 become attenuated. This specific finding confirms earlier observations reported by ourselves and others that the weakened association of the dye with a lipid bilayer is in direct proportion to the increased membrane content of negatively charged phospholipids [13,17,25]. The interaction of the anionic probe MC540 with a lipid bilayer of PS/PC vesicles may be directly attributed to the magnitude of surface membrane potential (Ψ_s) of PS/PC vesicles, which is determined by: (a) the concentration of negative PS molecules, underlying the surface charge density (σ) of the liposome membrane lipid bilayer and (b) the ionic strength (I), originating from the composition of the medium in which liposomes are suspended [6]. Under the conditions of the submolar ionic concentrations in PBS, the magnitude of surface potential vanishes in close proximity to the membrane surface due to effective screening [12,14]. Thus, although σ relates directly to the concentration of negative PS, I reduces the contribution of $\Psi_s = f(\sigma)$ responsible for the electrostatic repulsion between MC540 and the PS/PC lipid bilayer. In this part of our experiment I of the medium was maintained at a relatively constant level and therefore we may argue that it is merely PS concentration which the interactions of MC540 with liposomes fluctuate along with. Hence, we have confirmed that the interactions of MC 540 with a lipid bilayer are of electrostatic origin: we provide the evidence that they are the function of surface charge density (σ) relating to the concentration of PS in a lipid bilayer.

In natural biological membranes one cannot discriminate well between two inter-related effects: membrane lipid fluidity and PL translocation between inner and outer bilayer leaflet (apparently differing also in the degree of fatty acid saturation). It seems reasonably certain that the ongoing symmetrization of membrane PL might be often accompanied by the alterations in membrane lipid packing and fluidity. However, our present study was designed to maintain the dynamic parameters of PS/PC vesicle membranes at a constant level in order to avoid changes in membrane lipid fluidity with changing PS content. Using the ESR spectrometry, we showed that manipulating PS concentrations in PL vesicles have apparently influenced neither the lipid order parameter (relevant to lipid packing) nor the membrane lipid fluidity along with the increasing PS content in vesicle membranes, regardless of the depth of a lipid bilayer. Therefore, the potential lipid fluidity effects on MC540 binding were not pertinent to our experiments. Overall, we may argue that in our experimental system the surface membrane potential (Ψ_s) and the lipid fluidity/ordering are not the inter-related covariates originating from PS concentration in PL vesicles: it is merely the value of Ψ_s, which PS content contributes to, and which determines MC540 binding.

We have also revealed that higher $[K^+]_{out}$ makes PS/PC vesicles bind more MC540, which implies that TMP—which is positive inside the vesicles—favours the interactions of MC540 with a phospholipid bilayer. There is a substantial

bulk of literature pointing out that MC540 binding is sensitive to TMP: the more negative is the inside of a vesicle, the less vulnerable is the membrane surface to interact with MC540 molecules [2,9,20]. Obviously, the generated TMP depends on the equilibrium potential of all the permeable ions, and also on the inter-related surface membrane potential [8,9]. In our experimental model, under conditions when valinomycin is absent and there is no K^+ flux across a lipid bilayer, the MC540 interactions with a lipid bilayer of PS/PC vesicles are influenced merely by Ψ_s as a function of σ and the magnitude of I in the K^+ solution outside liposomes. Upon the addition of valinomycin, while considering that $\Delta\Psi_s = \Psi_s - \Psi_{s(val)}$ is close to 0 at the conditions of the vast excess of K^+ [3,6,14], the MC540 interactions with liposome PL membrane are no longer determined merely by σ and I, but might be potentially governed also by the additional [6]. We showed, however, that the variability attributed to such a binding/interaction(s) of MC540 with PS/PC vesicles was explained only to a minor extent by the generated TMP and largely by the variations in PS content in vesicle membranes. The compounding support for the above reasoning may also originate from purely theoretical calculations: for artificial PL bilayers and biological membranes the measured specific capacitance values are around 1 $\mu F/cm^2$, which corresponds to a dielectric constant of 2 and distance of about 25 Å. Hence, the amount of K^+ translocated inward to create the TMP of 100 mV (considered the upper value in the majority of nonexcitable living cells) is roughly one per 250 anionic PL, which will obviously not alter the surface charge density much [6].

Overall, it seems justified to conclude that the content of negatively charged PL and the resultant surface charge density are very significant contributors influencing the interactions of MC540 with PS/PC membranes, whereas TMP plays only a minor role. Thus, our present findings may introduce a novel qualification about the interpretations of any changes observed in biological studies using MC540 dye.

Acknowledgements

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References

[1] P. Armitage, G. Berry, Statistical Methods in Medical Research, Blackwell, London, 1994.
[2] B.P. Bammel, J.A. Brand, R.B. Simmons, D. Evans, J.C. Smith, Biochim. Biophys. Acta 896 (1987) 136.
[3] J. Barber, Biochim. Biophys. Acta 594 (1980) 253.
[4] M.K. Callahan, P. Williamson, R.A. Schlegel, Cell Death Differ. 7 (2000) 645–653.
[5] E.A. Disalvo, A.M. Campos, E. Abuin, E.A. Lissi, Chem. Phys. Lipids 84 (1996) 35.
[6] R.B. Gennis, in: C.R. Cantor (Ed.), Interactions of Small Molecules with Membranes: Partitioning, Permeability and Electrical Effects, 1989, pp. 235–269, Springer Verlag, New York.
[7] H.L. Jenssen, K. Redmann, E. Mix, Cytometry 7 (1986) 339.
[8] J.W. Lagerberg, K.J. Kallen, C.W. Haest, J. VanSteveninck, T.M. Dubbelman, Biochim. Biophys. Acta 1235 (1995) 428.
[9] J.W. Lagerberg, J. VanSteveninck, T.M. Dubbelman, Photochem. Photobiol. 67 (1998) 729.
[10] F. Lupu, M. Calb, A. Fixman, Thromb. Res. 50 (1988) 605.
[11] F. Lupu, M. Calb, C. Scurei, N. Simionescu, Lab. Invest. 54 (1986) 136.
[12] K. Masamoto, K. Matsuura, S. Itoh, M. Nishimura, J. Biochem. (Tokyo) 89 (1981) 397.
[13] A. Mateasik, L. Sikurova, D. Chorvat Jr., Bioelectrochemistry 55 (2002) 173.
[14] S. McLaughlin, Curr. Top. Membr. Transp. 9 (1977) 71.
[15] P.X. Petit, J.E. O'Connor, D. Grunwald, S.C. Brown, Eur. J. Biochem. 194 (1990) 389.
[16] E. Pipili, Thromb. Haemost. 54 (1985) 645.
[17] M. Rozalski, I. Waczulikova, J. Rievaj, K. Nagyova, M. Bryszewska, C. Watala, Cell. Mol. Biol. Lett. 7 (2002) 297 (26-5-2002 9th Mejbaum-Katzenellenbogen Seminar: Liposomes: From Models to Applications, Wroclaw/Szklarska Poreba/Piechowice, Poland).
[18] B.M. Salzberg, A.L. Obaid, F. Bezanilla, Jpn. J. Physiol. 43 (Suppl. 1) (1993) S37–S41.
[19] L. Sikurova, R. Frankova, J. Fluoresc. 3 (2002) 259.
[20] J.C. Smith, Biochim. Biophys. Acta 1016 (1990) 1.
[21] W. Stillwell, S.R. Wassall, A.C. Dumaual, W.D. Ehringer, C.W. Browning, L.J. Jenski, Biochim. Biophys. Acta 1146 (1993) 136.
[22] R.C. Vossen, M.C. Dam-Mieras, G. Hornstra, R.F. Zwaal, Lipids 28 (1993) 857.
[23] C. Watala, K. Gwozdzinski, E. Pluskota, E. Dzieciatkowska, C.S. Cierniewski, Eur. J. Biochem. 235 (1996) 281.
[24] C. Watala, I. Waczulikova, B. Wieclawska, M. Rozalski, P. Gresner, K. Gwozdzinski, A. Mateasik, L. Sikurova, Cytometry 49 (2002) (in press).
[25] B. Wieclawska, I. Waczulikova, M. Rozalski, L. Sikurova, C. Watala, XVIth International Symposium on Bioelectrochemistry and Bioenergetics, June 1–6, 2001, Bratislava, Slovakia, #86, 2001.
[26] P. Williamson, K. Mattocks, R.A. Schlegel, Biochim. Biophys. Acta 732 (1983) 387.