Disperse Orange 3 as a resonance Raman probe for measuring membrane order

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Abstract

Resonance Raman spectra of azobenzene derivatives were examined in the presence of lipid membranes to find a probe that can distinguish different membrane phases. The NO$_2$ symmetric stretching band of 4-(4-nitrophenylazo)aniline, also known as Disperse Orange 3 (DO3), is downshifted by about 4 cm$^{-1}$ on the phase transition of phosphatidylcholine membranes from the liquid crystalline to the gel phase. A comparable downshift also occurs when DO3 is bound to cholesterol-containing membranes in the liquid-ordered phase. Our results demonstrate that Raman spectrum of DO3 is a unique tool for measuring the molecular order of lipids in membranes.

1. Introduction

Cell membranes, composed of a complex mixture of glycerophospholipids, sphingolipids and cholesterol are considered to be basically in the fluid liquid disordered phase due to a high content of low melting point lipids with unsaturated acyl chains. However, a considerable body of evidence indicates that there is marked heterogeneity in the distribution of lipids and proteins in cell membranes [1–3]. It is now widely believed that sphingolipid- and cholesterol-rich microdomains in the tightly packed state occur in cell membranes [4]. Membrane microdomains, commonly called lipid rafts, are considered to be submicrometer-sized and highly dynamic assemblies of lipids [5,6], but they can be stabilized to form larger long-lasting domains through protein–protein and protein–lipid interactions and serve as platforms for signal transduction and membrane trafficking [4,7–9]. A crucial role of microdomains in the pathogenesis of Alzheimer’s disease has also suggested by a number of findings [10–12].

Optical imaging techniques, in particular fluorescence microscopy coupled with image analysis, are powerful tools to investigate membrane microdomains in living cells. One of the earliest probes to label microdomains is the B subunit of cholera toxin (CT-B). This protein binds specifically to ganglioside GM1 which has been found to be enriched in microdomains [13]. Therefore, microdomains can be visualized as GM1-enriched regions of cell membranes by using fluorescently labeled CT-B [14]. On the other hand, fluorescent probes sensitive to lipid packing order rather than lipid composition have also been used to study heterogeneity in both artificial and natural membranes. For example, the fluorescence emission from Laurdan exhibits a large blue shift on going from the disordered to the ordered phase of the lipid membrane in which the probe is embedded [15,16]. Membrane microdomains with a high packing density can be distinguished from the surrounding fluid membrane by using Laurdan and its related compounds [17].

Raman spectroscopy is another potentially powerful tool for elucidating the structural and functional bases of heterogeneity in cell membranes due to recent developments in high throughput micro-Raman instruments. For example, a Raman spectrum from a desired portion of a single-cell can be obtained with spatial resolution in the range of less than 1 μm [18,19]. Unlike widely used fluorescence imaging techniques, however, a packing order-sensitive probe applicable to Raman spectroscopic study on the membrane structure has not yet been found. Here we report the discovery of an azo dye–based molecular probe whose Raman spectrum is sensitive to changes in the molecular order of lipid membranes. Visible resonance Raman spectra of 4-(4-nitrophenylazo)aniline,
termed Disperse Orange 3 (DO3), were obtained in membrane-associated states. A Raman band assignable to the NO2 symmetric stretching vibration appears at 1338 cm$^{-1}$ when the dye molecule is bound to phosphatidylcholine membranes in the liquid crystalline phase. This band is downshifted by about 4 cm$^{-1}$ on the phase transition of the membrane to the gel phase. A comparable downshift of the NO2 stretching band also occurs when DO3 is bound to cholesterol-containing phosphatidylcholine bilayers in the liquid ordered phase.

2. Materials and methods

2.1. Preparation of liposomes

DO3 was purchased from Sigma–Aldrich. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol were purchased from Avanti Polar Lipids. Concentrations of DO3 was determined in ethanol by using molar extinction coefficient $\varepsilon_{444} = 22,900$ M$^{-1}$ cm$^{-1}$ [20]. Liposomes of lipid bilayer containing DO3 were prepared by a sonication method previously described [21].

2.2. Raman spectroscopy

Raman spectra of a suspension of liposomes consisting of 5 mM lipids and 5–100 μM DO3 was measured on a micro-Raman spectrometer (NRS-3100, JASCO, Japan) equipped with a thermoelectrically cooled CCD detector (DU401-BV-120, Andor, UK) [21]. The 488-nm line of a diode laser (Cyan-488-150, Spectra-Physics, Santa Clara, CA) was used for excitation. The temperature of the sample in a glass capillary tube was controlled with a circulating water bath. The wavenumber axis was calibrated with indene, and peak positions were reproducible within 0.5 cm$^{-1}$ for sharp bands such as the NO2 stretching band of DO3.

3. Results

3.1. Raman bands of DO3 sensitive to the gel–liquid crystalline phase transition of membranes

The temperature dependence of Raman spectrum of DO3 was examined in the presence of DPPC liposomes to find Raman bands that are sensitive to the gel–liquid crystalline phase transition of lipid bilayer membranes. DO3 is a highly lipophilic molecule that is poorly soluble in water by itself, but a clear reddish solution could be obtained by mixing with liposomes even in aqueous medium. Fig. 1a shows a 488-nm excited Raman spectrum of a DO3/DPPC mixture at a 1:50 M ratio (100 μM DO3 and 5 mM DPPC). Raman bands from the lipid, which are seen in the control spectrum of DPPC liposomes without DO3 (Fig. 1c), are negligibly weak in the spectra of DO3-containing membranes, because the π-π* electronic transition of DO3 in the 450–500 nm region is resonantly excited with 488-nm light. Thus, all the bands observed at a 1:50 DO3/DPPC ratio are ascribed to DO3. The Raman bands of DO3 still can be clearly seen at a 1:1000 DO3/DPPC ratio (5 μM DO3 and 5 mM DPPC), though DPPC bands are also seen at 1440, 1297, 1128 and 1063 cm$^{-1}$ (Fig. 1b).

The 1450–1280 cm$^{-1}$ interval of Raman spectra of a 1:50 DO3/DPPC mixture below and above the main phase-transition temperature ($T_m$) of DPPC (di-C16:0, $T_m = 41^\circ$C [22]) are compared in the inset of Fig. 1. The wavenumber shifts from the gel (10°C) to the liquid crystalline phase (60°C) of the membrane are within ±1 cm$^{-1}$ for most of the observed bands of DO3 including the 1423 and 1388 cm$^{-1}$ bands in the inset. However, a strong band at 1334 cm$^{-1}$ in the spectrum at 10°C is upshifted by 4 cm$^{-1}$ at 60°C. This band is assignable to the NO2 symmetric stretching vibration based on previous vibrational analyses of 4-nitrozobenzene and its derivatives [23,24]. A plot of the peak wavenumber of the NO2 stretch band versus temperature produces a sigmoidal curve (Fig. 2). A steep increase in wavenumber occurs at a temperature corresponding to the $T_m$ of DPPC. A similar experiment was performed by using phosphatidylcholine with different $T_m$ from that of DPPC. The plot also results in a sigmoid curve in the presence of DMPC liposomes, but the inflection point shifts to a lower temperature which coincides with the $T_m$ of DMPC (di-C14:0, $T_m = 23^\circ$C [22]) (Fig. 1S, Supplementary material). On the other hand, both a sigmoidal feature and a steep wavenumber shift of the NO2 stretch band are lost in the presence of POPC liposomes (Fig. 2). The POPC membranes are in the liquid crystalline phase in the temperature range examined (10–60°C) because of a low $T_m$ of this lipid (C16:0/C18:1, $T_m = -2.5^\circ$C [22]). These results indicate that the NO2 stretch band of DO3 shifts toward higher wavenumber on the gel to liquid crystalline phase transition of lipid in the membrane-associated state. The peak wavenumber of this band is insensitive to temperature in an ethanol solution (Fig. 2S, Supplementary material). This confirms that the observed wavenumber shift of the NO2 stretch band of DO3 in lipid membranes is not simply ascribed to the temperature change, but reflects the phase transition of the membranes.

Similar experiments were also performed with several other azobenzene derivatives such as 4-aminoazobenzene, 4-aminazo
benzene-4'-sulfonic acid, and 4-amino-4'-dimethylaminoazobenzene. However, Raman bands sensitive to the gel to liquid crystalline phase transition could not be found for these azobenzene derivatives which lack the nitro group (data not shown).

3.2. Raman spectra of DO3 in cholesterol-containing membranes

Temperature dependence of Raman spectrum of DO3 has been examined in the presence of DPPC liposomal membranes which contain cholesterol. The DPPC/cholesterol membranes are known to be in the liquid ordered phase over the whole bilayer at cholesterol contents above approximately 35 mol% [25]. In order to obtain a Raman spectrum of DO3 in the liquid ordered phase membranes, DPPC/cholesterol liposomal membranes were prepared at 40 mol% of cholesterol. The NO2 stretch band gradually shifts toward higher wavenumber with increasing temperature even in the presence of the DPPC/cholesterol membrane. However, the plot does not show a sigmoidal feature (Fig. 2), which is in agreement with the lack of phase transition in the membrane with the high cholesterol content. The peak wavenumber of the NO2 band in the DPPC/cholesterol membranes is 2–3 cm\(^{-1}\) lower than that in the POPC membranes in the liquid crystalline phase at every temperature examined, but rather close to that in the DPPC membranes in the gel phase at about 40°C. The wavenumber of the NO2 stretch band of DO3 may be sensitive to changes in the molecular order of lipid membranes, because both the gel and liquid ordered membranes are characterized by an ordered packing of lipid molecules. These results demonstrate that the membrane in the liquid ordered phase can be distinguished from that in the liquid crystalline (or liquid disordered) phase using Raman spectrum of DO3.

3.3. Binding mode of DO3 to lipid membranes

The wavenumber of the NO2 stretching band of DO3 has been examined in n-eicosane which mimics a hydrocarbon chain environment of lipid membranes. Eicosane is a 20-carbon alkane with a melting point of 37°C. The NO2 stretching band appears at \(\sim 1341 \text{ cm}^{-1}\) in the liquid n-eicosane, but shifts to \(\sim 1337 \text{ cm}^{-1}\) on the liquid to solid phase transition (Fig. 3). The phase-dependent wavenumber shift is reproduced in alkane, indicating that the wavenumber of the NO2 stretching vibration reflects the degree of order of the hydrocarbon chain region rather than that of the hydrophilic head group moiety of lipid bilayer membranes. DO3 may be buried, at least partly, in the hydrocarbon chain region of membranes.

In order to gain further insight into the membrane binding-mode of DO3, visible absorption spectra of DO3 were measured in DPPC membranes, ethanol, and n-heptane, respectively (Fig. 4). n-Heptane (melting point, \(-91^\circ\text{C}\)) is in the liquid phase in the temperature range examined (10–60°C). A strong band assignable to the lowest energy \(\pi-\pi^*\) transition is observed at about 400 nm in n-heptane solution (Fig. 4c). In contrast, the \(\pi-\pi^*\) transition appears at around 440 nm as a broad band in ethanol solution (Fig. 4b). The absorption spectra of DO3 in DPPC membranes are similar to those in ethanol rather than in n-heptane, although the peak wavelength and the band width (Fig. 4a). These results suggest that the DO3 molecules mainly reside in the hydrophilic head group region rather than the hydrocarbon chain region. Taking into account the observations that the phase-dependent wavenumber shift of the NO2 stretch band of DO3 occurs in both DPPC membranes (Fig. 2) and n-eicosane (Fig. 3), at least the nitro group moiety may be buried in the non-polar region of the membrane. It is reasonable to assume, therefore, that the DO3 molecules wedge into the hydrophilic head group region of the membrane so as to orient the amino group to the surface and the nitro group to the nonpolar region of the membrane.

The \(\pi-\pi^*\) transition band exhibits a blue shift with increasing temperature in both ethanol and n-heptane. As the plot of \(\lambda_{\text{max}}\) versus temperature shows, however, the wavelength shift has a larger temperature dependence in ethanol than in n-heptane (Fig. 4b and c, insets). This suggests that the peak wavelength of the \(\pi-\pi^*\) transition is sensitive to a polar interaction between DO3 and solvent, most likely hydrogen bonding between DO3 and the hydroxyl group of ethanol. Although a similar wavelength shift of the \(\pi-\pi^*\) transition is observed in the presence of DPPC liposomes, the slope of the plot is critically dependent on the phase of the membrane (Fig. 4a, inset). The slope is slightly larger than that in ethanol solution at higher temperatures, but becomes small...
obviously at temperatures below the \( T_m \) of DPPC. These results are consistent with that water molecules penetrate less to membranes in the gel phase than those in the liquid crystalline phase [15,16].

4. Discussion

Accumulating evidence suggests that assemblies of sphingolipids, cholesterol and proteins in cell membranes may generate local heterogeneity in not only composition but also physical properties of membrane such as lipid packing order and fluidity [3]. According to recent studies with new microscopy and other techniques, membrane microdomains can be classified into short-lived nanoscale assemblies and long-lasting larger domains [7–9,26]. The molecular mechanism of conversion of small assemblies into larger domains are of particular interest, because the latter may mainly serve as platforms for signal transduction and membrane trafficking [3,4]. Although relatively large and stable microdomains can be visualized in living cells by fluorescence microscopy using phase-sensitive fluorescent probes [17,27], these probes do not provide structural information on cell membranes.

In the present study, Raman spectra of azobenzene derivatives were examined in membrane-associated states to find a new tool that can be used to investigate physical properties of membranes. The obtained results show that the wavenumber of NO\(_2\) stretch band of DO3 is sensitive to changes in the lipid packing order of membranes. When micro-Raman spectroscopy is applied to the analysis of a single living cell that is stained with DO3, the membrane order will be measured with spatial resolution in the range of less than 1 \( \mu \)m [18,19]. There are several advantages to the use of DO3 for a Raman spectroscopic characterization of cell membranes. For example, DO3 has a potential to become a powerful tool for studying heterogeneity in cell membranes which are considered to be basically in the liquid disordered phase but may contain microdomains in the liquid ordered phase. The NO\(_2\) stretch band appears at a lower wavenumber in the liquid ordered phase as well as the gel phase membranes compared to in the liquid crystalline (or liquid disordered) membranes (Fig. 2). Thus, microdomains will be distinguished from the surrounding membrane by using Raman spectrum of DO3 if lipid molecules are orderly packed in the microdomains. Even if a clear liquid–liquid phase separation seen in model membrane systems does not occur in living cells, the lipid packing order of cell membranes will be measured with sub-micrometer spatial resolution.

The probe molecule that has an electronic transition in a visible wavelength range offers another advantage for studying the membrane structure. Resonantly enhanced Raman bands of DO3 are much stronger than non-resonant bands of lipid molecules, and thus can be observed at a very low concentration in a spectrum of membrane-bound DO3. A DO3/DPPC ratio of 1/1000 is high enough to observe the NO\(_2\) stretch band of DO3 without severe disturbance by Raman scattering from the lipids (Fig. 1b). A low probe/lipid ratio condition may be preferable to avoid the probe-induced perturbation of the membrane structure. More importantly, Raman bands of lipid molecules that become detectable on lowering DO3 concentration are able to provide valuable information on the membrane structure [28]. When DO3 is applied to cell membranes, a much wider variety of information may be given by Raman bands of membrane constituents such as membrane-associated proteins, in addition to information on the lipid packing order from the DO3 band. Therefore, a spectrum of a DO3-stained cell membrane is expected to offer a unique opportunity to gain insight into the origins and functions of membrane microdomains.

Author contribution

TM conceived and designed the project, YN acquired the data, TM and YN analyzed and interpreted the data, and TM wrote the paper.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research (Grant 24590043) from Japan Society for the Promotion of Science. We are also grateful to Prof. Takakazu Nakabayashi (Tohoku University) for fruitful discussion.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.10.008.
