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MINI-REVIEW

FRET studies of lipid–protein aggregates related to amyloid-like fibers

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Abstract

Acidic lipids are known to both catalyze amyloid fiber formation by amyloidogenic peptides/proteins and induce formation of 'amyloid-like' fibrils by non-amyloidogenic proteins. In this work, we describe the application of state-of-the-art time-resolved Förster resonance energy transfer methodologies to the characterization of the supramolecular structure of the aggregates formed by both a cationic peptide (hexalysyltryptophan) and a basic non-amyloidogenic protein (lysozyme) upon their interaction with negatively-charged fluid membranes (mixtures of zwitterionic phosphatidylcholine and anionic phosphatidylserine). It was concluded that both the peptide and protein induce the formation of multistacked lipid bilayers. Furthermore, upon using conditions that are described in the literature to cause the formation of amyloid-like fibers, lysozyme was found to induce the formation of a 'pinched lamellar' structure, with reduced interbilayer distance in the regions where there is bound protein, and increased interbilayer distance (stabilized by hydration repulsion) outside these areas. No significant lateral domains (lipid demixing) were induced in the membrane by either the cationic peptide or lysozyme.

Keywords: amyloid, anionic phospholipid, FRET, lipid bilayer, lipid-protein interaction.

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A variety of human degenerative diseases is now thought to be associated with the deposition in tissues of proteinaceous aggregates known as amyloid fibrils or plaques (Chiti and Dobson 2006). In addition to share the ability to form fibrillar aggregates with a common stacked cross-β-sheet structure (Chiti and Dobson 2006), several amyloidogenic proteins also interact with lipid membranes, and it has become increasingly recognized that membrane interaction may well be involved in the pathogenesis of their respective amyloid diseases (for recent reviews see Gorbenko and Kinnunen 2006; Munishkina and Fink 2007; Hebda and Miranker 2009). These results have been ascribed to the fact that membranes, particularly those containing negatively charged lipids, provide a unique heterogeneous chemical environment that can facilitate a surface crowding effect and destabilization of protein native structure, induce orientational anisotropy and conformational changes, alleviate electrostatic repulsion between charged monomers, and even drive an ordered polymerization of the protein (Gorbenko and Kinnunen 2006). As a result, formation of amyloid fibrils has been proposed to be catalysed by protein interaction with negatively-charged membrane interfaces, both for several amyloidogenic (Gorbenko and Kinnunen 2006; Munishkina and Fink 2007), as well as non-amyloidogenic proteins like cytochrome *c* and lysozyme among others (Zhao *et al.* 2004, 2005; Alakoskela *et al.* 2006). In addition, uptake of lipids in forming fibers has been shown to occur for a variety of

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Abbreviations used: A, FRET acceptor; D, FRET donor; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine; FRET, Förster resonance energy transfer; K_6W, hexalysyltryptophan.

water-soluble proteins (Zhao *et al.* 2004), and also for islet amyloid polypeptide (amylin) (Sparr *et al.* 2004; Domanov and Kinnunen 2008). At the same time, chemical analysis of *ex vivo* amyloid deposits revealed substantial amounts of lipids in aggregated material (Gellermann *et al.* 2005), which may point to a possible role of lipids in amyloid aggregation and/or stabilization of the preformed deposits against the natural clearance systems of the affected organism. Taken together, the above findings suggest that interactions with lipid membranes could contribute to the rate of formation and stability of amyloid deposits *in vivo*.

The results obtained with the non-amyloidogenic proteins were intriguing, and prompted us to study the structure of these lipid-protein supramolecular complexes, which are typically formed when using both a low ionic strength buffer and a high protein/lipid ratio in the experiments. Our team has developed state-of-the-art methods of analysis for time-resolved Förster resonance energy transfer (FRET) measurements, which have been applied to diverse problems in membrane biophysics such as lateral membrane domains, lipid/protein selectivity and peptide-induced morphological alterations (reviewed in Loura *et al.* 2010a,b; Loura and Prieto 2009, respectively). Most relevant to the subject pertinent to the present report, we recently used FRET to study the interaction between a basic peptide and negatively-charged membranes (Loura *et al.* 2006) and we further extended this methodology to the characterization of the supramolecular structure of the aggregates formed by a basic non-amyloidogenic protein (lysozyme) and negatively charged membranes, which putatively consisted of amyloid-like fiber structures (Coutinho *et al.* 2008). In this work, we describe the essential features of our methodology, the main results and implications of our recent work, and indicate questions that remain to be answered, and for which elucidation FRET and other fluorescence techniques can represent a valuable tool.

FRET in lamellar and multilamellar structures

Förster resonance energy transfer is a photophysical process by which an electronically excited species, the donor (D) transfers its excitation energy to another species, the acceptor (A) which effectively quenches the donor's excited state. As a consequence of FRET, the donor's fluorescence decay ($i_{DA}(t)$) becomes faster and highly dependent on the acceptor concentration and distribution. For randomly distributed probes scattered in parallel planes such as lipid bilayers, $i_{DA}(t)$ is given by

$$i_{DA}(t) = i_D(t)\prod_{j=1}^{N}\left[\rho(t,h_j)\right]^{N_j} \quad (1)$$

where

$$i_D(t) = \sum_i A_i \exp(-t/\tau_i) \quad (2)$$

is the donor decay in the absence of acceptor. The j^{th} term on the product on the right-hand side of eqn 1 represents the contribution of FRET to acceptors located on planes at a distance h_j to the plane of donors (see Figs 1 and 2), and is calculated as (Davenport *et al.* 1985)

$$\rho(t,h_j) = \exp(-tkCh_jF(t,h_j)) \quad (3)$$

where

$$C = \Gamma(2/3)n\pi R_0^2 \quad (4)$$

$$F(t,h_j) = \int_0^\infty \frac{1-\exp\left[(-t/\tau)\left(R_0/h_j\right)^6\alpha^6\right]}{\alpha^3}\,d\alpha \quad (5)$$

In the preceding equations, R_0, the so-called Förster radius, is a measure of the spatial scale probed by FRET (typically, 1 nm < R_0 < 6 nm) and can be calculated from spectral data, whereas $k = 2/R_0^2$, n is the surface density of acceptors, τ is the average donor lifetime in the absence of acceptor and Γ is the complete gamma function. $n_j = 1$ or 2 in eqn 1 is the number of equivalent planes of acceptors located at a distance h_j to that of the donors, and n is the number of different h_j values considered in a given geometrical model (for example, for the bilamellar model depicted in Fig. 1(b), $n = 3$, $n_1 = 2$, and $n_2 = n_3 = 1$).

Interaction of K_6W peptide with DPPC/DPPS membranes

We first studied the effect of the model basic peptide hexalysyltryptophan (K_6W, 0–150 μM) on the organization of fluid ($T = 60°C$) large unilamellar vesicles composed of an equimolar mixture (750 μM total lipid) of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, zwitterionic) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS, anionic) (Loura *et al.* 2006). For this purpose, two fluorescent probes mimicking DPPC were used {1-palmitoyl-2-[3-(diphenylhexatrienyl)propanoyl]-*sn*-glycero-3-phosphocholine (DPH-PC), D; 1-palmitoyl-2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC), A}, with $R_0 = 4.0$ nm. This system has allowed us to derive and test the FRET equations adequate for describing the formation of high-order association complexes driven by electrostatic interactions established between basic peptides/proteins and negatively-charged liposomes.

Whereas in the absence of peptide the time-resolved FRET data could be successfully analyzed using random distribution of probes in a bilayer geometry (Fig. 1a), this was no longer the case when K_6W was added in the studied

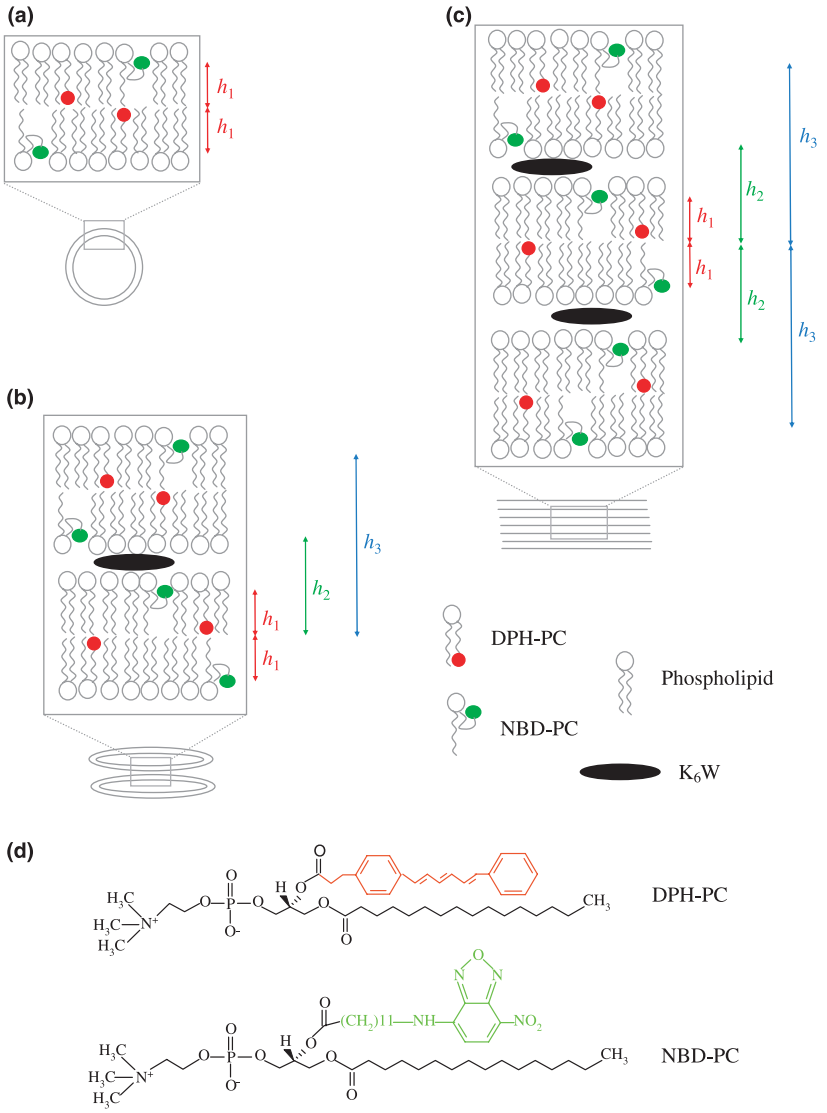

Fig. 1 Schematic diagrams of bilayer-based structures considered in the FRET models, illustrative of the aggregates formed between K_6W and DPPC/DPPS vesicles. (a) *Bilayer geometry*: Two planes of acceptors (NBD-PC) per donor plane (DPH-PC), both at distance h_1. (b) *Bilamellar geometry*: for each donor plane, there are two acceptor planes at distance h_1 and a single acceptor plane at distance h_2. The additional plane at distance h_3 was neglected in the analysis. (c) *Multilayer geometry*: for each donor plane, there are now two acceptor planes at distances h_1 and h_2, respectively. The two acceptor planes at distance h_3 were also neglected in the analysis. (b) and (c) depict situations corresponding to peptide-induced lipid vesicle aggregation driven by electrostatic interactions. The picture is not drawn to scale. (d) Chemical structures of the donor and acceptor probes, DPH-PC and NBD-PC, respectively. Adapted from Loura *et al.* (2006).

concentration range (20 μM ≤ [K_6W] ≤ 150 μM; see Fig. 2b and c, left). We anticipated that the efficiency of FRET would rise upon addition of K_6W to vesicles containing the probes, as a consequence of formation of peptide-induced DPPS-enriched domains that would exclude both D and A (and thus increase their proximity outside these domains). An increase in FRET was indeed observed (as seen from the faster decay of curve c in comparison with curve b, Fig. 2a), but this was not because of domain formation, as analysis of decays using a lamellar biphasic probe distribution was not successful (Loura *et al.* 2006). For [K_6W] ≤ 80 μM, statistically acceptable fits were obtained when considering a bilamellar geometry (Fig. 1b, $n = 3$, $n_1 = 2$, $n_2 = n_3 = 1$), which would result from peptide-mediated aggregation of two bilayers. However, for higher concentrations of peptide, this model could no longer describe the experimental decays (Fig. 2b and c, middle), and adequate fitting required consideration of a multilayer model (Fig. 1c, $n = 3$, $n_1 = n_2 = n_3 = 2$; Fig. 2b and c, right), corresponding to stacked lipid bilayers with peptide sandwiched between them. In all cases, there was no need to consider non-random distribution of probes, excluding the possibility of formation of domains in the FRET length scale. From the fitting procedure, a converging value $h_2 = 4.0$ nm is recovered. Because of the reduced sensitivity of FRET to larger distances, the value of h_3 could not be recovered, and FRET to acceptors located at this transverse distance is effectively masked by the much more efficient energy transfer to the closer acceptor planes (those at distances h_1 and h_2).

This study confirms that although a short polylysine peptide is unable to induce lateral phase separation in fluid phase lipid vesicles, apart from short-range clustering of anionic phospholipids, it causes widespread aggregation of DPPC/DPPS vesicles as a consequence of the expected

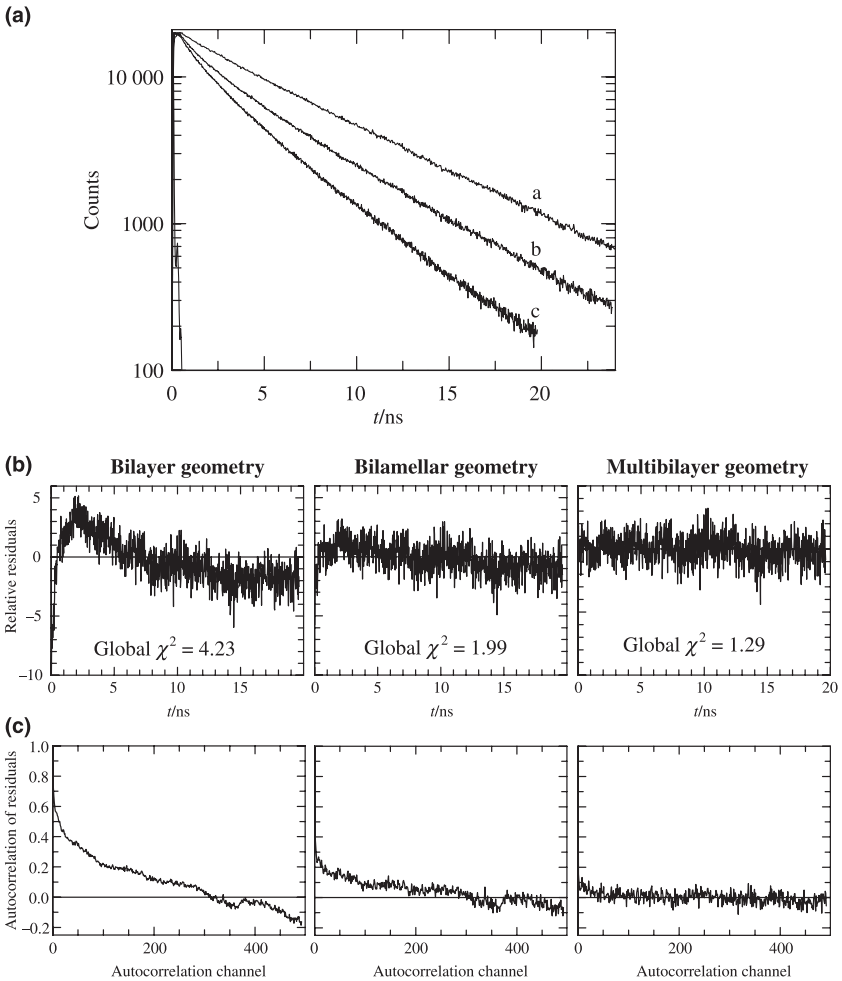

Fig. 2 (a) Fluorescence decays of DPH-PC in 50 : 50 DPPC/DPPS 0.75 mM LUV at 60°C. (a) No acceptor, [K_6W] = 0 μM; (b) NBD-PC : total lipid = 1 : 250, [K_6W] = 0 μM; (c) NBD-PC : total lipid = 1 : 250, [K_6W] = 150 μM. The excitation profile is also shown. (b, c) Residuals (b) and autocorrelation of residuals (c) of the fits to the fluorescence decay of DPH-PC, NBD-PC : total lipid = 1 : 250, [K_6W] = 100 μM. The models used were, from left to right, in both panels: bilayer geometry (Fig. 1a), bilamellar geometry (Fig. 1b) and multilayer geometry (Fig. 1c). The global (combining donor only and donor/acceptor decays) chi-square values are also shown in panel (b). Adapted from Loura *et al.* (2006).

strong electrostatic interaction between the membranes' anionic groups and the basic peptide. When peptide binding reaches saturation (at ∼ 100 μM in our experiments), there is extensive formation of stacked lipid bilayers, bridged by the anionic peptide and with a reduced intermembrane separation of $\sim h_2–h_1 = 2.3$ nm.

Interaction of lysozyme with POPC/POPS membranes

In an effort to contribute to the elucidation of the structure of the so-called 'amyloid-like' aggregates reported to result from addition of several non-amyloidogenic proteins to negatively charged membranes (Zhao *et al.* 2004, 2005; Alakoskela *et al.* 2006), we investigated the interaction of natively folded lysozyme (final concentration 0–9 μM) with 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/ 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) 4 : 1 (mol : mol) lipid vesicles (215 μM total lipid) in a low ionic strength buffer (20 mM Hepes, 0.1 mM EDTA, pH 7.4) using FRET (Coutinho *et al.* 2008).

In this study, two distinct set-ups were used:

(i) *Donor and acceptor are labelled membrane probes.* This scenario corresponds to the schemes depicted in Fig. 2(a) (lysozyme binds to the vesicles without inducing aggregation) and Fig. 2(b) (lysozyme induces vesicle aggregation upon binding). A first set of experiments was carried out with the FRET pair previously used in the K_6W/DPPC/DPPS study described above (DPH-PC/NBD-PC). It was found that FRET using this pair was insensitive to lysozyme concentration. This was interpreted as stemming from the relatively low R_0 value, which, coupled with the fact that multilayer aggregates involving lysozyme would have a repeat distance larger than measured for K_6W, would explain the insensitivity of this pair to report eventual structural alterations. For this reason, we switched to the 2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (D)/*N*-(lissamine™-rhodamine B)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (A) pair (R_0 = 5.5 nm). Using these probes and the absence of lysozyme, the donor decay could be analyzed using the single bilayer model (Fig. 2a), and h_2 = 3.8 nm was recov-

ered as a fitting parameter. Upon increasing the concentration of lysozyme, a small but significant increase in FRET efficiency is observed for 1.0 μM < [lysozyme] < 2.0 μM. In the same protein concentration range, an extensive increase in turbidity of the lipid suspension is observed. Analysis of the $i_{DA}(t)$ curves revealed that a significant improvement of fitting goodness was obtained by allowing a third plane of acceptors for [lysozyme] ≥ 1.0 μM, located at a distance h_3, which value converges to 6.7 nm in the saturation limit. This is consistent with a multilayer arrangement with a lamellar repeat distance of ≅ 9–10.5 nm.

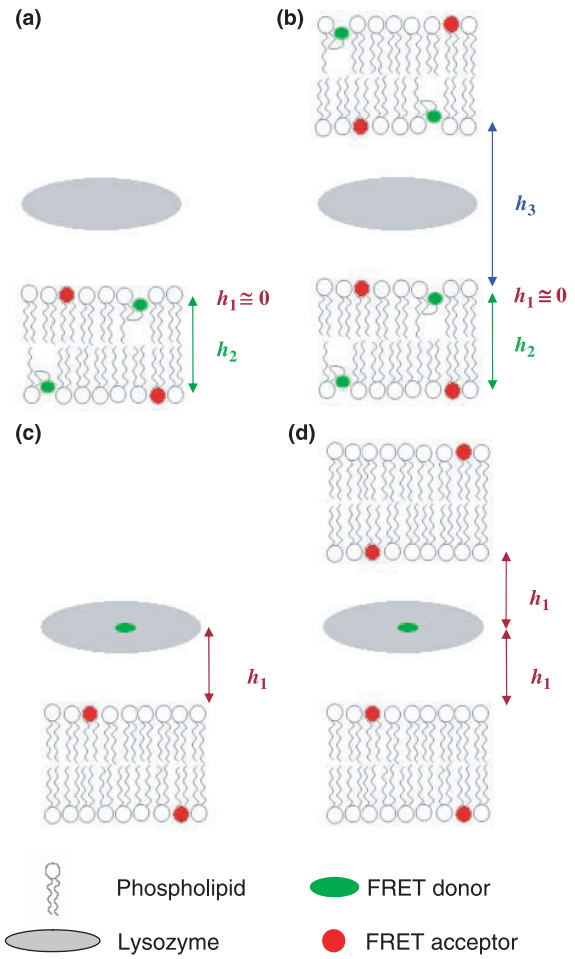

Fig. 3 Schematic representations of the FRET models used for both experimental setups explored in the lysozyme/POPC/POPS system. Panels (a) and (b) refer to FRET between two membrane probes, whereas in panels (c) and (d) donors are located in the protein and the acceptor is a membrane probe. Panels (a) and (c) illustrate topological models for protein interaction with a single lipid bilayer, whereas panels (b) and (d) describe the structure of a multibilayer arrangement with protein molecules sandwiched between adjacent bilayers. Only two bilayers are depicted because FRET to further acceptor planes is negligible. h_1, h_2 and h_3 are distances between planes of donors and acceptors taken into account in the FRET models. Reprinted from Coutinho *et al.* (2008). Copyright 2008 Biophysical Society.

(ii) *Donor fluorophore is located in the protein, whereas acceptor is a labelled membrane probe.* This alternative set-up is represented in Fig. 2(c) (lysozyme binds to the vesicles without inducing aggregation; $n = 1$; $n_1 = 1$) and Fig. 2(d) (lysozyme induces vesicle aggregation upon binding; $n = 1$; $n_1 = 2$). Two FRET pairs were used: wild-type lysozyme tryptophan residues as donors to 1,6-diphenyl-hexatriene as acceptor and Alexa488-labelled lysozyme as donor to *N*-(lissamine™-rhodamine B)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine as acceptor, respectively. Both pairs gave consistent results, with a transition from the model of Fig. 2(c) to that of Fig. 2(d) occurring for slightly lower protein concentrations (0.5 μM < [lysozyme] < 1.5 μM range) than observed in the FRET experiments involving only membrane probes. Another difference between the FRET setup with a membrane probe donor and that with donors in the protein was that, in the latter, much smaller lamellar repeat distances were recovered (≅ 5–7 nm), not larger than the sum of the thickness of one bilayer plus one protein molecule.

These differences can be rationalized by recognizing that FRET between protein-bound donor and a membrane probe allows estimation of the distance between adjacent bilayers in the regions where protein is located, whereas the value obtained from FRET between two membrane probes is probably an average of the distances between the two bilayers in the protein-excluded regions. In this way, a 'pinched lamellar' model for the lipid/lysozyme aggregates, depicted in Fig. 4, was proposed: lysozyme would connect two adjacent bilayers, probably causing short-range sequestration of acidic

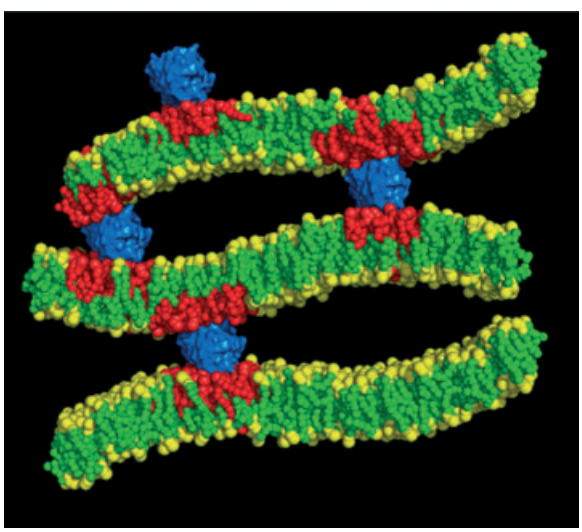

Fig. 4 Schematic representation of the 'pinched lamellar model' for the interaction between lysozyme (blue) and lipid bilayers containing anionic lipids (red). The model is drawn to scale taking into account the repeat distances obtained using FRET. For the sake of clarity, only three bilayers are shown of this multilamellar structure. Reprinted from Coutinho *et al.* (2008). Copyright 2008 Biophysical Society.

lipids (no extensive protein-induced phase separation is observed, even in the nanometer range). In this region, there is a reduced lamellar repeat distance of no more than ≅ 5–7 nm (as revealed by FRET from protein to membrane probe acceptor). Between these 'pinched regions', large pockets of water are contained, stabilized by hydration repulsion, and larger lamellar repeat distances (≅ 9–10.5 nm) are measured (by FRET between two membrane probes). For low protein concentrations (≤ 0.5 μM in the studied system), protein may bind to one vesicle without bridging adjacent bilayers, or even if such bridges occur, they will be rare and the overall interbilayer distance, as recovered by FRET from both set-ups is expected to be large. For intermediate protein concentrations (between 0.5 μM and 1.5 μM in the studied system), bridging occurs at the protein 'pinches'. These are still not widespread in the membranes' surface, and therefore FRET from membrane probes still senses a large interbilayer distance overall. However, because protein donors are necessarily located at the pinches, FRET from these donors already senses a greatly reduced interbilayer distance, and acceptors from both bilayers are felt in equal measure. For higher protein concentrations (≥ 2.0 μM in the studied system) bridging at the pinches is so widespread (because of the higher protein surface concentration) that even the interbilayer distance sensed by FRET from membrane probe donors is now somewhat reduced (because of obvious restriction in the extent of membrane bending between neighboring pinches), though not nearly as much as that reported by FRET from protein donors. Additionally, for these higher concentrations, it is expected that lysozyme inserts into the bilayer to some extent (Yuan *et al.* 2007; Gorbenko *et al.* 2008), reducing the lamellar spacing even further. It is hypothesized that the decrease in the dielectric constant experienced locally by lysozyme at a high protein/lipid ratio upon extensive protein-induced liposome aggregation might be responsible for the loosening of its tertiary structure, ultimately causing its disruption and formation of amyloid-like fibers. Work is under progress to gain insights about the factors that control the formation of these protein-membrane complexes by monitoring the changes undergone by the fluorescence properties of the Alexa488-labelled lysozyme upon interaction with a wide range of concentrations of liposomes containing acidic phospholipids. In addition to ensemble average studies, advanced microscopic techniques (fluorescence correlation spectroscopy and fluorescence lifetime imaging microscopy) are being employed in order to obtain a full characterization of the fibers' structure.

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