Enhanced pHLIP (e-pHLIP) for Targeted Cytoplasmic Delivery of Polar Payloads

Marissa Iraca, Craig Klumpp, Anna Moshnikova, Oleg A. Andreev, Yana K. Reshetnyak
Physics Department

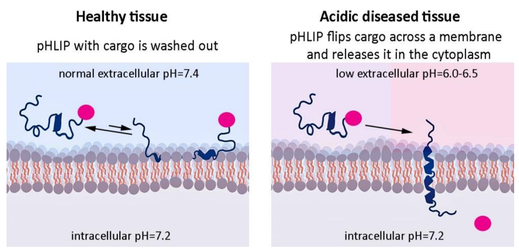
Abstract

pH Low Insertion Peptide (pHLIP) technology is employed in clinics for targeting of tumors and delivery of imaging and therapeutic agents. Here we introduced the novel enhanced pHLIP (e-pHLIP) system for efficient cytoplasmic delivery of large, polar payloads, including small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs). Our e-pHLIP delivery system wasdesigned, synthesized, and then tested for pH-dependent interactions with liposomal membranes and cultured cells, as well as tumor targeting and biodistribution in mouse tumor models. Fluorescently labeled siRNAs and ASOs for silencing of green fluorescent protein (GFP) were assessed in GFP expressing HeLa cells using fluorescent microscopy. Cellular uptake and distribution of the fluorescent siRNAs and ASOs delivered by e-pHLIP were monitored, and the decay of GFP fluorescence over time was quantified.
The e-pHLIP consists of two pHLIP peptides linked via a short PEG linker. The first pHLIP peptide serves as a membrane-anchoring domain and carries no payload molecule at its membrane-inserting terminus. This stabilizes membrane associate for a second step where the second pHLIP peptides serves a a payload-transporting domain, which carries a payload at its membrane-insertin terminus for transport of a payload across a membrane. We demonstrated improved interactions of e-pHLIP with membranes, no cytotoxicity, and enhanced targeting of acidic tumors by e-pHLIP (compared to pHLIPS) labeled with indocyanine green near infrared fluorescent dye, ICG. e-pHLIP exhibited successful cytoplasmic delivery of siRNAs and ASOs when tested on GFP-expressing HeLa cells.
Conclusions: We introduced the novel e-pHLIP delivery system comprised of one membrane insertion pHLIP peptide linked to another membrane insertion pHLIP peptide by a non-cleavable linker for cytoplasmic delivery of large polar payloads, which opens an opportunity for targeted delivery of gene-therapeutics.

pHLIP Technology

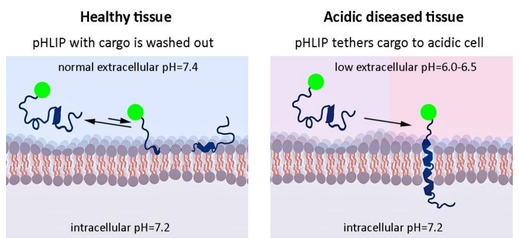
- A hallmark characteristic of solid tumors is an acidic tumor microenvironment from metabolically active cells.
- The negatively charged residues of pHLIP peptides bind to the hydrogen ions in this acidic environment and insert across cancer cells.
- This mechanism provides a method of targeted delivery, enhancing the therapeutic window.

Intracellular Delivery



- pHLIP has been studied to deliver cargo intracellularly, such as cytotoxic molecules (exatecan, calicheamicin) and cell regulating agents (STINGa, dexamethasone)

Extracellular Delivery

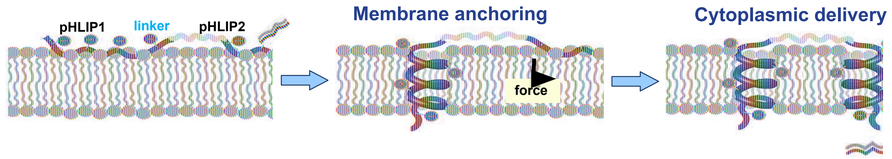


- pHLIP can also deliver agents extracellularly, allowing tumor cells to be tagged with molecules (cytokines, c-Myc) and imaging agents (fluorescent, SPECT, MRI).
- Currently, pHLIP-ICG is in clinical trials at Memorial Sloan Kettering Cancer Center for image-guided lumpectomy.

Novel Enhanced pHLIP (e-pHLIP) for siRNA and ASO delivery

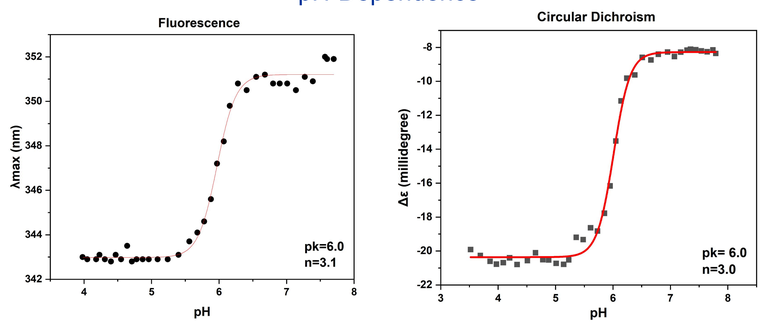
- In addition to targeted delivery, a current obstacle being investigated is how to “drug the undruggable” protein. These are proteins that do not have consistent binding sites.
- Gene therapy addresses the root of this problem by either making edits to the genome (DNA level) or adding/silencing mRNA for protein synthesis (RNA level).
- Silencing can be accomplished with small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs).
- However, gene therapeutics are not considered “drug-like,” i.e., they are significantly large and polar.
- Here we introduce enhanced-pHLIP (e-pHLIP), which contains two pHLIPs connected via a peg-linker. The first pHLIP has no cargo, readily inserting into the cellular membrane, acting as an anchor for the second pHLIP, which contains the polar payload. This anchoring increase “membrane-residence time,” allowing for enhanced probability of cytoplasmic delivery.

Enhanced pHLIP (e-pHLIP) = pHLIP1 – linker – pHLIP2 – siRNA



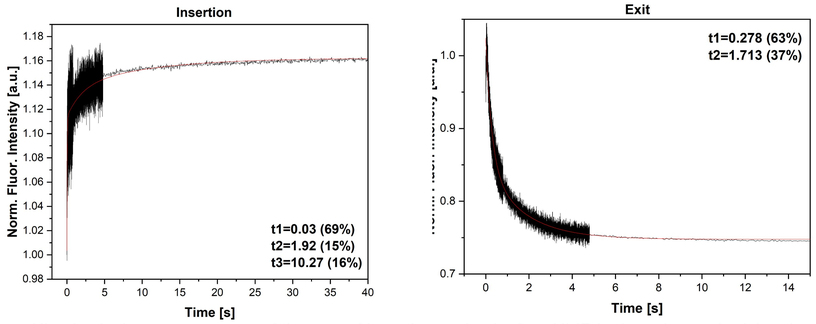
Biophysical Results for e-pHLIP

pH-Dependence



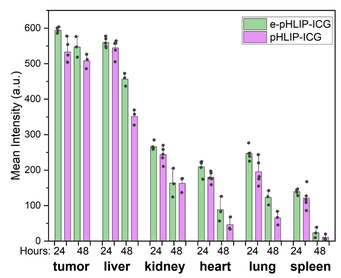
- Using liposomes as cellular models, biophysical interactions with e-pHLIP were performed.
- The pH dependence of e-pHLIP was measured to determine the pk value, which is where there is 50% insertion of e-pHLIP across the membrane. This confirms physiological relevance.

Kinetics



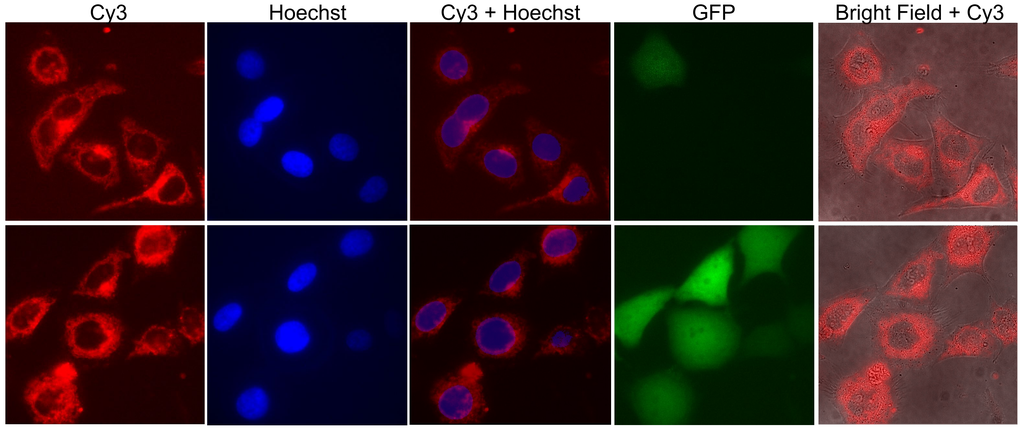
- Kinetics is the measurement of the rate of insertion and exit of e-pHLIP by lowering and raising the pH, respectively.
- e-pHLIP was determined to cross the membrane and equilibriate at a relatively quick time, with slower exit.

Tumor Targeting & Biodistribution



- Using a mouse model, mice were sacrificed 24 and 48 hours post-injection of constructs.
- Imaging was performed using Stryker endoscopy camera systems, and mean intensity was measured with ImageJ.

e-pHLIP-siRNA-Cy3 Treated Cells



- HeLa-GFP cells were treated with e-pHLIP-siRNA-Cy3.
- Nuclei of cells were stained with Hoechst.
- Cells were treated with trypan blue, which suppresses extracellular fluorescence.
- Imaging was performed using 100x objective, 16 hours post treatment.
- 2 views are shown.

Main conclusions

- e-pHLIP forms an alpha helical structure and inserts into liposomal membranes at a pk of 6.0, which is physiologically relevant. Relatively fast insertion rates were measured with slower exit rates. Tumor targeting and biodistribution studies on mouse models confirmed enhanced targeting of tumor as e-pHLIP-ICG cleared quickly from other major organs.
- Intracellular delivery in GFP expressing HeLa cells of siRNA via e-pHLIP was confirmed by use of trypan blue. This proof of concept is the beginning of exploration in gene therapeutics that are physiologically stable.

References

Boedtkjer, E. & Pederson, S. F. (2020, February 10). The Acidic Microenvironment as a Driver of Cancer. *Annu Rev Physiol, 82*, 103-126. https://doi.org/10.1146/annurev-physiol-021119-034627,.

Crawford, T., Moshnikova, A., Roles, S., Weerakkody, D., DuPont, M., Carter, L. M., Shen, J., Engelman, D. M., Lewis, J. S., Andreev, O. A., & Reshetnyak, Y. K. (2020). Phlip ICG for delineation of tumors and blood flow during fluorescence-guided surgery. *Scientific Reports*, 10(1). https://doi.org/10.1038/s41598-020-75443-5.

Reshetnyak YK, Andreev OA and Engelman DM (2024) Aiming the magic bullet: targeted delivery of imaging and therapeutic agents to solid tumors by pHLIP peptides. Front. Pharmacol. 15:1355893. doi: 10.3389/fphar.2024.1355893.

Taniguchi H, Suzuki Y, Imai K, Adachi Y. Antitumoral RNA-targeted oligonucleotide therapeutics: The third pillar after small molecule inhibitors and antibodies. Cancer Sci. 2022 Sep;113(9):2952-2961. doi: 10.1111/cas.1546.

Weerakkody, D., Andreev, O. A., & Reshetnyak, Y. K. (2016). Insertion into lipid bilayer of truncated phlip ® peptide. *Biochemistry and Biophysics Reports*, *8*. 290–295. https://doi.org/10.1016/j.bbrep.2016.10.001.