

Nanolayers for diagnostics of proteins involved in degenerative amyloidosis

M.-R. Martina, E. Mercatelli, P. Baglioni, and G. Caminati *

Department of Chemistry/CSGI, University of Florence, Via della Lastruccia 3, I-50019, Sesto Fiorentino, Italy

E-mail: gabriella.caminati@unifi.it

Abstract. FK-506 binding protein (FKBP12) is a protein of the family of immunophilins, involved in many neurodegenerative diseases such as Alzheimer's syndrome where FKBP12 is found to be overexpressed in early stages of the disease. We design and built Langmuir-Blodgett nanostructures incorporating ligands with high affinity for FKBP12: Tacrolimus (FK506) and Rifaximin as possible nanosensors to detect low FKBP12 concentration in the initial phase of the amyloidosis. The binding process of the different ligands has been studied by means of photophysical measurements investigating the fluorescence quenching of the tryptophan residue in the binding pocket of FKBP12 by addition of the ligand in solution. A biomimetic strategy was followed for the immobilization of the ligands: we selected different phospholipid nanoarchitectures differing in lipid composition, fluidity, number of layers and method of production (incubation versus co-spreading). Insertion of LB mono and bilayers in both FK506 and Rifaximin solutions have shown that both ligands penetrate the lipid matrix either as monomers or as aggregates depending on their initial concentration. More importantly, the experiments demonstrated that the ligands in the LB scaffolds efficiently quench FKBP12 fluorescence in solution as a consequence of ligand-binding to the protein.

Introduction

The FK506 binding proteins (FKBP) are a family of immunophilins with domains of protein-protein interactions and various cellular functions. It was recently proposed a direct involvement of FKBP12 in neurodegenerative diseases such as Alzheimer and Parkinson diseases, Multiple Sclerosis proliferation disorders and cancer [1] including a decrease of β -amyloid formation when the FKBP12 protein was inhibited by efficient ligands. Furthermore, the studies also demonstrated that FKBP12 is overexpressed in early stages of neurodegenerative diseases [1].

All FKBP12s contain a peptidyl-prolyl isomerase domain (PPI) catalyzing cis-trans isomerization of proline aminoacids. The binding of immunosuppressive ligands to this domain inhibits the PPIase activity and leads to immunosuppression. Immunosuppressive compounds such as FK506, rapamycin and cyclosporine A are high affinity ligands for FKBP12. FK506 binds FKBP12 (a known member of FKBP12s) and this complex leads to immunosuppression by inhibition of the phosphatase activity of calcineurin, a protein involved in the activation pathway of T-cells.

In the present work, we investigated the possibility of building nanostructures incorporating ligands with high

affinity for FKBP12. The long term goals of these studies are the design of nano-functionalized sensors for the detection of the protein in the initial phase of the amyloidosis and the study of optimal biomimetic systems for ligand delivery in the therapeutic stage.

In parallel, we are studying FKBP12 ligands newly synthesized with selected spacers and chemical functions for anchoring to surfaces. An FKBP sensor device can be envisaged by attaching to the ligands a suitable polymeric or alkyl chain ending with an anchoring group for biochemical sensing in Self-Assembled Monolayers or Supported Lipid Bilayers [2,3].

Furthermore, the information on this class of FKBP12 ligands will provide an effective mean for elucidating the role of this protein in amyloidogenesis [4,5]. Results in these directions will be presented in forthcoming studies. In the present work, we examined two ligands of FKBP12, namely FK506, a natural ligand also known as Tacrolimus and Rifaximin, an antibiotic molecule selected for its analogy of cyclic structure with FK506. FK506, a 23-membered macrolide lactone (Fig.1a), is an immunosuppressive drug used in treating patients after organ transplant. Rifaximin (Fig.1b) is a family member of rifamycins antibiotic molecules with broad-spectrum activity against Gram-positive bacteria. The action mechanism of these drugs is based on the inhibition of

RNA synthesis. Another important application of these antibiotics relates to the field of anti-cancer therapies because rifamycins inhibit MRP (Multidrug Resistance-associated Protein) activity responsible of unsuccessful of therapies anti-cancer.

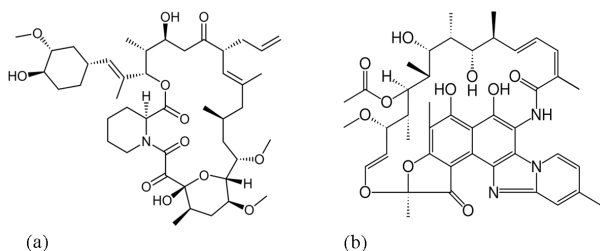


Fig. 1. Chemical structure of Tacrolimus (FK506) (a) and Rifaximin (b)

In particular, we examined Langmuir-Blodgett (LB) mono and bilayers, incorporating the ligand either by immersion of the lipid layers in the ligand solution or by co-spreading in the preparation of the precursor monolayer. The inclusion of the ligand in the LB structure was investigated by means of absorption and emission spectroscopy that allowed for the description of the dependence of ligand uptake on bulk concentration, LB sequence, layer number and phospholipid composition of the layers. Fluorescence quenching of FKBP12 in the presence of LB scaffold with immobilized ligands was also studied, the results enabled to assess the occurrence of a binding process at the outer layer of the LB nanostructures.

Materials and methods

Dipalmitoyl phosphatidylglycerol sodium salt (DPPG-Na, purity >99%) and Tacrolimus (FK506) were supplied by Sigma, Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and Palmitoyl Oleoyl-sn-glycero-3-phosphoglycerol sodium salt (POPG) were supplied by Avanti Polar Lipids, Rifaximin (Rfx, purity 99%) was a gift from FATRO-Pharmaceutical Veterinary Industry (Bologna, Italy). Chloroform/methanol (10:1 v/v) mixtures were used for DPPG-Na spreading solutions, Sigma Aldrich (Italy) supplied chloroform, methanol and dimethylsulfoxide (DMSO). Stock solutions of FK506 and Rifaximin were prepared dissolving the ligands in dimethylsulfoxide. Water was obtained from a Milli-RO coupled with a Milli-Q set-up (Millipore, resistivity=18.2 MΩcm⁻¹, pH=5.6 at 20°C). Phosphate buffer 0.1M (pH 7.4, 0.15 M NaCl), used for aqueous solutions was prepared using di-potassium hydrogen phosphate (K₂HPO₄) and sodium dihydrogen phosphate (NaH₂PO₄), purchased by Fluka. QS quartz slides (Hellma, Germany) were used as solid supports for LB films.

LB films were prepared using a KSV 3000 film balance apparatus (KSV, Finland). DPPG-Na and DPPC/POPG (8:2)+FK506 29% mixture were spread from chloroform/methanol solution on phosphate buffer subphase (T=25°C, pH: 7.4). Twenty minutes were allowed for solvent evaporation before monolayer

compression. The compression rate was 6 mm/min⁻¹. Monolayers were transferred from water–air interface to the solid slides at constant surface pressure, 20 min were allowed for monolayer equilibration at the selected target surface pressure. The dipper rate was 3mm/min⁻¹ for LB transfer. Incorporation of Rfx in the transferred DPPG-Na layers was achieved incubating the LB films in aqueous solution of the antibiotic for 15 h. Incorporation of FK506 was achieved by co-spreading of DPPC/POPG (8:2) + FK506 29% mixture in chloroform/methanol (10:1 v/v). Absorption spectra were recorded on a Lambda900 spectrophotometer (Perkin Elmer, Italy) with cuvette cell holder connected to a Haacke thermostatic bath. Fluorescence spectra were recorded on a LS50B spectrofluorimeter (Perkin Elmer, Italy), excitation and emission slits were set to 3.5 or 5 nm depending on the optical path (OP) length of the cell employed for solutions, 4 nm for LB. QS cell with 0.3 cm optical path length from Hellma (Hellma GmbH & Co. KG, Muellheim, Germany) were used for quenching experiments, whereas 1 cm OP cuvettes were used for absorption measurements of solutions and for absorption and fluorescence measurements of LB. Cuvettes quartz slides were cleaned with piranha solution and carefully rinsed with water and ethanol. The cuvettes were dried by nitrogen flushing prior to each measurement. All fluorescence measurements were run at 15 °C unless otherwise stated in the text, a temperature probe was inserted in the cuvette to ensure optimal temperature control. The concentration of stock FKBP12 solutions in PBS buffer (pH 7.4, 0.15 M NaCl) was determined by UV/Vis ($\epsilon_{280} = 9970 \text{ M}^{-1}\text{cm}^{-1}$). Aliquots of stock solution of ligands were used to prepare adequate concentrations for quenching experiments by dilution with standard phosphate buffer. Fluorescence quenching assays were performed measuring the decrease of intrinsic tryptophan fluorescence at 330 nm as a function of ligand concentration ($\lambda_{\text{exc}}=280 \text{ nm}$). The final FKBP12 concentration used for all measurements was 1 μM. The desired ligand concentration in the sample was obtained by appropriate dilution of ligand stock solution. The emission spectrum of the corresponding blank solution containing only the solvent was always recorded separately and subtracted. In all cases the final DMSO concentration in the sample was kept lower than 0.3% V/V. Emission spectra of the corresponding FKBP12-free samples were subtracted from the corresponding FKBP12-ligand fluorescence spectra. The apparent dissociation constant for each ligand was calculated from the decrease of fluorescence intensity values at $\lambda_{\text{em}}=325 \text{ nm}$ as a function of ligand concentration as previously reported [6].

Results

The binding process of the ligands was preliminary studied by means of photophysical measurements investigating the fluorescence quenching of the tryptophan residue in the binding pocket of FKBP12 [7]. With addition of the ligand in solution we observed an efficient fluorescence quenching of FKBP12. The

dissociation constants, K_d , for FK506 and Rfx were calculated according to literature and recent studies in our group [8,9]; the results are reported in table 1 together with the K_d value obtained for Rapamycin, a well known high affinity compound for FKBP, using literature data [9]. Interestingly, the data reveal that Rfx binds almost as efficiently as FK506 to the protein.

Tab.1. Dissociation constant, K_d of examined ligands

Ligands	K_d (nM)
FK506	6.2 ± 0.2
Rifaximin	13.8 ± 0.3
Rapamycin ^a	5.5 ± 0.2

The ligand were embedded in a planar scaffold using a biomimetic approach: that is to say preparing Langmuir-Blodgett films of phospholipids. We used two different procedures: i) incubation of 1 or 2 LB films of DPPG in solutions of the ligand at different concentrations or ii) inclusion of the FK506 ligand by co-spreading with a mixture of DPPC/POPG as reported also by other authors [10].

Nano-layers of DPPG-Na and DPPC/POPG (8:2) + FK506 29% were transferred onto a solid support by means of the Langmuir-Blodgett technique. We transferred 1 layer for DPPC/POPG (8:2) + FK506 29% mixture and 3 nominal layers for DPPG-Na. We always selected a transfer surface pressure, $\pi=25\text{mNm}^{-1}$, that was shown in a previous paper to ensure optimal interactions between the DPPG phospholipid and Rfx [11]. The transfer ratio was found to be close to unity for the first layer for both systems but in the case of DPPG-Na we always obtained z type LB films.

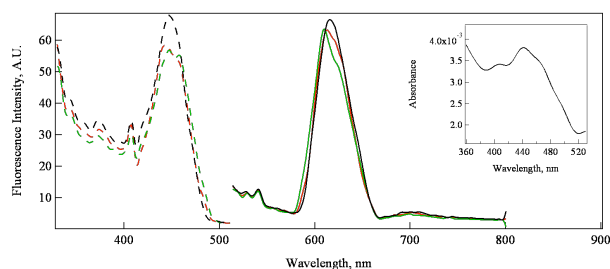


Fig. 2. Fluorescence excitation (dotted line) and emission (solid line) spectra of LB incubated in [Rfx] = $1 \times 10^{-5}\text{M}$ (red), [Rfx] = $0.8 \times 10^{-5}\text{M}$ (green), [Rfx] = $1 \times 10^{-6}\text{M}$ (black). $\lambda_{\text{exc}}=450\text{nm}$ and $\lambda_{\text{em}}=620\text{nm}$. Inset: UV-Vis spectra of LB incubated in Rfx.

Fluorescence emission and excitation spectra (see figure 2) for one DPPG LB layer incubated in Rfx solution unambiguously evidence the inclusion of the ligand in the nanolayer. This assignment is further supported by the presence of the characteristic absorption band at 450 nm of Rfx in the UV-Vis spectra of the same system (inset fig. 2). Increasing Rfx concentration results in a corresponding increase of absorption paralleled by a slight decrease in fluorescence emission, a behaviour that can be correlated to self-quenching phenomena. Conversely, increasing the number of LB layer for a same Rfx concentration induces only a minor increase in the

absorption and emission signal implying a preferential localization of the ligand in the outer layer of the LB films as proposed in the cartoon in figure 3. Therefore, in the present work only the 2-LB layers system was considered.

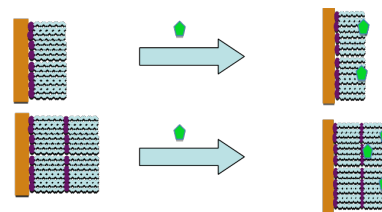


Fig. 3. Cartoon for ligand penetration in 1 and 2 LB layers.

Spreading isotherms for monolayers at water-air interface obtained for co-spreading are reported in fig. 4 together with the results for pure DPPC/POPG 8:2 mixture. The difference in area between the two isotherms was calculated for several surface pressures providing an average value of $31 \text{ \AA}^2/\text{molecule}$. This difference refers to the contribution of FK506 in the monolayer, since the values are uniform in the entire pressure range screened, we conclude that FK506 penetrates the lipid matrix and safely exclude migration of the ligand from the monolayer to the subphase also in the condensed phase chosen for LB transfer.

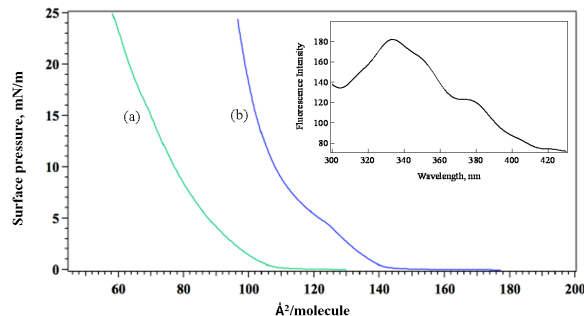


Fig. 4. Spreading isotherms of DPPC/POPG 8:2 (a) and DPPC/POPG 8:2 with FK506 (0.29%) (b). Inset: fluorescence emission spectra of FK506 in 1LB layer of DPPC/POPG 8:2.

Monolayers obtained from co-spreading were successfully transferred onto quartz slides and further characterized collecting their fluorescence emission. The spectra reveal the specific FK506 emission band at 330 nm (see inset Fig. 4) confirming the presence of the ligand in the LB scaffolds.

FKBP12-ligands interactions in the LB nanolayers were assessed from fluorescence quenching experiments in analogy to bulk solution studies. Fluorescence spectra were recorded for the FKBP12 protein solution both alone and in the presence of LB nanolayers containing the ligands. Typical results, reported in Figure 5 for 1 LB layer, show an efficient fluorescence quenching of FKBP fluorescence with both ligands. Higher quenching was found for the Rfx ligand (Fig.5), a result likely related to

the larger ligand concentration obtained with the incubation procedure.

We tested two bulk concentrations of Rfx: 9×10^{-7} M and 9×10^{-6} M and we observed a 20% decrease of fluorescence intensity of FKBP12 for the highest concentration. Whereas, we observed a decrease of 70% at low concentration, a result that reflects the inclusion of aggregated Rfx [11,12] molecules that are unavailable for binding for higher concentrations.

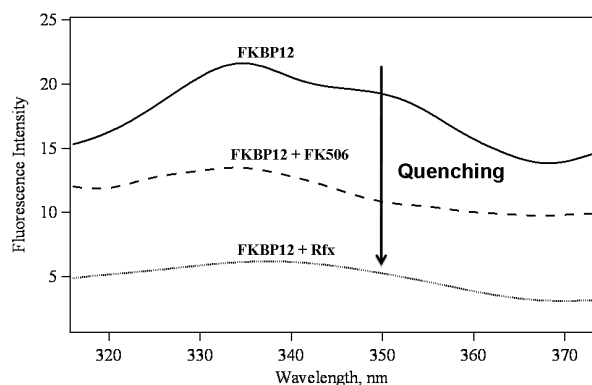


Fig. 5. Fluorescence emission spectra of FKBP12+LB (solid line), FKBP12+LB+FK506 by co-spreading of ligand (dashed line) and FKBP12 LB+Rfx by incubation with ligand (dotted line). [FKBP12]=1 μ M.

Furthermore, fluorescence emission spectra of Rfx in LB film show an emission band centred at 650 nm during and post-incubation with FKBP12. Such band is absent for the Rfx containing nanolayer in buffer solution where a single band at 620 nm is present. The band splitting and the blue shift suggest a change in the molecular microenvironment of Rfx upon protein binding and support the presence of Rfx aggregates. (Fig.6)

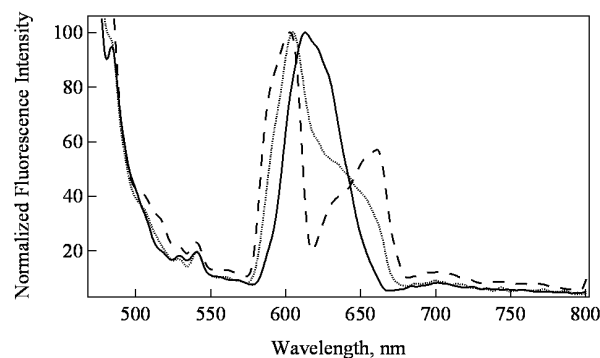


Fig. 6. Fluorescence emission spectra of Rfx in LB in buffer solution (solid line), Rfx in LB during incubation with FKBP12 (dashed line) and Rfx in LB post-incubation with FKBP12 (dotted line). FKBP12=[1 μ M]

Conclusions

Solution quenching studies of the FKBP12-Rfx system evidenced how the commonly used Rfx antibiotic is very effective in binding to the FKBP12 protein with a dissociation constant only two times larger than the K_d found for Tacrolimus, the most widely used FKBP12

inhibitor. The body of the results also indicate that both Rfx and FK506 penetrate easily biomimetic systems such mono and bilayers of phospholipids although incubation procedures yield good incorporation results only for Rfx whereas co-spreading was demonstrated to produce efficiently stable LB structures containing FK506. Moreover, quenching studies proved that the binding to the FKBP12 protein was effective also when the ligand where immobilized in the outer layer of the LB films provided that the molecules were not aggregated. This opens the way to important applications of these nanostructures, to name a few phospholipid formulation of liposomal systems for drug delivery as well as sensitive and specific nanosensors for FKBP12 for the early diagnosis of neurodegenerative amyloidosis.

Other potential ligands with different functional groups have recently been [6] proposed, quenching studies for these novel ligands, functionalized with suitable spacers and anchors, are currently in progress for LB films, Supported Lipid Bilayer and bare surfaces to be employed as sensors transducers.

Acknowledgements

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References

1. W. Cao, M. Konsolaky, J. Biosci. **36** (3), pp 493-498 (2011)
2. F. Gambinossi, L. Lorenzelli, P. Baglioni, G. Caminati, Colloids Surf. A Physicochem. Eng. Asp., **321**, 87-93 (2008)
3. S. Morandi, M. Puggelli, G. Caminati, Colloids Surf. A Physicochem. Eng. Asp., **321**, 125-130 (2008)
4. M. Gerard, A. Deleersnijder, J. Demeulemeester, Z. Debyser, V. Baekelandt, Mol. Neurobiology, **44**, 13-27 (2011)
5. T. Al-Kayal, E. Russo, L. Pieri, G. Caminati, D. Berti, M. Bucciattini, M. Stefani, P. Baglioni, Soft Matter, **8**, 9115-9126 (2012)
6. M. Bizzarri, E. Tenori, M. R. Martina, S. Marsili, G. Caminati, S. Menichetti, P. Procacci, J. Phys. Chem. Lett. **2**, 2834-2839 (2011)
7. C. H. Roehrig, C. Loch, J.-Y. Siegal, M. Overhand, Chem. Med. Chem. **2**, 1054-1070 (2007)
8. M.R Martina, E. Tenori, M. Bizzarri, S. Menichetti, G. Caminati, P. Procacci, submitted to J. Am. Chem. Soc. (2012)
9. C. Kozany, A. Maerz, C. Kress, F. Hausch, ChemBioChem, **10**, 1402-1410 (2009)
10. O. Canadas, R. Guerriero, R. Garcia-Canero, G. Orellana, M. Menendez, C. Casals, Biochemistry, **43**, 9926-9938 (2004)
11. S. Morandi, M. Puggelli, G. Caminati, Intern. J. Environ. Anal. Chem. **85**, 665-673 (2005)
12. S. Martini, A. Magnani, P. Corti, G. Corbini, R. Lampariello, M.P. Picchi, M. Ricci, C. Bonechi. Spectrosc. Lett., **35**, 581 (2002)