Experimental report

18/10/2017

Proposal: 9-13-662 **Council:** 4/2016

Title: Novel transmembrane recognition and immobilization phenomena in lipidmonolayers at the air/water interface regulated by fluorocarbon gases

Research area: Soft condensed matter

This proposal is a new proposal

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Samples: Nitrosoimidazoles EF3, EF5, EF9
Perfluorohexane
Perfluorobutane
Phospholipids DPPC, DMPC, DiC8PC

Instrument	Requested days	Allocated days	From	To
FIGARO Adsorption troughs	4	4	01/12/2016	05/12/2016

Abstract:

The recruitment of fluorinated therapeutic agents and biomarkers at interfaces is an important new focus in drug delivery research with implications in the treatment of cancers and neurodegenerative diseases. We have shown recently that the adsorption in phospholipid monolayers of soluble fluorinated compounds can be strongly enhanced by hydrophobic interactions at the air/water interface with fluorocarbon gases. In collaboration with Colleagues who synthesize the biomarkers, we propose to extend our recent work to a study of the interaction kinetics and composition of the interfacial film using the FIGARO reflectometer at the ILL. First, we will quantify the extent of irreversible recruitment of biomarkers in condensed-phase lipid monolayers. Then, through selective deuteration of spacer groups in the biomarker, we will resolve the partitioning of the biomarker and the gas in fluid-phase lipid monolayers. Neither of these aims has been realized by ourselves or others to date, and answers to these questions by exploiting the unique high flux of FIGARO at low q will pave the way for future work on the design of novel microbubble-based vectors for drug delivery applications.

Determination of the interfacial film composition responsible for a novel transmembrane recognition and immobilization phenomenon

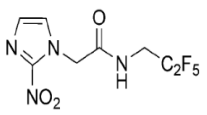
INTRODUCTION

The recruitment of *fluorinated* therapeutic agents and biomarkers at interfaces is an important new focus in drug delivery research with implications in the treatment of cancers and neurodegenerative diseases. Recently, we have shown (Yang, *Angew. Int. Ed.* **2015**, *54*, 8402) that the adsorption in phospholipid monolayers of water-soluble fluorinated biomarkers, that otherwise do not adsorb at this interface, can be empowered when a fluorocarbon gas is introduced in the gas phase. This provides a novel simple method to immobilize fluorinated therapeutics in the shell of medical microbubbles with potential in drug delivery for theranostic purposes.

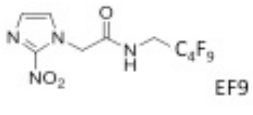
AIMS

The objective of our neutron reflectometry experiment on FIGARO at the ILL was to determine the composition of mixed monolayers formed at the air/water interface by a monolayer-forming phospholipid, a fluorocarbon gas (*F*-hexane), and a fluorinated nitrosoimidazole biomarker (*F*-biomarker). The *F*-biomarker is recruited at the interface by *F*-hexane and either is immobilized (i.e. trapped) in the monolayer or is expelled following removal of the gas.

The *F*-biomarkers (EF5, EF9) are cell hypoxia biomarkers that are presently investigated in clinical trials. FIGARO is the only neutron reflectometer in the world that is optimized for the precise quantification of sub-monolayer coverage of fluorinated moieties in weakly reflecting monolayers, thanks to its low-Q interfacial composition method (Campbell, *Soft Matter* **2016**, *12*, 5304).



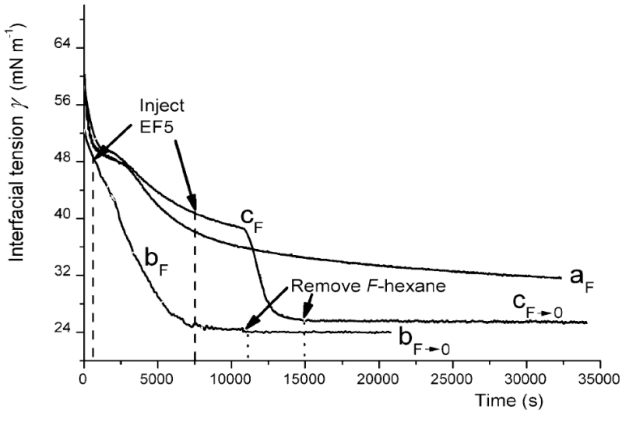
EF5



EF9

PRELIMINARY RESULTS

Using tensiometry we have shown that when a phospholipid forms a liquid condensed (LC) Gibbs monolayer, which is the case for 1,2-dipalmitoylphosphatidylcholine (DPPC), the *F*-biomarker recruited by contacting the film with *F*-hexane remains trapped in the monolayer after *F*-hexane is removed (Fig. 1). On the other hand, when the monolayer is in a liquid expanded phase (LE, *e.g.* with dioctanoylphosphatidylcholine, diC_8PC), the *F*-biomarker is expelled from the interface when *F*-hexane is removed (Fig. 2).



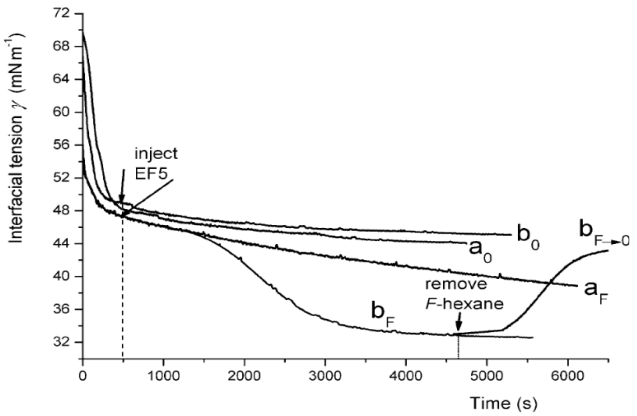


Fig. 1 (left). Kinetics of a) adsorption of DPPC, b) and c) sequential adsorptions of DPPC followed by EF5 (injected after 500 s (b), or 7000 s (c)). The experiments were achieved in the presence ($_F$), or absence ($_0$), of *F*-hexane. The subscript ($_{F\to0}$) refers to an experiment in which *F*-hexane is displaced by air, while keeping constant the bubble volume. Concentrations: 1 x 10^{-3} mol L^{-1} for both DPPC and EF5; 37°C. **Fig. 2 (right).** Kinetics of a) adsorption of diC_8PC and b) sequential adsorption of diC_8PC followed by EF5 (injected after 500 s, arrows). Concentrations: 5 x 10^{-6} mol L^{-1} for diC_8PC; 1 x 10^{-3} mol L^{-1} for EF5; 25°C.

Subsequent experiments established that microbubbles could be prepared with shells made of phospholipids in which *F*-hexane and EF5 were embedded. *In order to investigate the medical potential of these microbubbles it is essential to quantify the interfacial concentrations of the three interacting components.*

FIGARO EXPERIMENT: PLAN

An experiment (Dec. 2016) was granted 4 days of beam time on FIGARO. Reference data were first recorded with pure phospholipids: 8 and 30 mN m^{-1} for DPPC and 30 mN m^{-1} for 1,2-dimyristoylphosphatidylcholine (DMPC). The atmosphere in the adsorption troughs was then saturated with vapor of *F*-hexane. A homologous series of nitrosoimidazoles carrying two fluorinated moieties (EF5 and EF9) and two different hydrocarbon spacers (C_2H_4 and C_4H_8) was investigated. These compounds and their deuterated analogues were synthesized in the group of Prof. V. Gouverneur (Oxford, UK). When *F*-hexane saturation was reached, the lids of the adsorption troughs were opened, allowing replacement of the fluorocarbon-saturated atmosphere by pure air. The samples were prepared in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH adjusted to 7 by 1N NaOH) in ACMW (8.1% v/v D_2O in H_2O).

FIGARO EXPERIMENT 1: **Quantification of *F*-hexane in phospholipid monolayers**

We succeeded in quantifying the amount of fluorocarbon taken up in the phospholipid monolayers first in the absence of the *F*-biomarkers. We investigated three different phospholipids: DMPC, DPPC, and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC).

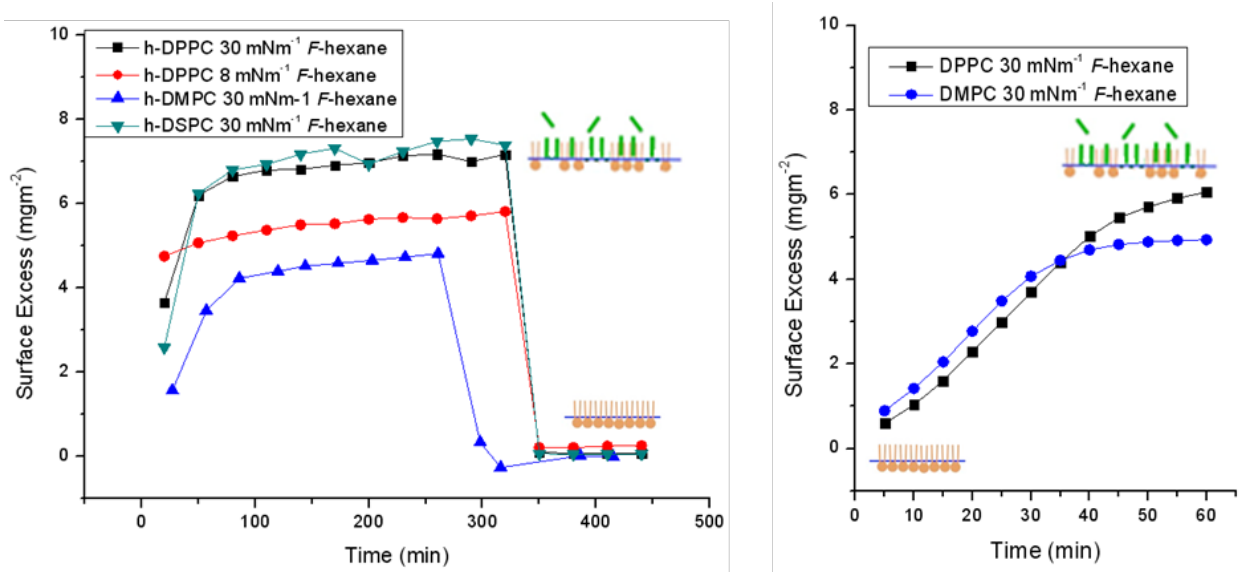


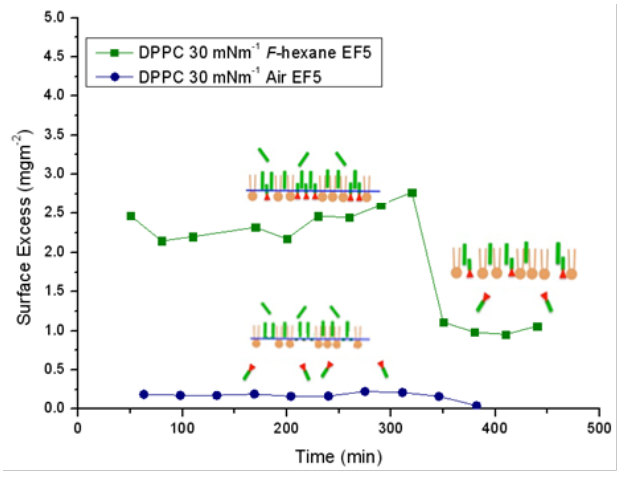
Fig. 3 (left): Surface excess of *F*-hexane in DPPC at 30 mNm^{-1} (black), 8 mN m^{-1} (red), in DMPC at 30 mN m^{-1} (blue), in DSPC at 30 mN m^{-1} (green) at as determined by neutron reflectivity measurements using FIGARO. **Fig. 4 (right):** Adsorption kinetics of *F*-hexane in DPPC (black) and DMPC (blue).

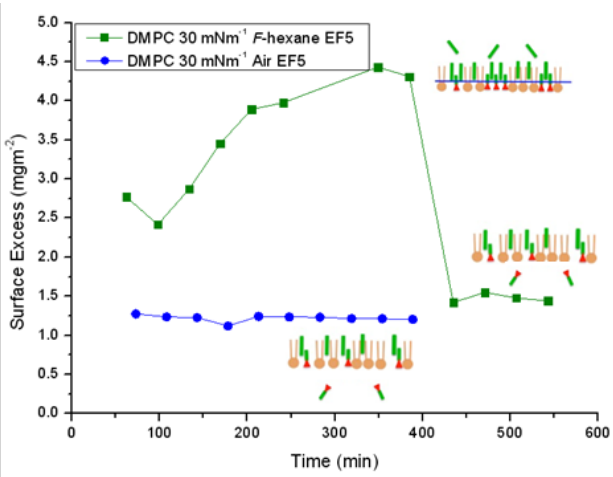
When the plateau is reached, the surface excess of *F*-hexane was maximum for DPPC and DSPC in the LC phase (~7 mg m^{-2}, Fig. 3), a value confirmed by ellipsometry (Beaglehole Picometer Light ellipsometer, Partnership for Soft Condensed Matter); it was ~5 mg m^{-2} for DPPC in the LE state, and ~4 mg m^{-2} for DMPC (LE). When saturation was reached, the lids of the adsorption troughs were opened, allowing replacement of the *F*-hexane-saturated atmosphere by air. The *F*-hexane surface excess was found to decrease rapidly to zero, meaning that no fluorocarbon remains trapped in the phospholipid monolayer. A study of the *F*-hexane adsorption kinetics at short times showed that *F*-hexane adsorbs in the DPPC and DMPC monolayers at similar rates (Fig. 4). This is the first time that the adsorption of *F*-hexane in phospholipid monolayers has been quantified, and these results will be collected in a publication. This finding is also of interest in the design of innovative lung surfactant substitutes based on fluorocarbons (Krafft, *Soft Matter*, **2015,** *11*, 5982).

FIGARO EXPERIMENT 2: **Quantification of *F*-biomarkers in phospholipid monolayers in the presence or absence of *F*-hexane**

Having determined the concentration of *F*-hexane in the phospholipid monolayers, and assuming that *F*-hexane concentration does not change in the presence of the *F*-biomarkers, we were able to calculate the concentration of the *F*-biomarker in the phospholipid monolayer by subtracting the corresponding contributing scattering excesses. With a DPPC monolayer under air, the EF5 surface excess is close to zero (Fig. 5), showing that EF5 does not adsorb in the phospholipid film. This situation is changed dramatically when the air phase is saturated by *F*-hexane. The EF5 surface excess then increases with time and plateaus at ~3 mg m^{-2}. When the lid of the trough is open, the surface excess drops but remains clearly above zero. This result demonstrates that a significant amount of EF5 (1 mg m^{-2}) stays trapped in the DPPC monolayer even after the fluorocarbon gas is removed. For DMPC, the EF5 surface excess strongly increases (up to ~5 mg m^{-2} versus 1 mg m^{-2}), Fig. 6, showing that *F*-hexane efficiently recruits EF5 to the interface. However, when *F*-hexane is replaced by pure air, the EF5 surface excess decreases and plateaus back to ~1 mg m^{-2}, that is, its value measured in the absence of the fluorocarbon gas. This means that EF5 is not immobilized in the phospholipid monolayer in this case. Thus, we have confirmed that immobilization of the *F*-biomarkers in phospholipid monolayers depends critically on the physical state, LC versus LE, of the phospholipid, and have succeeded to quantify the effects for the first time.

Immobilization of the *F*-biomarkers also depends on their molecular structure. Thus, EF9 is recruited at the phospholipid interface (both for DMPC and DPPC) when *F*-hexane is present. However, it is released in the water subphase when *F*-hexane is removed. The presence of a hydrocarbon linker also strongly influences the extent of immobilization of the *F*-biomarker once the fluorocarbon gas is removed. These results will provide fundamental information for the *in vivo F*-biomarker delivery. These results will be reported in a further publication.





Surface excess of the *F*-biomarker EF5 in DPPC (**Fig. 5 (left)**) and in DMPC (**Fig. 6 (right)**), as determined by neutron reflectivity measurements using FIGARO.

CONCLUSIONS

The FIGARO experiments allowed us to assess and quantify the concentrations of *F*-hexane and *F*-biomarker trapped in an interfacial phospholipid monolayer. The influences of the physical state of the monolayer and that of the molecular structure of the biomarker were substantial, providing useful insight for elaborating microbubbles designed to carry and deliver *F*-biomarkers. A continuation proposal was granted 3 days of beam time. This experiment, planned for Nov. 2017 was cancelled due to ILL reactor shut down and will be rescheduled as soon as possible.