Membrane processes and biophysical characterization of living cells decorated with chromatic polydiacetylene vesicles

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The structural complexity of the cell membrane makes analysis of membrane processes in living cells, as compared to model membrane systems, highly challenging. Living cells decorated with surface-attached colorimetric/fluorescent polydiacetylene patches might constitute an effective platform for analysis and visualization of membrane processes in situ. This work examines the biological and chemical consequences of plasma membrane labeling of promyelocytic leukemia cells with polydiacetylene. We show that the extent of fusion between incubated lipid/diacetylene vesicles and the plasma membrane is closely dependent upon the lipid composition of both vesicles and cell membrane. In particular, we find that cholesterol presence increased bilayer fusion between the chromatic vesicles and the plasma membrane, suggesting that membrane organization plays a significant role in the fusion process. Spectroscopic data and physiological assays show that decorating the cell membrane with the lipid/diacetylene patches reduces the overall lateral diffusion within the membrane bilayer, however polydiacetylene labeling does not adversely affect important cellular metabolic pathways. Overall, the experimental data indicate that the viability and physiological integrity of the surface-engineered cells are retained, making possible utilization of the platform for studying membrane processes in living cells. We demonstrate the use of the polydiacetylene-labeled cells for visualizing and discriminating among different membrane interaction mechanisms of pharmaceutical compounds.

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1. Introduction

The plasma membrane constitutes the platform for fundamental biological processes, such as ligand recognition [1], drug action [2], vesicle fusion and endocytosis [3], pore-formation by membrane-active peptides [4], and others. Numerous studies of membrane properties and membrane-associated processes have been reported, primarily through the use of varied model systems, such as vesicles, lipid monolayers and films, multilamellar systems, artificial lipid bilayers, and others. There are naturally clear advantages in the analysis of membrane events in the real molecular environments of living cells, rather than model membrane systems. The plasma membrane, however, is a highly complex biological entity, posing considerable challenges for application of bioanalytical techniques to elucidate detailed and specific molecular aspects. While many fluorescent probes (both small fluorescent dyes as well as large biological macromolecules) have been used for imaging cellular membranes [5], structural or dynamical analyses of membrane processes in living cells have been rare. Recently we have demonstrated that cells labeled with chromatic polydiacetylene (PDA) patches constitute a useful platform for studying varied membrane events in live cells [6,7].

Membrane labeling of living cells with PDA facilitates exploitation of the unique chromatic properties of this conjugated polymer [8]. PDA-based vesicles and thin films have been shown to undergo distinct blue–red colorimetric changes owing to conformational transitions in the conjugated (ene–yne) polymer backbone, induced by external structural perturbations [9–13]. Furthermore, PDA also exhibits interesting fluorescence properties; no fluorescence is emitted by the initially polymerized blue-phase PDA, while the red-phase PDA strongly fluoresces at 560 nm and at 640 nm (excitation at 485 nm) [14]. The chromatic transformations of PDA have been exploited also in
biological contexts: recent studies have demonstrated that blue-red transitions could be induced by membrane-active compounds in vesicle assemblies of phospholipid bilayers incorporated within the PDA matrices [15,16]. In the case of PDA-labeled living cells, we have demonstrated that local disruptions within the plasma membrane induce chromatic transformations of membrane-attached PDA patches, thus making the modified cells an effective “live cell sensor” for analysis of membrane processes.

Here we present a biophysical and physiological analysis of PDA-labeled cells, aiming to elucidate the parameters contributing to membrane incorporation of the lipid/diacetylene vesicles and the physiological consequences of cell labeling with PDA. The fundamental questions we address in this study concern the extent of modifications of the plasma membrane environment following PDA labeling, and the effect of cell-surface modification upon crucial intracellular processes. Several bioanalytical techniques and physiological assays have been applied, providing a comprehensive description of lipid/diacetylene vesicle fusion and the resultant modified cells, overall indicating that the polymer patches do not adversely and significantly affect cell properties. De picting the practical utilization of the PDA-labeled cell platform, we applied the assay for distinguishing mechanisms of drug–membrane interactions.

2. Materials and methods

2.1. Materials

Lipids, including 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) (sodium salt) (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), sphingomyelin (egg, chicken) (SpH), and cholesterol (Chol) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). The diacylceromonomer 10,12-tricosadiynoic acid was purchased from Alfa Aesar (Karlsruhe, Germany), washed in chloroform, and passed through a 0.45 µm nylon filter prior to use.

The fluorescent dyes 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,13-benzocadiazol-4-yl) (ammonium salt) (NB-DPE) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lisamine rhodamine B sulfonyl) (ammonium salt) (Rd-DPE) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL), 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), Fura-2AM (acetoxymethyl ester), and 5,5′,6,6′-tetracloro-1,1,3,3′-tetrachlorobenzimidazolocarbocyanine iodide (JC-1) were obtained from Molecular Probes, Inc. (Eugene, Oregon).

Tri(hydroxymethyl)aminomethane (Tris-base buffer), sodium dithionite, 2,4-dinitrophenol (DNP), methyl-j/-cytocdalin (MBD), IMDP (Met-Leu-Phenylalanine, and TMD were purchased from Sigma-Aldrich. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega Corporation (Madison, WI, USA). The pharmaceutical substances imipramine hydrochloride, amitryptiline hydrochloride, nortriptyline hydrochloride, acetobutol hydrochloride, (−)-metoprolol (+)-tartrate salt, propranolol hydrochloride, and dichloran sodium salt were purchased from Sigma-Aldrich.

2.2. Vesicle preparation

Preparation of vesicles containing the diacylceremonomer 10,12-tricosadiynoic acid and the lipid components (DMPC/diacetylene = 2:3 mole ratio, DMPE/DMPG/diacetylene = 1:1:3, SpH/cholesterol/diacetylene = 1:1:3, DMPG/cholesterol/diacetylene = 1:2:3) was carried out through the following protocol: all vesicle constituents were dissolved in chloroform/ethanol (1:1) and dried together in vacuo up to constant weight, followed by addition of deionized water to final concentration of 1 mM, and subsequently probe sonicated at 70 °C for 3 min. The vesicle solution was then cooled at room temperature and was kept at 4 °C.

2.3. Cell culture

Human promyelocytic leukemia cells HL-60 were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM-L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified 5% CO2 atmosphere at 37 °C. The cell lines were maintained at a density of 0.7×10⁶ cells/ml before harvest and were subcultured three times weekly.

2.4. Cholesterol depletion by methyl-j/-cytochelin treatment

HL-60 cells were washed and incubated in HEPES buffer (1×10⁶ cells/ml, pH 7.4) containing 10 mM methyl-j/-cytocdalin (MBD) for 1 h at 37 °C; following incubation, the cells were washed three times with the buffer and labeled with the lipid/diacetylene vesicles.

2.5. Vesicle fusion with cells

Cells were harvested from the growth medium and washed in a HEPES buffer (20 mM HEPES, 137 mM NaCl, 2.7 mM KC1, 1 mM KH2PO4, 2 mM-L-glutamine, and 5 mM α-glucose (pH 7.4)) by centrifugation at 400 g for 7 min. 2×10⁴ cells per sample were kept in 2 ml buffer to which the different lipid/diacetylene vesicles were added (final lipid concentrations 0.1–0.7 mM, depending on the experiment) and incubated for 45 min with slow shaking. Following incubation the cell suspensions were irradiated for 20–30 s at 254 nm (50–500 µJ/cm²) to polymerize the polydiacylene backbone, resulting in the appearance of blue color. The vesicles hybridized were washed three times for removal of non-associated vesicles and re-suspended in the same buffer before experiments. All experimental steps were carried out at 25 °C.

2.6. Fluorescence resonance energy transfer (FRET)

Lipid/diacetylene vesicles (concentration 1 mM) were prepared by the procedure described above. Prior to drying, the lipid/diacetylene vesicles were additionally supplemented with N-NBD-PE and N-Rh-PE at a 100:1 (phospholipid: N-NBD-PE:N-Rh-PE) mole ratio. Fluorescence emission spectra were acquired (excitation 469 nm) in the range of 500–700 nm on a FL920 spectrofluorimeter (Edinburgh, UK), using a 1 cm optical path cell. Three ratios of fluorescence emission peaks were recorded for each sample: R0 (initial) — ratio of fluorescence emissions of N-NBD-PE (530 nm)/N-Rh-PE (585 nm) immediately after cell and vesicle mixing, Rf (final) — after 45 min incubation with cells, and RF — following the addition of 10% Triton X-100 to the vesicles (Triton X-100 is a detergent causing vesicle destruction and maximum dispersion of the fluorescent dyes). The percentage of fusion was determined by the equation [17]:

\[
\%\text{Fusion} = \left(\frac{R_f - R_0}{R_f} - R_i\right) \times 100.
\]

All experiments were performed three times at 25 °C.

2.7. Fluorescence quenching

HL60 cells labeled with the fluorescent dye NBD-PE were prepared as follows: NBD-PE was dissolved in chloroform and dried prior to dissolution in absolute ethanol (1 mM). An aliquot of the NBD-PE/ethanol solution (1% v/v) was injected into a cell suspension containing 2×10⁶ cells in 1 ml cold HEPES buffer (pH 7.4) under vigorous stirring and kept on ice for 30 min (final concentration of NBD-PE 2 µM). The cells were subsequently washed three times by centrifugation for removal of excess NBD-PE and re-suspended in HEPES buffer. Lipid/diacetylene vesicle fusion was then carried out as described above.

The fluorescence quenching reaction was initiated by addition of sodium dithionium from a 0.6 M stock solution prepared in 50 mM Tris-base buffer (pH 11) to a final concentration of 10 mM. The decrease in fluorescence (467 nm excitation, 530 nm emission) was recorded for 240 s at 25 °C on an Edinburgh FL920 spectrofluorimeter. Anisotropy values (r) were automatically calculated by the spectrofluorimeter software using conventional methodology [18]. Fluorescence anisotropy measurements were performed at least three times.

2.9. Viability and cell proliferation

The fluorescence probe TMA-DPH was incorporated into the cell membrane by adding the dye dissolved in THF (1 mM) to cell suspension in HEPES buffer up to a final concentration of 1.25 mM. After 30 min of incubation at 37 °C with gentle stirring the cell samples were washed three times with HEPES buffer for removal of excess fluorescent probe. Lipid/diacetylene vesicles were then added to the cell suspension and incubated for 30 min at 37 °C. During all stages of preparation the cell density was 2×10⁶ cells per 1 ml buffer.

DPH fluorescence anisotropy was measured at 428 nm (excitation 360 nm) on an Edinburgh FL920 spectrofluorimeter. Anisotropy values (r) were automatically calculated by the spectrofluorimeter software using conventional methodology [18]. Fluorescence anisotropy measurements were performed at least three times.

2.9. Viability and cell proliferation

The cytotoxicity of the lipid/diacetylene vesicles has been assessed using a cell proliferation assay (Promega CellTiter 96A AQ, One Solution Cell Proliferation assay) [19]. Briefly, 7×10⁴ exponentially growing cells were seeded in 96-well microculture plates together with the vesicles (final concentration 0.1 mM) in volumes of 100 µL. Immediately, and after 24 h incubation at 37 °C, 20 µl aliquots of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium, inner salt (MTS) were added to each well and the samples were incubated for another hour at 37 °C. Plates were analyzed on a Jena Analytical ELISA reader at 490 nm. For each type of vesicles at least 3 replicates were analyzed.

Cell counts in the tested samples were based upon a calibration curve, in which 2×10⁴, 4×10⁴, 10×10⁴, and 20×10⁴ cells were seeded in a 96-well microculture plate in volumes of 100 µL. 20 µL MTS was added to each well and the samples were incubated for 1 h at 37 °C. The plates were then analyzed on a Jena Analytical ELISA reader at 490 nm.

2.10. Cytotoxic Cu²⁺ measurements

Measurements were carried out on differentiated HL60 cells. To induce granulocyte-like cell differentiation, HL60 cells at a density of 4×10⁴ cells/ml were cultured in the
presence of 1.25% (v/v) DMSO for 6–7 days [20]. The differentiated cells exceeded 86% of total cell count, as determined by the CD14 and CD11b differentiation markers (data not shown). Differentiated cells were harvested from the medium, washed in HEPES buffer, fused with the lipid/diacetylene vesicles and polymerized (see 2.5 vesicle fusion protocol above). The PDA-labeled cells (as well as control untreated differentiated cells) were then washed and re-suspended in Ca\(^{2+}\)-buffer [137 mM NaCl, 2.7 mM KCl, 20 mM HEPES, 1 mM KH\(_2\)PO\(_4\), 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 5 mM d-glucose, 2.5 mM probenecid, 0.1% BSA (pH 7.4)] and incubated with 2 μM Fura-2 AM dissolved in DMSO at 37 °C in the dark for 0.5 h under gentle shaking. Cells were extracted at distinct times, washed twice with buffer and re-suspended in the same Ca\(^{2+}\)-buffer. After additional 15 min of stabilization the cells were centrifuged and re-suspended in the same buffer at a concentration of 1 × 10\(^6\) cells/ml.

Cytosolic Ca\(^{2+}\) concentration was determined through recording the changes in Fura-2AM fluorescence using a Perkin Elmer LS 50 S luminescence spectrometer. Aliquots of 2 × 10\(^5\) cells (200 μl) per 2 ml of Ca\(^{2+}\)-buffer without BSA and probenecid were transferred to a temperature-controlled (25 °C) cuvette under continuous stirring. Excitations at 340 and 380 nm were recorded at a fixed emission (510 nm). At the end of each experiment the cells were lysed through addition of 0.1% Triton X-100 and supplemented by 20 mM EGTA to obtain the maximal and minimal Ca\(^{2+}\) levels, respectively. Calcium concentration was calculated by the equation [21]:

\[
\frac{[Ca^{2+}]}{[R]} = K_d \frac{(R - R_{min})}{(R_{max} - R)},
\]

where \(R_{max}\) and \(R_{min}\) are the Fura-2AM fluorescence excitation ratios at 340/380 nm for Ca\(^{2+}\)-lacking and Ca\(^{2+}\)-saturation concentrations, respectively. \(K_d\) for the Fura-2AM–Ca\(^{2+}\) complex was taken as 224 nM [21].

2.11. Mitochondria membrane potential

The fluorescent dye JC-1 was dissolved in DMSO to give a 5 mg/ml stock solution. This was further diluted 1/2000 in HEPES buffer and filtered using a 0.45-μm filter (Whatman). To assess the effect of PDA labeling on mitochondrial inner membrane potential, 50 μl of the labeled cell suspension containing 2 × 10\(^6\) cells were extracted and incubated for 30 min at 25 °C with 500 μl of the filtered JC-1 working solution (2.5 μg/ml). Cells were washed three times with HEPES buffer, deposited on a cover-slip and analyzed after PDA labeling at distinct time periods by fluorescent microscopy (Perkin Elmer’s spinning disc system: Ultra View ERS Rapid Confocal Imager). Unlabeled cells were washed with HEPES buffer and used as control. For comparison, both PDA-labeled and untreated cells after staining with JC-1 were incubated for 10 min with 0.5 mM DNP, a mitochondrial membrane uncoupling agent. Briefly, 1 μg of DNP was dissolved in 20 μl DMSO, this was further diluted 200 times in HEPES buffer (total concentration 1 mM) and was added to equal volumes of the cell suspension.

To view J-aggregates, excitation was 488 nm and green emission discrimination filter was used (498–552 nm); for monomeric JC-1, emitted light was collected through a red band-pass 580 nm to 615 nm filter (excitation 514 nm). A pseudo-color value was assigned to the acquisition of the red/green ratio and analyzed with Velocity™ 3-D/4-D visualization and analysis software. For each cover-slip three images of each condition were analyzed, and the intensity of the red/green fluorescence ratio signal from at least 10 cells in each image was determined.

2.12. Colorimetric analysis

Quantitative colorimetric determination was applied to cells labeled with DMPE/DMPC/diacetylene vesicles and polymerized (see preparation procedure above). Samples were prepared by adding the pharmaceutical compound examined, dissolved in water, to a 60 μl HEPES buffer (pH 7.4) solution containing 2 × 10\(^6\) labeled cells. Following addition, the solutions were diluted to 1 ml and UV-Vis spectra were acquired at 25 °C on a Jasco V-550 spectrophotometer, using a 1 cm optical path cell. The measurements were repeated three times for each sample.

A quantitative value for the extent of the blue-to-red color transitions within the PDA-labeled solutions is given by the colorimetric response (3CR), which is defined as follows [16]:

\[
%CR = \left(\frac{P_{\text{red}} - P_{\text{blue}}}{P_{\text{blue}}}\right) \times 100,
\]

where \(P_{\text{red}}/A_{\text{max}}\) and \(P_{\text{blue}}/A_{\text{max}}\) are the absorbance either at the “blue” component in the UV-Vis spectrum (640 nm) or at the “red” component (540 nm) (note: “blue” and “red” refer to the visual appearance of the material, not its actual absorbance). \(P_{\text{red}}\) is the blue-red ratio of the control sample (before addition of the pharmaceutical compounds), and \(P_{\text{blue}}\) is the value obtained for the PDA-labeled cells solution after induction of color change.

Further colorimetric analysis was carried out to compare the induced transitions in the PDA-labeled cells to the color transformations in cell-free DMPE/DMPC/PDA vesicles (1:1:3 mole ratio). Final lipid concentration of the vesicles was 0.7 mM. The vesicles were pre-incubated in HEPES buffer for 45 min prior to polymerization (for mimicking the preparation protocol of the PDA-labeled cells). The vesicle solution was diluted to insure that the initial intensity of the “blue” component in the UV-Vis spectrum (peak at 640 nm) of the vesicle sample (before addition of the pharmaceutical compounds) was identical to the corresponding signal in the PDA-labeled cells.

2.13. Fluorescence microscopy

Microscopy analysis was conducted for evaluating the effect of pharmaceutical compounds upon the PDA-labeled HL60 cells. Samples were prepared according to the procedure described above (colorimetric analysis) with minor changes: following addition of the pharmaceutical compounds, the solutions were diluted to 200 μl, from which 20 μl were deposited onto glass cover-slips. Fluorescence images of the PDA-labeled cells were acquired on a laser-scanning confocal microscope Axiovert-100 M (Zeiss, Germany) with a Plan-Neofluar 100×/1.3 oil objective. Excitation was 488 nm using an argon laser source. Emitted light was collected through a band-pass 625–655 nm filter.

3. Results

3.1. Biophysical characterization

Fig. 1 schematically depicts the procedure for membrane labeling of living cells with chromatic polymer patches [6]. The cells are incubated in a buffer solution containing vesicles comprising the diacetylene monomers and lipids (Fig. 1A). Subsequently, the lipid/diacetylene/cell constructs are polymerized through brief irradiation with UV light (254 nm), resulting in the creation of the chromatic sensor cells (Fig. 1B). The polymer patches attached to the cell surface undergo both blue–red transformations as well as emit intense fluorescence in response to processes occurring or affecting the plasma membrane.
cholesterol removal from the cell membrane significant within the plasma membrane in promoting vesicle fusion. Fig. 2B depicted in Fig. 2B was designed to examine the role of cholesterol in vesicle interactions efficient fluorescence energy transfer occurs between the vesicle-incorporated NBD (fluorescence donor) and Rh (fluorescence acceptor) [17]. However in the event of membrane fusion (with consequent lipid mixing among the vesicle and cell membrane bilayers) the fluorescence energy transfer is reduced due to the greater distance, on average, between the donor and acceptor molecules within the fused system [17].

Fig. 2A examines the extent of lipid fusion following incubation of the lipid/diacetylene vesicles with the HL60 cells, and the dependence of fusion upon specific vesicle compositions. The percentage values reported in Fig. 2A were derived from the ratios between the emission peaks of the donor (530 nm) and acceptor (585 nm), respectively, following incubation of vesicles and cells. The maximal donor/acceptor ratio is referred to Triton X-100 which induces complete dissolution of the vesicles, and thus minimal energy transfer between donor and acceptor [23]. The bar diagram in Fig. 2A clearly demonstrates that the most pronounced fusion occurred when DMPE/DMPG/diacetylene vesicles were incubated with the cells. In contrast, vesicles containing DMPC, DMPC/cholesterol, or sphingomyelin/cholesterol all exhibited significantly lower fusion values (Fig. 2A).

In addition to illuminating the relationship between membrane fusion and lipid composition of the vesicles, we also investigated the contributions of specific lipid components of the plasma cell membrane upon vesicle fusion (Fig. 2B). Specifically, the experiment depicted in Fig. 2B was designed to examine the role of cholesterol within the plasma membrane in promoting vesicle fusion. Fig. 2B compares the extent of fusion of DMPE/DMPG/diacetylene vesicles to normal HL60 cells (determined through FRET analysis), to vesicle fusion with HL60 cells pre-incubated with methyl-β-cyclodextrin (MBCD), known to extract cholesterol from the plasma membrane of cells [24]. Indeed, the bar diagram in Fig. 2B clearly indicates that cholesterol removal from the cell membrane significantly reduced fusion (from 50% to approximately 25%).

Application of other biophysical techniques complemented the FRET data in Fig. 2 and provided additional insight into the interactions and incorporation of the lipid/diacetylene vesicles within the cell membrane (Fig. 3). Fig. 3A depicts the results of fluorescence quenching analysis [25] utilizing HL60 cells incorporating the NBD-PE dye within the plasma membrane, and further incubated with lipid/diacetylene vesicles. In the experiments summarized in Fig. 3A, the fluorescence quencher sodium dithionite was added to the NBD-PE/lipid/diacetylene/HL60 constructs and the NBD fluorescence was monitored over time. The emission curves in Fig. 3A display the expected exponential quenching of the NBD fluorescence following interactions with the water-soluble dithionite. However, differences were observed among the rates of fluorescence quenching when different vesicles were incubated with the cells. These differences correspond to the relative exposure of the NBD residues to the aqueous solution — thus to the dissolved dithionite quencher [25]. In particular, the fastest quenching rate was recorded following addition of DMPE/DMPG/diacetylene to the NBD-PE/HL60 cells (curve iv in Fig. 3A). This result is most likely explained by the pronounced lipid rearrangement within the plasma membrane following interactions with the DMPE/DMPG/diacetylene vesicles, facilitating greater contact between the membrane-embedded NBD-PE and the dithionite. The pronounced fluorescence quenching following DMPE/DMPG/diacetylene vesicle addition, in comparison with the other lipid compositions examined, echoes the significant fusion between these vesicles and the cell membrane (Fig. 2A).

Fluorescence anisotropy measurements depicted in Fig. 3B and C shed more light on the dynamical consequences of vesicle interactions with the plasma membrane of the HL60 cells. The experiments in Fig. 3B–C employed HL60 cells containing the fluorescence dye TMA-DPH. The bar diagrams in Fig. 3B and C display the fluorescence anisotropy of TMA-DPH pre-incubated with the HL60 cells prior to addition of the lipid/diacetylene vesicles, and subsequent modifications of bilayer order induced by vesicle interactions. Indeed, the fluorescence anisotropy of lipid-embedded TMA-DPH, which is located in the bilayer close to the lipid/water interface, has been widely used as a probe of lipid acyl chain order and lateral diffusion within lipid bilayers [26].

Fig. 3B illuminates the changes of the fluorescence anisotropy of TMA-DPH induced by interactions of the cell plasma membrane with vesicles having different lipid constituents. Fig. 3B demonstrates that most lipid compositions yielded higher anisotropy — indicating increased rigidity of the membrane bilayer, most likely due to binding of the vesicles to the surface of the plasma membrane. Significantly, the most pronounced effect — highest fluorescence anisotropy — was observed after DMPE/DMPG/diacetylene vesicles were incubated with the cells (Fig. 3B, iv). The induction of highest bilayer rigidity following addition of DMPE/DMPG/diacetylene vesicles is most likely a consequence of the efficient vesicle/cell fusion, and is consistent with the experimental data presented in Figs. 2 and 3A.

Fig. 3C depicts the results of a titration experiment in which the TMA-DPH fluorescence anisotropy was recorded in solutions containing different concentrations of DMPE/DMPG/diacetylene vesicles.
Fig. 3C demonstrates that the fluorescence anisotropy of the lipid-embedded TMA-DPH gradually increases in the presence of greater vesicle concentrations, confirming the direct relationship between the induced bilayer rigidity and vesicle interactions with the plasma membrane.

3.2. Physiology of PDA-labeled cells

While Figs. 2 and 3 indicate that the fusion of the lipid/PDA vesicles onto the cell surface affects the molecular properties of the plasma membrane, the experiments do not provide insight into the overall physiological effects on the modified cells. Accordingly, Figs. 4–6 present application of biological assays designed to evaluate the effect of membrane labeling with PDA upon cell viability, and upon physiological processes occurring in the cell interior.

The bar diagram in Fig. 4 depicts the results of an MTS-based cell proliferation assay designed to determine the effect of incubation with the DMPE/DMPG/diacetylene vesicles upon overall cell viability. Live cell counting was carried out immediately after lipid/diacetylene vesicle addition (white bars in Fig. 4) and following 24 h incubation (grey bars). The data presented in Fig. 4 clearly show that the relative increase in the number of cells in the solution containing lipid/diacetylene vesicles was similar to the control cells (to which vesicles were not added). This proliferation is an indication of a vital cell population that kept dividing even through incubation and fusion of the lipid/diacetylene vesicles to the plasma membrane.

In addition to the overall viability and normal proliferation of the surface-labeled HL60 cells (Fig. 4), we investigated the effects of vesicle treatment upon important intracellular cell processes. Fig. 5 depicts an in situ analysis of changes in cytosolic Ca$^{2+}$ in PDA-labeled and unlabeled differentiated HL60 cells (dHL60). The differentiation of HL60 cells to granulocytes is important, since only the differentiated cells can be rapidly activated through plasma membrane receptors and intracellular Ca$^{2+}$ stores that trigger responses to chemotactic factors such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) [27]. The experiment depicted in Fig. 5 monitors the high sensitivity of cytosolic calcium...
Ca\textsuperscript{2+} concentration in human leukemia dHL60 cells to stimuli by the chemotactic peptide fMLP. This peptide is known to increase the concentration of intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]i), which is mobilized from both intracellular stores and by uptake from extracellular sources\[28\]. Cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i), was assessed using the fluorescent calcium indicator Fura-2AM\[29\]. Determination of [Ca\textsuperscript{2+}]i was performed after cell incubation with DMPE/DMPG/diacetylene vesicles and further polymerization through brief irradiation at 254 nm. Fig. 5A demonstrates that following activation by 1 \mu M fMLP, both untreated (control) cells, as well as the PDA-labeled cells exhibited elevated levels of free cytosolic Ca\textsuperscript{2+}. Importantly, Fig. 5A shows that even though the [Ca\textsuperscript{2+}]i peak appeared somewhat reduced in the PDA-labeled cells (curve b) compared to the untreated cells (curve a), the shape of the [Ca\textsuperscript{2+}]i curve was indicative of normal (functional) response of the calcium storage cell machinery [29]. Furthermore, Fig. 5A suggests that most fMLP membrane receptors retain their functionality even when PDA is also loaded on the cell surface; the small difference between the [Ca\textsuperscript{2+}]i response in the PDA-labeled cells and control cells is probably due to the lipid/PDA vesicle fusion onto the membrane. Fig. 5B further demonstrates that the ratio between the [Ca\textsuperscript{2+}]i peak following fMLP stimulation in normal and in PDA-labeled cells remained almost constant even 3 h after fusion and polymerization, indicating that this fundamental signaling pathway is not disrupted within this time period.

Another important functional assay performed on the PDA-labeled cells was a mitochondrial membrane potential analysis [30] (Fig. 6). Fig. 6 depicts the fluorescence emission images obtained using the cationic lipophilic dye JC-1 which selectively enter mitochondria [30]. In solution, free JC-1 exists in monomers emitting green fluorescence (excitation 490 nm, emission 527 nm), however when the mitochondrial membrane is functionally viable (polarized) the dye forms J-aggregates (multimers) which exhibit a large shift in emission (590 nm, red-orange). Thus, JC-1 fluorescence is highly sensitive to mitochondria membrane potential, changing from red to green upon events leading to loss of mitochondrial transmembrane potential as a result of mitochondrial damage. Overall, the ratio of red (J-aggregate)/green (monomeric JC-1) emission reflects the physiological state of mitochondria [31].

The representative fluorescence microscopy images of the HL60 cells in Fig. 6 confirm that no significant membrane depolarization occurred following PDA labeling. The image of the control (untreated) cells (Fig. 6A) depicts the red fluorescence from the J-aggregates accumulated within the mitochondria. PDA-labeled cells feature mainly red and yellow fluorescence areas (Fig. 6B), indicative of minimally disrupted mitochondrial membrane potential. The fluorescence images in Fig. 6A–B stand in stark contrast to the microscopy picture in Fig. 6C corresponding to complete mitochondrial membrane depolarization induced by 2,4-dinitrophenol (DNP) [32], resulting in the appearance of only the green JC-1 monomers. The fluorescence data presented in Fig. 6 are consistent with the [Ca\textsuperscript{2+}]i measurements (Fig. 5), overall indicating that plasma membrane labeling by the lipid/PDA do not exert adverse effects upon cell physiology.

3.3. Practical applications

Fig. 7 depicts a representative application of the PDA-labeled cell platform for analyzing membrane interactions of pharmaceutical compounds (Table 1). Evaluation of bilayer association and membrane permeation of pharmaceutical compounds is a critical component in
drug development because of the abundance in the human body of lipid barriers, such as the blood-brain barrier (BBB) or the gastrointestinal tract (GI), which might significantly affect drug delivery and efficacy [33]. Analysis of membrane interactions and permeation by pharmaceutical molecules is generally carried out through application of crude techniques, such as alcohol/water partition coefficients, that occasionally are not satisfactory as predictive tools.

Fig. 7A presents transmission and fluorescence microscopy images of PDA-labeled HL60 cells to which pharmaceutical compounds were added. Imipramine (Fig. 7Ai) and metoprolol (Fig. 7Aii) gave rise to the appearance of fluorescent spots on the cell surface; however metoprolol required an order-of-magnitude higher concentration for induction of fluorescence, compared to imipramine. Procainamide, on the other hand, did not induce fluorescence emission when added to the PDA-labeled cells even at a high concentration (Fig. 7Aiii). The fluorescent spots appearing on the cell surface correspond to the membrane-incorporated PDA patches undergoing the structural/physical changes induced by the binding of the drug molecules to the lipids.
chromatic transformation, induced through plasma membrane interactions of the tested compounds [6].

The significant differences in concentration-dependent fluorescence induction in Fig. 7A reflect the distinct membrane affinities and bilayer disruption properties of the pharmaceutical compounds examined. Imipramine (Fig. 7Ai) binds strongly to lipid bilayers and is believed to exhibit high affinity to membrane bilayers [34], consequently expected to induce significant reorganization of the bilayer surface and the corresponding chromatic transformations of the membrane-fused PDA patches, as depicted in Fig. 7Ai. Metoprolol (Fig. 7Aii) is a highly hydrophobic substance which inserts deeply into membrane bilayers [35] thus requiring a higher concentration to induce membrane perturbations and consequent chromatic transformation of PDA. In contrast to both imipramine and metoprolol, procainamide does not penetrate through lipid barriers through passive diffusion mechanisms [36] and thus did not affect fluorescence changes of the membrane-attached PDA patches (Fig. 7Aii).

Indeed, the concentration range in which the PDA chromatic transformations are induced by a tested pharmaceutical compound is an important parameter which can be ascribed to the mechanism of membrane binding and permeation. This observation is further exemplified in Fig. 7B which features the color transformations of the PDA-labeled cells. Essentially, Fig. 7B depicts the “colorimetric response” (%CR) dose–response curves recorded for several pharmaceutical compounds added to the PDA-labeled cell suspensions. Data points in the graph in Fig. 7B were calculated from the relative intensities of the blue and red peaks in the UV-Vis spectra of the PDA-labeled cell suspensions (see Materials and methods); high %CR values correspond to stronger red appearance of the cells, while low %CR reflects more blue color.

Fig. 7B indeed demonstrates that the color transformations of the PDA-labeled cells were induced in considerably different concentration ranges [note the logarithmic scale of the x axis]. Specifically, while imipramine, amitriptyline, and nortriptyline affected the blue–red transitions of the PDA-labeled cells in concentrations which were smaller than 0.1 mg/ml, metoprolol and acebutolol required significantly greater concentrations, of between 1 and 10 mg/ml. Fig. 7B also shows that procainamide and diclofenac yielded negligible color changes in the PDA-labeled cells, even when added in much higher concentrations to the PDA-labeled cell solution.

The experiments depicted in Fig. 7C were designed to assess whether the fluorescence (Fig. 7Ai) and color transformations (Fig. 7B) induced by the pharmaceutical compounds in the PDA-labeled cells were due to actual interactions with the plasma membrane of the cell, or rather the chromatic transitions corresponded to non-specific binding of the pharmaceutical compounds to the surface-attached PDA patches. This distinction is important, because PDA chromatic transitions induced by direct binding of the pharmaceutical substances will obviously be less informative from a biological point of view [6].

Specifically, Fig. 7C compares the dose–response curves induced by imipramine and by metoprolol in PDA-labeled cells and in cell-free DMPE/DMPC/PDA vesicles, respectively. The blue signals in the PDA-labeled cells and the lipid/PDA vesicles, respectively, were adjusted to be of equal intensity, and accordingly the differences apparent in the graphs between the colorimetric responses induced in the cells and in the vesicles point to different mechanisms responsible for the color transitions in each system. In particular, while imipramine induced much more pronounced blue–red transitions in the lipid/PDA vesicles compared to the PDA-labeled cells, the situation was the opposite for metoprolol (higher %CR induced when added to PDA-labeled cells, Fig. 7C). These results indicate that the chromatic transformations induced by the pharmaceutical compounds in the PDA-labeled cells originate from distinct interactions of the molecules with the plasma membrane of the cells, which consequently affect the structural transitions of the membrane-incorporated polymer patches.

4. Discussion

We presented a detailed biophysical and physiological investigation of newly-designed polydiacetylene-labeled cells and their application for studying membrane processes. A fundamental question addressed in this study was the following: to what extent the chromatic polymer patches were indeed embedded within the plasma membrane of the cells, and whether this cell-surface modification significantly (and adversely) affected the membrane properties and overall cell physiology.

The lipid components within the mixed lipid/diacetylene vesicles were found to be a critical parameter determining the extent of fusion with the cell plasma membrane. In particular, fluorescence resonance energy transfer (FRET) experiments depicted in Fig. 2 confirmed the occurrence of significantly greater lipid fusion between DMPE/DMPC/diacetylene vesicles (1:1:3 mole ratio) and the plasma membrane, compared to other vesicle compositions. Enhanced fusion phenomena in case of vesicles comprising phospholipids displaying the PE and PG headgroups have been previously observed [37]. Similar observations were also reported for other PDA-labeled cell systems [6]. Interestingly, while cholesterol in the vesicles did not appear to enhance lipid fusion (Fig. 2A), its presence within the cell membrane seems important (Fig. 2B). This result suggests that increased bilayer disorder decrease fusion of the lipid/diacetylene vesicles to the plasma membrane. The significance of lipid ordering in affecting vesicle fusion has been previously reported [38].

Application of additional biophysical techniques complemented the FRET analysis and provided additional insight into the molecular characteristics of vesicle–membrane interactions. Both the fluorescence quenching (Fig. 3A) and fluorescence anisotropy (Fig. 3B–C) data indicate pronounced effect upon the cell membrane following interactions with the DMPE/DMPC/diacetylene vesicles. Similar to the FRET results — DMPE/DMPC/diacetylene vesicles gave rise to much more significant effects compared to the other lipid compositions examined: marked lipid rearrangement (implied by the quenching experiments, Fig. 3A) and lesser lateral diffusion (TMA-DPH anisotropy, Fig. 3B–C). Both scenarios are consistent with a considerable lipid mixing between the DMPE/DMPC/diacetylene vesicles and the plasma membrane bilayers.

A crucial question pertaining to the usefulness of the new PDA–cell system for probing membrane processes in living cells concerns the effect of vesicle fusion on cell vitality and normal cell physiology. Cell viability assays (Fig. 4), clearly show that HL60 cells incubated with DMPE/DMPC/diacetylene vesicles continued to proliferate at a rate that was similar to untreated cells even after 24 h incubation with the lipid/diacetylene vesicles.

Physiological assays focusing on specific cell pathways confirmed that lipid/diacetylene labeling of the cell membrane had a minor effect on fundamental cellular processes. In that regard, the cytosolic Ca\(^{2+}\) stimulation experiment depicted in Fig. 5 underlies two important aspects pertaining to the PDA-labeled HL60 cells. First, intracellular calcium release triggered by extracellular ligands such as fMLP was not adversely affected in PDA-labeled cells. Second, the data in Fig. 5 suggests that attachment and polymerization of the lipid/PDA vesicles onto the cell surface did not significantly restricted access to the cognate receptors of fMLP — further indications for the minimal functional consequences of PDA labeling. Mitochondrial membrane potential measurements shown in Fig. 6 further illuminated the downstream effects of lipid/diacetylene fusion and polymerization, demonstrating that viability of the mitochondria was retained more than 90 min after irradiation of the cells to induce PDA polymerization.

The chromatic cell membrane assay can be employed for inspection and analysis of varied membrane processes, either by taking advantage of the intense fluorescence emission of the red-phase PDA patches for fluorescence microscopy analysis, or through UV-Vis spectroscopic examination based on the blue–red transformations of the polymer.
A representative example is given in Fig. 7 which depicts the application of the chromatic cells for detection of membrane interactions of pharmaceutical compounds. Indeed, the microscopic and spectroscopic experiments summarized in Fig. 7 reveal a close relationship between the bilayer affinity and insertion of the compounds examined and the induced fluorescence/color changes.

Specifically, amphiphilic molecules that exhibit high affinity to membrane bilayers induce chromatic transitions of the membrane-attached PDA patches at significantly lower concentrations compared to hydrophobic compounds like metoprolol and acebutolol. In contrast, pharmaceutical substances which exhibit negligible membrane interactions (or which do not permeate the plasma membrane through passive diffusion mechanisms) do not induce chromatic transformations of the attached PDA. Indeed, the chromatic response of the PDA-labeled cell assay can be roughly divided between these three groupings as depicted in Fig. 7B. The %CR curves in Fig. 7B highlight the potential application of the chromatic cells to distinguish among the membrane interaction profiles of tested compounds. Importantly, the application of the PDA-labeled cell platform does not require, a priori, knowledge on partition coefficients or binding constants of compounds tested. The relative ease of constructing the PDA-labeled cells and the fast chromatic response obtained (few seconds after mixing the cells and compound to be examined) point to the potential use of the cells in high throughput screening formats.

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References