Injection of Molecules into Cells using a pH-Triggered Molecular Nanosyringe

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ABSTRACT

We have engineered a molecular nanosyringe that enables injection of molecules into a target cell at acidic pH, but not at normal, physiological pH. The nanosyringe consists of a water-soluble membrane peptide (pHLIP – pH (Low) Insertion Peptide), a link and a cargo molecule. Injection is driven by pHLIP, which inserts as a helix across the cell membrane and translocates one of its termini into the cytoplasm. The peptide does not exhibit any elements of secondary structure in solution or when loosely bound on the cell membrane at neutral pH. We show the injection of cell-impermeable molecules into cancer cells at pH6.5. The nanosyringe offers a novel technology for selective delivery of cargo-molecules into cancer cells by exploiting the acidic extracellular environment that is a characteristic feature of many tumors.

Keywords: transmembrane peptide, delivery, nanosyringe, cancer, acidosis

1 INTRODUCTION

The problem of discovering therapeutic agents is often accompanied by the problem of delivering them in a specific way to their sites of action, accounting in part for the fact that so many drugs target receptors, binding specifically to regions exposed outside the cell membrane and so not requiring access to the cytoplasm. But, there are many useful targets inside cells, if only agents could be delivered internally, resulting in a growing effort to design new strategies to deliver molecules across plasma membranes. It has been discovered that cationic peptides such as TAT, antennapedia, arginine-rich peptides and their analogs facilitate the transport of a variety of biological molecules and even such large entities as liposomes, nanoparticles and adenoviruses. Despite the widespread interest in such molecular carriers, the mechanisms underlying cellular translocation by such cell-penetrating peptides are still under discussion. We have found a novel way to deliver molecules into the cells by using transmembrane peptides that respond to local environments and form transmembrane helices with one end exposed on the surface and the other end inserted into a target cell. The external part of the transmembrane peptide can be used to mark the cell surface for detection or imaging of target tissue, and the internal part can be used to translocate molecules into cells by conjugating them via a link that is cleavable in the cytoplasm.

2 RESULTS

Our work is based on the idea of application of transmembrane peptides for translocation of molecules attached to the peptide end that inserts across the lipid bilayer. We previously reported that a polypeptide, pHLIP, (AAEQNPIYWARYADWLFTTPLLLLDLALLVDADEG TCG) derived from the bacteriorhodopsin C helix is soluble in aqueous solution and spontaneously inserts across the membrane in a pH dependent manner [1]. The interaction of pHLIP with lipid bilayer at normal and low pH was monitored by the changes in tryptophan fluorescence and CD signals (Figure 1). At normal pH in solution and in presence of liposomes the peptide is a random coil. Lowering the pH triggers insertion of the peptide in the hydrophobic environment of membrane, which results in a shift of the tryptophan fluorescence spectrum maximum by more than 10 nm and an increase in intensity of emission of 1.5 times. Insertion is accompanied by the formation of a transbilayer alpha helix (Figure 1 b and data in [1]). The process of insertion is reversible, increasing of pH leads to the release of pHLIP and unfolding.

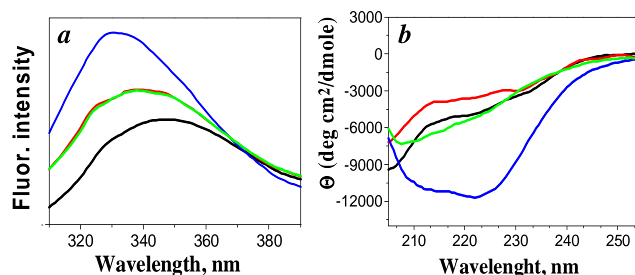

Figure 1. Fluorescence (a) and CD (b) spectra of pHLIP in solution in absence and presence of POPC liposomes. The black line represents pHLIP in phosphate buffer, pH7.4, then POPC liposomes were added (red line) and the pH was dropped (pH5.0) by adding HCl (blue line), and restored by addition of KOH to pH 7.4 (green line).

Our previous study shows that pHLIP translocates its C-terminus inside liposomes [2]. We attached various cargo molecules to pHLIP C-terminus via cleavable S-S and covalent links and tested the ability of the peptide to translocate molecules across a membrane of live cells. Among molecules were fluorescently labeled 1.1kDa byciclic peptide phalloidin (toxin from the *Amanita phalloides* mushroom) and a 12-mer peptide nucleic acid (PNA). Both molecules are membrane impermeable. pHLIP was able to translocate them at low pH (~6.5) (Figure 2a, b), but we saw no translocation at normal pH (7.4). When we performed fluorescence microscopy experiments and observed the translocation of cargo by pHLIP, we verified that labeled cells were alive using the dead cell marker SYTOX-Green. The quantification translocation was performed by FACS [2].

Phalloidin binds tightly to actin filaments [3] and it is commonly used as a specific marker of actin filaments in permeabilized cells. We observed the characteristic pattern of actin filaments staining inside the cell (Figure 2a), distinct from other cellular structures, organelles or membrane staining. The translocation of phalloidin was confirmed on several cell lines [2]. Also we documented that the phalloidin translocated by pHLIP at low pH leads to the inhibition of cytoskeleton dynamics and formation of multinucleated cells [2]. pHLIP-phalloidin might be considered as a potential anti-metastatic drug, which inhibits cell migration and movement.

PNAs are synthetic mimics of RNA and DNA, and are resistant to nuclease and protease degradation, and bind tightly to complementary DNA and RNA by Watson-Crick base pairing [4]. One of the most common applications of the PNA technology is its antisense application in which the PNA is designed to bind to messenger RNA (mRNA) to inhibit translation of the target genes. PNA is considered as one of the most promising molecules for gene therapy. However, the delivery of PNA, involving passage through the cell membrane, appears to be a general problem. We demonstrate that the pHLIP can translocate PNA through the cell membrane and release it in cytoplasm at low pH (Figure 2b).

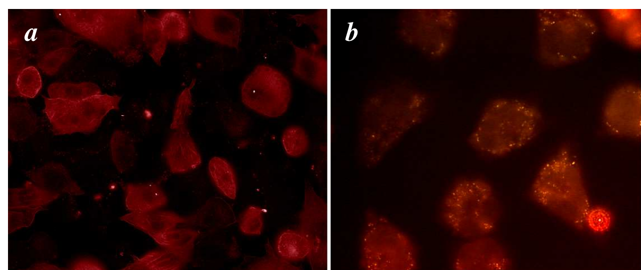

Figure 2. The delivery of phalloidin (a) and PNA (b) into cells by pHLIP at pH6.5. Fluorescence images of HeLa cells incubated (for 1 hour) with pHLIP-S-S-phalloidin-TRITC and pHLIP-S-S-PNA-TAMRA cleavable constructs (1 μM) at pH 6.5 are shown. No translocation was observed at pH7.4

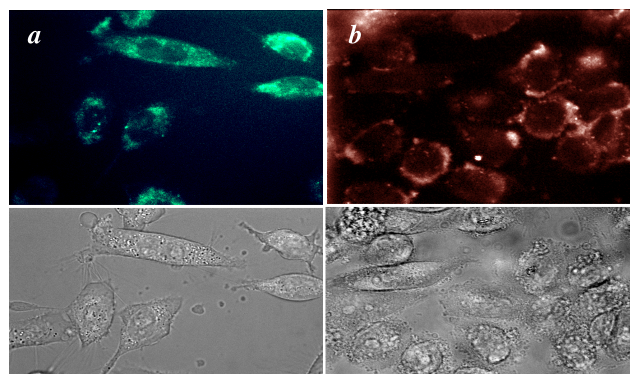

Figure 3. Fluorescence and phase contrast images of HeLa cells incubated (for 1 hour) with pHLIP-S-S-dansyl (a) and pHLIP-rhodamine (b) constructs at pH 6.5 are shown. Dansyl dye is distributed in cytoplasm, but rhodamine fluorescence is restricted to the cell membrane.

We prepared two additional constructs, where fluorescent dyes dansyl and rhodamine were attached to the C-terminus of pHLIP by S-S and covalent bonds, respectively. Figure 3a shows that dansyl dye was distributed in cytoplasm due to the cleavage of the disulfide link in the reducing environment of the cell and consequent release of the dye. In case of rhodamine covalently attached to pHLIP we observed fluorescence restricted to the cell membrane at low pH (Figure 3b). Washing the cells several times with buffer at normal pH led to the release of peptide from the cell membrane, leaving only traces of detected fluorescence. The results are in agreement with data obtained on liposomes, which suggest that the peptide does not enter the cell, but stays anchored to the membrane and can then be removed by raising the pH. pHLIP is not a cell-

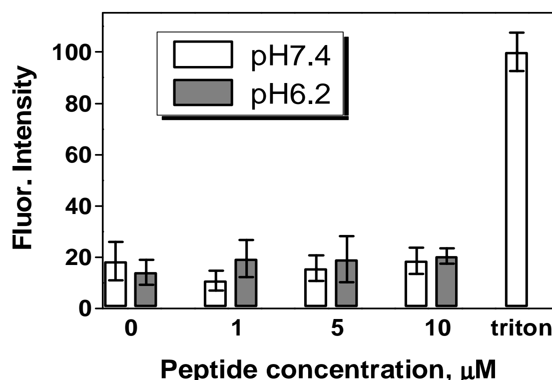

Figure 4. Membrane leakage was tested by incubation of HeLa cells at various concentrations of peptide (up to 10 μM) with nuclear-staining SYTOX-Orange (0.5 μM) at pH 7.4 and 6.5 for 1 hr followed by washing and measuring of fluorescence signal on a plate reader. The fluorescence intensity of cells incubated with SYTOX in the presence of 0.5% Triton-100, which disrupts the plasma membrane, was taken as 100%.

penetrating peptide.

Our previous study showed that pHLIP is not toxic for cells at concentrations of up to 16 μM [2], which is 8 times higher than the concentration used for molecule translocation. In all of our experiments we observed molecule translocation only when the cargo was linked to pHLIP. No translocation was detected during incubation with a mixture of unlinked cargo molecules and pHLIP. Also, we did not see staining of fluorescent cells by the dead cell marker, SYTOX. All of the data suggest that pHLIP does not induce pore formation. However, we performed additional experiments to check the ability of pHLIP to induce membrane leakage (Figure 4): HeLa cells were incubated at various concentrations of peptide (up to 10 μM) at pH 7.4 and 6.5 together with cell-impermeable nuclear-staining SYTOX-Orange for 1 hour, followed by washing and measurement of the fluorescence on a plate reader. The fluorescence signals of cells incubated with various concentrations of peptide and SYTOX were compared with those of cells incubated with SYTOX alone, and SYTOX plus 0.5% of the detergent Triton, which disrupts the membrane. The level of fluorescence in cells treated with the peptide was the same as in control cells, and about 6 times lower than the intensity of cells treated with Triton.

3 SUMMARY

We have engineered a molecular nanosyringe, employing a peptide (pHLIP) that inserts into a cell membrane only at low pH (<7.0), but not at normal physiological pH, translocating a molecule into a cell and releasing it in the cytoplasm. The peptide does not exhibit any elements of helical secondary structure in solution or on the cell membrane at neutral pH, however it becomes rigid (as a syringe needle), when it inserts into a lipid bilayer and forms a transmembrane helix. The spontaneous insertion is driven by protonation of Asp residues located in the transmembrane part of the polypeptide and occurs on a fast time scale (seconds) [1]. This insertion is consistent with the recent scale of Hesse et al. [5], where the membrane insertion scale is shown to be more favorable for the protonated form of Asp.

pHLIP offers a new technology for fast and efficient delivery of drugs, imaging probes, and cell and gene regulation agents into living cells *in vitro* and *in vivo*. The method allows translocation of certain cell impermeable molecules inside cell and attachment of a variety of functional moieties and particles to the cell surface at low pH. The pH-selective delivery properties of pHLIP provide a new approach for the diagnosis and treatment of tumors with low pH extracellular environments [6-7].

4 MATERIALS AND METHODS

4.1 Synthesis of peptide and peptide-cargo constructs

pHLIP was prepared by solid-phase peptide synthesis using standard Fmoc and purified by reverse phase chromatography (on a C18 column) at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. The peptide was labeled at its single C-terminal cysteine residue with dansyl and rhodamine by incubation with didansyl or tetramethylrhodamine maleimide in 10mM Tris-HCl, 20mM NaCl, 6M urea, pH8.0 in dark at 4ºC for 24 h. The conjugated peptides were purified on a G-10 size-exclusion column and transferred to PBS buffer, pH 7.4. The concentration of labeled peptide was determined by absorbance (dansyl: ε_{340}=4300 $M^{-1}cm^{-1}$, rhodamine: ε_{542}=65000 $M^{-1}cm^{-1}$).

pHLIP was conjugated to phalloidin-rhodamine (Ph-TRITC) using bi-functional photocrosslinker S-[2-(4-Azidosalicylamido)ethylthio]-2-thiopyridine (AET). AET makes an S-S bond with the C-terminus of the peptide and binds to Ph-TRITC under UV irradiation (pHLIP-S-S-Ph-TRITC). pHLIP was incubated with the crosslinker in PBS in the dark at 4ºC for 24 h. Excess crosslinker was removed using a G-10 size-exclusion spin column. 5x molar excess of Ph-TRITC was added to the pHLIP-crosslinker and illuminated at 340 nm for 30 min. PNA (TAMRA-o-o-CATAGTATAAGT-o-Cys-NH_2) was synthesized by Applied Biosystems. PNA-TAMRA was incubated with 4 times molar excess of pHLIP for 1 hr in PBS pH 7.4, 37°C. pHLIP-S-S-Ph-TRITC and pHLIP-S-S-PNA-TAMRA constructs were purified on G-10 column. The concentration was determined by measuring absorption of rhodamine at 546 nm (ε_{542}=65000 $M^{-1}cm^{-1}$).

4.2 Liposome preparation

Large unilamellar vesicles (LUVs) were prepared by sonication. The POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine, Avanti Polar Lipids, Inc.) phospholipid was dissolved in chloroform. Following removal of the solvent using a rotary evaporator, the phospholipid film was dried overnight and then rehydrated in 10mM Tris-HCl, 20 mM NaCl, pH 8.0 and vortexed. The suspension was sonicated using a Branson titanium tip ultrasonicator until the solution become transparent. The liposomes distribution was evaluated by dynamic light scattering.

4.3 Fluorescence and CD measurements

Tryptophan fluorescence and CD measurements were carried out on a digital phase-modulation spectrofluorometer ISS PC1 and Jasco 810 spectropolarimeter at 25°C, respectively, in a phosphate buffer. The concentration of peptide and lipids used in study was 7 μM and 1mM, respectively. The excitation wavelength was 295 nm for the excitation of only

tryptophan fluorophores in protein. The fluorescence spectra were recorded from 310 to 400 nm with the spectral widths of excitation and emission slits set at 4 and 2 nm, respectively. The polarizers in the excitation and emission paths were set at "magic" angle (54.7^o from the vertical orientation) and vertically (0°), respectively, in order to reduce Wood's anomalies from reflecting holographic grating. The emission spectrum of an aqueous solution of L-tryptophan was used as a standard for the correction of protein spectra for the instrument spectral sensitivity [8]. The intensities of the corrected spectra are proportional to the number of photons emitted per unit wavelength interval.

4.4 Cells

HeLa cells were provided by the Cancer Center of the Yale University Medical School. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

4.5 Fluorescence microscopy

For the fluorescence microscopy studies, the cells were grown in 35 mm dishes with 10 mm glass bottom windows coated with collagen. Cells were washed with PBS buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ with pH 6.5 or 7.4 and then incubated in PBS at the experimental pH in the absence or presence of varied concentrations (0.1 - 2 µM) of the pHLIP-S-S-dansyl, pHLIP-rhodamine, pHLIP-S-S-Ph-TRITC and pHLIP-S-S-PNA-TAMRA constructs. The medium pH was measured before and after incubation. The cancer cells can acidify the medium (PBS buffer) in a few minutes if the incubation volume is small, creating the problem of maintaining constant pH 7.4 in the PBS incubation buffer. We therefore preferred to use a low density of cells in the chamber, a larger volume and a higher phosphate concentration (up to 50 mM instead of the standard 10 mM PBS) and we routinely checked the pH before and after the experiments. The time of incubation was varied from 15 to 60 min. The incubation was followed by the replacement of the PBS buffer with Leibovitz's L-15 phenol free medium (supplemented with 5% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine) at the experimental pH for 1 hr and then at pH 7.4 (or pH6.5 for pHLIP-rhodamine). Fluorescent images were taken using an inverted epi-fluorescence microscope Olympus IX71. The images of cells stained with dansyl were taken on BioRad MRC-1024 two-photon confocal microscope with excitation at 740 nm.

4.6 Membrane leakage assay

HeLa cells were loaded in 96 well plates (2,000 cells per well), incubated for 24 hours in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The growth medium was then replaced with PBS buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at pH7.4 or 6.5 and increasing amounts of the peptide (1, 5 and 10 µM) and 0.5 µM of SYTOX-Orange. After 1 hour of incubation cells were washed with PBS buffer, pH7.4. The fluorescence was measured at 590 nm with excitation at 530 nm on plate-reader. In parallel, we analyzed the cells with membrane disrupted by adding 0.5% Triton-100 together with 0.5 µM of SYTOX-Orange followed by washing with PBS. All samples were done in triplicate.

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REFERENCES

[1] Hunt J.F., Rath P., Rothschild K.J., & Engelman D.M. (1997) *Biochemistry* **36**, 15177-151792.

[2] Reshetnyak Y.K., Andreev O.A., Lehnert U., & Engelman D.M. (2006) *PNAS*, in press.

[3] Wehland, J., Osborn, M., & Weber, K. (1977) *Proc. Natl. Acad. Sci. U.S.A,* **74**, 5613-5617.

[4] Nielsen P.E., Egholm M., Berg R.H., & Buchardt, O. (1991) *Science*, **254**, 1497-1500.

[5] Hessa T., Kim H., Bihlmaier K., Lundin C., Boekel J., Andersson H., Nilsson I., White S.H., & von Heijne G. (2005) *Nature*, **433**, 377-381.

[6] Stubbs M., McSheehy P.M.J., Griffiths J.R. & Bashford C.L. (2000) *Mol. Med. Today*, **6**, 15-19.

[7] Izumi H., Torigoe T., Ishiguchi H., Uramoto H., et al. (2003) *Cancer Treat. Rev*. **29**, 541-549.

[8] Burstein E.A., & Emelyanenko, V.I.. (1996) *Photochem. Photobiol*. **64**, 316-320.