Simultaneous characterization of lateral lipid and prothrombin diffusion coefficients by z-scan Fluorescence Correlation Spectroscopy

Martin Štefl, Anna Kułakowska, and Martin Hof

J. Heyrovský Institute of Physical Chemistry, v.v.i., Academy of Sciences of the Czech Republic, Dolejškova 3, Prague, Czech Republic

ABSTRACT☐ A new (to our knowledge) robust approach for the determination of lateral diffusion coefficients of weakly bound proteins is applied for the phosphatidylserine specific membrane interaction of bovine prothrombin. It is shown that z-scan fluorescence correlation spectroscopy in combination with pulsed interleaved dual excitation allows simultaneous monitoring of the lateral diffusion of labeled protein and phospholipids. Moreover, from the dependencies of the particle numbers on the axial sample positions at different protein concentrations phosphatidylserine-dependent equilibrium dissociation constants are derived confirming literature values. Increasing the amount of membrane bound prothrombin retards the lateral protein and lipid diffusion, indicating coupling of both processes. The lateral diffusion coefficients of labeled lipids are considerably larger than the simultaneously determined lateral diffusion coefficients of prothrombin, which contradicts findings reported for the isolated N-terminus of prothrombin.

Several enzymatic processes involving proteins containing γ-carboxyglutamic acid residues require those proteins to be associated with negatively charged membrane surfaces. A paradigm for such processes serves the membrane binding of the blood coagulation protein prothrombin followed by lateral diffusion to the prothrombinase enzyme complex . Since those proteins are only weakly binding to relevant membrane surfaces (apparent equilibrium dissociation constants K_d are in the μM range) , at physiological protein concentrations, there is a considerable amount of unbound protein and, thus, the lateral protein diffusion coefficients are difficult to access. To our knowledge, an application of total internal reflection with fluorescence pattern photobleaching recovery (TIR/FPPR) to bovine prothrombin fragment 1 (BPF1) binding to supported lipid bilayers (SLBs) is the only successful example of the translational mobility of a protein weakly bound to a membrane surface .

Conventional (single-spot) fluorescence correlation spectroscopy (cFCS) has been used recently for the comparison between basic peptides` diffusion with the associated lipid diffusion (). However, it is now accepted that, mainly due to intrinsic positioning and calibration problems, cFCS is not a reliable tool for the determination of 2-dimensional (2D) diffusion coefficients . Moreover, 2D cFCS measurements are relying on the assumption that the axial position of highest fluorescence intensity is equal to the laser waist. We have shown that this is not fulfilled when investigating plain SLBs (5). This mismatch might be even more severe when an unbound protein is contributing to the signal. The simplest way to overcome those positioning and calibration problems is the z-scan approach (5) that enables obtainment of artefact-free diffusion coefficients . The autocorrelation functions (ACFs) curves

are acquired at well-defined axial positions. For 2D diffusion, the divergence of the laser leads to a parabolic dependence of the diffusion times and particle numbers on the distances from the membrane, whereas in the case of 3D diffusion those parameters are position independent. Analysis of the axial dependence of the diffusion times and particle numbers allows the quantitative determination of 2D diffusion coefficients and surface concentrations (5).

In this article, we show that z-scan fluorescence correlation spectroscopy is a simple alternative to the sophisticated TIR/FPPR for the determination of 2D diffusion coefficients of those weakly bound proteins. Moreover, together with pulsed interleaved excitation (PIE) , we are able to simultaneously monitor lipid and protein diffusion and thus obtain direct information on possible coupling of both diffusion processes. Specifically, we investigated the calcium-dependent binding of alexa633-labeled bovine prothrombin (BP) to SLBs composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS). The SLBs of different lipid composition were labeled by atto488-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (atto488-DOPE).

BP was provided from Synapse b.v. (Maastricht, the Netherlands), DOPC, DOPS, and DOPE were purchased from Avanti Polar Lipids (Alabaster, AL). Alexa633 and atto488 were obtained from Invitrogen (Eugene, OR) and Attotec (Siegen, Germany), respectively. BP was covalently labeled by alexa633 via primary amines of lysine and arginine residues and then separated from free fluorescent dyes by gel filtration using Sephadex G-25 in 0.01M phosphate buffered saline (pH 7.2). DOPE was covalently labeled by atto488-NHS-ester and purified by adsorption chromatography on silica gel column (Kieselgel

60, Merck, Whitehouse Station, NJ) in chloroform/methanol/water (60/25/4) eluent. The SLBs were prepared by spreading small unilamellar vesicles (labeled to unlabeled lipid ratio 1:100,000) on freshly cleaved mica surface. Mica was attached to a holder 200 μm above the bottom of the cuvette (5). All BP (with a labeled to unlabeled ratio of 1:600) measurements were performed (10 mM Hepes buffer, 150 mM NaCl, 10 mM CaCl$_2$, pH 7.4) at 24 °C on a MicroTime 200 confocal microscope (PicoQuant, Berlin, Germany). We excited by two pulsed diode lasers (LDH-P-C-470, 470 nm and LDH-P-635, 635 nm) each of which with a 32 MHz repetition rate in PIE mode, double dichroic mirror Z473/635 RPC and band-pass filters HQ 515/50, HQ 685/50 (both Omega Optical, Brattleboro, VT), and a water immersion objective (1.2 NA, 60x) were used. Low power of 5 μW was chosen to minimize photobleaching and saturation.

ACFs were acquired at axial positions spaced 0.1 μm apart. Because at each axial position membrane bound and unbound BP contribute to the signal and intersystem crossing has to be taken into account, the ACFs were fitted to

$$G(\tau) = 1 + \frac{1}{PN(1-T)}\left(1 - T\left(1 - e^{-\frac{\tau}{\tau_0}}\right)\right) \cdot \left[\frac{FRa}{1+\frac{\tau}{\tau_{Da}}} \cdot \frac{1}{\left(1+\frac{\tau}{\tau_{Da}}\left(\frac{w_0}{w_z}\right)^2\right)^{\frac{1}{2}}} + \frac{1-FRa}{1+\frac{\tau}{\tau_{Db}}}\right] \quad (1)$$

where PN is the particle number of fluorescent molecules within the confocal volume, FRa is the percentage of unbound BP molecules within the PN value, τ_{Da} and τ_{Db} are diffusion times of unbound (3D) and bound (2D) BP, respectively, w_0 and w_z are spatial parameters of the confocal volume and T and τ_0 characterize the contribution and kinetics of intersystem crossing. τ_{Da} was determined before each z-scan by focusing on the solution above the SLBs (average value 450 μs ± 10 μs) and fixed in the z-scan evaluation. The resulting dependence of τ_{Db} on the z-position was analyzed as described elsewhere (5):

$$\tau_D = \frac{w_0^2}{4D_2}\left(1 + \frac{\lambda_0^2 \Delta z^2}{\pi^2 n^2 w_0^4}\right) \quad (2)$$

where D_2 is the lateral diffusion coefficient, n is refractive index, λ is the excitation wavelength and Δz is the distance between the sample position z_0 and the position of the beam diameter minimum. Since we used two-color PIE we obtained in a single measurement z-scans for alexa633-BP and atto488-DOPE, respectively (Fig. 1). The latter data set was obtained by the standard 2D z-scan (5). The obtained parabolic z-scan dependencies confirm that we indeed are characterizing 2D processes. In fact, membrane desorption of BP occurs on the second timescale and thus does not influence the detected ACFs.
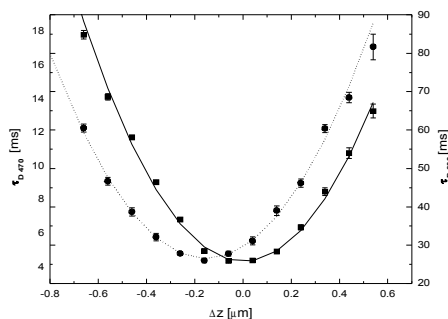
FIGURE 1 Dependence of diffusion times on the *z*-position of the focus for alexa633-BP (●; *dotted line*) and atto488-DOPE (■; *solid line*). Shown are the z-scans for 20 % DOPS and 8 μM BP. The distance between the z-focus positions created by the two lasers is ~192 nm ± 19 nm.

Z-scan fluorescence correlation spectroscopy was used to determine equilibrium dissociation constants (K_d) of the interaction of BP with the DOPS/DOPC SLBs. The number of bound BP molecules Q was plotted versus BP solution concentration [P] and analysed by Langmuir isotherms corrected for K_d:

$$Q = \frac{Q_{\lim} \times [P]}{K_d + [P]} \quad (3)$$

The Q values were calculated from z-scan minima of PN multiplied by FRa. Q_{lim} is the limiting value of labeled BP molecules bound to the membrane. Unspecific binding was determined in presence of EDTA and subtracted from the Q values. Obtained K_d values for SLBs composed of different DOPS/DOPC ratio are summarized in Table 1 and correspond to previously published data . As shown in Table 1, K_d were higher for lower molar fractions of DOPS within the membrane. These results confirm that BP binds to membranes in a phosphatidylserine-specific manner.

Atto488-DOPE lateral diffusion coefficients D_2 were determined for SLBs composed of a different DOPS/DOPC ratio. No significant differences in D_2 were observed with the increasing DOPS content within the membrane. In the range from 0 mol % to 30 mol % DOPS, the average lipid D_2-value was 4.3 ± 0.4 μm^2 s^{-1} (at 10 mM CaCl$_2$). Using the described approach we could investigate a), how the DOPS content and the BP concentration influence the BP lateral diffusion, and b), how is the latter related to the lateral lipid diffusion. Our data shows that with increasing Q (i.e., with increasing amount of DOPS or BP used) protein D_2 values decrease (Fig. 2). Similar behaviour was found for the BP

TABLE 1 Equilibrium dissociation constants of BP for different DOPS [mol %] membrane content

|	10%	15%	20%	25%	30%
K_d[μM]	4.0±0.9	3.2±0.3	2.6±0.5	2.0±0.3	0.8±0.1

Errors are represented by standard deviations. Note that ~10-15 mol % DOPS and a BP concentration of 1.4 μM are physiological relevant.
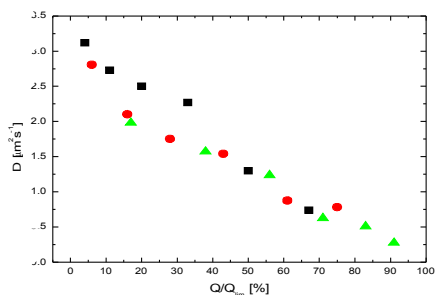
FIGURE 2 Dependence of BP lateral diffusion coefficients on the relative amount of bound BP Q/Q_{lim}. Data were determined for 10 mol % of DOPS (black), 20 mol % of DOPS (red) and 30 mol % of DOPS (green) containing DOPC SLBs. Q/Q_{lim} were calculated via Eq. 3 using K_d values from Table 1.

N-terminus BPF1 missing ~75% of the molecular weight of the native BP (). Above 30% Q/Q_{lim} the D_2 values obtained for three different DOPS contents overlap perfectly. In this range the observed dependency might be explained by a model based on bound BP molecules acting as impermeable diffusion obstacles (). Interestingly, in the physiological relevant range (Q/Q_{lim} < 30%) the BP diffusion weakly bound to 10% DOPS SLBs appears to be faster than on SLBs with higher DOPS content, indicating that at the same surface coverage the increasing amount of available DOPS headgroups is slowing down BP lateral diffusion.

Comparison of D_2 values for BP and lipids (Fig. 3) shows that BP diffuses significantly slower, which contradicts previously published TIR/FPPR data for BPF1 diffusion on SLBs claiming identical D_2 values for lipids and proteins . However, BP and lipid diffusion display qualitatively the same trends suggesting that both lateral diffusion processes are strongly related with each other. At low (10%) DOPS content and low BP concentration the difference between protein and lipid diffusion is considerably smaller than at higher DOPS contents.
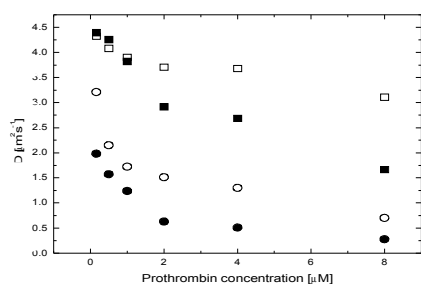
FIGURE 3 Comparison of BP (circles) and lipid (squares) D_2 values determined for membranes composed of 10 mol % of DOPS (*open symbols*) and 30 mol % of DOPS (*closed symbols*), respectively.

In summary, we found that the lateral diffusion of alexa633-BP is coupled with the diffusion of atto488-DOPE. However, BP is diffusing considerably slower than the lipids. No differences in the lateral diffusion within both layers of the used SLBs were observed . Thus, there is no evidence for significantly faster diffusion within the layer facing the support compared to diffusion within the lipid layer binding BP, suggesting that the reason for the difference in D_2 values between BP and lipids might be connected with the mechanism of BP diffusion. However, explanations based on hydrophobic interactions between the nonfragment part of BP and the SLBs (3,) or a possible exchange of DOPS molecules bound to BP with unbound ones appear at that point as too speculative.

ACKNOWLEDGEMENT

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