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Research paper

Detection of liposome membrane viscosity perturbations with ratiometric molecular rotors

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ABSTRACT

Molecular rotors are a form of fluorescent intramolecular charge-transfer complexes that can undergo intramolecular twisting motion upon photoexcitation. Twisted-state formation leads to non-radiative relaxation that competes with fluorescence emission. In bulk solutions, these molecules exhibit a viscosity-dependent quantum yield. On the molecular scale, the fluorescence emission is a function of the local free volume, which in turn is related to the local micro-viscosity. Membrane viscosity, and the inverse; fluidity, are characteristic terms used to describe the ease of movement withing the membrane. Often, changes in membrane viscosity govern intracellular processes and are indicative of a disease state. Molecular rotors have been used to investigate viscosity changes in liposomes and cells, but accuracy is affected by local concentration gradients and sample optical properties. We have developed self-calibrating ratiometric molecular rotors to overcome this challenge and integrated the new molecules into a DLPC liposome model exposed to the membrane-fluidizing agent propanol. We show that the ratiometric emission intensity linearly decreases with the propanol exposure and that the ratiometric intensity is widely independent of the total liposome concentration. Conversely, dye concentration inside liposomes influences the sensitivity of the system. We suggest that the new self-calibrating dyes can be used for real-time viscosity sensing in liposome systems with the advantages of lifetime measurements, but with low-cost steady-state instrumentation.

1. Introduction

Membrane viscosity is a characteristic term that describes the ease of movement within the phospholipid bilayer [1]. Recently, membrane viscosity has been investigated as a possible approach to indicate physiological processes within the cell [2–5]. Membrane protein functionality, carrier-mediated transport and membrane-bound receptors are directly influenced by membrane viscosity [6,7]. Increases in membrane viscosity have been reported with the onset of atherosclerosis [4], malignancy [8], diabetes [9,10], and hypercholesterolemia [11]. Conversely, a decrease in membrane viscosity has been linked with amyloid precursor protein production in patients with Alzheimer's disease [12]. Probing and quantifying changes in membrane viscosity may prove to be an exceptional tool to cell biologists and clinicians alike.

Mechanical methods such as magnetic microbead based rheometry [13] and micropipette aspiration [14] both induce a physical probing force which may impact the physiological state of the membrane. Both micropipette aspiration and magnetic rheometry return averaged values for a large region and cannot reveal local variations. Furthermore, these methods are surface-based and are unable to characterize inner core dynamics of the membrane. For the same reasons, these methods are limited in both temporal and spatial resolution. Fluorescence-based methods have become very popular for measuring membrane viscosity over the past 30 years. The most well-established and documented methods are fluorescence anisotropy and fluorescence recovery after photobleaching (FRAP).

FRAP experiments are considered the "gold standard" technique for monitoring protein mobility and activity within the cell. FRAP experiments are performed by photobleaching a population of fluorophores within a membrane, monitoring the diffusive recovery of intact fluorophores, and computing the rate constants for the recovery. The resulting rate constants are directly proportional to the membrane viscosity. The resulting rate constants have been used to calculate bulk viscosity values for lateral diffusion within the membrane. Viscosity values found by using this method provide an averaged metric for the area of the bleached spot, and the spot size limits the spatial resolution. FRAP has been used to characterize membrane-bound protein mobility [15,16] and the effects of

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mobility on gene expression [17]. Fluorescence anisotropy has been used, for example, to study DNA-protein interactions [18] along with the membrane viscosity altering effects of lipid peroxidation [19]. Both fluorescence anisotropy and FRAP require unique considerations when designing an experiment. A single FRAP experiment may take several minutes for bleaching and recovery, thus making real-time observations difficult. The accuracy of fluorescence anisotropy experiments is highly dependent on quality and alignment of the polarizers, to name two examples.

With molecular rotors, an alternative to the traditional fluorescence-based methods has emerged in recent years [20]. Molecular rotors are a unique class of fluorophores that exhibit a free volume-dependent quantum yield. This sensitivity results from two competing deexcitation paths, radiative photon release and a non-radiative deexcitation through intramolecular rotation. The rotation rate of these molecules is directly impeded by the local molecular free volume, which is a function of the local micro-viscosity. This behavior allows the inference of viscosity properties by measuring changes in the quantum yield. Quantum yield ϕ_F and viscosity η are related through a power-law [21],

$$\phi_F = \phi_0 \cdot \left(\frac{\eta}{\sigma}\right)^x \quad (1)$$

where ϕ_0 is the intrinsic quantum yield, i.e., the propensity of a specific dye to return to the ground state via the planar radiative pathway, σ reflects the dye's motion in the excited state and the associated electrostatic forces [22], and x depends on the dye and the local microenvironment. The dye constant σ has units of viscosity [22]. Because fluorescence emission is proportional to the quantum yield, simple emission intensity measurements advertise themselves for probing the local micro-viscosity. Previous research efforts have focused on derivatives of (2-carboxy-2-cyanovinyl)-julolidine (CCVJ), which show a single emission that obeys Equation (1). In particular, a farnesyl linked CCVJ derivative, abbreviated FCVJ, has been shown to exhibit local viscosity-dependent quantum yield in liposomes [23]. We have successfully incorporated FCVJ into a liposome model system and evaluated the effects of short and long chain alcohols, cholesterol, and various pharmaceutical compounds on membrane viscosity. Furthermore, FRAP experiments with increasing concentrations of cholesterol were used to compare the viscosity sensing capabilities. FRAP-derived viscosity and molecular rotor-derived intensity correlated highly, although FRAP reports long-range viscosities governed by translational diffusion, whereas molecular rotors provide intensity data governed by local micro-viscosity and rotational diffusion [21], two different metrics that cannot in all cases be directly compared.

Fluorescence intensity is primarily dependent on the local concentration of the fluorophore. Signal dependence on intensity values have led to the introduction of ratiometric sensing schemes to correct for concentration inconsistencies [24,25]. Luby-Phelps et al. [24] used a mixture of hydrophilic Cy3 and Cy5 dyes where size differences may lead to different local accumulation and therefore to different ratios. The styryl-based dye introduced by Wandelt et al. [25] exhibits polarity-sensitivity. We have developed a covalently linked ratiometric rotor that features a reference signal that is statistically viscosity-insensitive and a molecular rotor that is typically insensitive towards the polarity of the environment [26]. The ratiometric molecular rotors used in this study are composed of viscosity-insensitive reference fluorophore conjugated to a traditional molecular rotor [27]. The covalent linkage ensures equal local concentrations for the reference and the rotor, and we expect local concentration effects to be fully accounted for: At low dye concentrations and with negligible inner filter effect, the emission intensity I_{em} depends on a fluorophore's quantum yield ϕ_F according to Equation (2),

$$I_{em} = I_{ex} \cdot G \cdot c \cdot \phi_F \quad (2)$$

where I_{ex} is the intensity of the excitation beam, G is an instrument gain factor, and c is the dye concentration. By measuring the emission of the molecular rotor and dividing it by the emission of the reference fluorophore, the instrument-dependent factors in Equation (2) cancel out, and Equation (1) can be expressed in terms of the ratio of rotor intensity I_{Rotor} to reference intensity I_{Ref},

$$\frac{I_{\text{Rotor}}}{I_{\text{Ref}}} = \frac{\phi_0}{\phi_{\text{Ref}}} \cdot \left(\frac{\eta}{\sigma}\right)^x \quad (3)$$

where ϕ_{Ref} is the quantum yield of the reference fluorophore, which is usually close to unity. In this study, we examined to what extent these theoretical considerations can be applied to liposomes in practical experiments. For this purpose, we used a recently developed molecular rotor with a thiophene backbone [28] and covalently attached a coumarin reference unit. We examined this dye combination in an alcohol viscosity gradient to validate the assumption in Equation (3). Next, we integrated the dye into a liposome model and examined the liposome response to propanol. We finally assessed the concentration sensitivity of the ratiometric sensing system.

2. Materials & methods

2.1. Synthesis of ratiometric molecular rotor *1*

The ratiometric molecular rotor **1** was synthesized following the scheme provided in Fig. 1. The synthesis was achieved in three steps as follows.

Preparation of linker **4**: To a round bottom flask containing a solution of the BOC-protected amino alcohol **3** (5.41 mmol) and cyanoacetic acid (**2**) (3.44 mmol) in 10 ml of anhydrous DCM, EDC (5.43 mmol) and HOBT (5.43 mmol) were added. The formation of the product was monitored by TLC and was completed after overnight stirring at room temperature. The crude mixture was concentrated under reduced pressure and the product was purified via flash chromatography (10–30% EtOAc–hexanes).

Ester **4**: 68% yield; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.81 (s, 1H), 4.22 (t, 2H, $J = 6.2$ Hz), 3.47 (s, 2H), 3.16 (m, 2H), 1.81 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (100 MHz), $CDCl_3$ δ 163.0, 155.9, 113.0, 79.2, 64.1, 36.8, 28.7, 28.2, 24.6; HRMS calc for $C_{11}H_{18}N_2O_4Na$ $(M + Na)^+$ 265.1159 found 265.1163.

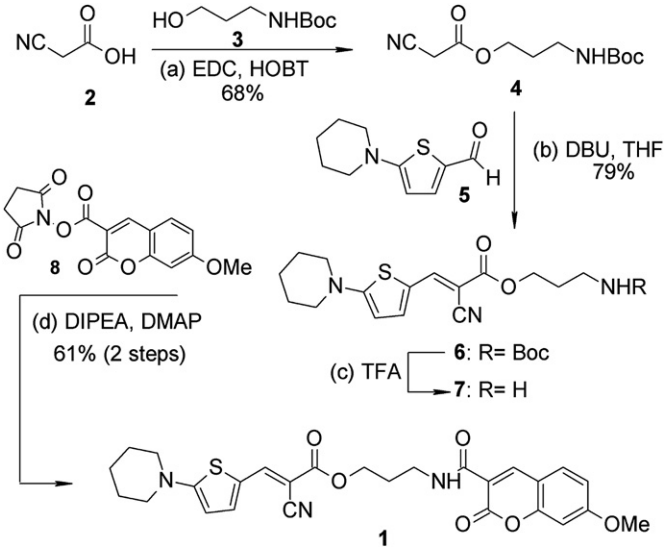

Fig. 1. Synthesis of the ratiometric molecular rotor **1**.