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# Bis-MSB, a laser dye as a polarization probe for microheterogeneous media

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**Abstract**

Bis-MSB, a UV laser dye with an elongated rigid structure possesses good spectral characteristics, high molar extinction coefficient and a high fluorescence quantum yield. As it is similar to DPH structurally with desirable fluorescence probe characteristics, its applicability as fluorescence polarization probe for microheterogeneous medium has been investigated. It shows a high and constant polarization value comparable to that of DPH in all the organized media. It is completely non-polar and partitions almost completely into the lipid membrane. Being cheaper than DPH, it proves to be a promising candidate to be used as a polarization probe for lipid membranes.

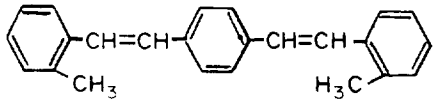
*Keywords:* Fluorescence polarization; Liposomes; Phase transition; Fluidity probe; Laser dye

## 1. Introduction

The studies on structure and dynamics of the microheterogeneous medium were most successfully pursued by fluorescence polarization techniques which have yielded indispensable information and concepts that immensely advanced the understanding of this area [1]. The fluorescence polarization technique has a series of important advantages over NMR and ESR [2]. It can be readily applied to complex systems like a biological membrane, polarized signal is highly sensitive and reproducible and the data are promptly interpretable. The fluorescence polarization signals can be converted to classical hydrodynamic expressions to describe the fluidity of heterogeneous matrix [3], which sets it apart from other techniques such as NMR and ESR.

Although a large number of fluorescence dyes have been employed as probes in membrane studies [4,5], the number of probes available for fluidity measurements is very limited as the probe has to possess a series of well-defined spectral and physical characteristics [6]. The fluorescence polarization fluidity probes can be divided into two main classes [1]. The first class consists of fluorescent dyes which can incorporate spontaneously into a well-defined lipid region of membrane. The fluorescence polarization characteristics of such probes relate directly to the microviscosity of the labelled region. To the second class belong fluorescent dyes which are covalently attached to fatty acids or phospholipids at different loci. Since some rotational modes of the fluorescent moiety in such compounds are markedly hindered, the correlation between microviscosity and fluorescence polarization can only be expressed by the relative scale. Only 2,5-diphenylhexatriene (DPH) and perylene which belong to the first type are used extensively for the overall fluidity measurements in microheterogeneous medium. Other fluidity probes such as DL-12-(9-anthroyl) stearic acid (12-AS) [7], 1-acyl-2-(*N*-4-nitrobenzene-2-oxa-1,3-diazole-aminocaproyl phosphatidylcholine (NBD-PC) [8] and parinaric acid [9] which belong to the second class, are used for fluidity measurement at a particular portion of the membrane.

In the present work polarization studies have been carried out on bis(*o*-methylstyryl)benzene (Bis-MSB) (I), a laser dye.

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Bis-MSB possesses, a very rigid polyene structure with a rod-like shape, of length 12 Å which is comparable to that of DPH which is of length 11 Å. For these kinds of polyenes only the all-trans form contributes to the fluorescence [10]. Bis-MSB, a UV laser dye [11], absorbs at 355 nm with a molar absorptivity of 48 800 l $mol^{-1}$ $cm^{-1}$ and emits at

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418 nm in ethanol. It has a very high fluorescence quantum yield of 0.98 in cyclohexane and a lifetime of 1.24 ns. Because of these desirable structural and spectral characteristics, its applicability as a fluorescence polarization probe has been evaluated in various microheterogeneous media like neutral, anionic and cationic micelles and in DMPC liposome. Comparison is made with the behaviour of DPH, an established polarization probe, in various environments.

## 2. Experimental

The probes DPH (Sigma) and Bis-MSB (Lambdachrome Laser Dyes) were used as obtained. The surfactants triton X-100, CTAB and SDS were purified by recrystallization from methanol. DMPC-lipid used for liposome preparation was obtained from Sigma and it was checked for its purity by looking out for a single spot in TLC. Solvents used were doubly distilled.

### 2.1. Liposome preparation [12] and labelling [13]

DMPC was dissolved in chloroform–methanol 2:1 v/v at the desired molar ratio. The solution was evaporated to dryness under nitrogen, followed by addition of 50 mM aqueous sodium chloride to yield a final concentration of 0.1 mM phospholipid. This was then subjected to high intensity ultrasonic irradiation for 3 min using a Vibronics Bath Sonicator. The almost clear suspensions of unilamellar vesicles were then used for further experiments.

Labelling was achieved by two methods: i) by adding a measured amount of dye to the lipid solution, before preparation of liposomes, in a molar ratio yielding a final lipid/probe ratio of 200, such that the probe is directly incorporated into the membrane, and ii) by adding an unlabelled liposome suspension to an aqueous dispersion of DPH and Bis-MSB in tetrahydrofuran [13]. After addition of the probe the solution was allowed to equilibrate for about an hour and was checked for constancy in the fluorescence intensity with time. Liposomes labelled by both the methods were found to give the same type of results. For each preparation, a control solution containing the same lipid components but lacking the probe was prepared under identical conditions to serve as a blank.

### 2.2. Micelle preparation and labelling

Aqueous micelle solutions were prepared by dissolving the micelle-forming substance in water to a concentration which is twice the critical micelle concentration (CMC). Labelling of the micelles were done by adding the surfactant to an aqueous dispersion of the probe in THF. To check the solubilization of the probe into the micelle, the concentration of the surfactant was varied from below to above the micelle concentration and a very sharp increase in fluorescence intensity was observed for concentration near the CMC.

### 2.3. Polarization measurements

Fluorescence polarization measurements were carried out by the conventional method [14] of measurements of $I_{\parallel}$ and $I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected through a polarize oriented parallel and perpendicular to the direction of polarization of excitation beam. The fluorescence polarization ($P$) of the probe was obtained using the relationship [14]

$$P=\frac{(I_{\parallel}-I_{\parallel s})-(I_{\perp}-I_{\perp s})}{(I_{\parallel}-I_{\parallel s})+(I_{\perp}-I_{\perp s})}$$

where s stands for the scattering component. Corrections for the scattering were obtained with dye-free controls. The contribution of error from control was found to be less than 0.5% of the polarization value.

### 2.4. Instrumentation

Absorption and fluorescence measurements were taken with Hitachi 2108 spectrophotometer and Hitachi F4500 spectrofluorimeter respectively. Polarization measurements were taken with polacoat filter polarizers. Temperature was controlled with a thermostat bath. A deviation of less than 0.5 °C in the recorded temperature was estimated for all the systems measured.

## 3. Results and discussions

Bis-MSB is used for the first time as a fluidity probe. Some of its spectral characteristics are described here. Figs. 1 and 2, show the absorption and emission spectra of Bis-MSB in cyclohexane at 25 °C and in various solvents of differing polarity. The spectral maximum did not change appreciably

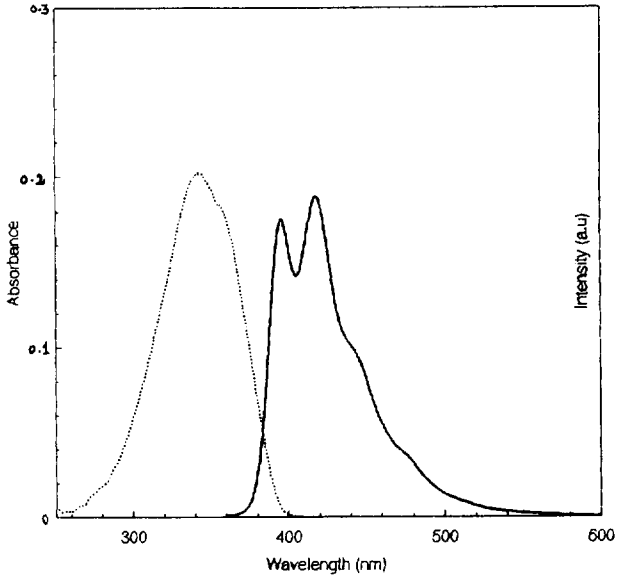


Fig. 1. Absorption (· · ·) and emission (— — —) spectrum of Bis-MSB in cyclohexane at 25 °C.

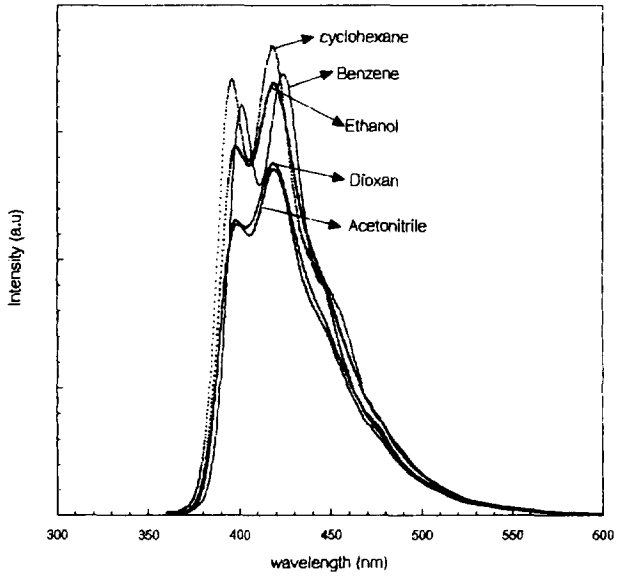


Fig. 2. Emission spectrum of Bis-MSB in different solvents of varying polarity at 25 °C. Concentration of Bis-MSB is $6.3 \times 10^{-6}$ M.

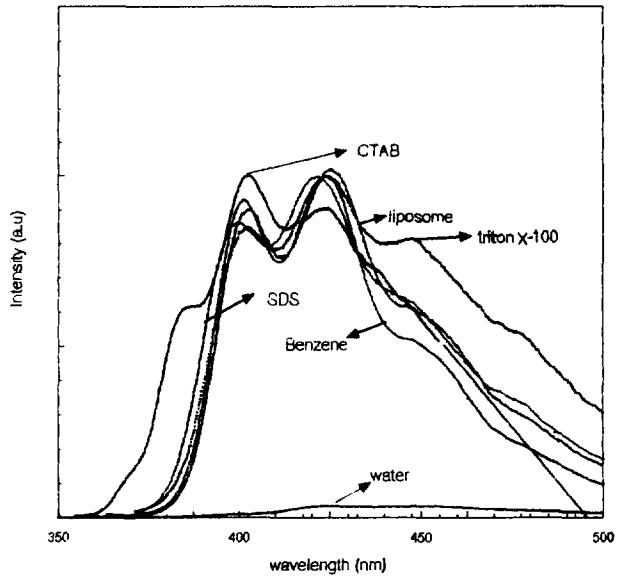


Fig. 3. Emission spectrum of Bis-MSB in DMPC liposome; triton X-100; CTAB; SDS micelles and in benzene. Concentration of Bis-MSB is $6.3 \times 10^{-6}$ M.

Table 1
Relative fluorescence quantum yield (relative to reported quantum yield in cyclohexane [11]) of Bis-MSB and emission maximum of Bis-MSB in various solvents

| Solvent | $\lambda_{max}$ (emission) (nm) | $\phi_f$ |
|---|---|---|
| Cyclohexane [11] | 418 | $0.98_0$ |
| Hexane | 418 | $0.97_7$ |
| Benzene | 425 | $0.91_3$ |
| Dioxan | 419 | $0.84_2$ |
| Acetonitrile | 418 | $0.83_9$ |
| Methanol | 418 | $0.90_5$ |
| Ethanol | 418 | $0.91_7$ |

with change in solvent. The emission maximum observed in different solvents and their relative quantum yield (with reference to the quantum yield in cyclohexane) is given in Table 1. The intense absorption maximum at 355 nm with a molar extinction coefficient of 48 800 l $mol^{-1}$ $cm^{-1}$ and the high fluorescence quantum yield of 0.98 in cyclohexane, facilitates the detection of a fluorescence signal at concentrations as low as $10^{-9}$ M. The well-separated absorption and emission bands reduce considerably the possibility of energy transfer from one fluorophore molecule to the other and the contribution of scattered excitation light to fluorescence signal. The emission spectrum of Bis-MSB in water, micelles and in liposome is given in Fig. 3. It is known that when a solution of DPH in THF is added to water, a dispersion of DPH microaggregates in water is obtained. This dispersion is non-fluorescent [15]. It is expected that Bis-MSB would also form a dispersion of microaggregates in water. This dispersion is found to be almost non-fluorescent. The incorporation of Bis-MSB into micelles and liposomes is witnessed by a sharp increase in fluorescence signal. This confirms the solubilization of Bis-MSB in the micelles and liposomes. The emission spectrum obtained in micelles and liposomes is structured and resembles that in benzene as solvent showing that Bis-MSB is present in the inner hydrocarbon region of these media. When micelle and liposome suspension is added to Bis-MSB dispersion in water a 2000-fold enhancement in fluorescence is observed.

### 3.1. Partition coefficient of Bis-MSB in liposome

The partition coefficient of Bis-MSB into liposomes was determined by using fluorescence measurement techniques

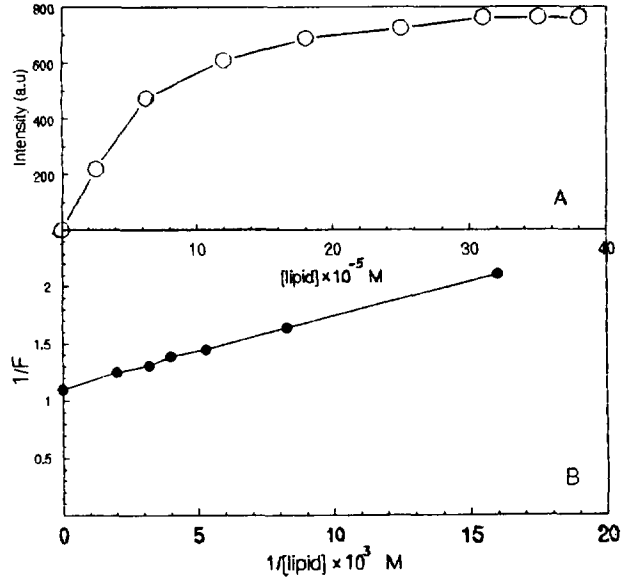


Fig. 4. (A) DMPC liposome titration against $1.525 \times 10^{-6}$ M Bis-MSB. 1 ml of varying concentration of DMPC liposome is added to 1 ml of the probe in phosphate buffered saline at pH 7. After equilibration at room temperature for 2 h fluorescence experiments were carried out. (B) Double reciprocal plot of fluorescence intensity vs. lipid concentrations.

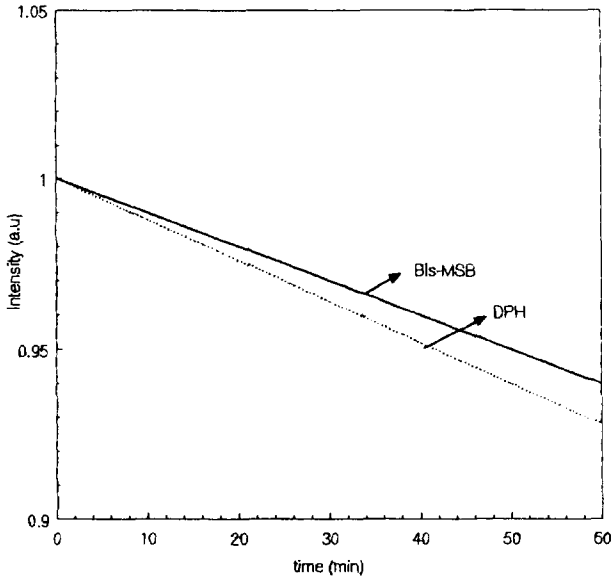


Fig. 5. Time scan spectrum for $3.85 \times 10^{-6}$ M Bis-MSB and DPH in cyclohexane at 25 °C, on continuous irradiation at 355 nm in a fluorimeter with 100 W xeon arc lamp using slits with a band pass of 5 nm.

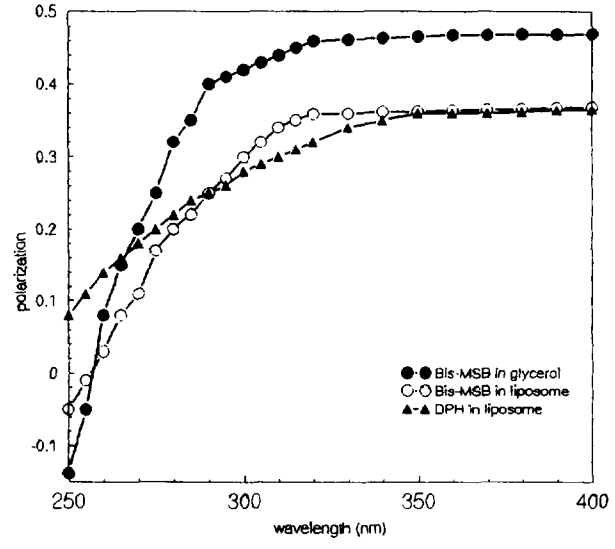


Fig. 6. Polarization spectrum of Bis-MSB, in glycerol and DMPC liposome; and that of DPH in DMPC liposome; all at 5 °C.

[16]. A sigmoidal curve was obtained on plotting fluorescence intensity versus lipid concentration (Fig. 4(A)). A double reciprocal plot (Fig. 4(B)) of $1/F$ against $1/L$, according to the equation

$$1/F = 55.6/(K_p F_0 L) + 1/F_0$$

was linear, from the slope of which the partition coefficient was calculated. In the equation $F$ and $F_0$ are the fluorescence intensities of Bis-MSB in the presence and the absence of liposome suspension respectively, $K_p$ is the partition coefficient and $L$ denotes the concentration of lipid. The partition coefficient ($K_p$) value for MSB in liposome was found to be $0.8 \times 10^6$ which is comparable to that of DPH ($1.3 \times 10^6$).

### 3.2. Cis–trans isomerisation

Conjugated polyenes are known to undergo photo-induced cis–trans isomerization reaction which is reversible and viscosity dependent [17]. For all practical purposes, it is considered that only the all-trans form contributes to fluorescence. To determine the extent of fluorescence intensity loss due to cis–trans isomerization a time-scan experiment was carried out. Same concentration solutions of Bis-MSB and DPH in cyclohexane was subjected to continuous irradiation in the fluorimeter with the excitation beam at 355 nm. The time scan was recorded at their emission wavelength maximum for a period of 1 h (Fig. 5). The extend of cis–trans isomerization in Bis-MSB is similar to that of DPH and on leaving the solution in dark, ($\leq 5$ min) the original intensity was reobtained.

### 3.3. Fluorescence polarization measurements

A high excitation polarization value of 0.436 is obtained for Bis-MSB in glycerol which remains fairly constant in the wavelength range 320–400 nm as shown from its polarization spectrum in glycerol at 5 °C (Fig. 6). This property provides a depolarization scale which is unaffected by small shifts in absorption spectrum. Polarization measurements were done in micelles at 25 °C, and in DMPC liposomes at various temperatures using both DPH and Bis-MSB under identical conditions and the results are given in Table 2. The value of $P$ measured at 355 nm, remained constant throughout the labelling process in micelles and in liposomes. This rules out the possibility of energy transfer between the fluorophore molecules or conformational changes in the liposome, processes which are often sources of error in polarization measurements. Corrections due to the contribution of scattered excitation light to the fluorescence signal was made with identical but unlabelled systems. The maximal contribution

Table 2
Polarization data obtained in various media using Bis-MSB and DPH as probes. [probe] = $6.3 \times 10^{-6}$ M, $\lambda_{ex} = 355$ nm; $\lambda_{em} = 425$ nm

| Medium | Concentration (mM) | Temperature(K) | Excitation polarization values | |
|---|---|---|---|---|
| | | | Bis-MSB | DPH |
| Triton X-100 | 0.28 | 298 | 0.256 | 0.202 |
| CTAB | 11.4 | 298 | 0.191 | 0.108 |
| SDS | 10.3 | 298 | 0.200 | 0.121 |
| DMPC liposome | 0.1 | 278 | 0.358 | 0.352 |

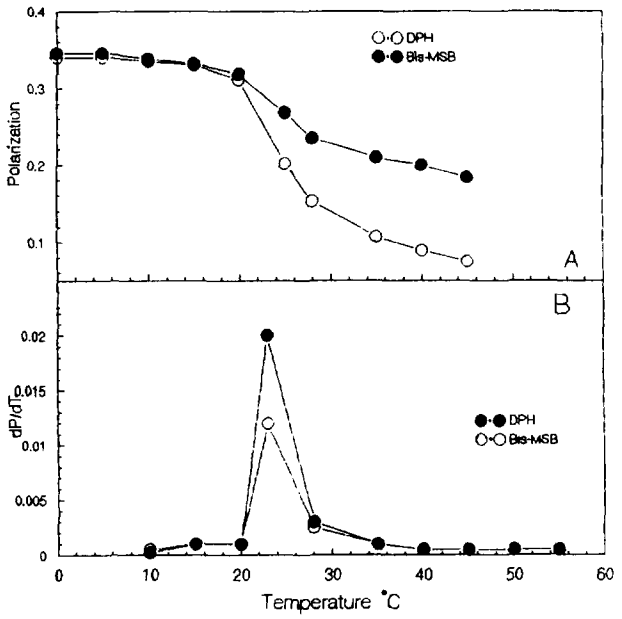


Fig. 7. (A) Temperature profile of polarization of Bis-MSB and DPH in DMPC liposome. (B) Plot of $dP/dT$ of Bis-MSB and DPH in DMPC liposome vs. temperature.

of scattered excitation light should be in the value of $I_{\parallel}$ which was found to be less than 0.5% for all the systems studied. Successive dilution of liposomes did not cause any substantial change in the measured degree of fluorescence polarization, eliminating the possibility of depolarization of fluorescence signal due to scattering. In general, the polarization values obtained are higher for Bis-MSB than DPH, in all the heterogeneous media. This is expected as the structure of Bis-MSB is more rigid than that of DPH. Also the rigid structure avoids the depolarization due to rotation of side groups. The polarization value obtained in triton X-100, a neutral micelle is higher than that obtained in charged micelles like SDS and CTAB, which can be explained as due to head-group repulsions [18].

### 3.4. Study of phase transitions in DMPC vesicles

DMPC liposomes undergo a phase transition ($T_c$) from solid gel to a liquid crystalline state at 23 °C [19]. Such phase transitions are accompanied by an abrupt change in fluidity of membrane which is revealed in the temperature profile of polarization of the probes like DPH and perylene [20]. To see whether polarization of Bis-MSB is sensitive to such phase changes, polarization measurements were carried out with Bis-MSB in DMPC liposomes, for the temperature range of 5–60 °C, covering both the solid gel and liquid crystalline phase. The temperature profile of polarization of Bis-MSB and DPH in DMPC liposome is given in Fig. 7(A). As can be seen, the polarization remains more or less constant in the solid gel phase of DMPC and there is a sudden drop in polarization near 23 °C, which is the phase transition temperature of DMPC liposome. For temperatures above $T_c$, a gradual decrease of polarization is observed. The temperature profile of polarization of DPH is also given in Fig. 7(A) for comparison. The temperature profile of Bis-MSB looks very similar to that of DPH except for a higher value of polarization in the liquid crystalline phase, which can be attributed to the more rigid structure of Bis-MSB. From Fig. 7(A), the limiting value of P for Bis-MSB is 0.358 in DMPC liposome, very close to that of DPH. The drop in polarization at the $T_c$ and the subsequent low polarization value in the liquid crystalline phase, clearly indicates the increase in the membrane fluidity with phase transition. The higher value of polarization observed in Bis-MSB compared to DPH, in the liquid crystalline phase is due to the greater structural rigidity of Bis-MSB.

A much more clear description of the phase changes in the DMPC liposome can be seen by the plot of $dP/dT$ versus $T$ (Fig. 7(B)), where a maximum polarization change is observed near 23 °C, the phase transition temperature of DMPC liposomes.

## 4. Conclusion

Bis-MSB possesses all the desirable characteristics to be used as a fluorescence polarization probe for the non-polar moiety in microheterogeneous medium. It is completely non-polar thus facilitating complete partitioning into the lipid phase ($K_p = 8 \times 10^5$ M) and into the hydrocarbon core of the micelles. It has an elongated, rigid structure thus giving a high and constant polarization ($P_0$) value. It possesses a high extinction coefficient at 355 nm ($S_0$–$S_1$ transition), and a high quantum yield, and hence a fairly high lipid/probe ratio is sufficient ( > 250). It has good spectral characteristics with a minimum overlap of absorption and fluorescence spectrum, thereby eliminating the possibility of depolarization due to resonance energy transfer. Its lack of fluorescence in water ensures that the observed polarization only reflects the non-polar moiety. Bis-MSB can easily be synthesized by the Meerwin arylation reaction [21], and its cost is only about 1/6th of that of DPH. Thus Bis-MSB proves to be a promising polarization probe for organized media. Further work is under way.

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