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DNA modification of live cell surface

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ABSTRACT

We report a novel approach for the attachment of DNA fragments to the surface of live cells. By using fluorescence microscopy and flow cytometry we demonstrated that our synthetic conjugates of fatty acid with oligonucleotides can be incorporated in plasma membrane and then hybridized with complementary sequences at the cell surface. Method permits to control amount of immobilized DNA on the cell surface. All procedures can be completed within minutes and do not alter cell viability. Using this approach we tethered floating myeloid HL-60 cells to adherent A431 epitheliocytes in a sequence specific fashion. Thus, this method allows rapid and simple DNA multicoding of the cell surface and, therefore, opens new opportunities in manipulating with cell–cell interactions.

INTRODUCTION

Manipulation and transformation of the cell structures and functions are of great import in the contemporary life science research, biotechnology and therapeutics. Delivery of the molecule of interest into the cell is a key element in the manipulation with cell interior. Numerous approaches are designed for the cell intervention including viral vectors, liposomes, cationic polymers, receptor-mediated delivery systems, cell-penetrating peptides, chemically functionalized nanoparticles, etc. In contrast to manipulation with cell genome and organelles, transformation of the cell surface has received significantly less attention. Nevertheless aims, which can be achieved via plasma membrane (PM) modification, include labeling of surface proteins, vehicle and drug delivery, targeted cell adhesion to specific surfaces and manipulation with cell–cell interactions. These tasks have been accomplished via cell transfection with DNA, via chemical modification of surface biomolecules and via fusion of lipid vehicles with PM (1–5). The latter approach has relatively low efficiency and is feasible for the lipid transfer into PM (4,5). DNA transfection is widely applied but it requires complex gene engineering procedures and affects cell genome. Thus, among these three approaches chemical modification of cell surface seems to be the most effective and general. In particular, a relatively simple and widely spread method to study membrane proteins and to enhance cell adhesion involves biotinylation of cell surface proteins followed by an attachment of the molecule of interest conjugated to streptavidin (6–8).

However, direct biotinylation of PM of live cells involves modification of functional groups in proteins and can be harmful. In addition, it permits only a binary coding of interactions: only one type of molecules can be bound to the cell and only one type of cells can be attached to a specific spot on the adhesive surface. Saxon and Bertozzi have developed an advanced technology by using methabolic incorporation of abiotic azidosugar into membrane-associated glycanes that was followed by coupling of azide with a biotinylated triarylphosphine via Staudinger reaction, which does not alter native PM components (9). Recently, group of Prof Francis has applied similar approach to link single stranded DNA to the surface of living cells. These strands were successfully used to anchor cells to supporting surfaces via sequence-depended DNA polymerization (3). The major advantage of this DNA-based system is the multicoding of all possible cell–molecule, cell–cell and cell–surface interactions. However, it necessitates extensive and elongated manipulations with cells, do not permit quantitative control for the extent of modifications and may produce cell damage.

We developed a new approach for a rapid non-covalent attachment of oligonucleotides to the cell surface. We synthesized conjugates of oligonucleotides with fatty acids (ONfa) and demonstrated that these conjugates can be inserted into PM of viable cells and then can be hybridized with complementary oligonucleotides on the cell surface. Our simple protocol permits rapid one-step remodeling of the surface of floating and adherent cells to create new binding properties encoded by nucleotide sequence and to induce artificial contacts between cells. Thus, this approach may be utilized in various cell-based biotechnology applications.

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MATERIALS AND METHODS

Reagents

CellTrace Far Red DDAO-SE (CTFR), 4,4-difluoro-1,3,5,7-tetramethyl-8-(4-maleimidylphenyl)-4-bora-3a,4a-diaza-*s*-indacene (BODIPY-508), 8-bromomethyl-4,4-difluoro-3,5-*bis*-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene (BODIPY-650), Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine [(Texas Red)-phosphoethanolamine], propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR, USA). 1-Palmitoyl-2--linoleoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti polar lipids (Alabaster, AL, USA). Phosphate buffer saline (PBS) tablets were from Helicone (Moscow, Russia). Methanol, acetonitrile, dimethylsulphoxide and ethyl ether were purchased from Panreac (Barcelona, Spain). Dulbeccos's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum and L-glutamine were purchased from Paneco (Moscow, Russia). 6-Carboxyfluorescein phosphoramidite (6-FAM-phosphoramidite), 3′-Amino-Modifier C7 supports and other chemicals required for routine DNA syntheses were purchased from Glen Research (Sterling, VA, USA).

Oligonucleotide synthesis

Oligonucleotides were synthesized by the automated solid phase phosphoramidite method on a DNA synthesizer ASM 800 (Biosset, Novosibirsk, Russia). Cleavage from the support and deprotection of oligonucleotides were achieved with 28% ammonia (5 h, 50°C). 5′-*O*-Dimethoxytrityl (DMTr) protecting groups were removed routinely by 80% acetic acid (20 min). All derivatives of oligonucleotides were purified by reverse-phase HPLC using a linear gradient of acetonitrile in 0.1 M ammonium acetate on an Agilent Chemstation 1100 Series (Agilent, Germany) equipped with fluorescence detector and Diasorb C16T 4×250 mm column (Elsico, Moscow, Russia).

The following set of oligonucleotides was prepared (Table 1): 5′-T_x-CH_2-$CH(CH_2OH)$-$(CH_2)_4$-NH_2-3′ (where $x = 18$ and 25 for oligonucleotides designated T18N and T25N, respectively), 5′-(6-FAM)-T_{18} (FT18), 5′-(6-FAM)-T_x-CH_2-$CH(CH_2OH)$-$(CH_2)_4$-NH_2-3′ (where $x = 18$ and 25 for oligonucleotides designated FT18N и FT25N), 5′-A_{25}-(6-FAM)-3′ (A25F), 5′-(GCCAAGTGTGGTCACCTGCAC)$_3$-T_8–N(C7)-Ste-3′ (XNSte) and 5′-(GTGCAGGTGACCACACTTGGC)$_3$-T_8–N(C7)-Ste-3′ (YNSte).

Synthesis of fatty acid *N*-oxysuccinimide derivatives

Fatty acid (1 mmol), *N*-oxysuccinimide (2 mmol) and *N*,*N*-dimethylaminopyridine (1 mmol) were dried by evaporation with dry pyridine, dissolved in 3 ml of mixture of dioxane/pyridine (3:1) and gently mixed with 1 ml of dioxane, which contained *N*,*N*-dicyclohexylcarbodiimide (2 mmol). This solution was stirred for 5 h, then water (0.2 ml) was added and mixture was stirred for additional 45 min and filtered. Supernatant was dried and dissolved in ether (10 ml), was washed three times with a 5% solution of $NaHCO_3$ (5 ml) and three times with water and then was dried over Na_2SO_4. Fatty acid derivatives were recrystallized from hexane and dried in vacuum. The yield of derivatives was 87–92%. Thin layer chromatography: R_f 0.35, Kieselgel 60 (Merck, Germany), chloroform-diethyl ether (10:1).

Table 1. The list of synthesized oligonucleotides

Oligonucleotide designation	Nucleotide sequence
FT18N	5′-(6-FAM)-T_{18}-alkyl-NH_2-3′[a] NCTCAACTTTACGTTTCTTTTTTTTCTTGTCA-TCGTCATCACC-3′
FT18NPal	5′-(6-FAM)-T_{18}-alkyl-NH-Pal-3′[b]
FT18NSte	5′-(6-FAM)-T_{18}-alkyl-NH-Ste-3′[c]
FT25NSte	5′-(6-FAM)-T_{25}-alkyl-NH-Ste-3′
FT18	5′-(6-FAM)-T_{18}-3′ NCTCAACTTTACGTTTCTTTTTTTTCTTGTCA-TCGTCATCACC-3′
T18N	5′-T_{18}-alkyl-NH_2-3′
T18NPal	5′-T_{18}-alkyl-NH-Pal-3′
T18NSte	5′-T_{18}-alkyl-NH-Ste-3′
A25F	5′-A_{25}-(6-FAM)-3′
XNSte	5′-(GCCAAGTGTGGTCACCTGCAC)$_3$-T_8–N (C7)-Ste-3′
YNSte	5′-(GTGCAGGTGACCACACTTGGC)$_3$-T_8–N (C7)-Ste-3′

[a]alkyl, $–CH_2–CH(CH_2OH)–(CH_2)_4–$.
[b]Pal, palmitoyl.
[c]Ste, stearoyl.

Synthesis of fatty acid oligonucleotide conjugates

3′-aminoalkyl oligonucleotides (5–7 μM) and *N*-oxysuccinimide derivative of fatty acid (100 μM) were dissolved in 40–60 μl of DMSO and incubated in the dark for 17 h at 60°C. The solution was mixed with water (0.5 ml) and ether (1 ml). Ether layer was removed after phase separation. Conjugates of fatty acids and oligonucleotides designated as T18NPal, T18NSte, T25NPal, T25NSte, FT18NPal, FT18NSte, FT25NPal, FT25NSte, XNSte and YNSte (Table 1) were separated by reverse-phase HPLC and stored in 50% ethanol.

Analysis of synthetic products

Synthetic products were analyzed by using HPLC and polyacrylamide gel electrophoresis (20% acrylamide, 1% *N*,*N*′-methylenebisacrylamide in 7 M urea, 0.5 M EDTA, 50 mM Tris–Borate buffer, pH 8.3 at voltage of 1200 V). Furthermore, analysis was carried out by UV spectroscopy on a Shimadsu UV-1650PC (Japan) spectrophotometer, fluorescence spectroscopy on a Hitachi F-4500 spectrofluorimeter and by matrix assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF mass spectra were acquired on a MicroFlex workstation and UltraFlex mass spectrometer (Bruker, Germany) equipped with UV lasers (Nd, 354 nm, and N_2, 337 nm) by using a standard MSP target polished steel (Bruker, Germany) and 3-hydroxypicolinic acid matrix. Mass spectra were obtained in a linear mode with detection of positive ions; the accuracy of measured masses was 0.1%.

Small unilamellar liposomes

Phosphatidylcholine, stored in chloroform, was dried under nitrogen, mixed in vortex in HEPES buffer (20 mM, pH 7.4) and then sonicated three times for 30 s on ice. Liposomes were used immediately after preparation.

Cell cultures

Jurkat human T lymphoblastic lymphoma cell and HL-60 myeloid cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine (2 mM), HEPES (25 mM) and gentomycin (50 μg/ml). Murine macrophage-like cell line J774 was grown in DMEM medium supplemented with 10% FBS and glutamine (4 mM); human squamous epithelial cell line A431 was grown in DMEM medium supplemented with 15% FBS. Cells were grown at 37°C in 5% CO_2 atmosphere. Dead cells were prepared by incubation in 50% ethanol for 30 min.

Cell viability

PI was used to assess cell viability by flow cytometry and fluorescence microscopy. Cells were washed once and incubated in PBS or serum free RPMI medium with PI at final concentration of 5 μM for 5 min at room temperature.

Incorporation of ONfa into cell PM

Jurkat and HL-60 cells were centrifuged (at $1000 \times g$, for 5 min), washed once and resuspended in PBS. Cells (10^6 cells/20 μl) were incubated with ONfa (0.1–1.0 μM) in PBS at 37°C for 15 min and then washed once with serum-free RPMI. J774 macrophages were seeded on cover-slips (5×5 mm) in 24-well plates (5×10^4 cells per well, confluence ~15%), cultured overnight and then labeled with FT18NSte as described above in final volume of 250 μl.

Hybridization of oligonucleotides on the cell surface

Jurkat cells (0.5×10^6 cells/20 μl) were washed once, resuspended and incubated in PBS with T18NSte (0.4–1.6 μM) at 37°C for 5 min to incorporate fatty acid-oligonucleotide conjugate into cell PM (PM). Treated cells were centrifuged, resuspended in PBS and incubated with A25F (0.2–1.6 μM), complementary to T18Nste, at 37°C for 5 min. Then cells were placed in water bath to cool down to 6°C over 20 min period. Fluorescence from FAM-labeled oligonucleotides hybridized on the cell surface was monitored by flow cytometry and confocal fluorescence microscopy.

In the separate set of experiments oligonucleotide duplex was incorporated into cell PM. Duplex A25F-T18NSte was prepared by coincubation of A25F and T18NSte in PBS for 5 min at 37°C and for 40 min at 4°C. Cells treatment with duplex was carried out in the same conditions as with T18NSte.

In some experiments, cells were counterstained with cell tracker CTFR. At the end of treatments Jurkat cells were incubated in PBS with CTFR (0.5 μM) for 10 min at room temperature, washed once and evaluated by fluorescence microscopy.

Manipulation with cell–cell interactions via ONfa hybridization

HL-60 cells were labeled with BODIPY-508 (0.5 μM) for 5 min in PBS and washed twice with PBS. T18NSte and YNSte (10 μM) were incorporated in PM of HL-60 cells as described above. A431 epithelial cells were seeded on cover-slips (5×5 mm) in 24-well plates (2×10^5 cells per well, confluence ~80%) and cultured overnight. Cells were labeled with BODIPY-650 (0.5 μM) for 10 min in PBS, washed twice and treated with XNSte (10 μM) in PBS. Then PBS was exchanged for ONfa-labeled HL-60 cells. A431 and HL-60 cells were coincubated for 5 min at room temperature and for additional 40 min at 8°C. Then cover-slips were rinsed with PBS to remove unbound cells.

Confocal laser scanning microscopy

Fluorescence microphotographs of cells were taken by using a Nikon ECLIPSE E-800 epifluorescence microscope (Tokyo, Japan) equipped with C1 confocal module, Argon and HeNe lasers. The green fluorescence for FT18N, FT18NSte and BODIPY-508 was acquired using excitation wavelength of 488 nm and emission bandpass filter of 545/50 nm. The red fluorescence for PI was monitored using excitation wavelength of 488 nm and emission longpass filter of 610 nm. The red fluorescence for CTFR and BODIPY-650 was detected using excitation wavelength of 594 nm and emission longpass filter of 610 nm. Data were acquired and stored as eight bit images by using EZ-C1 2.30 Software (Nikon Corporation). Images were analyzed by using ImageJ 1.34 Software (Wayne Rasband, National Institute of Health, USA, www.rsb.info.nih.gov/ij/).

Flow cytometry

Cells were analyzed by using Coulter Epix XL flow cytometer (Beckman Coulter, Miami, Florida) equipped with a 488-nm Argon laser. Green fluorescence for FT18N and FT18NSte and red fluorescence for PI were monitored by using 505–545 and 650–725 nm bandpass filters respectively.

Statistics

Data are expressed as means ± SEM. Standard procedures were used to calculate means and standard deviations. Differences among means were considered to be significant at $P < 0.05$.

RESULTS

Structure and synthesis of fatty acid derivatives of oligonucleotides

To attach oligonucleotides to the surface of live cells we constructed amphiphile conjugates of oligonucleotide and fatty acid. The hydrophobic moiety was aimed to anchor an entire molecule at the PM by incorporating into the outer leaflet (Figure 1). Oligonucleotides were synthesized

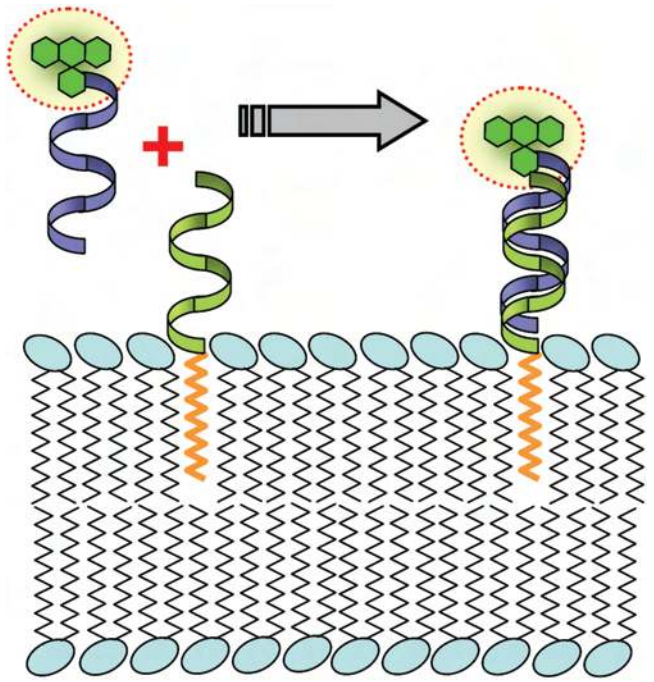

Figure 1. Incorporation of oligonucleotide-fatty acid conjugates (oligonucleotide, green color; fatty acid, brown color) into cell membrane and their hybridization with a complementary fluorescently labeled oligomer (complementary oligonucleotide, blue color; FAM-label, bright green color).

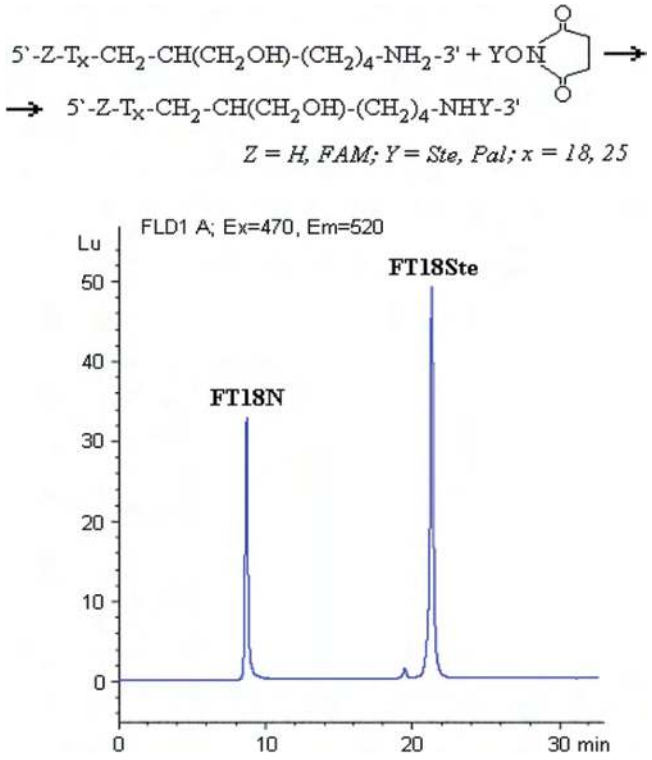

Figure 2. Scheme of synthesis and HPLC analysis of fatty acid oligonucleotide conjugates. Fatty acid oligonucleotide conjugate (FT18Ste) was prepared via condensation reaction of FAM-labeled oligomer (FT18N) with stearic acid *N*-oxysuccinimide. Products were analyzed by using HPLC system equipped with a fluorescent detector (excitation and emission wavelengths were 470 and 520 nm, respectively).

and purified as 3′-aminoalkyl phosphodiester derivatives with a final purity of ≥97% according to HPLC assay. The following condensation reaction of oligonucleotide with *N*-oxysuccinimide derivative of a fatty acid yielded 50–66% of the conjugate as detected by reverse-phase HPLC (Figure 2).

Relatively short T_{18} and T_{25} homosequences were prepared to avoid any steric hindrance during hybridization near the cell surface. Oligomers were labeled with fluorescent moiety (6)-FAM to visualize both binding of conjugates to PM and hybridization of DNA at the cell surface. Reaction products were analyzed by using HPLC, MALDI-TOF mass spectrometry, UV spectroscopy and fluorescence spectroscopy. A typical mass spectra of 5′-(6)-FAM$(T)_{18}$-OP(O)CH_2-CH(CH_2OH)-$(CH_2)_4NH_2$ (FT18N), 5′-(6)-FAM$(T)_{18}$-alkylaminostearate (FT18NSte) and 5′-(6)-FAM$(T)_{18}$-alkylaminopalmitate (FT18NPal) are presented at Figure 3. Molecular masses of products obtained by MALDI-TOF MS deviated by less than 0.3% from anticipated estimates.

In addition, we synthesized two complimentary oligonucleotides, which were comprised of three repeated blocks of 21 bp sequence, T_8 spacer and stearoyl acid (Table 1). To prevent self-aggregation, ONfa were dissolved in 40–50% ethanol at the concentration of 10–200 μM and stored at 4°C.

Labeling of cells with fluorescent derivatives FT18NSte and FT18N

Jurkat and HL-60 cells were co-incubated with 0.2 μM FT18NSte, a fatty acid derivative of FAM-labeled oligonucleotide. Fluorescence microscopic evaluation of cells revealed an efficient cell staining (Figure 4A and I). Co-staining of Jurkat cells with cell tracer CTFR, which covalently modifies intracellular proteins, further demonstrated that FT18NSte was localized in the PM (Figure 4C and E). In the presence of FAM-labeled oligonucleotide lacking fatty acid (FT18N) the majority of cells remained unstained, though few cells contained the stain, which was most likely localized in the nuclear region (Figure 4B, D and F).

To investigate features of FT18NSte and FT18N interaction with cells, we co-stained cells with propidium iodide, a membrane impearment dye, which can label only necrotic cells with a compromised membrane. FT18N labeled dead cells but not live ones as evident by colocalization of red and green fluorescence (Figure 4H). Necrotic cells were labeled by FT18NSte with heterogeneous distribution in cytosole and nucleus similar to FT18N (Figure 4G).

T lymphoblastic Jurkat cells and myeloid HL-60 cells are floating cells with a relatively smooth surface. In contrary, macrophage-like J774 cells have numerous pseudopods on the surface and grow attached to the solid support. Fluorescence microscopy has revealed bright staining of PM of macrophages with FT18NSte but not with FT18N (Figure 4K). Moreover, FT18NSte allowed observing multiple pseudopods at the cell surface.

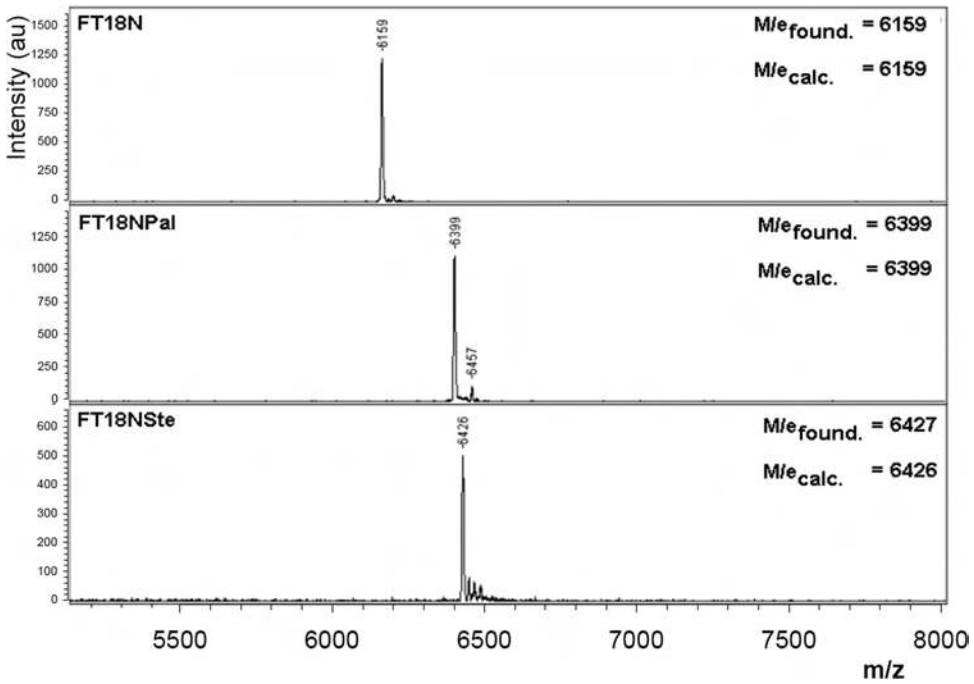

Figure 3. MALDI TOF mass spectra of oligonucleotide conjugates. Peaks at *m/z* 6159, 6399 and 6427 represent FT18N, FT18Pal and FT18Ste respectively.

These morphological features were similar to ones detected with (Texas Red)-phosphatidylethanolamine (Figure 4L). This fluorescent marker cannot be transferred between membranes and, therefore, it labels PM by incorporating into the outer leaflet or by a non-specific absorption (10).

Quantitative analysis of incorporation of fatty acid oligonucleotide conjugates into cell PM

We applied flow cytometry to quantitatively analyze incorporation of FT18NSte into cell PM. Cell distribution as measured by FAM fluorescence depended on the amount of FT18NSte added to cells, but not on the amount of FT18N (Figure 5A and B). Dependence of the fluorescence mean value on FT18NSte concentration was close to linear in the range of 0.05–0.2 μM (Figure 5C). Fluorescence intensity detected from FT18NSte-labeled cells was at least 300 times higher than from FT18N-labeled cells.

This dose dependence was confirmed by fluorescence microscopy in the range of 0.05–0.4 μM. Next, we estimated an amount of FT18NSte on the cell surface. By using latex beads (D = 9 μm) labeled with fluorescein in the range of 2×10^4 to 2×10^6 molecules per bead we obtained a standard calibration curve of fluorescence intensity detected at the bead surface by confocal microscopy. Assuming that Jurkat cells have a round shape, plain surface and $D = 12$ μm, we found that approximately 10^7 molecules were attached per one cell at the concentration of 0.4 μM FT18NSte.

Comparative flow cytometry analysis of the binding of 18-mer and 25-mer oligonucleotide fatty acid conjugates to cell membranes revealed no difference. Similarly, cells were successfully labeled with both palmitoyl and stearoyl conjugates of 18-mer oligonucleotides with onlyminor quantitative difference (data not shown).

Stability of cell labeling by FT18NSte

Incorporation of FT18NSte into cell PM is a fast process that occurs within first 3 min as revealed by time course studies of cell fluorescence by both flow cytometry and fluorescence microscopy (data not shown). In order to assess stability of FT18NSte labeling, cells were labeled, washed and cultured for 24 h in complete RPMI media. Flow cytometry and microscopy evaluation of cells at various time intervals showed gradual decay of the fluorescence signal: little changes occurred within first hour (<20%); ~50% fluorescence decay was observed at ~2.5 h; a very weak signal was detected at 24 h (Figure 6). Fluorescence microscopy analysis of cells during 2 h period showed a partial internalization of the dye. FT18NSte was only in PM during first 20 min incubation and appeared as an additional rare intracellular punctuate pattern at ~1 h. At 2 h incubation, internalized part of the dye was further concentrated in one region of cytosole, presumably Golgi apparatus (data not shown). These morphological features of staining suggest that FT18NSte was internalized via endocytosis.

Direct penetration of the membrane and the flipping from the outer to inner leaflet are two alternative mechanisms of ONfa uptake. To gain further insight into the interaction of ONfa with lipid membranes, we prepared small unilamellar liposomes from phosphatidylcholine, preincubated these liposomes with FTN25Ste and analyzed distribution of the conjugate inside and outside of liposomes by quenching fluorescence of external molecules

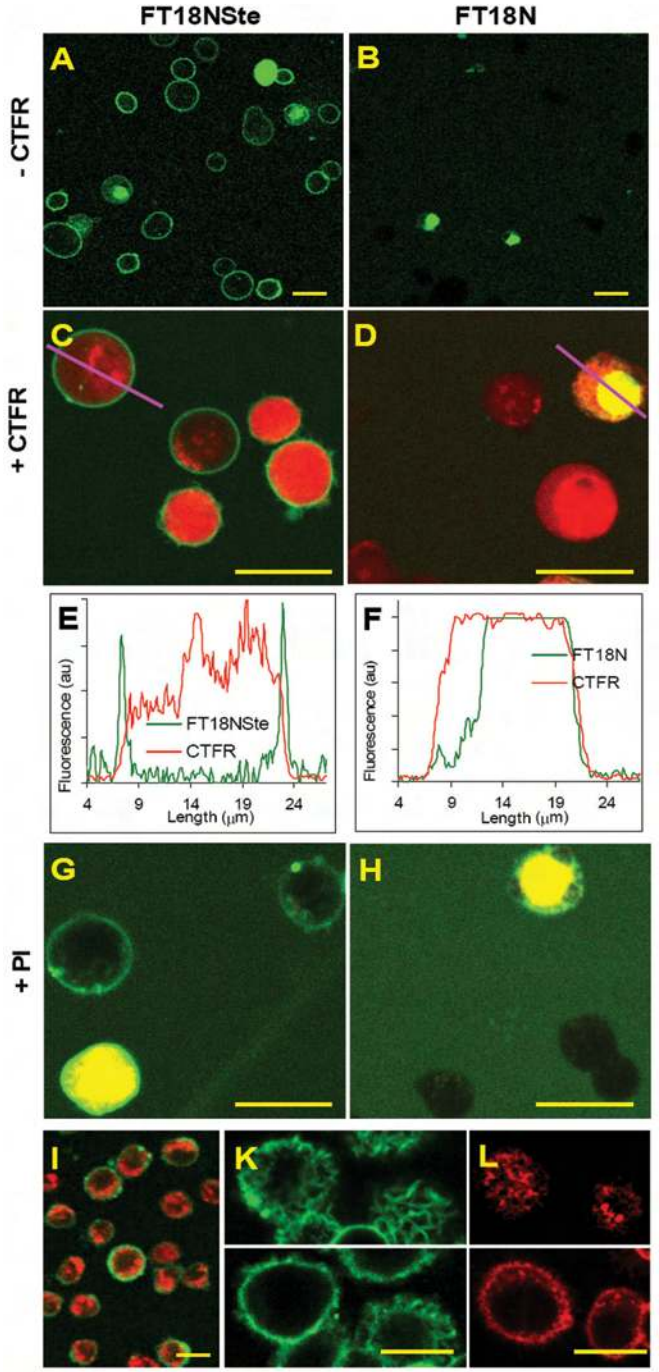

Figure 4. Fluorescence microscopy of Jurkat cells labeled with FT18NSte and FT18N. Cells were co-incubated with 0.2 μM FT18NSte (**A, C**) or FT18N (**B**, **D**) for 15 min at 37°C in PBS (green color). (C, D) Additionally, cells were labeled with 0.5 μM CTFR for 10 min at room temperature (red color). (**E, F**) Fluorescence profiles of single cells stained with CTFR and either FT18NSte (E) or FT18N (F); profiles were plotted via lines shown on panels C and D in violet color. (**G, H**) Cells were labeled with 5 μM PI for 5 min and with either FT18NSte (G) or FT18N (H). Dark spots on panel H represent unstained cells on the fluorescent background. Colocalization of green and red fluorescence is represented in the yellow color. (**I**) HL-60 cells were stained as in (C). (**K**) J774 cells were stained as in (A). (**L**) J774 cells were stained with 5 μM (Texas Red)-phosphatidylethanolamine. Photographs presented at panels K and L were taken at two focal plains: through the top (upper panel) and through the middle section of cells (bottom panel). The yellow marker size is 20 μm.

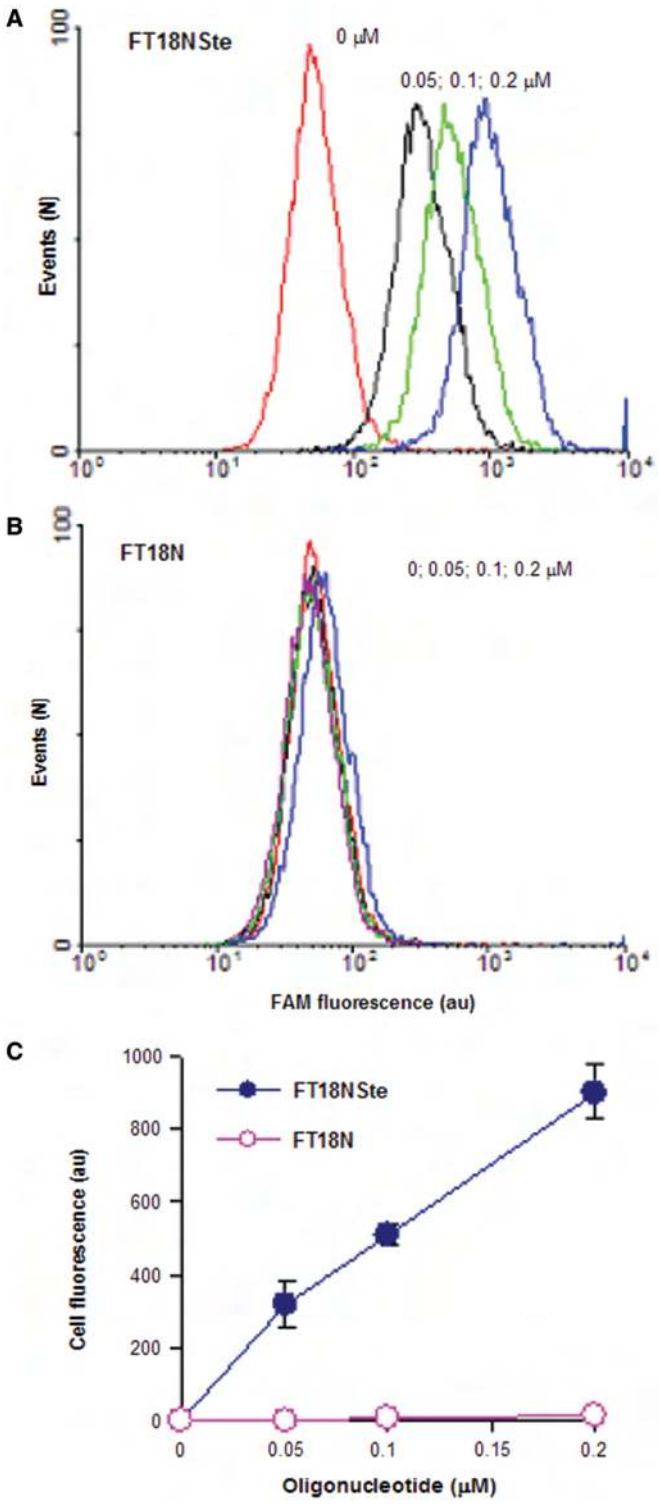

Figure 5. Flow cytometry detection of FT18NSte integration into Jurkat cells. Cell distribution by FAM fluorescence in log scale. Curves represent populations of cells treated with 0.0. 0.05, 0.1 and 0.2 μM concentrations of FT18NSte (**A**) and FT18N (**B**) (red, black, green and blue lines, respectively). (**C**) Mean values of FAM fluorescence of Jurkat cells recalculated from plots of cell distribution ($n = 4$). Incubation conditions are same as in Figures 4A and B.

(Supplementary Data, Figure S1). After 30 min coincubation, no FTN25Ste was detected inside liposomes thus demonstrating that conjugate does not readily penetrate the phospholipid membrane and flip from the outer to inner leaflet.

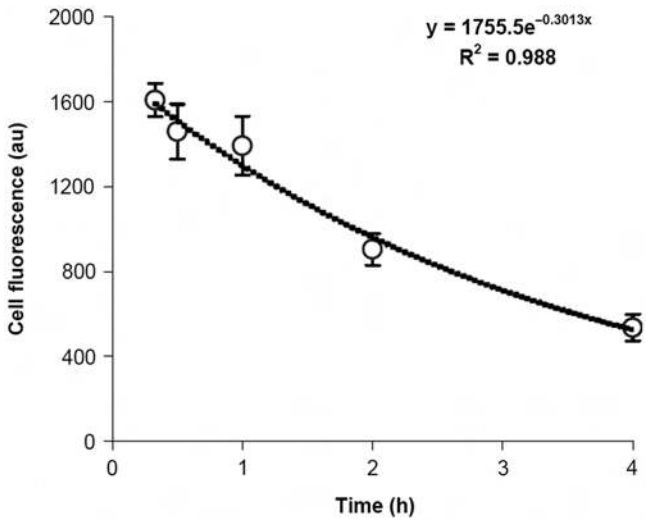

Figure 6. Time course of fluorescence quenching of FT18NSte in Jurkat cells. Cells were labeled with 0.2 μM FT18NSte for 10 min at 37°C in serum-free RPMI, washed and cultured in complete RPMI medium. Fluorescence was evaluated by flow cytometry.

All together these results suggest that FT18NSte incorporates rapidly into outer leaflet of PM and retains there at the substantial level for several hours. Subsequent slow internalization of the dye occurs predominantly via endocytosis.

Cytotoxicity of FT18NSte and FT18N

Fatty acids generally induce perturbation in the membranes and may produce detrimental effect on cell metabolism. Since rare necrotic cells were observed in the presence of FT18NSte and FT18N by fluorescence microscopy, we analyzed substantial cell populations (>10 000) by flow cytometry with PI staining. Assessment of cell viability from 15 min to 24 h after incubation with FT18NSte and FT18N in the range of 0.05–1.00 μM revealed no cytotoxic effects (Figure S2). Furthermore, flow cytometry analysis confirmed that cells were brightly stained with FT18N if only being necrotic.

Hybridization of oligonucleotides at the cell surface

To carry out a hybridization reaction of complementary oligonucleotides at the cell surface, we incorporated non-fluorescent T18NSte into cell PM and then co-incubated these cells with a complementary FAM-labeled oligonucleotide A25F. Fluorescence microscopy analysis revealed that only T18NSte modified cells became fluorescent (Figure 7C and D). Flow cytometry further confirmed

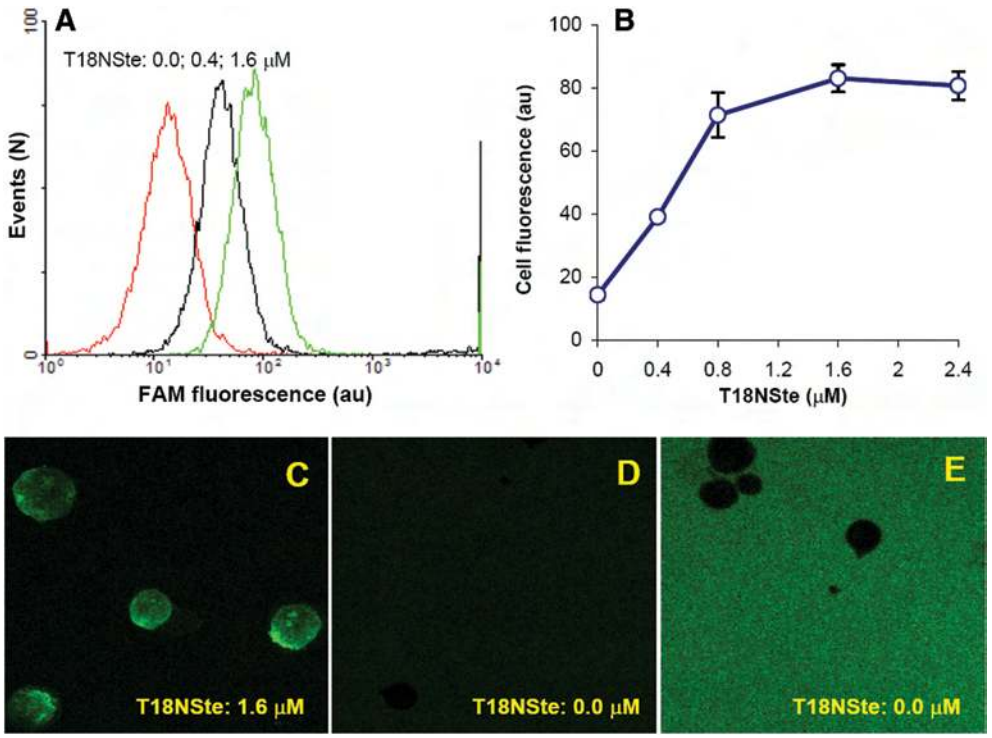

Figure 7. Hybridization of T18NSte integrated into PM with complementary A25F at the cell surface. Jurkat cells were labeled with T18NSte (0.4–2.4 μM) at 37°C for 5 min, washed and incubated with complementary A25F (0.4 μM) at 37°C for 5 min. Then cells were placed in water bath to cool down to 6°C during 20 min time period. (**A**) Histograms of cell distribution by A25F fluorescence pretreated with T18NSte at the concentration of 0.0, 0.4 and 1.6 μM (red, black and green lines, respectively). (**B**) Dose dependence of a mean A25F fluorescence intensity detected from cells on T18NSte concentration ($n = 3$). (**C–E**) Microphotographs of A25F fluorescence in Jurkat cells pre-incubated with 1.6 μM T18NSte (C) and without T18NSte (D, E). (C, D) Instrumental conditions are similar; (E)—same as (D), but PMT gain is enhanced to demonstrate the presence of unstained cells (dark spots) on the fluorescent background of A25F.

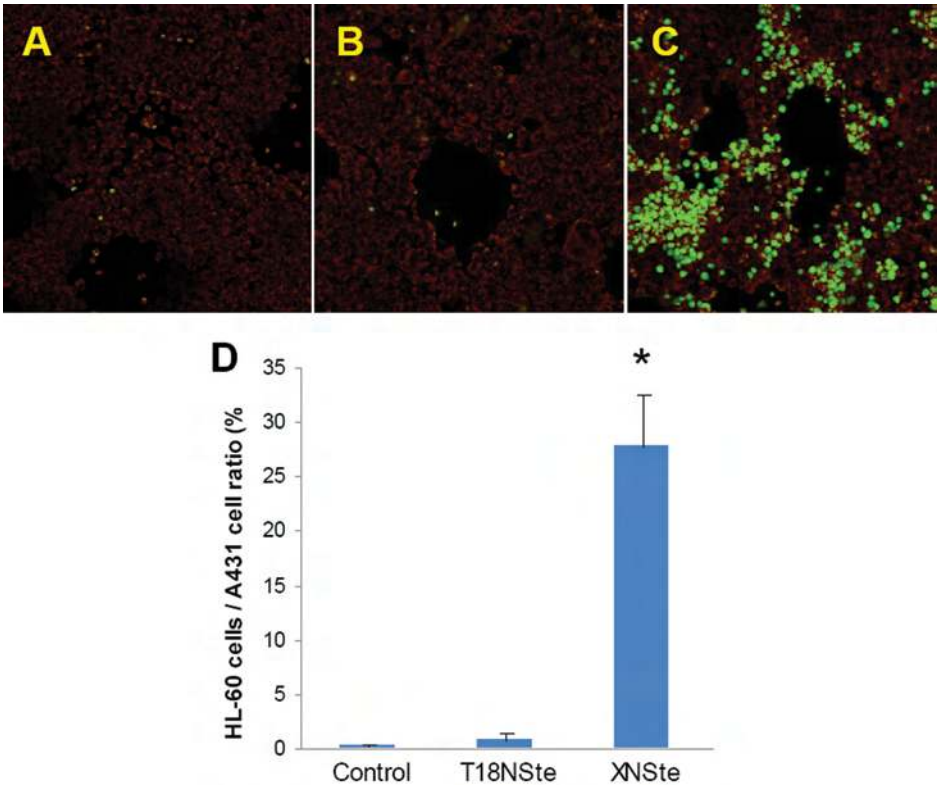

Figure 8. Sequence specific attachment of myeloid HL-60 cells to epithelial A431 cells. (**A–C**) Microphotographs of unlabeled, T18NSte- and YNSte-labeled HL-60 cells (A, B and C, respectively) after coincubation with XNSte-labeled A431 cells. A431 cells and HL-60 cells were prelabeled with thiol-reactive fluorescent dyes BODIPY-650 (red) and BODIPY-508 (green), respectively. (**D**) Relative number of HL-60 cells retained on the surface of A431 cells.

that the entire population of cells pre-labeled with T18NSte shifts to higher fluorescence signal after incubation with A25F as compared with non-labeled cells (Figure 7A). Next, we performed hybridization reaction using constant concentration A25F and cells with various amounts of incorporated T18NSte. Fluorescence signal detected from cells depended on the concentration of T18NSte used for the labeling, and hence, it depended on the surface density of T18NSte (Figure 7B). Moreover, this reaction nearly reached the saturation when T18NSte concentration (0.8 μM) was twice A25F (0.4 μM). Stoichiometry of complimentary oligonucleotide reaction is 1:1, hence, it is possible that at least 50% of T18NSte was incorporated into PM and was available for the hybridization.

Enhancement of cell–cell tethering via ONfa interactions

We applied ONfa conjugates to create new interactions between two cell lines, HL-60 and A431. A431 cells are adherent epithelial cells; in contrary, myeloid HL-60 cells grow in suspension and do not normally form contacts with surfaces and other cells. XNSte was incorporated in A431 cells grown on microscope slides, while complimentary YNSte and non-complimentary T18Ste were incorporated in HL-60 cells. Suspension of HL-60 cells was overlaid on A431 epitheliocytes, coincubated for 5 min at room temperature and 40 min at 8°C to allow hybridization of oligonucleotides. Then cover-slips were gently rinsed over 60 min and continuously monitored by fluorescent microscopy. Retention of T18Ste-labeled HL-60 cells was slightly but insignificantly increased, while retention of YNSte-labeled cells was stimulated by 200 times as compared to unlabeled cells (Figure 8). YNSte-labeled cells could be removed from A431 cell surface by an extensive washing with PBS thus showing that complimentary ONfa can induce reversible tethering of cells.

DISCUSSION

Remodeling of the cell surface is a powerful approach for the labeling of PM proteins, for the modulation of cell–cell interactions and for targeted cell adhesion. However, only few techniques are designed for these purposes including lipid vehicle fusion, chemical modification and metabolic integration of the molecule of interest in PM. These approaches do not satisfy all contemporary demands of cell biology and biotechnology. Thus, the development of a new general and simple method is needed.

Recent developments in this area include metabolic integration of a functional azido group in oligosaccharides of the cell surface by using long-term treatment of cells in culture with the derivatives of *N*-α-azidoacetylmannosamine. This synthetic sugar is metabolized into *N*-α-azidoacetyl sialic acid (11), which then is used for chemical modification of the cell surface oligosaccharides

via biologically inoffensive reaction. By using this method surface sialic azides were conjugated via covalent linkage with synthetic oligonucleotides modified with phosphine group (Staudinger ligation) (3). Although hybridization of cell-surface oligonucleotides was not demonstrated directly, Jurkat cells bearing amide-bound oligonucleotides were successfully immobilized on solid supports covered with complimentary oligonucleotides (3). Overall this is an excellent approach, but it requires three days to modify cell surface that significantly exceeds cell doubling time, which usually ranges from 20 to 36 h in transformed cell lines. As a result, variation in cell metabolism may produce very diverse population in respect to the amount of functional azido groups on the cell surface. Another obscurity is a quantitative control for a metabolic surface modification.

To achieve rapid and controlable immobilization of oligonucleotides on the cell surface we employed a non-covalent attachment of their derivatives to the PM via hydrophobic interactions. Normally, DNA fragments, which contain highly charged natural sequences, cannot incorporate and penetrate the membrane. To overcome this natural DNA property, various hydrophobic derivatives have been synthesized (12–17). In particular, steroid derivatives of oligonucleotides were attached to artificial lipid bilayers, leaving oligonucleotide domain outside. These derivatives rapidly entered cells probably via receptor-mediated pathway (12–14). Recently, conjugates of lipid with DNA [$(C_{18})_2$–DNA] have been prepared and used to attach liposomes to solid supports (15). However, this protocol involve harsh chemical modifications of lipids in the membranes and, thus, cannot be been tested on live cells.

Several DNA analogues have been synthesized via modification of a sugar–phosphate backbone including thiophosphoryl and thiophosphoramidate analogues, which have superior amphiphilic properties, extended half live *in vivo* and can penetrate the membrane (16,17). Palmitoyl derivatives of thiophosphoryl oligonucleotides can form stable complexes with low-density lipoproteins; their LDL-mediated delivery was suggested in gene therapy (17). Palmitation of 5′-position of well known thiophosphoramidate antagonist of telomerase (GRN163) strengthened antitumour properties of this oligonucleotide (16,17). However, alterations in sugar–phosphate backbone significantly enhances cytotoxicity and immunogenicity of the oligonucleotides.

Constructs described above were aimed for the application in gene therapy and were used to stimulate delivery of oligonucleotides into the cell, yet this experience appeared to be practical for the engineering of new oligonucleotide derivatives, which could be attached to the cell surface. We designed constructs of DNA and hydrophobic domains to utilize hydrophobicity of the later as a membrane anchor. The lipid domain was represented by palmitoyl and stearoyl residues, since these natural fatty acids cause less disturbance in PM as compared to cholesterol. The highly charged natural phosphodiester sequence composed a DNA moiety that could presumably prevent penetration and flipping of the molecule to the cytosolic side of PM.

We studied binding of ONfa to the PM, their cytotoxicity and hybridization at the live cell surface. It was found that PM of adherent cells (J774 macrophages) and floating cells (Jurkat T-limphocytes, myeloid HL-60 cells) was brightly stained with fluorescent ONfa. Moreover, fatty acid domain was critical for the binding. ONfa incorporation into cells was fast, dose-dependent and yielded a substantial amount of an oligonucleotide on the cell surface. Furthermore, fate of surface-attached oligonucleotides was associated with their internalization and nuclease degradation.

Internalization of ONfa can occur either by endocytosis or by transfer to cytosole—by protein-mediated transfer, by flipping from the outer to inner leaflet of PM and by direct membrane penetration. Fluorescence microscopy revealed punctuate staining in cytosole, but no staining of intracellular membranes (i.e. mitochondria and nuclear envelope) thus suggesting that endocytosis is likely to occur. Studies of ONfa interactions with phospholipid liposomes demonstrated that their membranes remained impermeable for the conjugate. These results advocate that ONfa metabolism is predominantly associated with an endosomal degradation, that ONfa cannot readily cross the membrane and, hence, in PM it is localized in the outer leaflet. Thus, in contrast to metabolic engineering of cell surface, this method permits rapid and controlable attachment of oligonucleotides to the surface.

Several factors have to be considered to minimize internalization of modified oligonucleotide during its immobilization and hybridization on the cell surface. First, calcium induces DNA transport into the cell, while magnesium ions stimulate uptake of DNA by liposomes (18,19). α-fetoprotein and albumin, which are involved in fatty acid transport into cell, may account for receptor-mediated internalization of oligonucleotide derivatives. α-Fetoprotein is one of the proteins of fetal bovine serum used for cell culture; most of transformed cell lines, including Jurkat cells, express α-fetoprotein receptor at a relatively high level (20). Thus, an incorporation of oligonucleotides in PM and their hybridization at cell the surface has to be carried out in the serum-free medium without divalent cations. Yet, even in the presence of serum and all cations required for normal cell growth half live of oligonucleotides in PM was about 3.5 h.

We were able to demonstrate hybridization of PM-attached oligonucleotides with fluorescently labeled complementary oligonucleotide. Efficiency of this reaction depended both on the amount of surface oligonucleotide and on the concentration of its complementary probe in the solution. This experiment provides an additional evidence for the location of ONfa in the outer leaflet of the PM.

Furthermore, we applied two complimentary 70-mer ONfa conjugates to create artificial contacts between cells and to tether floating HL-60 cell to adherent A431 epitheliocytes. Importantly, under physiological conditions, cell–cell and cell–surface interactions are multistep processes, which are initiated by tethering of the cell to the matrix and other cells via receptor-mediated interactions. These are temporary interactions; the following events usually depend on the involvement of additional specific

receptors, which promote formation of stable contacts and induce specific cell activity. Tethering is indispensable for (but is not limited to) the homing of stem cells to their nice, for the clearance of dead cells by phagocytes, for blood coagulation, for lymphocyte polarization and migration to an inflammatory site (21–25). Thus, tissue development and homeostasis are linked to cell tethering and the following formation of natural contacts. The failure to develop such interactions can lead to a programmed cell death known as anoikis (26). We believe that presented results verify applicability of our method for the induction of oligonucleotide specific cell tethering, which is required for the creation of physiological cell contacts. Hence, this approach can be used for the spatial targeting of cells.

Overall, we demonstrated that ONfa can be incorporated into several cell types (epitheliocytes, T-lymphocytes, macrophages and myeloid cells) and can be hybridized at the cell surface. An amount of surface-attached ONfa is sufficient for the measuring by simple fluorescence techniques as well as for cell tethering. Labeled cells have to be utilized within several hours due to relatively fast metabolism of ONfa. Binding of ONfa to PM is non-covalent and, hence, another potential limitation is in continuous transfer of ONfa between extracellular membranes. We were unable to detect cell to cell exchange of fluorescent FT18NSte (data not shown), but cannot also exclude this possibility. Thus, the development of new conjugates with improved binding properties may enhance stability of the labeling and specificity of interactions. This may include a synthesis of new lipid conjugates with better hydrophobic properties (i.e. an oligonucleotide–phospholipid conjugate or new ONfa, in which fatty acid is modified by an additional hydrophobic moiety) or a conjugation of an oligonucleotide with a protein (or protein domain) capable of specific binding to the membrane interface (i.e. annexins). In view of successful results obtained with ONfa, we believe that further development of the method based on the non-covalent attachment of ON to the cell surface can be very promising for the modification of cell surface and for the generation of sequence specific cell–cell and cell–surface interactions. The major advantage of this approach over receptor-mediated cell adhesion is in multicoding of interactions that presumably may permit to design complex cell patterns, cell networks and 3D structures. Therefore, this method may contribute to the development of various cell-based biotechnological applications including biosensors and tissue engineering.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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