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pH dependent transfer of nano-pores into membrane of cancer cells to induce apoptosis

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Proper balance of ions in intracellular and extracellular space is the key for normal cell functioning. Changes in the conductance of membranes for ions will lead to cell death. One of the main differences between normal and cancerous cells is the low extracellular pH_e and the reverse pH gradient: intracellular pH_i is higher than extracellular pH_e . We report here pH-selective transfer of nano-pores to cancer cells for the dis-regulation of balance of monovalent cations to induce cell death at mildly acidic pH_e as it is in most solid tumors. Our approach is based on the pH-sensitive fusion of cellular membrane with the liposomes containing gramicidin A forming cation-conductive β -helix in the membrane. Fusion is promoted only at low extracellular pH by the pH (Low) Insertion Peptide (pHLIP®) attached to the liposomes. Gramicidin channels inserted into the cancer cells open flux of protons into the cytoplasm and disrupt balance of other monovalent cations, which induces cell apoptosis.

The control of intracellular pH and balance of ions in a cell are essential for a number of important physiological processes, such as protein, DNA and RNA synthesis and control of the cell cycle¹. In cancerous tissue the balance of protons is disturbed, which leads to acidification of the extracellular milieu and reversing of the transmembrane pH gradient compared with normal tissue: the intracellular pH (pH_i) in tumors is higher than the extracellular pH (pH_e)^{2,3}. The equilibration of the extracellular and intracellular pHs as well as disruption of balance of other ions will lead to cell death. We propose to transfer in a pH-dependent manner nano-pores to the plasma membrane of cancer cells to disrupt intracellular and extracellular ion balance and induce cytosol acidification. Nano-pores will be formed by linear hydrophobic peptide, gramicidin A (gA), consisting of alternating L- and D-amino acids, which forms cation-conductive β -helix in the membrane^{4,5}. The diameter of gA β -helix channel is about 4–5 Å, which can conduct about 10^7 cations per second⁵. The high conductance is observed for the protons, sodium and potassium ions. The gA channels formed in the liposomes will be delivered to the membrane of cancer cells by fusion. The fusion between the lipid bilayer of liposome and cancer cells will be promoted by the pH (Low) Insertion Peptide (pHLIP®). The pHLIP inserts into the lipid bilayer and forms transmembrane helix at $pH < 7^{7,8}$, and therefore is able to bring in close contact liposome and cellular membranes only at low pH. We have demonstrated that the pHLIP-based optical, PET and SPECT imaging agents can target acidic diseased tissue^{9–13}, and we proved that pHLIP technology can substantially improve delivery of nanoparticles and liposomes to the acidic diseased tissue^{14,15}. The presence of pHLIP on the surface of PEGylated-liposomes enhanced membrane fusion and lipid exchange in a pH dependent fashion, leading to the increase of cellular uptake and payload release¹⁶. In the present study we demonstrate that the pHLIP-coated liposomes could be used to transfer nano-pores (peptide channels) into the membrane of cancer cells, disrupt transmembrane pH gradient and balance of sodium ions, which leads to the apoptosis of cancer cells.

Results

Preparation of liposomes and biophysical studies. We introduced pH-sensitive fusogenic liposomes containing 80 mol% of DOPC lipids, 5% of DSPE-maleimide lipids conjugated with Cys residue at the N-terminus of pHLIP peptide (ACEQNPIYWARYADWLFTPLLLDLALLVDADEGT), 5% of fluorescein (FITC) labeled lipids and 10% of gramicidin (gA: formyl-(L)W-(D)G-(L)A-(D)L-(L)A-(D)V-(L)V-(D)V-(L)W-(D)L-(L)W-(D)L-(L)W-(D)L-(L)W-ethanolamine) (Figure 1a). The DOPC pHLIP-coated liposomes containing gA formed single lamellar structures of the average size of about 120 nm in diameter (the polydispersity 0.07 ± 0.01 and zeta potential -30.7 ± 1.8 mV), which were stable within a month if kept at 4°C (Figure 1a, b SI). The liposome surface was stabilized by the negatively charged pHLIP peptides. Our previous data indicate that one pHLIP

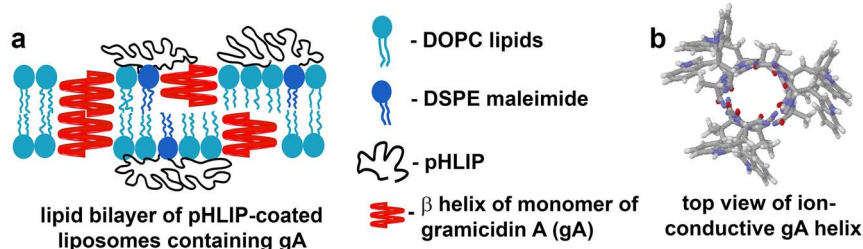


Figure 1 | Schematic presentation of lipid bilayer of the pHLIP-coated liposomes containing gramicidin A (gA) ion-conductive channels (a). Top view of the gA channel showing conductive pore of 5 Å in the diameter (the atomic coordinates of gA were visualized by the RasWin Molecular Graphics program using 1 MAG file from the Protein Data Bank).

molecule in its extended configuration can occupy about 120 lipids at pH 8¹⁷. Thus, 5% of pHLIP in liposome (about 3% on the outer surface) should fully cover liposome surface. Control liposomes containing gA, but no pHLIP and containing pHLIP, but no gA were also tested. Gramicidin A is a linear peptide spanning just half bilayer and capable of adopting different configurations. Single stranded helical form, when two monomers of gA located within inner and outer leaflets of bilayer form head-to-head dimer, corresponds to the channel (pore) configuration of 4–5 Å in the diameter (Figure 1b). Gramicidin in the channel configuration exhibits characteristic circular dichroism (CD) signal and short wavelength shift of the tryptophan fluorescence spectrum¹⁸, monitored by us and shown on Figure 2a and Figure 2b, respectively. To demonstrate conductance of protons by the gA channel we performed kinetics measurements on liposomes containing 2% DHPE lipids head groups of which were labeled with FITC. The FITC carries two negative charges at pH 9. As the pH is lowered the carboxyl groups of FITC are protonated and FITC is transferred to the non-fluorescent non-charged form. Thus, decrease of pH from 9 to 3 is associated with the decreased of fluorescence. The pH 8 → 3 transition was induced by fast mixing

(5 ms dead time) of the DOPC-FITC liposomes and HCl in the stopped-flow apparatus. The sudden decrease of about 75% of fluorescent signal was associated with the quenching of emission of FITC on the outer leaflet of bilayer. The quenching was completed within a dead time (5 ms) followed by the slow kinetics reflected penetration of the protons across a bilayer. It led to the quenching of fluorescence of FITC located at the inner leaflet of bilayer (red line on Figure 2c). The data are in an accordance with the previously published results^{19,20}. At the same time, when 2% or 10% of gA was incorporated into liposomes the decrease of FITC fluorescence was completed within less than 5 ms due to the conductance of protons by the gA channel (blue line on Figure 2c). We also followed pH equilibration after the addition of base to induce pH 3 → 8 transition, which leads to the increase of FITC fluorescence. For the liposomes containing gA the changes of fluorescence were completed within less than 5 ms. At the same time, the slow kinetics was observed for the liposomes containing no gA (Figure 2d).

Inhibition of cell proliferation. Biophysical studies indicated that gA is adopting conductive channel form in the liposomes. The main question we proposed to answer if the pHLIP-coated liposomes will be able to transfer gA channel to the cellular membranes of cancer cells, destroy ion balance and induce cell death. All experiments were carried out on the cancer cells growing in standard DMEM media pH 7.4 and on the cells adapted for low pH growth of 6.2 in DMEM. First, we tested ability of the pHLIP-coated liposomes containing 5 and 10% gA to inhibit proliferation of A549 lung carcinoma cells adapted for low pH growth (Figure 2a SI). Cells were treated at pH 6.2 with the constructs for four days. The liposomes containing 10% gA were more effective in inhibition of cell proliferation, since increase of the gA concentration in membrane significantly raise the probability of assembling of conductive channel, when both monomers come together and form head-to-head β-helix. The liposome composition containing 10% gA was selected for further experiments. A549 (human lung carcinoma), HeLa (human cervix adenocarcinoma) and M4A4 (human breast ductal carcinoma) cell lines were treated with the pHLIP-coated liposomes containing 10% gA and 5% pHLIP for 18 hrs at pH 7.4 or pH 6.2 in DMEM followed by the replacement of media with DMEM containing 10% FBS. MTS assay was performed on 4-th day after the treatment to establish number of viable cells (Figure 3a–c). More than 80% of cells were dead in the result of treatment with the liposomes of 10 μM of total lipid concentration at pH 6.2. At the same time, the treatment of cells with the same construct at pH 7.4 did not affect cell proliferation, except of M4A4 cells at the highest concentration of the construct. We also treated A549 cells for 1 day in DMEM of pH6.2 and then transferred cells into the pH7.4 DMEM with 10% FBS (Figure 3c). When cells treated at low pH were transferred to normal pH the cytotoxic effect was significantly less. In a control experiment, the same cell lines were treated at the same conditions with the pHLIP-coated liposomes with no gA at concentrations up to 100 μM at both pHs and no any effect on cell proliferation was observed (Figure 2c–d

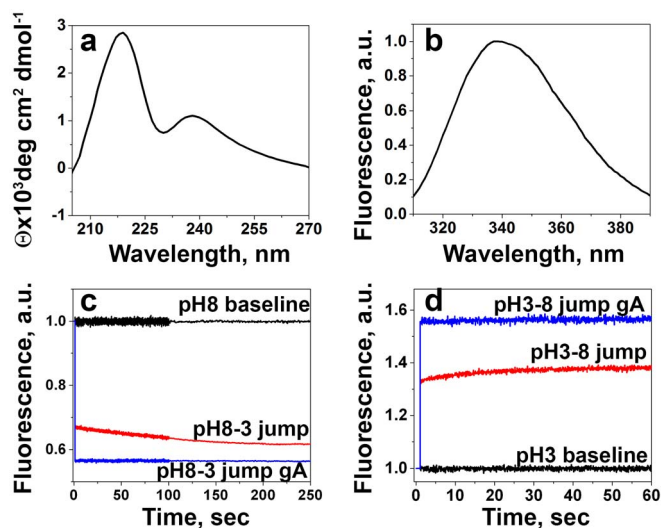


Figure 2 | Biophysical characterization of liposomes containing gramicidin A. The circular dichroism (a) and tryptophan fluorescence (b) of the DOPC liposomes containing 10% gA were shown (c–d) The DOPC liposomes containing 2% FITC-DHPE lipids and containing no gA (red line) or 2% gA (blue line) in phosphate buffer pH 8 or pH 3 were mixed with HCl or NaOH (dead time of mixing was 5 ms) to induce transition from pH 8 to 3 (c) or transition from pH 3 to 8 (d), respectively. The changes of FITC emission were observed in real time in the stopped-flow apparatus. The baseline signal obtained by mixing of liposomes at pH 8 or pH 3 with buffer of the same pH is shown by black lines.

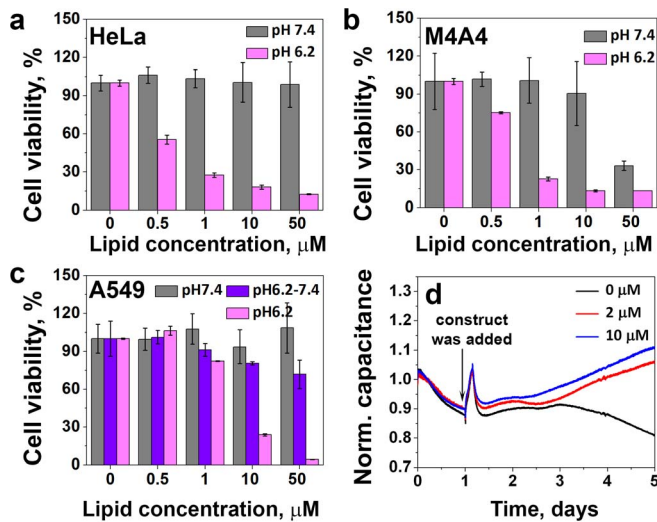


Figure 3 | Inhibition of cell proliferation. HeLa, human cervix adenocarcinoma, (a), M4A4, human breast ductal carcinoma, (b), A549, human lung carcinoma (c) cells grown in standard media of pH 7.4 and adapted for low pH growth of 6.2 were treated with the DOPC pHLIP-coated liposomes containing 10% gA and 5% pHLIP for 18 hrs at pH 7.4 or 6.2 in DMEM followed by the replacement of media with DMEM containing 10% FBS of the same pH as treatment or DMEM of pH 7.4, 10% FBS (blue columns). MTS assay was performed on 4-th day after the treatment. (d) About 25,000 of A549 cells were seeded in an 8W10E + ECIS array in DMEM at pH 6.2, next day cells were treated for 3 hrs with different concentrations of the DOPC pHLIP-coated liposomes containing 10% gA and 5% pHLIP liposomes in DMEM at pH 6.0 followed by addition an equal volume of the DMEM containing 20% FBS at pH 6.2. The kinetics of inhibition of cell proliferation was monitored by changes of capacitance within 5 days.

SI). No inhibition of A549 cells proliferation was also observed, when cells were treated for 4 days with the liposomes containing 10% gA and no pHLIP at concentration of up to 500 μM at both pHs (Figure 2b SI). Thus to induce inhibition of cell proliferation the liposomes need to be i) coated with pHLIP, ii) contain gramicidin channels and iii) incubated with cells at low pH. The kinetics of inhibition of proliferation of A549 cells treated with the pHLIP-coated liposomes containing 10% gA at pH 6.2 was monitored by

the changes of capacitance measured at AC frequencies using the electric cell-substrate impedance sensing (ECIS) technique (Figure 3d). The capacitance varies in a linear fashion with the fractional cell coverage of the bottom of special chamber coated with the thin film gold electrodes connect to the ECIS electronics²¹. When cells were shrinking and dying after the treatment with the construct the capacitance was increasing. The cell death happens couple days after the treatment.

Acidification of intracellular space. We assumed that the pHLIP-coated liposomes fuse with the cellular membrane and deliver gA channels to the plasma membrane. The efficient conductance of protons of gA channel from the extracellular space to cytoplasm leads to the acidification of intracellular space. The pH-sensitive fluorescent dye SNARF was used to measure pH in a cell²². SNARF in the ester form can freely diffuse across the membrane into cytoplasm, where in the result of esterification it is transferred to the cell-impermeable fluorescent carboxyl acid. Fluorescence of carboxy SNARF at 595 nm increases with pH changes from 8 to 5. Fluorescent spectra from the cells (Figure 4a) were recorded using an Andor spectrometer coupled with a fluorescent microscope. Nigericin, K^+/H^+ ionophore, was used to disrupt balance of the protons and equilibrate pH between the extracellular and intracellular spaces, which was needed for the calibration of the fluorescent signal of SNARF in cells²³. Ratio of SNARF fluorescent signals at 595 nm to 640 nm were calculated for each pH and plotted against the respective pH, which allowed constructing the calibration curve (Figure 4b). Then, fluorescent signal of SNARF was measured in A549 cells treated with the pHLIP-coated liposomes containing 10% gA and non-treated cells. First day after the treatment, the pH inside the non-treated (control) and treated cells was 7.08 and 7.02, respectively (Figure 4b, blue and magenta circles). However on the second day after the cells treatment the intracellular pH was dropped to 6.53 (Figure 4b, red circle), while the intracellular pH in the control group was around 7.23 (Figure 4b, green circle). After each measurement of intracellular pH, the nigericin was added to the cells, and the measured pH was found to be around 6.2–6.3 in each case as expected, as a pH of L-15 media used in the experiment.

Disruption of cellular balance of Na^+ ions. It is known that gramicidin pore conducts well not only protons, but all monovalent cations including sodium and potassium ions. We used sodium ion indicator, CoroNa Green dye, that exhibits an increase in green fluorescence upon binding Na^+ in presence of physiological

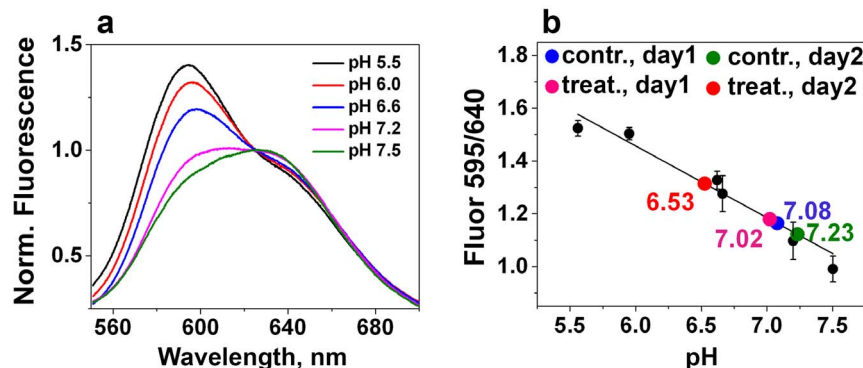


Figure 4 | Monitoring changes of intracellular pH. First, calibration curve was established: A549 cells grown at pH 6.2 were incubated with SNARF-5F followed by washing with 50 mM phosphate/citric acid solution of pH 5.5, 6.0, 6.6, 7.2 and 7.5. To equilibrate extracellular and intracellular pH, the ionophore nigericin was added to cells and the fluorescence spectra of SNARF-5F were recorded (a). Ratio (595/640 nm) of SNARF-5F fluorescent signal was plotted for each respective pH (black dots) and the linear fit was performed to establish calibration curve (b). The fluorescence spectra of SNARF-5F was obtained on A549 cells non-treated and treated with the DOPC pHLIP-coated liposomes containing 10% gA and 5% pHLIP at pH 7.4 or 6.2 for 3 hrs followed by addition an equal volume of the DMEM containing 20% FBS. The SNARF-5F emission ratio of 595/640 nm was calculated and the corresponding intracellular pH was obtained from the pH calibration curve (color dots on panel b).

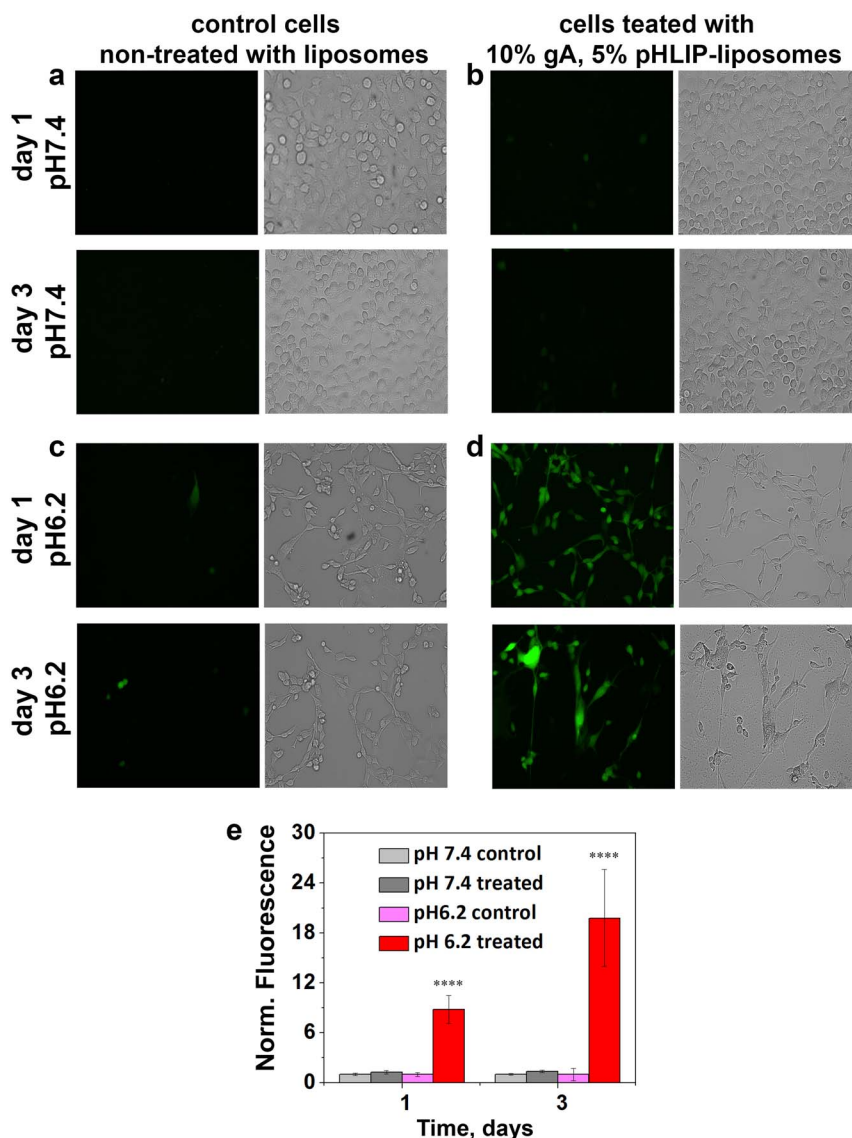


Figure 5 | Monitoring changes of intracellular concentration of Na^+ . A549 cells non-treated or treated for 1 day with the DOPC liposomes with 10% gramicidin and 5% pHLIP at pH 6.2 and pH 7.4 were incubated with CoroNaTM Green next and 3-rd days after the treatment followed by washing and imaging of cells. The representative fluorescent and phase-contrast images of cells are shown (a–d). The average per cell fluorescence intensity changes for control and treated cells are shown on panel e.

concentrations of other monovalent ions^{24,25}. A549 cells non-treated and treated with the pHLIP-coated liposomes containing 10% gA at both 7.4 and 6.2 pHs were loaded at different time points with the cell-permeable CoroNa Green AM, washed and imaged. We observed enhancement of the green fluorescence of cells treated with the liposomes at low pH on day 1 and even more significant increase of fluorescence on day 3 (Figures 5). It indicates the equilibration of concentration of sodium ions in the extracellular and intracellular spaces in the result of transfer of sodium ions into the cytoplasm via the gramicidin channel.

Mitochondria depolarization assay. Disturbance of the balance of monovalent ions and acidification of the intracellular space induces apoptosis, which was monitored by the depolarization of mitochondria. Membrane-permeable dye, JC-9, exhibits potential-dependent accumulation in the mitochondria monitored by the fluorescence emission shift from green (~530 nm) to orange (~590 nm)²⁶. Consequently, the mitochondrial depolarization is reflected by increase in the green/orange fluorescence intensity ratio (Figures 6). The ratio of green/orange fluorescence of JC-9

dye was measured on the A549 cells at different time points after cells treatment at both pHs of 7.4 and 6.2 with the pHLIP-coated liposomes containing 10% gA and on non-treated cells. Only cells treated with the liposomes at low pH demonstrate increase of green/orange fluorescence ratio at 3-rd and 4-th days after the treatment indicating to the mitochondria depolarization and cell apoptosis.

Discussion

The pHLIP-coated liposomes mimic highly efficient intracellular delivery systems utilized by the viruses and pathogenic organisms^{27–29}, since pHLIP at low pH performs function of the fusogenic peptides that brings membrane of the liposome and a cell in close contact¹⁶. There are two case scenarios: i) the liposome membrane fuse with the cellular membrane and transfer gramicidin conductive channel to the plasma membrane, or ii) the liposome enters a cell via endocytosis, most probably, macropinocytosis, in the result of simultaneous insertion of several pHLIP peptides into the plasma membrane, which can lead to the plasma membrane ruffling and blebbing and can trigger internalization of the liposomes (Figure 7). In the latter case, the pHLIP-coated liposome will fuse with the membrane

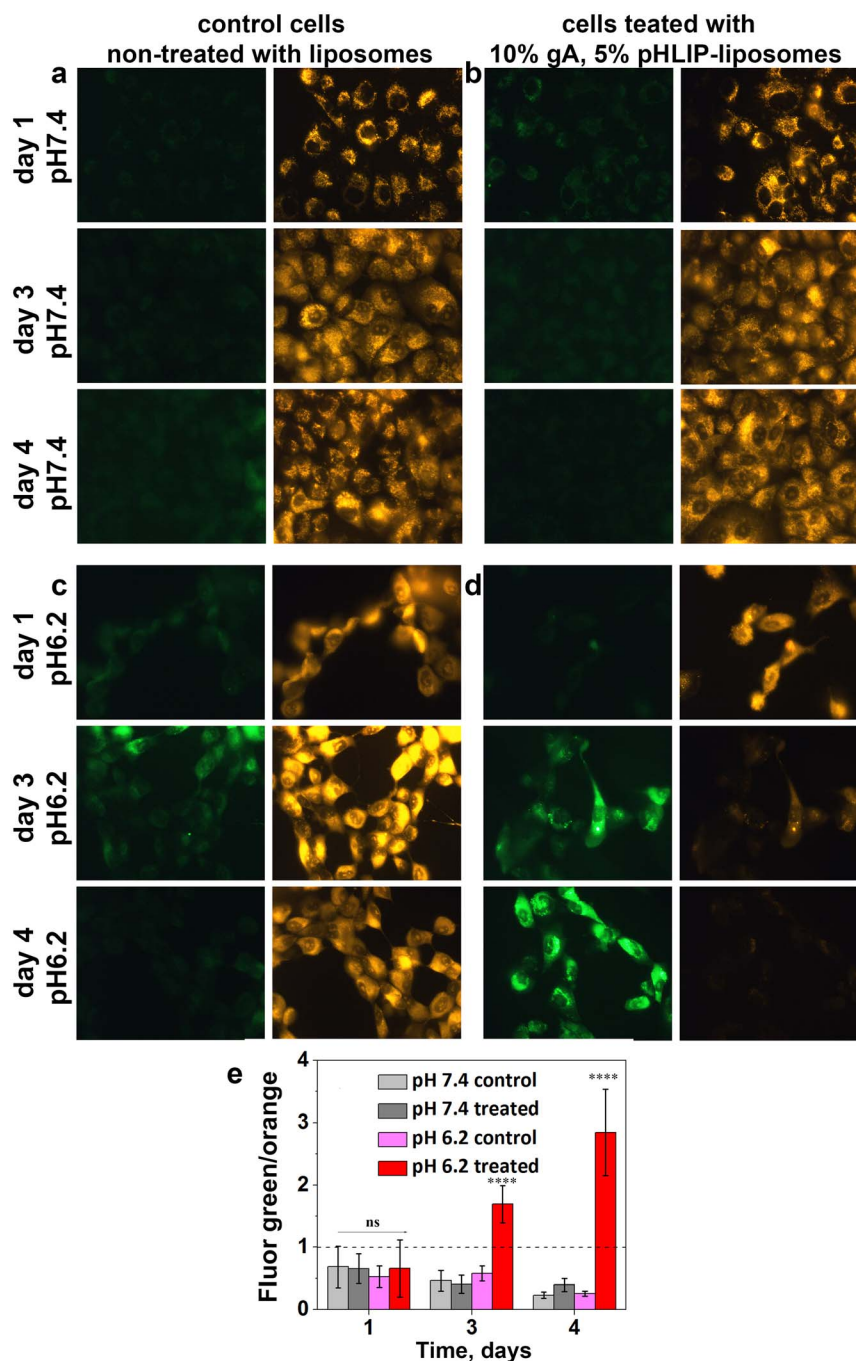


Figure 6 | Mitochondria depolarization assay. The representative fluorescent JC-9 images of A549 cells non-treated and treated with the DOPC pHLIP-coated liposomes containing 10% gA and 5% pHLIP at pH 7.4 and 6.2 are shown (a–d). Depolarization of mitochondria is characterized by changing color from yellow to green of JC-9 fluorescence. The cells images were taken at excitation and emission wavelengths centered at 490 nm and at 520 nm (green) or 540 nm and 580 nm (yellow), respectively, at different days after cells treatment with the liposomes. The changes in average ratio of green to yellow signals are shown on panel e.

of endosome at low pH. In both case scenarios, the gramicidin channels will disrupt the balance of monovalent cations. At the same time, proton pumps and sodium/potassium ion channels actively pump protons and sodium ions from the cytoplasm to the extracellular space and potassium ions to the cytoplasm. It takes time for complete equilibration of ions. Disturbance of ion balance leads to the mitochondria depolarization, which is a signal of early apoptosis. Interestingly, cells treated for 1 day at low pH with the pHLIP-coated liposomes containing gramicidin, but then transferred to the media of normal pH, demonstrated much less cytotoxicity compare with the cells growing at low pH all the time.

The pH-selective delivery of conductive pores to destroy balance of ions, which leads to cell death, could be considered as a potential novel way of treatment of acidic solid tumors. An example of selective delivery and proper assembly of the gA channels in cellular membranes opens an opportunity for the delivery of various membrane peptides and proteins. It might find wide application in biotechnology and medicine. The acidification of extracellular space could be transient, just to promote fusion between the liposome and cellular membrane. At the same time a number of pathological states, such as solid tumors, ischemic myocardium and others are associated with the development of acidic extracellular environment.

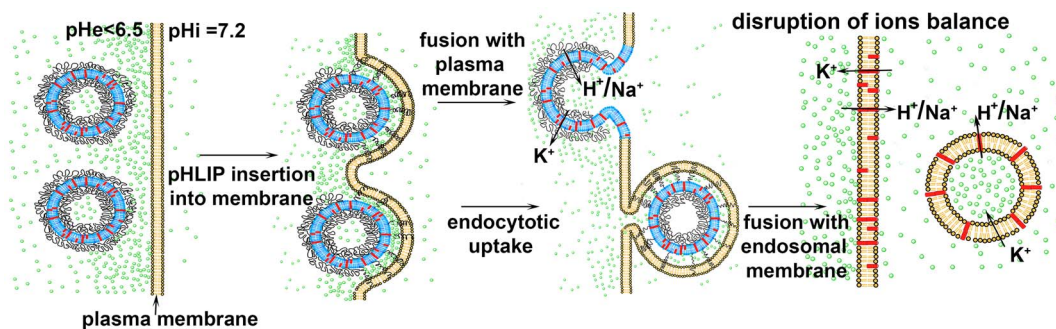


Figure 7 | Schematic presentation of interactions of lipid bilayer of the pHILIP-coated liposomes containing gA with plasma membrane of a cell, which leads to the transferring of gA channels (nano-pores) to the cellular membranes and disruption of the monovalent cations balance (presentation of liposome in the endosome is schematic and not in a scale).

We already demonstrated the pHILIP-mediated delivery and accumulation of optical, PET and SPECT imaging agents, gold-nanoparticles and liposomes to the acidic diseased tissue^{10,12,13,15}.

Methods

Materials. Gramicidin A (gA) (from *Bacillus brevis* $\geq 90\%$ (HPLC)), N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (FITC-DHPE) were purchased from Sigma-Aldrich. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (sodium salt) (DSPE-maleimide), were obtained from Avanti Polar Lipids. The pHILIP peptide (ACEQNPIYWARYADWLFTPLLLLDALLVDADEGT) was prepared by solid-phase peptide synthesis at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. 3,3'-dimethyl- α -naphthoxycarbonyl iodide (JC-9), nigericin, SNARF (seminaphthorhodofluors)-5F 5-(and-6)-carboxylic acid, acetoxymethyl ester, acetate and CoroNaTM Green, AM were purchased from Invitrogen. Water was purified using a Millipore Milli-Q system.

Synthesis of DSPE-pHILIP. DSPE-pHILIP was synthesized by conjugation of DSPE-maleimide with single cysteine residue at the N-terminus of pHILIP. In a typical coupling reaction, 1200 nmol of peptide was mixed with 1000 nmol of DSPE-maleimide in 400 μ L of degassed methanol and incubated overnight stirring at room temperature under argon.

Liposome preparation. Liposomes were prepared by extrusion: a chloroform solution of the 80–98 mol% of DOPC lipids mixed with 5% pHILIP conjugated lipids (pHILIP-DSPE), 2–5% fluorescent labeled lipids (FITC-DHPE) and 0–10% gA were evaporated using rotary evaporator producing an even thin film, followed by the additional overnight evaporation under the high vacuum to remove traces of organic solvents. This lipid layer was re-suspended in 1 mL 10 mM phosphate, 150 mM NaCl buffer and went through 10 freeze-thaw-vortex cycles. The resulting liposome solution was extruded 31 times through 50 or 100 nm filters, which were used for biophysical measurements and experiments on cultured cells, respectively. Liposomes were sterilized by filtering through 0.2 μ m filter before addition to cells. Solution of liposomes was kept at 4°C overnight before use to ensure gA channels formation. The concentration of lipids was calculated by measuring absorbance of FITC at 496 nm ($\epsilon = 89,000 \text{ M}^{-1} \text{ cm}^{-1}$) or gA and pHILIP at 280 nm ($\epsilon = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$ for gA and $\epsilon = 13,940 \text{ M}^{-1} \text{ cm}^{-1}$ for pHILIP) of the liposomes dissolved in methanol.

DLS and Zeta potential measurements. The size of liposomes was measured by the dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS instrument with Malvern disposable cuvettes (Malvern USA). Samples were typically 100 nmol liposomes in 1 mL of 10 mM phosphate, 150 mM NaCl, pH 7.4 buffer. The zeta potential was measured on the same instrument using folded capillary cells from Malvern.

Cryo-electron microscopy. About 5 μ L droplet of liposome solution was spread on a Lacey Formar/Carbon electron microscopy grid and preserved in a frozen-hydrated state by a rapid freezing in liquid ethane. The vitrification process was performed via FEI Vitrobot system with the setting of a single blot of 3 sec, an offset of 1, and drain and wait time of 1 sec. Samples were imaged using JEOL 2100 TEM with an accelerating voltage of 200 kV at magnifications of 20,000 \times and 40,000 \times .

Steady-state fluorescence and circular dichroism measurements. Tryptophan fluorescence and circular dichroism (CD) measurements were carried out on a PCL ISS spectrofluorometer (ISS, Inc.) and a MOS-450 spectrometer (Bio-Logic Inc.), respectively. All measurements were performed at 25°C. DOPC liposomes containing 0, 2% or 10% of gA were used in the study in phosphate buffer, pH 7.4. Fluorescence spectra of gA at excitation of 280 nm was recorded from 310 nm to 400 nm with the

spectral widths of excitation and emission slits set at 4 and 2 nm, respectively. CD spectra were recorded from 200 to 260 nm with 0.5 nm increment using a cuvette with an optical path length of 0.5 cm.

Kinetics of FITC quenching. DOPC liposomes containing 2% and 10% gA, 2% FITC-DHPE and liposomes containing no gA were used in the kinetics studies. All solutions were degassed several minutes under a vacuum to minimize air bubbles before loading into syringes of the stopped-flow apparatus (Bio-Logic, Inc.). Liposomes of 1.5 mM lipid concentration in 10 mM phosphate buffer pH 8 (or pH 3) with 150 mM NaCl were rapidly mixed (within 5 ms – dead time of the set up we used) with an equal volume of HCl (or NaOH) to have final pH of 3 (or pH 8). Changes of the fluorescence intensity of FITC at 519 nm excited at 496 nm were monitored in a real time. Baseline was measured by mixing the liposomes with equal volume of the buffer.

Cell lines. A549 (lung carcinoma), HeLa (human cervix adenocarcinoma) and M4A4 (human breast ductal carcinoma) cell lines were obtained from American Tissue Culture Collection (ATCC). Cells were authenticated, stored according to the supplier's instructions, and used within 3 months after frozen aliquot resuscitations. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1 μ g/mL of ciprofloxacin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. By serial passages some cells were adapted for the growth in low pH media (pH 6.2). The pH 6.2 media was prepared by mixing 13.5 g of dry DMEM powder with 0.1 g of sodium bicarbonate in 1 L of deionized water supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1 μ g/mL of ciprofloxacin.

Proliferation assay. About 3,000–4,000 of A549 cells were seeded in 96 well-plates at pH 7.4 and 6.2. After 24 hrs, cells were treated for 3 hrs with various concentrations of pHILIP-coated DOPC liposomes in DMEM at pH 7.4 and 6.0 followed by addition of an equal volume of DMEM with 20% FBS. In other experiments, cells were treated with liposomes for 18 hrs followed by the removal of the constructs and replacement of the media with DMEM containing 10% FBS. For the control experiments cells were treated at both normal and low pHs with the DOPC liposomes containing no gA or no pHILIP. Cells were grown until non-treated cells in control reached 80–90% confluence. Cell viability was assessed by the colorimetric reagent (CellTiter 96 AQueous One Solution Assay, Promega) of the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, which was added for 1 hour to cells followed by measuring absorbance at 490 nm. All samples were prepared in triplicate. Each experiment was repeated several times.

ECIS assay. Kinetics of inhibition of proliferation of A549 cells treated with the pHILIP-coated DOPC liposomes containing 10% gA and 5% pHILIP at pH 6.0 were monitored by the changes of capacitance measured at AC frequencies on a ECIS[®] 8Z (electric cell-substrate impedance sensing) instrument (Applied Biophysics, Inc.). Cells (~25,000 cells per well) were loaded in 8W10E+ 8-well plate (Applied Biophysics, Inc.) and incubated overnight. Next day, the cells were treated with different concentrations of liposomes in serum free pH 6.0 media for 3 hrs followed by addition of an equal volume of DMEM containing 20% FBS at pH 6.2. Each well had two sets of 20 circular 250 μ m diameter active gold electrodes. The changes of capacitance were monitored within 5 days after seeding of cells.

Intracellular pH measurements. First, pH calibration curve was established: about 40,000 A549 cells were seeded in collagen coated glass bottom dishes (MatTek, Corp.) at pH 6.2. Next day cells were incubated with 20 μ M of SNARF-5F in L-15 phenol red free media at pH 6.2 for 1 hr followed by washing three times with 50 mM phosphate/citric acid solution at pH 5.5, 6.0, 6.6, 7.2 or 7.5. Next, 25 μ L of 0.4 μ g/ml nigericin was added to cells and incubated for 15 min to equilibrate the intracellular pH with the pH of buffer. Fluorescent spectra of SNARF-5F before and after addition of nigericin to the cells were recorded in the range of 520–800 nm on a fluorescent inverted microscope IX71 (Olympus America, Inc.) with an attached Shamrock 303 (Andor



Technology, Inc.) spectrograph equipped with a Newton EMCCD (electron multiplied charged couple device) camera (Andor Technology, Inc.) using 480 ± 10 nm excitation an interference filter and a long pass emission filter with 520 cutoff wavelength. Ratio of SNARF-5F fluorescent signal at 595 nm/640 nm were calculated for each pH and plotted against the respective pH, and fitted with a linear function using Origin9 software (Origin Lab, Corp.) to obtain calibration curve. In separate experiments A549 cells were treated at pH 7.4 and 6.0 with 250 μ M of the DOPC pHLIP-coated liposomes containing 0 or 10% gA and 5% pHLIP for 3 hrs followed by the addition of an equal volume of 20% FBS in DMEM and incubated overnight. At various days after the treatment cells were incubated with 60 μ M SNARF-5F in 200 μ l of L-15 pH 6.2 for 1 hr, followed by washing with L-15 of pH 6.2 and recording SNARF-5F emission spectra. Next, nigericin was added to the cells to equilibrate pH and emission spectra of SNARF-5F were recorded once again. The SNARF-5F emissions signal ratio of 595/640 nm was calculated and the corresponding intracellular pH was established using pH calibration curve.

Intracellular Na⁺ measurements. About 30,000 A549 cells of pH 7.4 and 50,000 A549 cells adapted to pH 6.2 were seeded in the collagen coated glass bottom dishes. Next day, cells were treated with 1 mL of 250 μ M lipid concentration of the DOPC liposomes with 10% gramicidin and 5% pHLIP (control – equal volume of PBS 7.4) in pH 6.2 and pH 7.4 DMEM without FBS and incubated for three hours. After 3 hours 1 ml of 20% FBS in DMEM medium at pH 6.2 and pH 7.4, respectively, were added and incubated until the experiments were performed. Next day, the treatment solution from the control and treated dishes was removed and 100 μ l of 100 μ M CoroNa™ Green AM solution in L-15 at pH 7.4 was added and incubated for 45 min. After 45 mins, cells were washed three times using PBS and imaged in L-15 at pH 6.2 or pH 7.4 using green channel of the fluorescent microscope. The same procedure was repeated with the treated and control cells on 3rd day after the treatment. Quantification of the fluorescent signals from the cells was performed using an ImageProPlus software. Statistical analysis of the data was performed using the “Statistica 5.0” package. The p-level was computed based on the two-tailed test.

Mitochondria depolarization assay. About 30,000 A549 cells were seeded in the collagen coated glass bottom dishes. Next day, cells were treated at pH 7.4 and 6.2 with 1 mL of 250 μ M lipid concentration of the DOPC pHLIP-coated liposomes containing 10% gA and 5% pHLIP for 3 hrs followed by addition of an equal volume of DMEM containing 20% FBS at corresponding pH. At various days after the treatment cells were incubated with 10 μ g/mL JC-9 solution in L-15 at pH 7.4 or 6.2 for 15 min followed washing and imaging. Imaging was performed on a fluorescent inverted microscope IX71 (Olympus America, Inc.) using color Q-Color3 CCD camera (Olympus America, Inc.). The following excitation and emission filters were used: for green channel 490 ± 10 and 520 ± 10 nm; and for yellow channel 540 ± 10 and 580 ± 10 nm. Quantification of fluorescent signals from the cells was performed using an ImageProPlus software. Statistical analysis of the data was performed using the “Statistica 5.0” package. The p-level was computed based on the two-tailed test.

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Author contributions

O.A.A. and Y.K.R. designed the research; D.W., M.C.A.A. and A.L. performed experimental work; D.W., M.C.A.A., O.A.A. and Y.K.R. analyzed data; O.A.A. and Y.K.R. wrote the manuscript.

Additional information

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