Pulmonary surfactant is a lipid/protein mixture that reduces surface tension at the respiratory air–water interface in lungs. Among its nonlipidic components are pulmonary surfactant- associated proteins B and C (SP-B and SP-C, respectively). These highly hydrophobic proteins are required for normal pulmonary surfactant function, and whereas past literature works have suggested possible SP-B/SP-C interactions and a reciprocal modulation effect, no direct evidence has yet been identified. In this work, we report an extensive fluorescence spectroscopy study of both intramolecular and intermolecular SP-B and SP-C interactions, using a combination of quenching and FRET steady-state and time-resolved methodologies. These proteins are compartmentalized in full surfactant membranes but not in pure 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) vesicles, in accordance with their previously described preference for liquid disordered phases. From the observed static self-quenching and homo-FRET of BODIPY-FL labeled SP-B, we conclude that this protein forms homoaggregates at low concentration (lipid: protein ratio, 1:1000). Increases in polarization of BODIPY-FL SP-B and steady-state intensity of WT SP-B were observed upon incorporation of under-stoichiometric amounts of WT-SP-C. Conversely, Marina Blue-labeled SP-C is quenched by over-stoichiometric amounts of WT SP-B, whereas under-stoichiometric concentrations of the latter actually increase SP-C emission. Time-resolved hetero-FRET from Marina Blue SP-C to BODIPY-FL SP-B confirm distinct protein aggregation behaviors with varying SP-B concentration. Based on these multiple observations, we propose a model for SP-B/SP-C interactions, where SP-C might induce conformational changes on SP-B complexes, affecting its aggregation state. The conclusions inferred from the present work shed light on the synergic functionality of both proteins in the pulmonary surfactant system.

The respiratory surface is stabilized by pulmonary surfactant, which lowers the surface tension at the air–water interface, thus minimizing the work of breathing and preventing the alveolar collapse. Type II pneumocytes are responsible for the synthesis and secretion of pulmonary surfactant toward the alveolar surface, where surfactant is rapidly adsorbed to form a multilayered highly cohesive phospholipid-based film at the air–liquid interface. The lack, deficiency, or dysfunction of pulmonary surfactant cause severe respiratory disorders, such as neonatal respiratory distress syndrome in preterm babies lacking surfactant, or the pulmonary dysfunction associated with acute respiratory distress syndrome, where inflammatory processes in the lung lead to surfactant inactivation (1).

Lung surfactant is composed of ~90% of lipids and 10% of specific proteins including SP-A, SP-B, SP-C, and SP-D. The lipid fraction is mainly composed of phosphatidylcholine (PC), the most abundant species being dipalmitoylphosphatidylcholine with a 40% of the total mass of surfactant. Other important lipid components include the anionic phospholipids phosphatidylglycerol (PG) and phosphatidylinositol, which represent 8–15% by mass. Cholesterol, the main neutral lipid in surfactant, accounts for up to 8–10% by mass. The phospholipid fraction of surfactant is responsible for its ability of dramatically reducing surface tension at the alveolar air–liquid interface. The hydrophobic proteins SP-B and SP-C are absolutely required for interfacial adsorption, stability of the surface active film, and spreading activities of surfactant during inspiration–exhalation cycles (2).

In type II pneumocytes, surfactant proteins and lipids are assembled into highly packed membranous organelles called lamellar bodies, which are secreted to the alveolar subphase.
SP-B and SP-C supramolecular complexes

Hydrophobic proteins SP-B and SP-C are synthesized as much larger precursors, whose maturation is coupled with protein trafficking along the exocytotic pathway of surfactant. SP-B and SP-C precursors (pro-SP-B and pro-SP-C) consist of the mature protein sequence flanked by both N-terminal and C-terminal propeptides. Sequential processing of the 381-amino acid pro-SP-B into the 79 residues of mature SP-B involves the cleavage activity of Napsin A, Cathepsin H, and Pepsinogen C (3–5) proteases. On the other hand, little is known about the enzymes involved in SP-C processing and, up to date, only cathepsin H is known to cleave the N-terminal peptide of pro-SP-C (6). Furthermore, it has been well-established that SP-B is necessary for a proper processing of pro-SP-C (7).

Mature SP-B and SP-C are proteins permanently associated to lipids. SP-B is an 8.7-kDa protein with a highly hydrophobic content (\(\sim 40\%\)), which mainly adopts an \(\alpha\)-helical secondary structure (8, 9). It belongs to the saposin-like protein family, characterized by containing 6 highly conserved cysteines that establish intramolecular disulfide bridges. SP-B also presents a seventh cysteine responsible for the formation of the intermolecular disulfide bridge of the covalent homodimer (10). Hydrophobic regions of its amphipathic segments establish interactions with the acyl chains of phospholipids, allowing an orientation of SP-B parallel to the membrane surface (11–14). SP-B, which exhibits a positive net charge, is known to preferentially interact with anionic phospholipid, such as PG (12, 15). Purification of SP-B from detergent-solubilized porcine pulmonary surfactant revealed the existence of SP-B particles that adopt a ring-like structure of a defined size, with an inner pore of 10 nm of diameter (16). According to a proposed model of the tridimensional structure of SP-B based on the atomic structure of Saposin B resolved by X-ray diffraction (16), the native supramolecular structure of SP-B could consist of 5 or 6 covalent dimers assembled into a ring-shaped structure. These native SP-B rings would interact peripherically with the membrane, where N-terminal segments would be located deeper through insertion of tryptophan residues (Trp-9) into the bilayer. Regarding to its function, SP-B shows membrane-perturbing capacities inducing fusion, aggregation, and lysis of vesicles (17). These activities allow SP-B to promote the reorganization of monolayers and membranes, forming and stabilizing multilayer stacks through SP-B/SP-B interactions (18, 19). These features are critical for the biophysical activity of SP-B in the pulmonary system. SP-B is essential to promote the rapid adsorption of phospholipids into the air–liquid interface (20), and to maintain the stability of pulmonary surfactant films during successive respiratory cycles (21). This is achieved by SP-B through generation of a membranous reservoir formed by stacked membranes, which would be connected by apposed SP-B rings, from the alveolar subphase up to the interfacial monolayer. This reservoir provides a continuous supply of surface active molecules to the interfacial film during alveolar compression–expansion cycles. Moreover, SP-B plays a crucial role in the biogenesis of pulmonary surfactant, being necessary for the proper assembly of lamellar bodies (22) and for the processing of pro-SP-C (23) through mechanisms that are still unknown.

SP-C monomer is a 35-amino acid hydrophobic peptide with a primarily \(\alpha\)-helical secondary structure. Its 23-amino acid C-terminal segment is enriched with branched aliphatic residues, mostly valine, forming a highly hydrophobic \(\alpha\)-helix, which shows a transmembrane orientation (24, 25). The N-terminal segment of SP-C exhibits an amphipathic character with a positive net charge and two palmitoylated cysteines. These cationic residues favor an enhanced interaction of SP-C with anionic phospholipids such as PG. Furthermore, both palmitic chains allow a tight interaction of the N-terminal region with the bilayer, anchoring the protein to the membrane and preventing its exclusion from highly compressed interfacial films (26). SP-C also establishes and stabilizes membrane–membrane and membrane–interface interactions (27, 28), thus promoting lipid exchange (29). At the alveolar interface, SP-C is believed to enhance the insertion of phospholipids into the air–liquid interface at the initial formation of the film and at its re-spreading during alveolar expansion (20).

Both hydrophobic surfactant proteins SP-B and SP-C segregate into liquid-disordered phases in surfactant membranes, forming protein-rich patches where SP-B and SP-C might initiate bilayer–bilayer and bilayer–monolayer contacts (30). Some evidences point to a reciprocal modulation of SP-B and SP-C (31–33), suggesting possible interactions between both proteins that could be required to ensure a proper interfacial activity of pulmonary surfactant by the coordinated action of SP-B and SP-C. However, up to date, no SP-B/SP-C interactions have been yet identified.

In the present work, we confirm for the first time the existence of SP-B/SP-C interactions by FRET experiments and characterize the interaction between these two proteins by analyzing the effect of the presence of SP-C on the fluorescence properties of SP-B, in different model membranes, through time-resolved fluorescence spectroscopy, fluorescence anisotropy, and protein–protein-promoted quenching effects.

Results

Photophysical characterization of labeled SP-B and SP-C

Fig. 1 shows the absorption and emission spectra of Marina Blue SP-C and BODIPY FL-SP-B in 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) bilayers. Both spectral shape and maximal intensity wavelengths agree with those described in
Table 1
Fluorescence intensity and anisotropy global decay fitting parameters of fluorescent Marina Blue-SP-C and BODIPY FL-SP-B in POPC membranes

|                      | Marina Blue-SP-C | BODIPY FL-SP-B |
|----------------------|------------------|----------------|
| Fluorescence intensity decay parameters |                    |                |
| \(\tau_0/\text{ns} \) | 5.09 (0.76)       | 8.54 (0.64)    |
| \(\tau_1/\text{ns} \) | 3.44 (0.34)       | 6.08 (0.53)    |
| \(\chi^2 \)          | 1.29              | 1.08           |

SP-B and SP-C supramolecular complexes

The literature for these labels (34). There is a considerable overlap between the emission of Marina Blue and absorption of BODIPY FL, and therefore these fluorophores constitute an adequate pair for FRET (see section “SP-B/SP-C interactions from FRET” below).

As commonly observed for fluorophores in lipid bilayers, the fluorescence decays of BODIPY FL and Marina Blue, attached to SP-B and SP-C, respectively, are complex and best described by a sum of exponentials, even at low concentration (Table 1). Additionally, the fluorescence anisotropy of both labeled proteins could be adequately described by two rotational correlation times: a faster component, \(\phi_1 < 1\) ns, and a slower one, \(\phi_2 \approx 6–9\) ns (Fig. 2). This heterogeneity of both fluorescence emission and rotational depolarization kinetics of the labeled proteins points to an average interfacial location of both fluorophores, characterized by steep variations in polarity and microviscosity. A finite limiting value \(r_c\) could also be observed in both anisotropy decays, characteristic of restricted fluorophore rotational motions.

**SP-B self-aggregation revealed by concentration quenching and depolarization**

From the fluorescence decay of BODIPY-FL we further investigate the possibility of self-aggregation of SP-B, by measuring the concentration dependence of different photophysical parameters. The decay kinetics of BODIPY-FL SP-B fluorescence becomes faster upon increasing the concentration of labeled protein, and the variation of the decay parameters was analyzed with a Stern-Volmer equation for dynamical (collisional) quenching,

\[
\tau_0/\tau = 1 + k_q(\tau)[Q]
\]

where \(Q\) is the concentration of quencher (which, for self-quenching, identifies with that of fluorophore), \(\tau\) is the so-called lifetime-weighted quantum yield (amplitude-averaged mean fluorescence lifetime),

\[
\tau = \sum \alpha_i \tau_i
\]

\((\tau)\) is the true (intensity-averaged) mean fluorescence lifetime, and the subscript “0” indicates values extrapolated for infinite dilution. The fitting parameter \(k_q\) is the bimolecular collisional quenching constant, which according to the approximation of Umberger and Lamer (35), is given by Equation 4.

\[
k_q = 4\pi N_A (2R_c)(2D) \left(1 + \frac{2R_c}{\tau_0 < \tau_0 > D} \right)
\]

In this equation, \(R_c\) is the collisional radius (taken as the sum of the fluorophore and quencher radii, or twice the fluorophore radius for self-quenching), and in this way the self-diffusion coefficient \(D\) can be obtained. Note that the use of average lifetimes (instead of individual decay components) in Equation 1 constitutes an approximation, which is reasonable given the difficulty in addressing the quenching of each individual complex decay component (36). From the slope of the fitting straight line \((r^2 = 0.914)\) to the data of Fig. 3A, \(k_q = (2.64 \pm 1.25) \times 10^3\) M\(^{-1}\) s\(^{-1}\) (and assuming \(R_c = 1.0\) nm) \(D = 1.4 \times 10^{-7}\) cm\(^2\) s\(^{-1}\) (90% confidence interval: [0.43 \times 10^{-7} cm\(^2\) s\(^{-1}\), 2.6 \times 10^{-7} cm\(^2\) s\(^{-1}\)]) are recovered. As visible in the figure and reflected in the estimated uncertainties, the data are affected by scattering, which is related to the small extent of quenching. Still, they indicate that the protein is in the membrane, with diffusion coefficient similar to that of lipid.

However, as inferred from Fig. 3B, the steady-state fluorescence intensity \(I_F\) displays a highly nonlinear variation. To elucidate whether this was solely due to statistical pairs existing at the moment of excitation, we analyzed this variation in the context of a combination of dynamical and active-sphere quenching processes.

\[
I_F = \frac{C[F]}{1/\tau_0 + k_q[F]} \exp(-VN_A[F])
\]

In Equation 5, \(C\) is a multiplying factor, \(N_A\) is the Avogadro constant, and \(V\) is the active sphere volume. From the latter, the active sphere radius \(R\) is obtained, because we already determined the dynamic contribution as described above, (Equation 1). In case that \(R\) is significantly larger than the sum of the collisional radii of fluorophore and quencher (\(~10\) Å for typical molecular pairs), a static quenching model considering the formation of a molecular complex should be invoked. That is clearly the case here, as analysis with Equation 5 leads to an unrealistic value (\(r = 37\) Å; not shown).

This led us to consider a model including formation of nonfluorescent BODIPY FL dimers \((F_2)\) in equilibrium with fluorescent monomers \((F_1)\).

\[
2F_1 \leftrightarrow F_2, K = [F_2]/[F_1]^2, [F] = [F_1] + 2[F_2]
\]

Here \([F]\) denotes the total fluorophore concentration. Combination of this equilibrium with Equation 5 (replacing the linear emission term with the solution to the quadratic equation in Equation 6; in the other terms, which are related to active
sphere (exponential) and dynamical quenching (denominator) contributions, the total fluorophore concentration is kept to lead to Equation 7.

$$I_F = \frac{C \exp(-V N A F)}{1/\tau_0 + k_q[\text{F}]} \frac{1 + 8K[F]}{4K}$$

(Eq. 7)

To obtain an estimate for the dimerization constant $K$, we fixed $k_q$ and $\tau_0$ to the values obtained from analysis of the time-resolved data with Equation 1, and used $V = (4\pi/3)(1.0 \text{ nm})^3$ as a reasonable value for the active sphere volume. We found that a clear correlation existed between $C$ and $K$, and an adequate description of the whole concentration range could not be obtained with a dimerization model. The curve in Fig. 3B corresponds to $K = 1.05 \times 10^8 \text{ M}^{-1}$, but the quality of the fit is similar for other $K$ values of the same (or higher) order of magnitude. Despite the high values of $K$ thus retrieved, it is apparent that the experimental variation is still not well-described, and $I_F$ actually plateaus for lower concentrations than predicted by a dimerization model. Because of the large correlation between aggregation order and association constant, we did not attempt further refinement of this analysis. In any case, extensive BODIPY FL SP-B self-aggregation can be safely inferred, very likely involving high-order aggregates.

Another evidence of BODIPY FL SP-B aggregation is provided by measuring steady-state anisotropy ($r$) as a function of protein concentration. In the absence of aggregation, $r$ would be expected to decrease markedly in the 0–7 $\mu\text{M}$ concentration range from its infinite dilution value $\langle r \rangle_{\text{d}}$, because of intermonomer homo-FRET. This is illustrated by the theoretical curve displayed in Fig. 3C, obtained using the Snyder and Freire model (37) for two-dimensional energy migration among randomly distributed dipoles (37) (see text for details).

Figure 3. Concentration dependence of BODIPY FL SP-B fluorescence lifetime (A), steady-state intensity (B), and anisotropy (C). The lines in A and B are best fits of Equations 1 and 7, respectively. In C, the squares and circles are values obtained in the absence and in the presence of Marina Blue SP-C (0.5 mol %), respectively, whereas the curve was obtained using the Snyder and Freire model for two-dimensional energy migration among randomly distributed dipoles (37) (see text for details).
with depolarized emission, as evidenced by the reduced \( \langle r \rangle \) chromophore units.

**SP-B/SP-C interactions from fluorescence quenching in full surfactant lipids**

We now address the possibility of interactions between SP-B and SP-C, but in a real surfactant/lipid mixture. Fig. 4A displays tryptophan emission spectra of WT SP-B (7 \( \mu \)M) in the presence of increasing amounts of WT SP-C, both reconstituted in full surfactant lipids (1 mM). Although no spectral changes are apparent, it is clear that the maximal emission intensity undergoes significant changes upon addition of SP-C. Curiously, small amounts (<1 \( \mu \)M) of SP-C increased the emission of SP-B, whereas higher concentrations lead to a marked decrease, to less than half the intensity recorded in the absence of the former. As depicted in Fig. 4B, this biphasic behavior is also observed in time-resolved measurements: both \( I_{F0}/I_F \) and \( \tau_{0}/\tau \) ratios undergo a slight decrease for [SP-C] up to 1 \( \mu \)M, and increase for higher concentrations. From the slope of the \( 1/\tau \) variation with [SP-C] in the linear range (Equation 1), \( k_Q = 3.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \) is recovered, which in turn leads to \( D = 6.2 \times 10^{-6} \text{ cm}^2/\text{s} \) after application of Equation 4 assuming \( R_e = 1.0 \text{ nm} \). This is an absurdly high value for lateral diffusion in lipid bilayers, which probably reflects protein compartmentalization, i.e., a considerably larger effective SP-C concentration for quenching of SP-B than expected from the bulk concentration in the lipid. The \( k_Q \) value recovered from Fig. 4B is \( \sim 15 \) times higher than that obtained for SP-B self-quenching (Fig. 3A), which means that the proteins are located in membrane regions that amount to only \( \sim 1/15 \) of the entire bilayer. This agrees with the previous observation that SP-B and SP-C distribute exclusively in regions of liquid-disordered phase, with some accumulation close to the phase boundaries in surfactant membranes showing liquid ordered/liquid disordered coexistence (30, 39). The density of both proteins in those regions would then be higher than that calculated considering uniform distribution in the whole membranes (Fig. 4C). The fluorescence data presented here agrees with this heterogeneous distribution of the proteins, and suggest that SP-B surroundings are actually enriched of SP-C patches.

**Figure 4. Quenching of WT SP-B by WT SP-C and protein compartmentalization in full surfactant lipid bilayers.** A, emission spectra of the tryptophan residue of SP-B (7 \( \mu \)M) (excited at 280 nm) in surfactant lipid membranes (1 mM). B, time-resolved (overbar\( \tau \)) and steady-state (\( \tau \)) blue and steady-state (\( I_{F0}/I_F \), red) experimental quenching ratios (points) and fitting curves to the linear region (from 0.8 mM SP-C onwards) using a simple Stern-Volmer formalism (Equation 1), and a Stern-Volmer formalism combined with sphere of action (variation with [SP-C] in the linear range (Equation 1), which means that the proteins are located in membrane

\[
\frac{I_{F0}}{I_F} = \frac{\tau_{0}}{\tau} \exp(VN_{A}[Q])
\]

(Eq. 8)

where \( I_{F0} \) is the value of SP-B fluorescence intensity extrapolated to [SP-C] = 0 from the SP-B concentration range considered for this analysis ([SP-C] > 0.8 \( \mu \)M). A best fit sphere radius of \( r = (3V/(4\pi))^{1/3} = 1.1 \text{ nm} \) is thus obtained, which is probably an unreasonably high value, indicative of static quenching due to complex formation. To account for this possibility, one may fix \( R \) to a more feasible value, and allow for the formation of SP-B/SP-C heterodimers with equilibrium constant \( K_s \). This leads to the following expression:

\[
\frac{I_{F0}}{I_F} = \frac{\tau_{0}}{\tau} \exp(VN_{A}[Q])(1 + K_s[Q])
\]

(Eq. 9)
In this way, and again assuming $R \sim 1.0 \text{ nm}$, $K_q = 10.5 \text{ m}^{-1}$ is obtained, indicative of a moderate degree of static quenching of SP-B by SP-C.

We also addressed the possibility of quenching of Marina Blue SP-C fluorescence by WT SP-B. Fluorescence emission spectra of 5 $\mu\text{M}$ Marina Blue SP-C, reconstituted in POPC membranes (1 mM), in the absence or presence of increasing amounts up to 16 $\mu\text{M}$ native unlabeled SP-B, are shown in Fig. 5A. The corresponding fluorescence intensity and lifetime data, transformed for Stern-Volmer analysis, are represented in Fig. 5B. The experiments were carried out in POPC bilayers to avoid potential heterogeneous distributions of the proteins in the membranes as we showed before. From the line dependence of Marina Blue SP-C $\tau_0/\tau$ with increasing SP-B concentration (Equation 1), $k_Q = 1.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ was recovered. In turn, from this bimolecular quenching rate value, a diffusion coefficient of $D = 7.3 \times 10^{-8} \text{ cm}^2/\text{s}$ was obtained from Equation 4. This value is compatible with no protein compartmentalization, as expected in pure POPC membranes.

Although the steady-state data are affected by considerable scatter, it is apparent that the extent of quenching observed in these conditions is significantly higher than that apparent in the time-resolved experiment. Analysis with Equation 8 results in the best fit green line in Fig. 5B, which accounts for the curvature of the data, but leads to a nonphysical sphere radius ($r = 3.3 \text{ nm}$). Using Equation 9 and assuming $R \sim 1.0 \text{ nm}$, a value of $K_q = 1.9 \times 10^2 \text{ m}^{-1}$ is now obtained (red curve in Fig. 5B). Because of the quantitative uncertainty resulting from the dispersion in the experimental data points, this should be viewed as an indicative value. In particular, an improved description of the data could be obtained by considering higher-order aggregation of SP-B and SP-C (not shown). Still, our data clearly point to a significant degree of static quenching of SP-C by SP-B in this system.

An independent piece of evidence for SP-B/SP-C association was observed upon measuring the steady-state anisotropy ($r$) of BODIPY FL SP-B. Unlike the above discussed data obtained in the absence of the latter protein, those measured in the presence of Marina Blue SP-C (Fig. 3C, triangles) follow closely the theoretical expectation of the Snyder and Freire model (37) for energy homo-FRET among randomly distributed BODIPY FL fluorophores. The lower extent of BODIPY FL depolarization indicates that addition of SP-C to SP-B reduces the proximity among SP-B molecules. Therefore, these data are compatible with a scenario where SP-C binds to SP-B aggregates, disrupting them.

### SP-B/SP-C interactions from FRET

An adequate verification of this hypothesis may be provided by time-resolved FRET measurements. From the Marina Blue SP-C emission and BODIPY FL SP-B absorption spectra of Fig. 1, we calculated the Förster radius $R_0$, according to Equation 10.

$$R_0 = 0.02108 \left[ \kappa^2 \Phi_0 \eta^{-4} \int_0^\infty \lambda^4(\lambda) \epsilon(\lambda) \, d\lambda \right]^{1/6} \tag{Eq. 10}$$

In this equation, $\kappa^2$ is the so-called orientation factor (see Refs. 40 and 41 for extensive discussions), $\Phi_0$ is the fluorescence quantum yield of donor in the absence of acceptor, $n$ is the refractive index of the medium, and the integral term is a measure of the overlap between the donor normalized emission ($I$) and acceptor absorption ($\epsilon$). The constant value in the equation above assumes that nanometer units are used for both $\lambda$ and $R_0$. Using the dynamic isotropic limit value $\kappa^2 = 2/3$, $n = 1.4$ for the water/lipid interface, and $\Phi_0 = 0.89$ for Marina Blue (42), together with the aforementioned BODIPY FL molar absorptivity, we obtained $R_0 = 4.93 \text{ nm}$.

In our previous works employing time-resolved FRET (43), donor decays in the absence and presence of varying amounts of acceptor were globally analyzed, linking the intrinsic donor decay parameters, as they apply to all samples. Because the decay of Marina Blue SP-C emission becomes faster in the presence of SP-B due to dynamic quenching (as described in the previous subsection), this strategy had to be adapted to the system at hand. To this purpose, for each SP-B concentration, we linked the decay of Marina Blue SP-C in the presence of BODIPY FL SP-B (donor + acceptor sample) with that of Marina Blue SP-C in the presence of WT SP-B (donor sample), the latter having the same concentration as that of SP-B in the donor + acceptor sample (Fig. 6). In this way, we could uncouple the FRET and collisional quenching, and recover the parameters pertinent to the former process without interference of the latter.

In an increasingly complex modeling approach, we started by considering donor and acceptors all in the same plane, with uniform distribution of acceptors surrounding each donor (Model 1) (44, 45).
In this equation, $i_D(t)$ is the donor decay in the absence of FRET, and

$$c = n \Gamma(2/3) \pi R_0^2 / \langle \tau_D \rangle^{1/3} \tag{Eq. 12}$$

where in turn $n$ is the numerical acceptor density (number of acceptors per area unit), $\Gamma$ is the complete $\gamma$ function, and $\langle \tau \rangle$ is the mean fluorescence lifetime in the absence of FRET. $c$ is therefore expected to be proportional to the acceptor concentration, and the recovered fitting values can be compared with theoretical expectations calculated using Equation 12 (46, 47). Table S1 shows the best fit parameters and $\chi^2$-square values obtained with this model, whereas fits, residuals, and residual autocorrelations are plotted in Fig. S1. It can be promptly concluded that this model is not satisfactory even for low acceptor concentration, and becomes totally inadequate for high acceptor concentrations.

As a first refinement, we considered that there were two populations of nonequivalent donors, sensing different local acceptor concentrations ($c_1$ or $c_2$) (45, 47), which are model parameters (Model 2).

$$i_{DA}(t) = i_D(t) \left[ A_1 \exp(-c_1 t^{1/3}) + A_2 \exp(-c_2 t^{1/3}) \right] \tag{Eq. 13}$$

Figure 6. Fluorescence decays of Marina Blue SP-C in POPC membranes in the presence of WT SP-B or BODIPY FL SP-B. The fluorescence decay of Marina Blue-SP-C (5 μM) in POPC vesicles (1 mM) have been measured in the presence of WT SP-B (blue lines) or BODIPY FL-SP-B (red lines). The SP-B concentration (either WT or labeled with BODIPY FL) were, from top row to bottom row, 1.25 μM; 2.5 μM; 5 μM; 7.5 μM; 15 μM. In the left panels, the fitting curves (Model 3, see also Fig. 7) to each data set and the instrumental response function are also shown (in black). The middle and right plots in each row show the residuals and residual autocorrelation plots for the corresponding concentration of WT (blue) or BODIPY-labeled (red) SP-B.
An additional parameter in Equation 13 is \( q = A_2/A_1 \), the ratio between the amounts of donor in the two populations (which are proportional to pre-exponential factors \( A_2 \) and \( A_1 \)). Assuming that distribution of fluorescent donors is still uniform (and the invoked behavior would be due to acceptor non-uniform distribution), the overall acceptor concentration \( \langle c \rangle \) is obtained by averaging over \( c_1 \) and \( c_2 \) (each weighted by the relative amount of donors in the respective population).

\[
\langle c \rangle = (c_1 + q c_2)/(1 + q)
\]  
(Eq. 14)

Again, \( \langle c \rangle \) can be compared with theoretical expectations calculated using Equation 12. Table S2 displays best fit values and \( \chi \)-squares resulting from this analysis, whereas best fit, residual, and autocorrelation plots are shown in Fig. S2. An interesting behavior may be hinted from these results. There appears to be one population of isolated donors (with \( c_1 = 0 \)), which is dominant (\( q < 1 \), or \( A_1 > A_2 \)) for low SP-B concentrations, but vanishes with increasing \( [SP-B] \) (\( q \) becomes \( \gg 1 \), or \( A_2 \gg A_1 \) in the high acceptor concentration limit). The other donor population is small for low \( [SP-B] \) but dominant in the high acceptor concentration limit. This model constitutes a clear improvement over Model 1, which is apparent both in the increased goodness of fit and in the agreement between the recovered \( \langle c \rangle \) and the theoretical \( c \). However, there is still room for statistical improvement, especially for higher \([SP-B]\) values.

As Marina Blue and BODIPY-FL are polar molecules that should be located at the bilayer interface, we then considered the possibility that, instead of distributing in a single plane, the real scenario would imply the presence of each fluorophore in either bilayer leaflet (Fig. 7). This situation would agree with the heterogeneous fluorescence intensity and anisotropy decays of Fig. 2. Therefore, for each donor, two planes of acceptors would be available: half of the total acceptors would be located in the same leaflet of a given donor (\( h = 0 \), where \( h \) is the interplanar distance), whereas the other half would be located in a parallel plane, on the opposing leaflet (\( h = 3.4 \) nm, similar to the average transverse distance between the lipids headgroups in the leaflets (48)),

\[
i_{dA}(t) = i_d(t)\left[A_1\rho_{cis,1}(t)\rho_{trans,1}(t) + A_2\rho_{cis,2}(t)\rho_{trans,2}(t)\right]
\]  
(Eq. 15)

where

\[
\rho_{cis}(t) = \exp(-ct^{1/3})
\]  
(Eq. 16)

are the intraplanar FRET terms for the first (\( i = 1 \)) and second (\( i = 2 \)) donor populations, identical to those considered in Equation 13, and

\[
\rho_{trans}(t) = \exp\left[-\frac{2c_i}{\Gamma(2/3)-b} \int_0^t \left[1 - \exp\left(-tb^3\alpha^2\right)\right] \alpha^{-3}d\alpha\right]
\]  
(Eq. 17)

are the corresponding interplanar terms, with \( b = (R_0/h)/<r>^{1/3} \) (49). In this formalism (Model 3), the theoretical concentrations are halved, because the total surface area is doubled. The fitting parameters obtained with this model are shown in Table 2. Models 2 and 3 are qualitatively similar, and the above considerations regarding the two donor populations and associated acceptor concentrations recovered with Model 2 still hold for Model 3. However, allowing for two planes of fluorophores instead of a single one leads to considerable improvement in the goodness of fit, which is now acceptable for all acceptor concentrations (see Fig. 6 for fits, residuals plots, and residual autocorrelation plots within the framework of Model 3). A verification of the adequacy of Model 3 is the excellent accordance between the \( \langle c \rangle \) obtained from fitting and the theoretical \( c \) values, observed over the whole studied concentration range (Fig. 8). Note that the straight line in this figure is the theoretical expectation calculated from the acceptor surface concentrations and the spectroscopic \( R_0 \) value, without any fitting parameters.

**Discussion**

Previous studies had confirmed that the interfacial activities of the pulmonary surfactant complexes are optimal when both proteins SP-B and SP-C are present in their physiological ratio (20). *In vivo* experiments confirm this evidence, concluding that both proteins might be absolutely required for a proper stabilization of the alveoli at the end of expiration (50), specially at long term.

The possibility that SP-B and SP-C could cooperate through formation of possible SP-B-SP-C complexes in lipid membranes has been explored in the present work by quenching and FRET analysis. The quenching methodology has been carried out with both labeled and nonlabeled proteins, to rule out labeling as a potential cause of artifacts. Moreover, we have confirmed the same effects in membranes with the full lipid compositional complexity of surfactant and in pure POPC vesicles, where no membrane domain compartmentalization effects are to be expected. Alternative FRET experiments have been carried out using fluorescently derivatized versions of both proteins. The existence of SP-B/SP-C interactions have been detected in all the combinations of WT and fluorescently labeled proteins used and in simple (POPC) or complex (full surfactant lipids) membranes, suggesting that SP-B-SP-C complexes do really exist. This is the first time, as far as we are aware, that this possibility is really supported. Previous FRET analysis between SP-C bearing a dansyl group (acceptor) and SP-B’s Trp (donor) concluded that there was no specific interaction between SP-B and SP-C (51). The results presented here incorporate measurements of time-resolved fluorescence, which is a more reliable
technique than steady-state fluorescence because it is not affected by light scattering due to the presence of vesicles or protein aggregates, and also does not rely on absolute intensity data. The occurrence of SP-B/SP-C interactions is clearly demonstrated by the existence of static quenching of SP-B by SP-C, and by the model of interaction required to interpret the Marina Blue-SP-C/BODIPY-FL SP-B FRET data, indicating that donor and acceptor molecules are not evenly distributed. Possible SP-B/SP-C interactions had also been previously proposed in interfacial monolayers (31), where it was concluded that a SP-B-SP-C complex could be formed with the participation of anionic phospholipids.

The experiments described here also propose the existence of SP-B/SP-B interactions with strong indications of formation of supramolecular oligomers of SP-B. These interactions have been deduced from both self-quenching analysis of SP-B derivatized with BODIPY-FL and measurements of its steady-state anisotropy. These results are consistent with the finding of the presence of oligomeric structures of SP-B, whose structure, revealed by electron and atomic force microscopy, consists of ring-shaped particles with an inner diameter of 10 nm. A model of oligomerization has been proposed suggesting assemblies of multiple SP-B dimers (16).

The combined analysis of the data regarding SP-B/SP-C and SP-B/SP-B interactions allows us to draw a last group of conclusions. A careful inspection of the dependence of quenching of SP-B by SP-C on the concentration of SP-C in the membrane reveals that at low SP-C densities (0.437 and 0.875 μM) there is an unexpected but reproducible slight increase of Trp fluorescence intensity and lifetimes. Once demonstrated that homooligomerization of SP-B occurs and that it is associated with self-quenching of the protein fluorescence, the data reflecting an apparently contradictory increase in the fluorescence of SP-B produced by low amounts of SP-C could be explained if those small quantities of SP-C were able to dissociate preformed SP-B oligomers that were previously self-quenched in the absence of SP-C. This biphasic behavior of the quenching of SP-B by SP-C could actually be accommodated in the framework of a complex model that involves n-order dark aggregates of SP-B, as well as SP-B/SP-C heterodimers that retain part of the fluorescence of the monomer (not shown). This was not included here as it would lead to a considerable increase in complexity, without significant quantitative insight.

Along this line, dissociation of SP-B homoaggregates could be responsible for the increase of fluorescence anisotropy of BODIPY-FL caused by SP-C. In this way, at low SP-C:SP-B ratios, the first few molecules of SP-C could contribute to partial dissociation of SP-B oligomers but not that much to the extensive quenching of SP-B fluorescence by SP-C, producing as a net effect an increase of the overall fluorescence of SP-B (see Fig. 9). As the amount of SP-C in the membrane progressively increases, most SP-B molecules would then take part in quenched SP-B/SP-C complexes producing the overall quenching effect modeled by the Stern-Volmer approach. The stoichiometric analysis of quenching of SP-B by SP-C suggests that saturation is reached at equimolecular SP-B-SP-C complexes. In this scenario, we speculate that a competition could exist between SP-B/SP-C and SP-B/SP-B interactions, and therefore, that SP-C could somehow modulate the oligomerization state of SP-B and the functional properties related with defined structural organizations of the protein.

A similar picture can be drawn from the FRET analysis. This experiment revealed the existence of two limiting scenarios. “Under-stoichiometric” acceptor concentrations ([SP-B]:[SP-C] < 1) is the first. In an excess of SP-C, most of BODIPY FL SP-B will probably be involved in Marina Blue SP-C/BODIPY FL SP-B aggregates. Most fluorescing Marina Blue SP-C molecules (i.e. those which are not part of aggregates with SP-B, and are not statically quenched) will sense an acceptor-depleted environment. Those that by chance are located close to aggregates may sense a higher than expected local concentration (hence \( c_1 = 0 \), and \( c_2 \) > calculated theoretical \( c \)).

“Over-stoichiometric” acceptor concentrations ([SP-B]:[SP-C] > 1) is the second. In an excess of BODIPY FL SP-B, the acceptor concentration is large enough so that all fluorescent donors become essentially equivalent regarding acceptor distribution, and sense a local acceptor concentration close to the theoretical expectation.

In the light of these results, we propose that SP-B/SP-C interactions would trigger a conformational change in the supramolecular structure of SP-B. It is known that the synergic action of

### Table 2

**Analysis of time-resolved fluorescence data of Fig. 6, in the framework of Model 3**

| [BODIPY SP-B] (µM) | 1.25 | 2.5 | 5 | 7.5 | 15 |
|--------------------|------|-----|---|----|----|
| Recovered \( c_1 \) | 0.037 | 0.0206 | 0.0446 | 0 | 0 |
| Recovered \( c_2 \) | 0.782 | 1.034 | 0.744 | 0.856 | 1.423 |
| Recovered \( q \) | 0.221 | 0.406 | 0.616 | 5.385 | 17.67 |
| Calculated \( <r> \) (Eq. 14) | 0.172 | 0.313 | 0.311 | 0.722 | 1.347 |
| Theoretical \( c \) (calculated from actual [BODIPY SP-B] and spectroscopic \( R_0 \)) | 0.122 | 0.244 | 0.488 | 0.732 | 1.464 |
| Global \( \chi^2 \) (considering both donor-only and donor-plus-acceptor decay for each [BODIPY SP-B]) | 1.321 | 1.297 | 1.145 | 1.189 | 1.351 |

**Figure 8.** Overall acceptor concentrations \( c \) recovered from fitting of Model 3 to the time-resolved FRET data of Fig. 6 (black circles) and theoretical values calculated from the actual acceptor concentrations with Equation 12 (straight line), using the spectroscopic \( R_0 = 4.93 \text{ nm} \) value, and area/POPC lipid = 0.66 nm\(^2\) (48) were calculated.

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**J. Biol. Chem. (2018) 293(24) 9399 –9411**
SP-B and SP-C are responsible for the permeability of surfactant membranes, where SP-B pores would allow rapid lipid transfer between the leaflets of the bilayer and also polar molecules flow through the pore (2). Additionally, it has been suggested that SP-B would mediate interconnection of bilayers by apposition of two rings (16). According to this line of evidence, SP-C interaction with SP-B would lead to a structural change in the SP-B pore, which might cause the uncoupling of the two connected SP-B rings, and therefore, the detachment of the bilayers. A recent publication showed that SP-C had the ability to fragment large vesicles into smaller ones, whereas SP-B prompted fusion between membranes, generating liposomes of high polydispersity. These activities were related with the ability of each of the two proteins to form supramolecular assemblies. However, vesicles containing SP-B and SP-C did not undergo size changes, indicating that the fusion/fission activities of both proteins were counteracted when they are together (33). The effect of SP-C to modify the structural organization of SP-B homo-oligomers, as deduced from our experiments, provides a structural basis to that mutual functional interference.

Dissociation of SP-B homo-oligomers mediated by SP-C might be different in the alveolar environment. SP-B/SP-C interactions might be modified by the presence of other proteins, such as SP-A. In vitro, SP-A influences on the regional organization of phospholipid monolayers containing SP-B or SP-C (52–54). Also, SP-A and SP-B are involved in the reorganization of surfactant membranes into tubular myelin (55). A functional study has determined the existence of a different behavior of proteoliposomes prepared from isolated SP-B and SP-C and the behavior of those from the hydrophobic protein fraction of the native pulmonary surfactant (containing both SP-B and SP-C), the latter being the one that mimics better the functional surface active properties of native surfactant (20). This would suggest that there are some interactions between SP-B and SP-C that might not be re-established when the isolated proteins are mixed. The experiments of the present work have been performed with a mixture of isolated SP-B and SP-C. Therefore, although our results clearly claim that SP-B and SP-C interact in surfactant membranes, some aspects of this protein–protein interaction might differ from a genuine native pulmonary surfactant context.

An important question is related with the physiological implications of the SP-B/SP-C interaction in the pulmonary surfactant system. Upon touching the air–liquid interface, lamellar bodies immediately unravel and surfactant membranes are instantaneously adsorbed into the interface. Phospholipids are quickly and effectively transferred due to the cooperative action of SP-B and SP-C. At the respiratory surface, SP-B generates a network of membranes interconnected among them and to the interfacial monolayer. In this way, SP-B contributes to film stability and to achieve the optimal lipid packing that allows a minimum surface tension (20, 56). On the other hand, SP-C seems to have a destabilizing effect by producing local perturbations on lipid layers (2). SP-C could then contribute to the extracellular metabolism of surfactant by fragmentation of large surfactant membranes into small lipoprotein vesicles, which would allow its uptake by macrophages or type II pneumocytes. It is tempting to speculate that complexes of SP-B/SP-C are maintained in the secreted surfactant and, upon its adsorption into the interface, these protein complexes might disassemble allowing each protein to display its defined and specific activity at the peri-interfacial environments. Concerted conformational changes of SP-B and SP-C
such as the ones deduced from our work could be essential to ensure the proper functioning of the pulmonary surfactant.

**Experimental procedures**

**Materials**

Most of the experiments have been carried out with POPC, which forms simple uniformly disordered fluid bilayers at room temperature. All synthetic and derivatized lipids were purchased from Avanti Polar Lipids (Birmingham, AL). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany). Both hydrophobic proteins and surfactant lipids were isolated from porcine lungs by an adaptation of the method of Curstedt et al. (57), as described elsewhere (58). The purity of protein fractions was routinely checked by electrophoresis in SDS-polyacrylamide gels and quantitated by amino acid analysis. The lipid fraction of surfactant, used in experiments involving both WT proteins, was obtained by gel filtration chromatography in LH-20 matrix of the bulk organic extract of porcine surfactant, purified from bronchoalveolar lavage. Lipid concentration was determined by phosphorous quantification.

**Protein labeling and reconstitution in membranes**

SP-B and SP-C were derivatized with BODIPY-FL and Marina Blue (purchased from Invitrogen). The labeling was carried out following a similar procedure to that published elsewhere (59), as follows. The apparent pH of a solution containing pure SP-B or SP-C in chloroform/methanol (2:1, v/v) was adjusted to 6.6 for SP-B and 7.1 for SP-C by the addition of an appropriate volume of a methanolic 50 mM Tris-HCl solution. The protein solutions were incubated at 4 °C overnight in the presence of five times excess (mol/mol) of the desired probe. The reaction was stopped by the addition of HCl until the apparent pH decreased to 2. The unreacted probe was removed by Sephadex LH-20 chromatography. The elution profile of BODIPY FL SP-B was monitored by measuring the absorbance of the fractions at 280 and 504 nm and Marina Blue SP-C elution profile was monitored measuring the absorbance at 240 and 365 nm. The quantification of the degree of labeling was estimated after amino acid analysis and spectroscopic determination of probe incorporation. The molar extinction coefficients used for the probes in chloroform/methanol (2:1, v/v) were ε(BODIPY-FL, 504 nm) = 82 × 10^3 M⁻¹ cm⁻¹ and ε(Marina Blue, 365 nm) = 19 × 10^4 M⁻¹ cm⁻¹ (34). The probe: protein ratio (mol/mol) determined for the labeled proteins was BODIPY-FL/SP-B = 0.92 and Marina Blue:SP-C = 0.5. Adequate amounts of stock solutions of host lipids, proteins, and probes were mixed in chloroform/methanol solutions, dried under a stream of nitrogen, and suspended in 50 mM HEPES with 150 mM NaCl, pH 7, buffer at room temperature. Multilamellar vesicles were made by freeze-thaw cycling 5 times, with vortexing between each cycle.

**Absorption and fluorescence spectroscopy**

All measurements were carried out at room temperature. Absorption spectra were obtained in a Shimadzu UV-3101PC spectrophotometer using spectral bandwidths of 2.0 nm, and corrected for turbidity using the procedure of Castanho et al. (60). Steady-state fluorescence measurements were obtained with an SLM Aminco 8100 Series 2 spectrofluorimeter (Rochester, NY) with double excitation and emission MC400 monochromators in a right-angle geometry. The light source was a 450-W Xe arc lamp and for reference a Rhodamine B quantum counter solution was used. 5 × 5-mm quartz cuvettes were used. Fluorescence decay measurements were carried out with a time-correlated single-photon timing system, which is described in reference (61). Excitation and emission wavelengths are specified in each experiment depending on the fluorophore. Data analysis was carried out using a nonlinear, least-squares iterative convolution method based on the Marquardt algorithm (62). The goodness of the fit was judged from the experimental χ² value (χ² < 1.5 indicating adequate fitting), weighted residuals, and residual autocorrelation plot.

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SP-B and SP-C supramolecular complexes