 Highly Efficient Cell Membrane Tracker Based on a Solvatochromic Dye with Near-Infrared Emission

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ABSTRACT: The cell membrane is composed of a phospholipid bilayer with embedded proteins and maintains cell homeostasis through dynamic changes. An abnormal cell membrane shape could be a sign of unhealthy cells. Probes for subcellular fluorescence imaging that can identify the abnormal plasma membrane and record the dynamic changes are needed. Based on a solvatochromic dye with a near-infrared emission strategy, the amphiphatic molecule (E)-2,2′-(4-(2-(4-(dicyanomethylene)-4H-chromen-2-yl)vinyl)phenyl)azanediyl)bis(ethane-1-sulfonic acid) (MRL) contained a hydrophilic sulfo group and a hydrophobic chromone group, which was designed and synthesized for staining the cell membrane and monitoring the morphology of the membranes under different conditions. MRL exhibited an excellent photostability and low cytotoxicity; when cells were incubated with MRL, cell membranes were specifically labeled. MRL is capable of long-term monitoring of the morphological changes of cell membrane.

INTRODUCTION

The plasma membrane is composed of phospholipid bilayers, including hydrophilic and hydrophobic parts. It comprises the main structure and function of cells and plays an important role in cellular processes, such as cellular apoptosis, cell adhesion, cell proliferation, endocytosis, exocytosis, signal transduction, and cell junction, as well as in other diseases.1–7 Real-time visualization and detection of the dynamic changes of the plasma membrane in situ has critical importance in elucidating the function of the cell membrane, which serves as an effective approach for fundamental cell biology research and medicine development.

Fluorescence spectroscopy is a powerful and reliable technique, and it has been extensively used for cell membrane imaging with superior sensitivity and resolution, including cell membrane fusion, apoptosis, and phagocytosis.5,9 Recently, a variety of fluorescent materials with different properties and functionalities have been extensively developed for staining cell membranes. Nevertheless, these fluorescent materials have some drawbacks, which hinder their application. For example, the free lipophilic DiD family (such as DiO, DiI, DiL, and DiR molecules) is just appropriate for dynamically tracing short-time events or whole cells rather than plasma membranes, possibly due to their generated background fluorescence when suspended in a solution, resulting in a low signal-to-noise (S/N) ratio;10–15 organic quantum dots (QDs) fluorescent materials have high cytotoxicity;16,17 the organic fluorescent dye CellMask is susceptible to photobleaching under laser exposure and not suitable for long-term observation or real-time tracking;18 as well as aggregation-induced emission (AIE) probes have high working concentrations and low dyeing efficiency, which are inappropriate for monitoring the dynamic changes of the cell membrane in vivo.19–21 In contrast, the emitting luminescent agents with a long emission wavelength are more suitable for imaging in vivo because they can minimize autofluorescence interference and optical self-absorption.

The longer emission wavelength of near infrared (NIR) can reduce light scattering, tissue absorption, and autofluorescence for in vivo imaging.22–25 Besides, NIR has the advantages of high tissue penetration depth, high-resolution imaging, high sensitivity, high fluorescence quantum yield, good photostability, and biocompatibility.26–31 Thus far, NIR has been extensively applied in the fields of sensing, imaging, biological diagnosis, and therapy.32–37 However, some dyes still show a green fluorescent signal in the field of cell membrane imaging. Therefore, developing a high signal-to-noise (S/N) ratio, low cytotoxicity, good photostability, high dyeing efficiency, and...
near-infrared emission fluorescence probes is highly needed to achieve monitoring the dynamic changes of plasma membrane.

In this study, we designed and constructed an amphipathic fluorescent probe, \((E)-2,2'-(\{(4-(2-(4-(dicyanomethylene)-4H-chromen-2-yl)vinyl)phenyl)azanediyl\}bis(ethane-1-sulfonic acid)\) (MRL), based on a solvatochromic dye with near-infrared emission for imaging and monitoring the cell membrane (Figure 1). The 2-(2-(4-aminostyryl)-4H-chromen-4-ylidene)malononitrile dye has a lipophilic and a hydrophilic moiety. When cells were incubated with an MRL aqueous solution, the lipophilic moiety of MRL preferred to bring the probe MRL into cells, but the sulfonic group detested being close to lipids due to its hydrophilic property. Moreover, the repelling action between the sulfonic acid group with negative charges and phosphoric acid further prevented MRL from entering the cell. Thus, MRL probes have to embed in the lipid bilayer of the cell membrane, and the 2-(2-(4-aminostyryl)-4H-chromen-4-ylidene)malononitrile fluorophore showed strong fluorescent emission because of its solvatochromic property. MRL exhibited high universality to different cells and excellent sensitivity to the cell membrane. Furthermore, it showed low toxicity and strong stability, which can be used as a long-term and real-time tracker. We also demonstrated that MRL can monitor the morphological changes in the cell membrane of living cells.

**RESULTS AND DISCUSSION**

A novel fluorescent probe, \((E)-2,2'-(\{(4-(2-(4-(dicyanomethylene)-4H-chromen-2-yl)vinyl)phenyl)azanediyl\}bis(ethane-1-sulfonic acid)\) (MRL), was constructed to dye the cell membrane by using the benzene-incorporated dicyanomethylene-4H-chromene derivative (BDCM) as a fluorophore, which has an excellent solvatochromic property. We propose a hypothesis about the cytomembrane-staining mechanism in Figure 1. When the probe has contact with the cell membrane, the moiety A of MRL is combined with the cytomembrane as the lipophilic part. At the same time, the moiety B is combined with the water molecule as the hydrophilic part; it drags the MRL in water, and the negative charges of the sulfonic acid group are repelled with phosphoric acid, which prevent MRL from entering the cell. The small-molecule MRL dye emits strong red-NIR light with emission at about 670 nm under 561 nm excitation. Thus, the cell membrane can be stained well.

We evaluated the optical characteristics of MRL in different solvents. As shown in Figure 2A,B, the emission peak of MRL shifts from 620 to 690 nm and the absorption peak in a range from 475 to 530 nm in different solvents. Then, we investigated the fluorescence emission intensity of MRL in different volume ratios of DMSO and water (Figure 2C) or glycerin and water (Figure 2D). It had almost no fluorescence in water, and the fluorescence intensity increases with a red shift from 670 to 690 nm when the volume fraction of DMSO
increased. The fluorescent intensity of MRL increased with a blue shift from 655 to 670 nm until the glycerin fraction reached 80%. MRL had no fluorescence in water, but it showed strong fluorescence in DMSO and glycerin/H_{2}O (8:2, v/v) under a 365 nm fluorescent lamp (Figure 2C,D insert). These results confirmed our hypothesis that MRL showed no fluorescence as a free state in a solution, while MRL can generate strong fluorescence and light the cell membrane when it embedded into the cytoskeleton.

To investigate the properties of MRL as a cell membrane tracker, we incubated it with HeLa cells. When viewed by confocal laser scanning microscopy (CLSM), red fluorescence was observed on the cytomembrane, and no fluorescent signal was visible in the nucleus and cytoplasm after incubating HeLa cells with MRL (Figure 3A). These results illustrated that MRL had the potential of an ideal fluorescent bioprobe as it can specifically stain cell membranes.

The concentration and incubation time of membrane dyes are important. The high concentrations and long staining time of dyes could lead to higher fluorescence intensity, whereas the dyes can penetrate into the cytoplasm, which reduces the S/N ratio. Therefore, low working concentrations and fast staining are necessary for membrane staining. To study the influence of the tracking molecule concentration on cell membrane staining, HeLa cells were incubated with 2, 4, and 10 μM MRL solutions for 5 min, respectively (Figure 3A). The
When the concentration was increased to 10 μM, the outlines of the cell membrane were clearly observed. Lipid compositions of cell membrane, and species maybe HEK-293 T, and CHO cells. Next, we investigated the effects of staining time by incubating HEK-293, HEK-293 T, and CHO cells with MRL at different concentrations for 24 h. The fluorescence intensity of the cell membrane of CHO cells was observed (Figure 3A). Afterward, we estimated the effects of staining time by incubating HEK-293 cells with MRL (2 μM) at different concentration range from 0.3 to 10 μM, respectively.

To investigate whether the probe developed here could be universally applicable for different cell lines, we incubated HEK-293, HEK-293 T, and CHO cells with MRL at different concentrations and time points. HEK-293 T cells were most easily labeled with MRL. When HEK-293 T cells were incubated with 2 μM MRL, the fluorescence signal in the plasma membrane was uneven but bright. Meanwhile, the fluorescence distribution of HEK 293 cells was dotted in the cell membrane. However, the same concentration of the membrane dye cannot illuminate the cell membrane of CHO cells. When the concentration was increased to 10 μM, the red fluorescence of the cell membrane of CHO cells was observed (Figure 4A). Compared with HEK-293 and HEK-293 T cells, CHO cells are less likely to be lit up by MRL. The different cell types, lipid compositions of cell membrane, and species maybe lead to the different staining ability of MRL for HEK-293, HEK-293 T, and CHO cells. Next, we investigated the effect of staining time by incubating HEK-293, HEK-293 T, and CHO cells with MRL at 2 μM from 2 to 12 min (Figure 4B). It demonstrated that the fluorescence intensity of the cell membrane was higher, and the distribution was more uniform as the labeling time increased.

Cell adhesion is the process in which cells attach to another and/or to an extracellular matrix substrate in their immediate environment through cell adhesion molecules (CAMs). In addition, adherent cells can be isolated by treatment with trypsin, which is a protease used to cleave the peptide bonds of CAMs. After the digestion with trypsin, the adherent cells will leave the surface of the matrix and recover to a spherical shape.

From this speculation, we used confocal microscopy to image HEK-293 cells stained with MRL to explore the possibility of using MRL to monitor the detachment process of adherent cells. The HEK-293 cells were cultured in trypsin (0.125%), and the photos of the cell membrane were captured every 0.2 s using confocal microscopy for 20 min. From Figure 5A and Video S1, we can clearly see the digestion process when the cell membranes were stained with MRL. The results indicate that MRL is a potential candidate for long-term monitoring of plasma membrane morphological changes and detection of microevents.

Finally, we evaluated the cytotoxicity of MRL by MTT assay. The cell viabilities of HeLa cells incubated in MRL in the concentration range from 0.3 to 10 μM had no difference from the blank group (Figure 5B). This result indicated that MRL possesses low cytotoxicity at the different concentrations; hence, it is suitable for imaging applications.

CONCLUSIONS

In summary, an amphiphilic molecule MRL consisting of a 2-(2-((4-aminostyryl)-4H-chromen-4-ylidene)malononitrile dye as a lipophilic group and sulfonic salt as a hydrophilic group with near-infrared emission characteristics was designed, synthesized, and utilized for staining cell membranes. Confocal fluorescent imaging data indicate that MRL could efficiently and quickly track cell membranes and has universal applicability and high photostability in different cell types. MRL could monitor the morphological changes of the cell membrane stimulated by circumstance, such as detecting the morphological changes of the cell membrane by treating HeLa cells with trypsin. MRL showed strong near-infrared fluorescent emission on cell membranes, so MRL has potential application in probing deeper into the tissue/body.
Scheme 1. Chemical Structure of Amphiphilic MRL and Its Synthetic Route

EXPERIMENTAL SECTION

Materials and Instruments. Reagents for synthesis were purchased from Macklin. Water was purified by a Cascade PE reservoir system. 1H NMR (500 MHz) and 13C NMR (125 MHz) spectra were acquired on a Bruker Avance-500 spectrometer (Germany) with CDCl3 and d6-DMSO used to dissolve samples. High-resolution mass spectrometry (HRMS) was obtained through a Q-TOF6510 spectograph (Agilent). The absorbance of MTT was measured by a microplate reader (Tecan Austria GmbH A-5082). UV spectra were performed on a UV-2600 spectrophotometer, and fluorescent measurements were operated on an F-4600 FL spectrophotometer. The confocal images of HeLa cells were taken by Nikon A1R MP. All the experiments were performed at room temperature unless otherwise specified.

Synthesis of MRL. The synthetic route of MRL is shown in Scheme 1. 2-(2-methyl-4H-chromen-4-ylidene)-malononitrile (500 mg, 2.4 mmol) and 4-acetamidobenzaldehyde (470 mg, 2.88 mmol) were dissolved into 40 mL of toluene, and then, pyrrolidine (0.5 mL) and AcOH (0.5 mL) were added into the solution. The reaction was refluxed for 72 h. After the reaction was completed, DMF was removed under reduced pressure to obtain the crude product. Then, compound 1 (380 mg, 1.2 mmol), 2-bromoethanesulfonic sodium salt (508 mg, 2.4 mmol), K2HPO4 (482 mg, 2.8 mmol), and KI (239 mg, 1.4 mmol) were dispersed in 20 mL of methanol to obtain MRL (274.7 mg, 43.6%).

Solution Preparation. The fluorescence emission spectrum recorded the value of emission at a wavelength of 540–850 nm with excitation at the wavelength of 561 nm. The UV absorption spectroscopy was recorded at a wavelength of 300–800 nm. The stock solution of MRL (1 mM) was prepared by dissolving it in DMSO. Then, the stock solution was diluted in a series of different concentrations with PBS. The test solutions were prepared by adding 20 μL of the stock solution into 2 mL of deionized water and DMSO or glycerol and water with different volume ratios. The stock solution (20 μL) was diluted with 2 mL of different solutions (H2O, DMSO, DMF, MeOH, EtOH, THF, acetonitrile, 1,4-dioxane, 80% glycerol, and water). The trypsin solution (0.25%) was diluted with PBS to a mass fraction of 0.125%.

Cell Imaging. The cells needed for the experiment were cultured in 35 mm dishes. After 12 h of cell culture, the cells were incubated with MRL at different concentrations in a phosphate buffer solution (PBS pH = 7.2–7.4). After incubation, the cells were imaged directly without washing, and the pictures were obtained at a time interval of 2 min. After incubation of HeLa cells with MRL (2 μM) in PBS for 5 min, the liquid in the dish was removed, 0.125% trypsin with MRL (2 μM) was added, then the dish was imaged immediately. Fluorescence imaging was collected by Nikon A1R MP with a 40× water objective under excitation at 561 nm.

Cell Culture. HeLa cells were maintained in MEM supplemented with 10% FBS at 37 °C with 5% CO2. HEK-293 T, HEK-293, and CHO cells were cultured in DMEM supplemented with 10% FBS at 37 °C with 5% CO2. Before the experiment, the cells were precultured until confluency was attained.

Cytotoxicity Study. The 2-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazole bromide (MTT) assay was used to assess the cytotoxicity of MRL. HeLa cells were seeded into a 96-well plate at a density of 5000 cells per well. After the cells adhered, the cells were exposed to a series of doses of MRL (0–10 μM) in a culture medium at 37 °C. After 24 h, 10 μL of MTT solution (final concentration: 0.5 mg/mL) was added to each well to produce insoluble compounds. After 4 h of incubation, the culture medium was removed, and 100 μL of DMSO was added to each well. After 1 h, the absorbance at 490 nm was recorded using a microplate reader. The experiment was performed at least three times.
ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acsomega.0c01416.

**H NMR, **13C NMR, and MS (PDF)
Process of digesting cells labeled by MRL (MP4)

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Notes
The authors declare no competing financial interest.

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