Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

© Wiley-VCH 2005

69451 Weinheim, Germany

Visualization of membrane processes in living cells by surface-attached chromatic polymer patches**

Zulfiya Orynbayeva, Sofiya Kolusheva, Etta Livneh, Alexandra Lichtenshtein, Ilana Nathan, Raz Jelinek*

Corresponding author: Dr Raz Jelinek, Department of Chemistry, Ben Gurion University, Beersheva 84105, Israel. Tel: +972-8-6461747. Fax: +972-8-6472943. E-mail: razj@bgu.ac.il

1. Methods

Cell culture.

Human monocytic U937 leukemic cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 1% penicillin/streptomycin/nystatin at 37°C in a humidified 5% CO_2 standard cell culture incubator. Cells were maintained at a density of 0.7-1×10^6 cells/ml before harvested.

Vesicle preparation.

Phospholipid/PDA vesicles.

The diacetylenic monomer, 10,12-tricosadiynoic acid (GFS Chemicals, OH) was washed in chloroform, and filtered through a 0.45 μm filter prior to use. 10,12-tricosadiynoic acid, dimyristoylphosphatidylglycerol (DMPG), and dimyristoylphosphatidylethanoleamine (DMPE) (Sigma-Aldrich Co.) at molar ratios of 3:1:1 were diluted in chloroform and dried under vacuum. The dry films of polymer/lipids were probe-sonicated in deionized water at ~70^{o}C. The vesicle solution was then cooled and kept at 4^{o}C overnight. Total lipid concentration was 7.3 mM.

DMPC-cholesterol vesicles.

Multilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) and cholesterol were prepared by dilution of the lipid constituents at 2:1 molar ratio in chloroform:ethanol (1:1 v/v) and drying under vacuum. The lipid-mixture film was re-suspended in 2 ml of HEPES buffer. Total lipid concentration in the resulting suspension was 1 mM.

Preparation of PDA-labeled cells.

Cells were harvested from the growth medium and washed in a HEPES buffer containing 20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1mM KH_2PO_4, 2 mM L-glutamine, and 5 mM D-glucose (pH 7.6) by centrifugation at 400g for 7 minutes. Cells ($2x10^6$) were kept in 2 ml buffer to which were added the DMPE/DMPG/PDA vesicles (final lipid concentration 0.3 mM) and incubated for 30 minutes with slow shaking.

Following incubation the cell suspension was irradiated for 10-20 seconds at 254 nm (30-50 mJ/cm^2) to polymerize of the PDA backbone, which resulted in the appearance of blue color. The vesicle/cell hybrids were washed three times for removal of non-associated vesicles and re-suspended in the buffer before conducting measurements. All experimental steps were carried out at 25^{o}C.

Cell survival assay

Cell viability was estimated by the trypan blue dye exclusion method after 2 min incubation cells with 0.4% trypan blue. Percentage of dead (blue stained) and live (not stained) cells was determined using a haemocytometer at the transmission microscope. Cell viability was also assessed by acridine orange (produces green nuclei appearance in live and early apoptotic cells) and ethidium bromide (produces orange nuclei appearance in necrotic and late apoptotic cells) staining: 2 μl of dye mix (1:1) (v/v) in buffer were added to a 25 μl cell suspension to a final concentration of 100 μg/ml. Apoptotic cells were characterized by nuclear condensation of the chromatin and/or nuclear fragmentation. Cell viability was calculated with a fluorescence microscope (Olympus IX70, Japan) using 20 (NA 1.5) objective, 470-490 nm BP (band pass) excitation and 520 nm LP (long pass) emission filters. About 300 cells were evaluated for the appearance of apoptosis and/or necrosis for each sample.

Fluorescence energy transfer assay.

A resonance energy transfer experiment was performed to determine the extent of fusion between the DMPE/DMPG/PDA vesicles and the cell plasma membrane. [7] Vesicle/cell hybrids were prepared by the same procedure described above, excluding the polymerization step. DMPE/DMPG/PDA vesicles were additionally supplemented by *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (*N*-Rh-PE) (Molecular Probes, OR) at a 100:1 (phospholipid:fluorophore) molar ratio. Final pellets were re-suspended in 1 ml buffer and emission spectra were acquired (excitation 469 nm) at 25°C on a FL92 spectrofluorimeter (Edinburgh, UK).

Following vesicle preparation, 94 μl vesicles were added to 2 ml of buffer containing $2x10^6$ cells. The fusion time was 0.5 h at 25^{o}C under continuous slow shaking. Following three washings (centrifugation for 7 min at 400 g) the pellets were re-suspended in buffer and emission spectra were recorded. The peak at 530 nm was assigned to *N*-NBD-PE, while the signal at 585 nm was ascribed for *N*-Rh-PE (excitation wavelength for *N*-NBD-PE 469 nm). As a control sample (minimal energy transfer), Triton X-100 (1% final concentration) was added to the vesicles. Fluorescence emission spectra were acquired at 25°C on an Edinburgh FL92 spectrofluorimeter (Edinburgh, UK). Total sample volume was 1 ml, and the solutions were placed in a quartz cuvette having a 1 cm optical path length.

Scanning electron microscopy (SEM).

Preparation of samples for SEM experiments was carried out as follows: cell suspension in buffer was kept on a tissue culture dish to achieve attachment to the plastic surface. Fixation was carried out with a warm fixative (2.5% glutaraldehyde, 2% parafolmaldehyde in 0.2 M phosphate buffer) for 30 minutes. Following fixation, samples were washed twice with PBS, dehydrated in a graded ethanol, and immersed in graded series of hexamethyldisilazane (HMDS). Following HMDS evaporation, cells were gold-coated and viewed using a QUANTA 200 (FEI Inc.) SEM microscope in the secondary electron mode.

Transmission electron microscopy (TEM).

TEM measurements were carried out with a JEM-1230 model microscope (JEOL, Japan) operating at 80 kV. Cell pellets were fixated with the fixative solution (same as used in the SEM experiments) at 4°C overnight. Cells were then washed three times with PBS and post-fixated in 1% OsO_4, dehydrated stepwise in a graded ethanol series, and were stained during dehydration with saturated uranyl acetate. Cells were embedded in an epoxy resin, sectioned and stained with lead citrate before examination.

Laser confocal microscopy.

Transmission differential interference contrast (DIC) and fluorescence images of the cells were acquired on a laser scanning confocal microscope (Olympus IX70) with an PlApo 60x objective using oil (NA 1.4). Excitation was at 488 nm using argon laser source. Emitted light was collected through a LP 560 nm filter.

Visible spectroscopy.

Compounds (oleic acid or poly-L-lysine) were added to samples containing $2x10^6$ PDA-labeled cells hybrids or approximately 10μM DMPE/DMPG/PDA vesicles at 1 ml HEPES buffer (the vesicle concentration chosen yielded the same intensity at 640 nm as the cell suspension). Measurements were carried out at 25°C on a Jasco V-550 uv-vis spectrophotometer, using a 1 cm optical path cell. Spectra were recorded at wavelength range between 450 and 700 nm.

2. Evaluation of membrane fusion by an energy transfer experiment

To determine whether and to what extent the DMPE/DMPG/PDA vesicles incubated with the U937 cells did actually fuse with the plasma membrane, we carried out fluorescence resonance energy transfer (FRET) measurements utilizing DMPE/DMPG/PDA vesicles that also contained the fluorophores *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (*N*-Rh-PE**)** (D.K. Struck, D. Hoekstra, R.E. Pagano, *Biochemistry* **1981**, *20***,** 4093-4099).

This type of experiment has been widely used to determine vesicle fusion. Initially, efficient fluorescence energy transfer occurs between donor and acceptor within vesicles containing the two fluorophores (which are in close proximity). However, when fusion of the fluorphore-containing vesicles with another (un-labeled) membrane takes place, the extent of fluorescence energy transfer is reduced. Figure 1_{SI} clearly shows that following incubation of the fluorophores-containing vesicles with the U937 cells, the ratio between the fluorescence peaks at 530 nm (*N*-NBD-PE - energy donor) and 585 nm

(*N*-Rh-PE, energy acceptor) significantly increased (Figure 1_{SI}, broken line). This result points to occurrence of lipid fusion between the vesicles and the cell membrane, which lead to redistribution and increasing distances between the fluorophores in the *cell* membrane. Importantly, when labeled and un-labeled *vesicles* were mixed (without cells present), no change in the energy transfer was observed – indicating that vesicle-vesicle fusion was negligible.

We further estimated the extent of vesicle-cell fusion from the ratio of the fluorescence peak intensities. [7] The percent of vesicle fusion to the cell membrane was calculated from the equation:

$$\% \text{ fusion} = (R_f - R_i)/(R_t - R_i) \times 100$$

where R = fluorescence NBD(530nm)/RhB(585nm), the indices f, i, t stand for final, initial, and after addition of Triton X-100, respectively. The calculation yielded values of 50-60%, underlying a significant incorporation of the DMPE/DMPG/PDA vesicles within the cell membrane.

Figure 1_{SI}

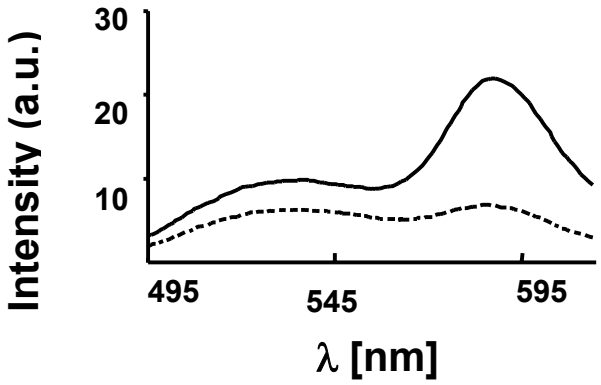

Figure 1_{SI}: Fusion of phospholipid/PDA nano-patches with the cell membrane. Fluorescence emission spectra of DMPE/DMPG/PDA vesicles containing *N*-NBD-PE and *N*-Rh-PE. *Solid line*: vesicles in an aqueous solution; *broken line*: vesicles incubated for 30 minutes with U937 cells. Fusion of vesicles with the cell membrane resulted in a change of the ratio between the fluorescence peaks at 530 nm (NBD) and 585 nm (rhodamine).