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Depth-dependent analysis of membranes using benzophenone-based phospholipids

Bino John, E. Ravi Kumar, Anil K. Lala*

Biomembrane Laboratory, Department of Chemistry and Biotechnology Center, Indian Institute of Technology Bombay, Powai, Bombay 400 076, India

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Abstract

Any attempt to probe the membrane hydrophobic core with chemical reagents necessitates the use of reactive intermediates like carbenes and nitrenes, which can insert into C–H bonds. Several photoactivable reagents based on carbenes and nitrenes have been reported. However, the high reactivity of these reagents, often leads to very low insertion yields. We report here a high degree of cross-linking (35–40%) achieved with three benzophenone-based phospholipids and analyze the carbon functionalization data using a multiple Gaussian function. These phospholipids are so designed so as to permit depth-dependent labeling in membranes. Single bilayer vesicles were prepared from these phospholipids and dimyristoylphosphatidylcholine. The cross-linked product was isolated and characterized by mass spectroscopy. The results obtained indicated that the cross-linked product was dominated by dimeric product formed by intermolecular cross-linking. The Gaussian analysis used here provides insight into the relative depths of the probes inside the membrane.

Keywords: Depth-dependent analysis; Cross-linking; Benzophenone-based phospholipids; Gaussian

1. Introduction

The main objective of the membrane hydrophobic core labeling is to provide information on the nature of lipid and protein components embedded in the membrane. A photochemical approach involving the use of photoactivable reagents has been effectively used to study membrane-embedded domains of transmembrane proteins [1]. Both carbene and nitrene precursors, e.g. 5-iodo-naphthyl-1-azide [2], adamantyl diazirine [3], 3-trifluoromethyl-3-(*m*-iodophenyl) diazirine [4] and diazofluorene [5] have been used

* Corresponding author. Tel.: +91-22-5784383; fax: +91-22-5767152/5784383/5783480.

E-mail address: anillala@chem.iitb.ernet.in (A.K. Lala).

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to probe the chemically inert environment in the membrane hydrophobic core. In order to obtain structural information at different depths in a membrane, fatty acid and phospholipid probes based on *m*-nitro-phenylazide- [6], α-diazo-β-trifluoropropionoxy- [7], trifluoromethylphenyldiazirino- [8] and diazofluorene [9] groups have been used. These approaches involve the attachment of a photoactivable group to the ω-position of a fatty acid, which is often acylated at the C2 position of phospholipids. The results obtained in artificial membranes, using some of these photoactivable fatty acids and phospholipids, indicate that on photolysis these probes cross-link to the fatty acyl chains of the neighboring phospholipid [9,10] at a site which is proximal to the resulting carbene. Using phospholipids prepared from benzophenone-based fatty acids (Fig. 1), we have been able to show earlier that these lipids lead to functionalization of the neighboring phospholipid in a depth-dependent manner [11]. However, in order to use these benzophenone-based probes, for cross-linking studies in the membrane, it is important to quantify the degree of cross-linking. Here we report that a high degree of cross-linking (35–40%) can be achieved using these phospholipids. Analysis of the carbon functionalization data using a multiple Gaussian function provided insight into the depth-dependent labeling.

2. Experimental details

2.1. Materials and general procedures

All chemicals and solvents used were commercial grades of highest purity available. The benzophenone-based probes used were, 1-myristoyl-2-[6-(1′-phenyl-4′-benzoyl) hexanoyl]-*sn*-glycero-3-phosphorylcholine (C6ABzPC), 1-myristoyl-2-[6-(1′-phenyl-4′-(4″-*n*-hexyl-benzoyl)hexanoyl]-*sn*-glycero-3-phosphorylcholine (C6ABzC6PC), 1-myristoyl-2-[8-(1′-phenyl-4′-(4″-*n*-butyl-benzoyl) octyl]- *sn*-glycero-3-phosphorylcholine (C8ABzC4PC) and were prepared, as described earlier [11]. Single bilayer vesicles were prepared from 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (DML) and the benzophenone-based probes. UV-visible spectra were recorded on a Shimadzu UV-265 spectrometer. Mass spectra were recorded on a Shimadzu QP-1000 spectrometer. Sephadex LH-20 column chromatography was carried out on a pharmacia SIR column (2.5×45 cm) using chloroform/methanol as the mobile phase.

2.2. Photo-cross-linking of C6ABzPC-DML (1:2) vesicles

Single bilayer vesicles were prepared from DML (65.3 mg, 94 μmol) and C6ABzPC (34 mg, 47 μmol) in deoxygenated phosphate buffer (18 ml). The vesicle preparation was photolysed at 28°C using an APP 40 annular photoreactor fitted with a 400 W medium pressure mercury lamp for 2 h, the phase transition temperature for DML being 26°C. The photo-insertion was followed by UV-absorption spectroscopy by monitoring the intensity of the characteristic 260 nm band for the benzophenone-based phospholipids. The photo-inserted product was extracted using chloroform/methanol (1:1 v/v; 2×18 ml). The crude product was loaded on Sephadex LH-20 column and eluted with chloroform/methanol (1:1 v/v) at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and analyzed for the phosphate content and absorbance at 245 nm. The high molecular weight fractions corresponding to the cross-linked product were pooled together and assayed for phosphate, which corresponded to 43 μmol.

2.3. Transesterification of cross-linked phospholipid (DML-C6ABzPC)

The cross-linked phospholipid (40 μmol) fraction was dissolved in anhydrous methanol (6 ml) and 200 μl of 0.5 M sodium methoxide in methanol was added to it. This solution was stirred at room temperature for 12 h. The pH was then adjusted to 7.0 and the crude product was extracted with *n*-hexane/ether (8:2 v/v). The organic layer was washed with water till neutral, and distilled to get a residue corresponding to a

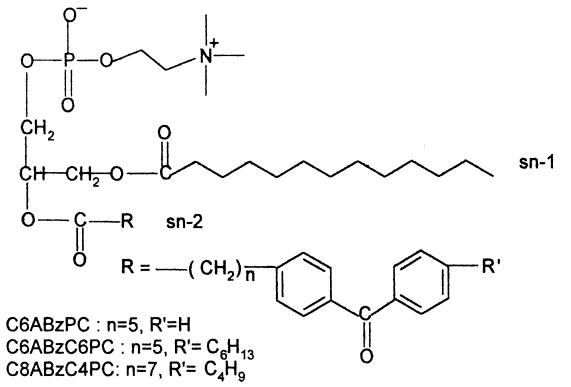

Fig. 1. Schematic structure of DML and the benzophenone-based probes.

mixture of fatty acid methyl esters. This material was further treated with excess of diazomethane in order to ensure complete esterification.

3. Results and discussion

3.1. Cross-linking studies

Benzophenones on exposure to UV radiations undergo a $n \rightarrow \pi^*$ transilion to the excited singlet, which rapidly decays to the excited triplet state. The excited triplet state does not react with water nor does it undergo intramolecular rearrangement like carbenes and nitrenes, thus lowering the extent of non-productive reactions of the reactive intermediate to products which are not useful from the point of view of inter-molecular cross-linking.

3.2. Photo-cross-linking of C6ABzPC, C6ABzC6PC and C8ABzC4PC in DML and isolation of the cross-linked product

Vesicles from C6ABzPC-DML,C6ABzC6PC-DML,C8ABzC4PC-DML (1:2 molar ratio) were prepared and photolysed. The photo-cross-linked product was chromatographed on Sephadex LH-20. The cross-linked dimeric phospholipids eluted first, followed by monomeric phospholipids. The degree of cross-linking was estimated by phosphate assay using these phospholipids and is given

Table 1
Degree of cross-linking of benzophenone-based phospholipids with DML in vesicles prepared from them on a larger scale

Phospholipids[a]	Cross-linked product[b] (%)
DML/C6AbzPC (2:1)	40.91
DML/C6ABzC6PC (2:1)	34.92
DML/C8ABzC4PC (2:1)	42.47

[a] The values given in the parentheses refers to the molar ratio of the phospholipids used in vesicle preparation.

[b] The cross-linked fraction isolated by Sephadex LH-20 chromatography were analyzed for phosphate and are expressed as percent of total phosphate.

in Table 1. The data indicate that in all the three cases, over one-third of the benzophenone-based phospholipid is cross-linked to the neighboring phospholipid. The cross-linked fraction was subjected to transesterification and analyzed by mass spectroscopy, which gave a significant peak at m/z 552 corresponding to the molecular weight of the cross-linked diester (Fig. 2) in the case of C6ABzPC-DML. The high degree of cross-linking obtained suggests that these probes can be efficiently used for the depth-dependent analysis of membranes and, hence, could be useful in probing the membrane bound proteins.

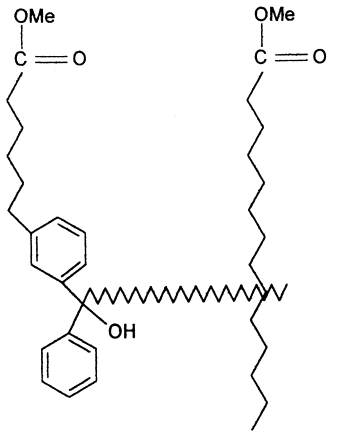

Fig. 2. Structure of cross-linked diester obtained on photo-cross-linking followed by transesterification of C6ABzC6PC-DML vesicles. The wavy lines indicate the site of cross-linking.

Once it was established that the benzophenone-based phospholipids undergo intermolecular cross-linking to the neighboring phospholipid molecule and reasonable quantities of cross-linked products were available, it was decided to establish the actual cross-linking by these phospholipids using the previously reported methodology [11]. It involves dehydration of the cross-linked diester followed by ozonolysis. The oxidative cleavage of the resulting ozonide leads to two products, one of them being the parent benzophenone-based fatty acid. The second product obtained corresponds to a series of *n*-keto acids, because the benzophenone chromophore inserts at more than one site in the neighboring fatty acyl chain of the phospholipid. The mass spectral analysis of the thioketal derivatives of these *n*-keto acids permits one to establish the degree, of cross-linking associated with a particular carbon atom [11].

3.3. Analysis of the carbon functionalization data

From Fig. 3, it can be seen that the *sn*-1 and *sn*-2 chains of DML are non-equivalent since the *sn*-1 chain trails by two to four carbon atoms with respect to the *sn*-2 chain [12] and, hence, would have different cross-linking sites. This would give rise to two different distributions for the extents of inter-molecular cross-linking. Gaussian functions have been successfully used to obtain information about the depth of various probes in the membrane [13,14]. The carbon functionalization data reported earlier for the benzophenone-based phospholipids and DML [11] resembled a Gaussian type of distribution but a single Gaussian analysis failed to give an adequate fit. Hence, we decided to carry out an analysis based on a linear combination of two Gaussian functions [Eq. (1)]. The method of curve fitting (Fig. 4) was used to determine the unknown parameters.

$$F(i) = \frac{S_1}{\sigma_1\sqrt{2\pi}}\exp\left[\frac{-(i-i_{m1})^2}{2\sigma_1^2}\right] + \frac{S_2}{\sigma_2\sqrt{2\pi}}\exp\left[\frac{-(i-i_{m2})^2}{2\sigma_2^2}\right]\ldots. \quad (1)$$

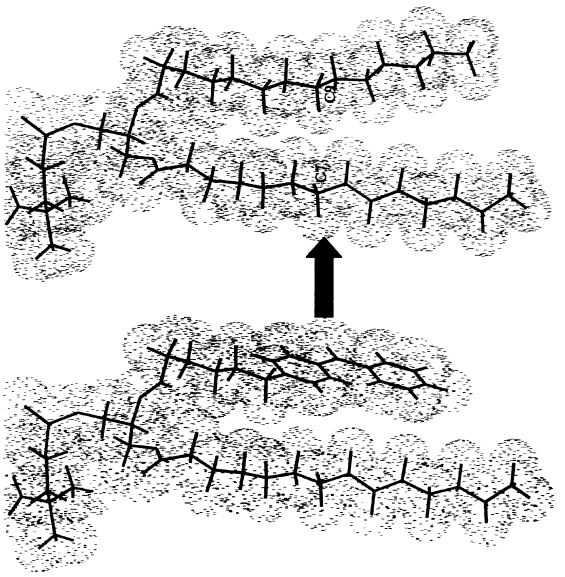

Fig. 3. The extended conformation of C6ABzPC (left) and DML (right). The arrow indicates the most probable site of cross-linking.

In Eq. (1), $F(i)$ denotes the functionalization of the ith carbon atom. S_1 and S_2 are the area under the Gaussians, σ_1, and σ_2 are the dispersions, i_{m1}, and i_{m2} are the carbon atoms around which the Gaussians are centred. These parameters (Table 2) should in effect give an estimate of the position of benzophenone relative to the two alkyl chains of DML. In the case of C6ABzC6PC, the difference between the values of i_{m1} and i_{m2} is close to 1.9 methylene units. This difference is close to 1.7 methylene units in the case of C6ABzPC. These values should be close to the difference in depth of the two different chains in the membrane. This is a result of the *sn*-2 fatty acyl chain being shorter than the *sn*-2 chain of the glycerol backbone. It is evident from Table 2 that the depth for the C6ABzPC and C6ABzC6PC are almost the same and are approximately equal. Molecular modeling of the probes in an extended conformation as shown in Fig. 3 indicates that the main site of cross-linking for the probe C6ABzPC is located between C8 and C9 for the *sn*-2 chain, C7 and C8 for the *sn*-1 chain. The values given in Table 2 are in excellent agreement with this

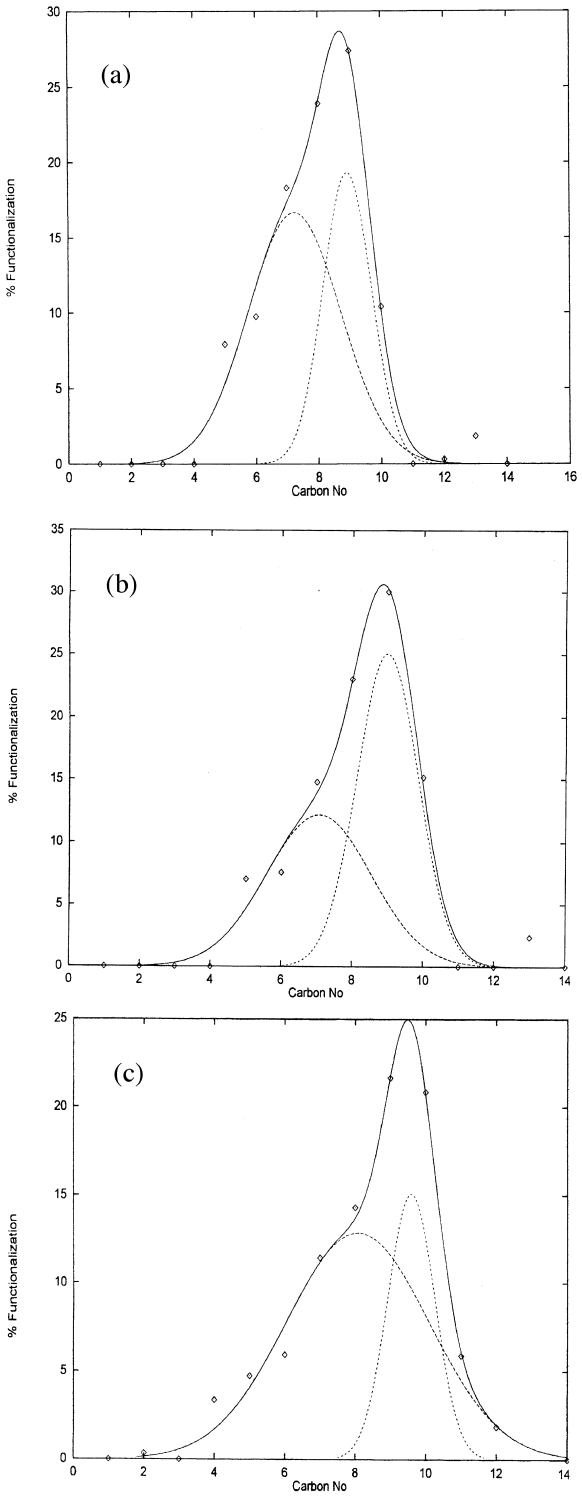

Fig. 4.

observation. The depth of the probes obtained from the Gaussian analysis suggests that the probes are a bit deeper than the 7th carbon atom of the *sn*-1 chain. The values of i_{m1} and i_{m2} for C8ABzC4PC are each, approximately 1.5 methylene units deeper than the corresponding values for C6ABzC6PC. Here, it should be noted that the probe does not go deeper by two methylene units compared to C6ABzPC as would be expected from the number of methylene groups. This is because, as the probe goes deeper, the wobbling motions increase due to the increased fluidity going down the membrane, which in turn cause a decrease in the average depth of the probe. This suggests that increasing the tail length does not always help substantially in probing the membrane deeper. Hence, there might exist an optimum length for the probe for effective cross-linking. A similar conclusion was reported earlier, based on fluorescence studies with fatty acids [15]. The parameter would reflect the freedom of motion for each chain. It is seen that σ_1 is greater than σ_2 in all cases, which suggest that the *sn*-2 chain (Fig. 3) is more ordered than the *sn*-1 chain. This conclusion is consistent with the order parameters reported by molecular dynamics simulation of the DMPC bilayer [16].

The Gaussian distribution analysis presented here provides an insight into the relative depth of the probes in the membrane. Thus, these probes can be effectively used in depth-dependent analysis. The major advantage associated with the use of benzophenones as photoactivable reagents is their ability to form a benzophenone excited triplet and the triplet state's inertness towards water. The electrophillic nature of other commonly used photoactivable reagents like carbenes or nitrenes and the resulting high reactivity towards water is probably one of the main drawbacks associated with these reagents. While benzophenone-based phospholipids have been re-

Fig 4. The multiple Gaussian fits for the probes [Eq. (1)]. The solid line represents the curve fit obtained using Eq. (1). The dotted lines represent the individual Gaussians of the multiple Gaussian function (◇) represents the carbon functionalization data. (A) C6AbzPC; (B) C6ABzC6PC; and (C) C8ABzC4PC.

Table 2
Parameters of the multiple Gaussian function[a]

Probe	S_1	i_{m1}	σ_1	S_2	i_{m2}	σ_2
C6AbzPC	61.7	7.2	1.5	37.5	8.9	0.8
C6ABzC6PC	45.4	7.1	1.5	54.3	9.0	0.9
C8ABzC4PC	65.2	8.1	2.0	25.5	9.6	0.7

[a]*Abbreviations:* S_1 and S_2 are the area under the Gaussians [Eq. (1)]; σ_1 and σ_2 are the dispersions; i_{m1}, and i_{m2} are the carbon atoms around which the Gaussians are centred.

ported in the past [17,18] the low labeling yields associated with them, i.e. $< 0.1\%$ [17] have not been encouraging, especially in view of the fact that other benzophenone-based photoactivable reagents give rise to quite reasonable cross-linking yields [19,20]. Furthermore, these phospholipid probes were prepared using only 4-carboxy benzophenone, which strongly limits the use of these probes for depth-dependent labeling of the membrane hydrophobic core. It is quite possible that such shallow probes may only label proteins near the membrane surface and it is not surprising that a report on a similar phosphatidyl ethanolamine-based azide probes indicated selective labeling of spectrin in erythrocytes [5]. The present work indicates that the suitably designed benzophenone-based probes can be effectively used for depth-dependent analysis of lipids, and proteins in membranes.

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