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Photoactivatable Phospholipids Bearing Tetrafluorophenylazido Chromophores Exhibit Unprecedented Protonation-State -Dependent ^{19}F NMR Signals

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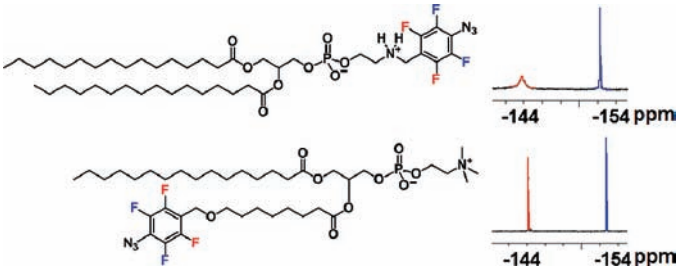
ABSTRACT



Phospholipids bearing tetrafluorophenylazido chromophores were synthesized with perfectly conserved amphiphilicity and photochemical activity. Interestingly however, those phospholipids harboring the amine-linked chromophores exhibited unusual ^{19}F NMR signals which depended on the protonation state of the lipid headgroup. These probes may serve as powerful tools for studying various pH-dependent events in biomembranes.

Biomembranes are of considerable importance not only due to their function as the cell boundary regulating the exchange with and protection from the outside environment but also due to their multiple roles in intra- and extracellular processes.[1] However, studying these biomembranes is extremely challenging due to their heterogeneous mosaic composition and limited solubility. Photoactivatable phospholipids are useful tools to investigate biomembranes *via* a photolabeling approach.[2,3] In our effort to develop stable and efficient photoactivatable phospholipidic probes, we are particularly interested in using tetrafluorophenylazide moieties as photoactive chromophores.[4,5] This is due to much more efficient photolabeling yielded by the fluorinated aryl azides compared to their nonfluorinated counterparts thanks to considerable stabilization of the reactive arylnitrene intermediates by the electronegative fluorine atoms and the resulting longer half-life.[6] Moreover, the presence of fluorine atoms[7,8] provides a unique advantage in ^{19}F NMR based investigations of biological processes thanks to the absence of fluorinated compounds in most biological samples which could otherwise give rise to interfering background signals.

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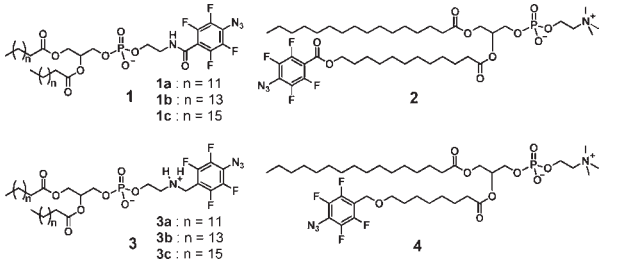
The high sensitivity of ^{19}F NMR results additionally from the 100% natural abundance of ^{19}F as well as the sensitive resonance signals distributed over a wide spectral width upon even minor chemical changes.[9]

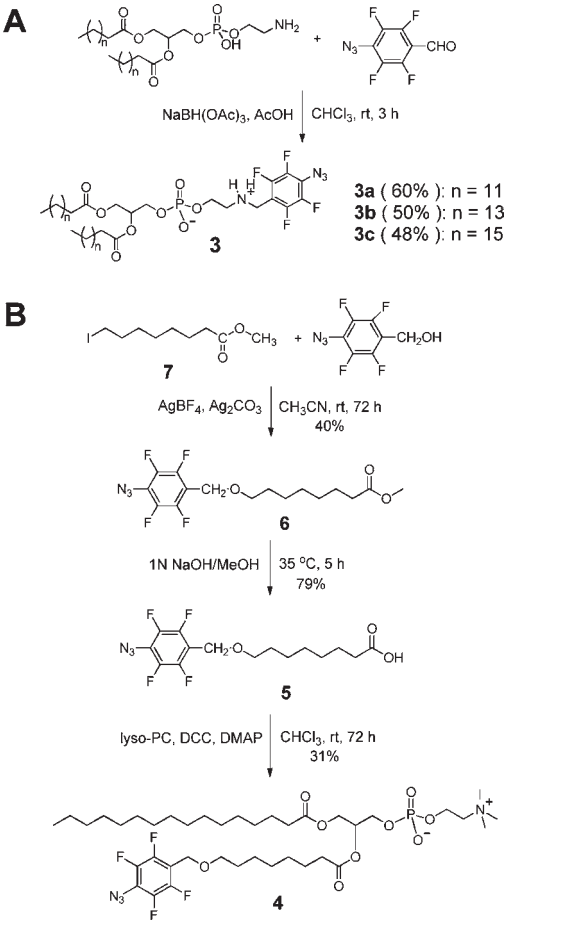
Scheme 1. Photoactivatable Phospholipidic Probes Containing the Tetrafluorophenylazido Group



In our previous work, we synthesized two photoactivatable phospholipidic probes **1** and **2** containing the photoactive tetrafluorophenylazido group at the polar head and in the fatty acid chain, respectively (Scheme 1).[4] However, probe **1** has the chromophore coupled to the phosphatidylethanolamine *via* amide linkage, leading to the abolishment of the positive charge of the phospholipid head amine group at physiological pH. This may alter the related structure and functions of phospholipids when incorporated within biomembranes. Meanwhile probe **2**, which has the tetrafluorophenylazido group attached to the fatty acyl chain *via* an ester linkage, may be hydrolytically or enzymatically labile and thus limit its potential applications. We therefore wanted to introduce the tetrafluorophenylazido group to the phospholipids *via* a more reliable amine and ether linkage at the polar head and in the fatty acid chain respectively (probes **3** and **4** in Scheme 1). The amine linkage between the chromophore and the amine head of the phospholipids was expected to ensure that the phospholipid heads of the probes **3a**–**c** retained their protonation ability at physiological pH, while the ether linkage between the tetrafluorophenylazido group and the fatty acid was expected to make probe **4** resistant to chemical and enzymatic hydrolysis. Here we report the synthesis and characterization of these two probes. Interestingly, probe **3** exhibits unusually broad ^{19}F NMR signals arising from the different protonation states of the adjacent amine function at the phospholipid headgroup. This finding could be useful when studying the biological processes involving pH variation within biomembranes that are important and yet difficult to investigate due to the absence of suitable and sensitive methods.

Probe **3** was synthesized by the reductive amination of 4-azidotetrafluorobenzaldehyde with the corresponding

Scheme 2. Synthesis of Probes **3** (A) and **4** (B)



phosphatidylethanolamine (Scheme 2A) using sodium triacetoxyborohydride [$NaBH(OAc)_3$].[10,11] In addition to the expected product **3**, we also obtained two side products arising from the direct reduction of 4-azidotetrafluorobenzaldehyde and the double reductive amination of 4-azidotetrafluorobenzaldehyde, respectively (Scheme S1). In order to diminish these side reactions, we reduced the excess amount of 4-azidotetrafluorobenzaldehyde and shortened the reaction time. To further improve the reaction, we chose $CHCl_3$ as the solvent for its good solubility of phospholipid compounds. Altogether, these efforts allowed us to obtain probes **3a**–**c** with good and satisfactory yields.

Synthesis of probe **4** was then achieved by conjugating the tetrafluorophenylazido-containing fatty acid surrogate **5** with lyso-phosphocholine (lyso-PC) (Scheme 2B). Compound **6**, the ester form of **5**, was prepared by coupling **7** with 4-azidotetrafluorobenzyl alcohol, with **7** being obtained conveniently using a one-step procedure developed by our group (Scheme S2). We obtained **6** in only moderate yield, with the best yield being attained using the $AgBF_4$/Ag_2CO_3 system.[12] This was mainly due to the low reactivity of **7** caused by its long alkyl chain, because the shorter alkyl iodides such as CH_3I and CH_3CH_2I could give

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satisfactory yields.[12] The obtained **6** was then hydrolyzed to give **5** (Scheme 2B). Coupling of **5** with lyso-PC led to the desired probe **4**. Despite numerous attempts to improve the yield of **4** *via* various means such as performing the reaction under strictly anhydrous conditions, or using different activating or coupling reagents, it proved difficult to achieve higher yields mainly due to the incomplete coupling reaction but also the extremely difficult purification process by performing flash chromatography on silica gel and the notorious instability of the phospholipid compound.

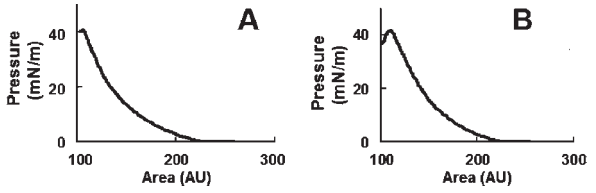

Figure 1. Pressure–area isotherms of surface monolayers of the synthesized probes **3b** (A) and **4** (B).

All the synthesized probes gave excellent NMR, IR, UV, and HRMS analytical data. We then wished to test whether they had conserved the amphiphilic characteristics of natural phospholipids. All the probes were poorly soluble in water; however, their solubility could be considerably increased in the presence of the detergent, sodium dodecylsulfonate (Figure S1). In addition, these probes could form a Langmuir monolayer film at the air/water interface.[13] Their pressure–area isotherm curves (Figure 1) are strikingly similar and resemble that obtained with the natural phospholipid, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Figure S2). This suggests that the differences in their structure do not influence the area occupied by each molecule or the intermolecular interactions. Collectively, these data confirm that probes **3** and **4** are, like natural phospholipids, amphiphilic.

We next studied the photochemical properties of these phospholipid probes. Both **3** and **4** were stable in the dark and underwent a rapid photochemical reaction (Figure 2). Irradiation of these probes at 300 nm quickly led to the disappearance of the maximal absorption band of the tetrafluorophenylazide chromophore (Figure 2). In addition, the observed isosbestic points indicate that the photochemical reaction was a clear-cut photodecomposition process. Overall, these data indicate that our lipid probes retain the photochemical properties of fluorinated phenylazide and are potentially promising tools in photolabeling studies of biomembranes.

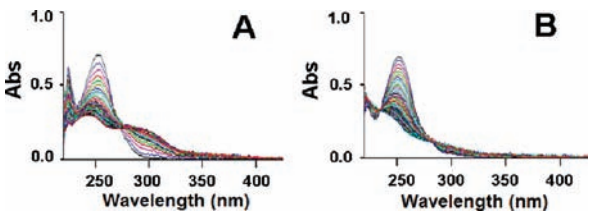

Figure 2. UV spectral recording of the photochemical reaction of **3b** (A) and **4** (B) upon irradiation at 300 nm at 20 °C.

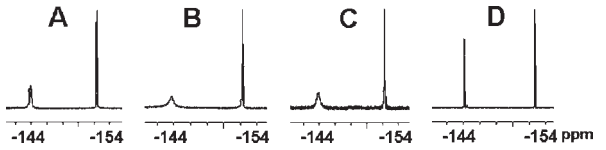

Figure 3. ^{19}F NMR spectra of the photoactivatable phospholipidic probes **3a** (A), **3b** (B), **3c** (C), and **4** (D) were recorded in $CDCl_3$.[15]

We went on to analyze the ^{19}F NMR of these probes (Figure 3) as ^{19}F NMR is an alternative and complementary approach to photolabeling to investigate biomembranes.[14] To our surprise, probe **3** showed an unusually broad ^{19}F signal around −144 ppm (Figure 3A–3C), in addition to the normal and sharp singlet peak recorded at −152 ppm. This was completely different from what we previously observed with lipid probes containing a 4-azidotetrafluorophenyl chromophore including probes **1**, **2** (Figure S3), and **4** (Figure 3D), all of which show two sharp ^{19}F NMR signals around −153 and −144 ppm, corresponding to the two pairs of fluorine atoms on the phenyl ring in the chromophore. Similar to the phospholipids **1**, **2**, and **4**, the lipid probes **3a**–**c** are well soluble in the NMR solvent and, therefore, exclude the formation of micelles or vesicles, which might be the origin of the observed unusual NMR signals.

We know that the amine functionality can be easily protonated in physiological media at a pH less than its pK_a value. Based on this knowledge, we hypothesized that the free and protonated states of the amine function in probe **3** may cause different chemical shifts of the adjacent F atoms in the tetrafluorophenylazido chromophore; the equilibrium between these two states could lead to the merge of the two distinct ^{19}F NMR signals into one broader peak (Figure 3A–3C). In order to confirm this, we performed ^{19}F NMR analysis on a model compound in its free amine **8** and protonated amine **8′** states (for their preparation see Supporting Information), rather than on **3** directly. This is because the phospholipid probe **3** is relatively fragile in its protonated state at low pH during the ^{19}F NMR experimental process. With the aid of this model compound, we indeed observed that the ^{19}F nuclei in the chromophore adjacent to the amine function had two sharp and distinct chemical shifts at −145 and −139

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(15) Two or three drops of CD_3OD were added to 300–500 μL of $CDCl_3$ in order to improve NMR resolution.

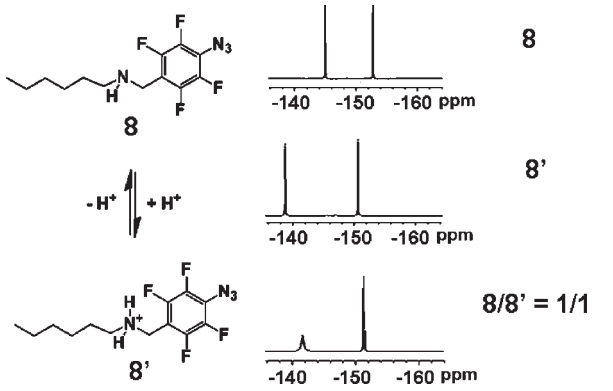

Figure 4. ^{19}F NMR spectral recording of **8**, **8′**, and their mixture at a ratio of 1/1 were recorded in $CDCl_3$.

ppm in free amine **8** and protonated amine **8′**, respectively (Figure 4). By mixing **8** with **8′** in a 1:1 ratio,[16] the ^{19}F NMR signals at −145 ppm (**8**) and −139 ppm (**8′**) disappeared and coalesced into a single, exchange broadened NMR signal at −142 ppm (Figure 4). This clearly indicates that there is chemical exchange between the two amine forms **8** and **8′**, with an exchange rate in the order of the chemical shift separation (expressed in Hz). Indeed, slow chemical exchange on the NMR time scale would have led to the observation of two distinct ^{19}F signals, one due to the free (−145 ppm) and the other to the protonated amine (−139 ppm). In contrast, fast chemical exchange would lead to the observation of a single line, the chemical shift of which would be the average of the chemical shifts of the two forms (i.e., around −142 ppm for a 1:1 molar ratio). Only intermediate chemical exchange rates can yield exchange broadened signals similar to those depicted in Figures 3 and 4.

It is known that the electron-withdrawing character of the fluorine atom can affect the pK_a values of the corresponding benzylic amines, as shown by the pH back-titration of the compound **8** and benzyl amine (Figure S4). This can be interesting as the fluorinated benzylic amines may also serve as ^{19}F NMR pH indicators for an investigation on pH dependent biological events.[17,18] Cell adhesion, biomembrane fission and fusion, lipid−protein binding, etc. depend critically on intracellular pH and the protonation state of the phospholipid headgroup.[19,20] The photoactivatable phospholipid probe **3** developed here could constitute a useful means to study these processes by ^{19}F NMR in addition to the photolabeling approach. Moreover, ^{19}F NMR has the unique advantage of being highly sensitive and informative for the study of biological systems using fluorinated probes[21] because of the absence of natural fluorinated compounds in most biological systems, the high natural abundance of fluorine, and the wide range of chemical shifts sensitive to the chemical environment.

In conclusion, we have synthesized novel photoactivatable phospholipidic probes containing tetrafluorophenylazido groups either at the polar head *via* an amine bridge or in the fatty acid chain *via* an ether linkage. The lipid-like amphiphilic characteristics and excellent photochemical properties of these probes forecast their potential application in photolabeling studies of biomembranes. Furthermore, when linked to the amine function at the phospholipid headgroup, the tetrafluorophenylazide chromophore exhibited protonation state-dependent ^{19}F NMR signals, which could prove useful in investigating pH-dependent membrane processes. These are often difficult to study but are nevertheless involved in important phenomena including lipid−protein interactions, membrane fission/fusion, drug delivery, etc.

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Supporting Information Available. Experimental procedures and spectroscopic data for all new compounds as well as Schemes S1−S3 and Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

(16) Due to the poor solubility of probe **3** and compound **8** in water, we could not undertake a reliable pH titration process of these probes using ^{19}F NMR.

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