Cell Imaging

Visualization of Membrane Processes in Living Cells by Surface-Attached Chromatic Polymer Patches**

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The plasma membrane constitutes a critical platform for diverse biological processes, such as ligand recognition,[1] drug action,[2] vesicle fusion and endocytosis,[3] and pore-formation

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[**] We are grateful to Dr. Rina Jeger, Dr. Aviva Kiriaty (Ben Gurion University), and Dr. Vladimir Kiss (Weizmann Institute of Sciences) for assistance with the microscopy experiments. Financial assistance from the Reimund Staedler Minerva Center for Mesoscopic Macromolecular Engineering, funded through the BMBF, is acknowledged.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200462393

by membrane-active peptides.[4] Many of these membrane processes involve perturbations that affect the structural or dynamic properties of the phospholipid bilayers, or result in changes to the cell membrane or its morphology.[5] Numerous membrane studies employing model systems have been reported,[6] and varied techniques have been developed for imaging surface phenomena in living cells. Almost all the imaging methods are based on radioactive or fluorescent labels[7] or employ semiconductor quantum dots[8] that target specific molecules or biochemical pathways. However, microscopic imaging of localized plasma-membrane interactions and bilayer perturbations in living cells has not been achieved. Such information would greatly contribute towards understanding diverse cell-surface processes and their effects on membrane constituents.

Herein we show that attachment of polydiacetylene (PDA) nanopatches onto cell surfaces facilitates visualization and analysis of membrane-perturbing events in living cells. Conjugated PDA assemblies exhibit unique chromatic properties. PDA vesicular aggregates and 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] 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.[10] The chromatic transformations of PDA have also occurred 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 matrixes.[11]

As a proof-of-concept for construction of PDA-labeled living cells, monocytic U937 leukemic cells were incubated with vesicles composed of dimyristoylphosphatidylethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), and the diacetylene monomer 10,12-tricosadyinoic acid (mole ratio among the components 1:1:3), and subsequent short (10–20 s) irradiation at 254 nm induced polymerization of the PDA nanopatches. The phospholipid constituents were found to be essential for PDA attachment to the cell membrane, and a fluorescence resonance energy transfer (FRET) experiment confirmed the occurrence of significant fusion between the DMPE/DMPG/PDA vesicles and the plasma membrane (see Supporting Information). Surface attachment of DMPE/DMPG/PDA vesicles that were irradiated and polymerized prior to addition to the cell is generally feasible (thus eliminating the UV-irradiation step which is detrimental to the cells). This procedure, however, yielded a much smaller degree of vesicle surface attachment (possibly owing to the rigidity of the already-polymerized PDA framework) and furthermore induced chromatic transformations within the vesicles upon cell binding.

A critical question pertaining to the usefulness of the new PDA–cell system concerns the effect of vesicle attachment on cell survival. We carried out both a trypan-blue exclusion assay[12] and acridine-orange/ethidium bromide double staining[13] to evaluate the percentage of live PDA-treated U937 cells following the incubation and polymerization (Figure 1). The cell vitality data obtained from the two assays clearly show that most of the PDA-labeled cells survived for more than three hours after the treatment. At longer time periods the percentage of dead and pre-apoptotic cells increased, probably owing to the vesicle fusion processes, the UV irradiation, and the absence of various necessary nutrients in the buffer medium.

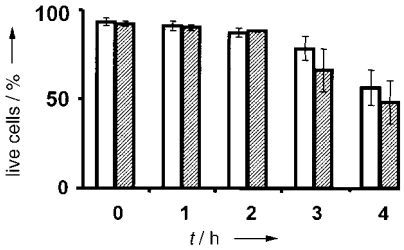

Figure 1. Percentage of live PDA-labeled cells, calculated at different times after incubation of the U937 cells in the presence of phospholipid/polymer nanopatches and subsequent polymerization. Cell viability was determined by the trypan-blue assay (white bars)[12] and the acridine-orange/ethidium bromide assay (shaded bars).[13]

The surface topography and membrane morphology of the PDA-treated U937 cells were examined by scanning electron microscopy (SEM, Figure 2A,B) and transmission electron microscopy (TEM, Figure 2C). Magnification of the membrane surface by SEM reveals an abundance of small bright patches of 50–100 nm in size localized at the cell surface (Figure 2A). These nanopatches appeared only when the U937 cells were placed in suspensions containing phospholipid/PDA vesicles and were not observed on surfaces of untreated cells (Figure 2B). A representative thin-section TEM image of a PDA-labeled cell in Figure 2C clearly shows irregular patches protruding from the membrane leaflet, most likely corresponding to the dense PDA particles.[14] These darker structures were conspicuous on membranes of the

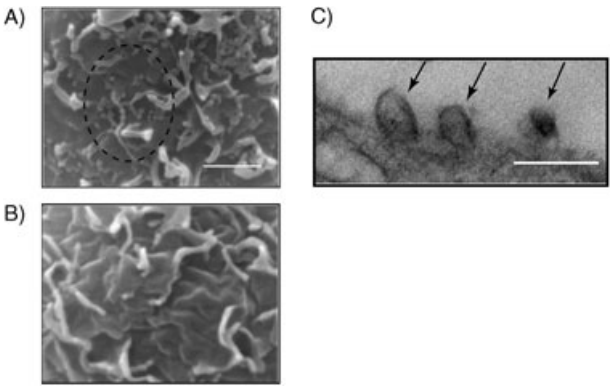

Figure 2. SEM images depicting: A) part of the membrane surface of the PDA-labeled cells showing nanopatches. The broken oval highlights a region with a large number of the patches (scale bar: 1 μm); B) part of the membrane surface of an untreated U937 cell (same scale as (A)); C) TEM image depicting a thin section of the PDA-labeled cell membrane showing fused PDA particles (indicated with arrows; scale bar: 200 nm).

PDA-labeled cells but were not observed in TEM experiments of native U937 cells.

PDA-labeled U937 cells were used to investigate membrane interactions of biological and pharmaceutical molecules (see Figure 3 and Figure 4). Figure 3 depicts laser confocal microscopy images of the chromatic cells. Unperturbed PDA-labeled cells did not emit any fluorescence (Figure 3A) since the surface-incorporated PDA assemblies in their initial blue phase are not fluorescent.[10] However, addition of lidocaine, a local anesthetic, to the cell suspension resulted in the appearance of fluorescent spots concentrated exclusively on the cell surface (Figure 3B). Lidocaine, similar to other anesthetic substances, is believed to increase bilayer fluidity,[15] which induces the structural transformation of the PDA nanopatches into the fluorescent red phase (Figure 3B). Modification of bilayer fluidity by lipophilic substances was shown to induce dramatic chromatic transitions in model phospholipid/PDA vesicles.[16] Importantly, bilayer fluidization induced by the lidocaine employed in this case did not give rise to immediate cell lysis, as determined by the trypan-blue cell viability assay (data not shown). Similar to lidocaine, membrane perturbations by polymyxin-B (PMB), a cytotoxic membrane-active peptide,[17] produced striking fluorescence images (Figure 3C). The extended fluorescence view shown in Figure 3C (containing superposition of cell-surface slices extracted over a 2-μm width) depicts abundant spots distributed on the cell surface, most likely corresponding to locations of plasma-membrane disruption induced by the peptide.

The chromatic cell platform was used to study other membrane-perturbing processes, such as the interaction of lipid vesicles with cellular membranes.[18] Figure 3D and E feature the confocal microscopy images recorded at different times after addition of vesicles composed of dimyristoylphosphatidylcholine (DMPC) and cholesterol to the PDA-labeled cells. The microscopy image in Figure 3D was recorded approximately five minutes after mixing the PDA-labeled cells with DMPC/cholesterol dispersion and shows faint fluorescent patches most likely indicating the onset of interactions between the vesicles and the bilayer membrane of the U937 cells. Greater abundance of fluorescent areas with higher intensities was recorded approximately thirty minutes after addition of DMPC/cholesterol dispersion to the chromatic cells (Figure 3E). The appearance of increasingly intense fluorescent domains on the membrane is ascribed to the local destabilization of the membrane through cholesterol-promoted binding.[19]

The unique colorimetric properties of PDA can be further exploited for the analysis of membrane interactions. Figure 4A depicts photographs of the PDA-labeled cells before and after interaction with oleic acid, a membrane-fluidizing agent.[20] The sedimented cells before treatment had the intense blue color of the polymer patches attached to the cell membrane (Figure 4A, left image). However, immediately after incubating the cells with oleic acid, the cell suspension turned red (Figure 4A, right image) owing to fluidization of the plasma membrane. Importantly, examination of the cells treated with oleic acid under a microscope (data not shown) indicated significant swelling on the cells, most likely a result of membrane disruption by the fatty acid, which is consistent with the colorimetric data in Figure 4.

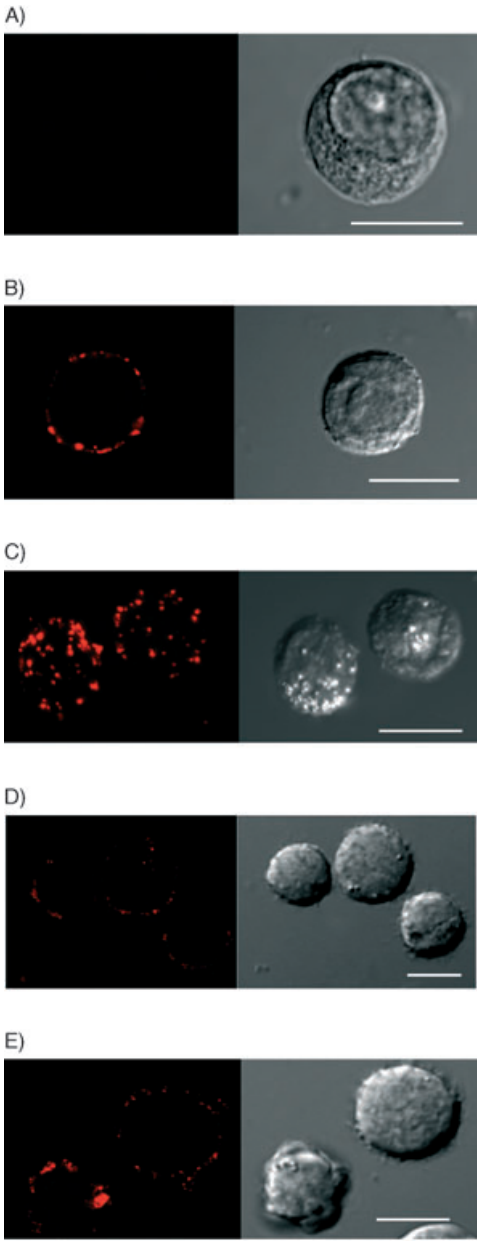

Figure 3. Confocal microscopy fluorescence images recorded using an LP 560 nm filter (left) and transmission differential interference contrast (DIC) microscopy images (right) of PDA-labeled U937 cells. Scale bars in all images correspond to 10 μm. A) Initially prepared unperturbed PDA-labeled cells: no fluorescence emitted from the blue-phase PDA patches; B) Addition of lidocaine (1 mM) to the hybrid cell suspension. C) Addition of PMB (50 μM). An extended fluorescence image is shown (superposition of four membrane slices). D and E) Interaction of DMPC/cholesterol vesicles (0.1 mM final lipid concentration) with the PDA-labeled cells either 5 min (D) or 30 min (E) after mixing of DMPC/cholesterol vesicles with PDA-labeled cells.

Figures 4B–D indicate that visible spectroscopy could be employed for studying cell-membrane processes by using the PDA-labeled cells. The spectra of the parent blue PDA-labeled cells displayed the typical maxima at around 650 nm (solid lines, Figure 4B and C). Addition of oleic acid to the

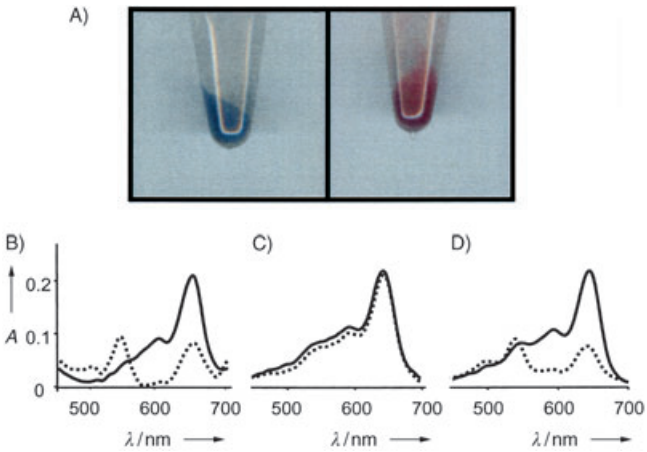

Figure 4. Photographs of tubes containing PDA-labeled cells. The cells were sedimented to improve visible observation. Left tube: blue cells before treatment; right tube: cells after incubation with oleic acid (180 μM). UV/Vis spectra: B) PDA-labeled cell suspension before (solid line) and after (broken line) addition of oleic acid, final concentration 180 μM; C) PDA-labeled cell suspension before (solid line) and after (broken line) addition of poly-L-lysine, final concentration 1 $\mu g\,mL^{-1}$; D) Cell-free DMPE/DMPG/PDA vesicle suspension before (solid line) and after (broken line) addition of poly-L-lysine, final concentration 1 $\mu g\,mL^{-1}$.

PDA-labeled cells gave rise to a pronounced blue-to-red transition and accordingly the emergence of a dominant peak at around 540 nm (broken line, Figure 4B). The color transition of PDA is most likely related to the increased fluidity of the lipid membrane induced by oleic acid.

The visible spectroscopy data in Figure 4C and D further facilitate evaluating the critical issue of whether the chromatic transitions observed in the PDA-labeled cells were due to plasma-membrane perturbations induced by membrane-active compounds, rather than induced by direct interactions with the phospholipid/PDA particles attached to the cells (these interactions would clearly attract much less biological interest). Figure 4C depicts the spectrum resulting from addition of high-molecular-weight (84 kDa) poly-L-lysine (PL), a polypeptide with a high positive charge,[21] to the PDA-labeled cells. Figure 4C clearly demonstrates that no colorimetric change was observed when PL was added to the suspension of PDA-labeled cells. In contrast, PL gave rise to an immediate and dramatic blue–red transformation of free lipid/PDA vesicles (not attached to cells) in an aqueous solution (Figure 4D). This color change arises from the strong electrostatic interactions between the positively charged polypeptide and the phospholipid/PDA vesicles.[11] The comparison between the spectroscopic results of PDA-labeled cells (Figure 4C) and cell-free vesicles (Figure 4D) indicates that PL only marginally interacted with the plasma-membrane-embedded PDA moieties, which are protected by cell-surface carbohydrate residues that hinder access of the large polypeptide to the membrane. Furthermore, the absence of immediate colorimetric change after mixing the cells with PL (Figure 4C) suggests that PL does not induce significant initial disruption of the cell membrane, even though this polypeptide is known to eventually give rise to cell lysis.[22]

In summary, we have constructed new chemically engineered cells through attachment of chromatic polydiacetylene nanopatches onto the plasma membrane. These hybrids facilitated visualization and investigation of surface processes in living cells. The membrane-incorporated polymer patches did not adversely affect cell vitality for several hours and responded to structural perturbations of the plasma membrane both through induction of fluorescence as well as by undergoing blue–red color changes. The new chromatic cell platform is generic by nature and conceptually different from other cellular imaging techniques. In essence, the fluorescent/colorimetric polydiacetylene patches do not report upon specific biomolecular targets within the cell surface (or cell interior) but rather respond to processes and surface interactions that give rise to structural and dynamic modifications of the plasma membrane. This approach makes possible microscopic imaging and quantitative spectroscopic analyses of biological events occurring in real time within the membranes of living cells. PDA-labeled cells were constructed in our laboratories using other cell types, including epithelial MCF-7 cells, Chinese hamster ovarian (CHO) cells, erythrocytes, and others (manuscript in preparation). The new chromatic cell system could find wide applicability including studying plasma-membrane fusion and vesicle budding processes.

Received: October 22, 2004
Published online: January 10, 2005

Keywords: cell imaging · fluorescent probes · membrane processes · scanning probe microscopy · vesicles

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