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Synthesis, Characterization, and Tumor Targeting of Novel pH Sensitive Nanoparticles

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SYNTHESIS, CHARACTERIZATION, AND TUMOR TARGETING
OF NOVEL PH SENSITIVE NANOPARTICLES

BY

JENNIFER L. DANIELS

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
PHYSICS

UNIVERSITY OF RHODE ISLAND

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DOCTOR OF PHILOSOPHY DISSERTATION
OF
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2016

ABSTRACT

When particles approach a nanoscale size, they exhibit novel properties not seen in their bulk counterparts. Therefore, nanoparticles are widely used in the diagnosis and treatment of cancer as imaging, delivery, and therapeutic agents. However, the main challenge is to obtain stable, biocompatible, and tumor-targeting nanomaterials so that they preferentially accumulate in cancerous tissue.

Many contemporary therapeutic and imaging approaches to cancer treatment are based on specific tumor-targeting biomarkers such as antibody or vitamin binding. Although there are significant successes, the inter- and intra-heterogeneity of tumors, their complexity, and their ability to adapt to new conditions often create parts of a tumor that do not express enough of the biomarker to be targeted. On the other hand, extracellular tumor acidity, resulting from the metabolic alterations of cancer cells, attracts a lot of attention as a novel general tumor biomarker. pH (Low) Insertion Peptides (pHLIP® peptides), discovered in our laboratory, represent a novel class of pH-responsive tumor-targeting delivery agents.

The molecular mechanism of a pHLIP® peptide's action is based on the protonation of Asp/Glu residues, which enhances the peptide's hydrophobicity and promotes the membrane-associated folding and insertion of a transmembrane helix. It was shown that pHLIP® peptides have excellent targeting of acidic tumors and specific delivery of imaging and therapeutic agents to cancer cells within tumors. Therefore, it is expected that coating nanoparticles with pHLIP® peptides would enhance the stability of

nanomaterials, promote uptake of nanomaterials by cancer cells, and/or facilitate in the release of therapeutic and diagnostic molecules to the cytoplasm of cancer cells.

The main goal of this work was the development of novel pHLIP® peptide coated nanomaterials for cancer therapy. More specifically, we introduced and investigated:

- a family of pHLIP® peptides (along with K-pHLIP, the pH-insensitive counterpart to pHLIP®);
- fusogenic, PEGylated liposomes coated with pHLIP® peptide for the delivery of therapeutic molecules;
- specific delivery to tumors of nanogold® clusters conjugated to the pHLIP® peptide;
- novel pHLIP® and PEG coated spherical and spiked gold nanoparticles

A variety of rationally designed pHLIP® variants were characterized. It was shown how tuning the biophysical properties of the peptide–lipid bilayer interactions alter: i) tumor-targeting, ii) distribution in organs, and iii) blood clearance. Also presented, is the current understanding of the main principles of pHLIP® design. All pHLIP® coated nanomaterials in this work that have been administered to cancerous cells have shown a pH-dependent interaction *in vitro* and/or *in vivo*.

A novel type of pH-sensitive, “fusogenic” pHLIP® liposomes was developed. These pHLIP® liposomes displayed enhanced membrane fusion in a pH-dependent fashion

that leads to the increase of cellular uptake and payload release *in vitro*, highlighting their future use as drug delivery agents.

The uptake and retention of nanogold® clusters in acidic tumors was significantly enhanced by conjugating the clusters with pHLIP® peptides, further supporting the significance of pHLIP® peptide's presence in nanomaterial coating for tumor-targeting. Furthermore, K-pHLIP (the pH-insensitive version of pHLIP®) failed to target tumors *in vivo*.

A novel approach for the synthesis of pHLIP® and PEG coated spherical gold nanoparticles was introduced. These nanoparticles showed enhanced stability in solution and homogeneous tumor uptake with no staining of the surrounding muscle tissue. Potentially, they could be used for the enhancement of x-ray radiation. Novel spiked gold nanoparticles coated with pHLIP® and PEG were also created. Spiked particles exhibited plasmonic resonance in the near infrared region. The particle solutions were heated by an 805 nm laser, thereby demonstrating a potential application in photothermal therapy.

We believe that pHLIP® coated nanomaterials will find a great range of applications in the treatment of cancerous tumors.

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Also, I greatly appreciate my teachers, professors, instructors, and all others who have contributed to my education. Finally, I would like to thank my family (including Joshua Hassell, my better half) for the support and encouragement they have provided over my academic career.

PREFACE

This dissertation is written in the “Manuscript Format” using the Thesis/Dissertation template of University of Rhode Island. There are four manuscripts, each organized into a chapter. The figures and tables of each manuscript are listed under the corresponding chapter in the list of figures and tables.

The results of our studies presented here in the first three chapters were published in three papers:

1. D. Weerakkody, A. Moshnikova, M. S. Thakur, V. Moshnikova, **J. Daniels**, D. M. Engelman, O. A. Andreev, Y. K. Reshetnyak, “Family of pH (Low) insertion peptides for tumor targeting,” *Proced. Natl. Acad. Sci U.S.A.*, 110, 5834-5839 (2013).
2. L. Yao, **J. Daniels**, D. Wijesinghe, O. A. Andreev, Y. K. Reshetnyak, “pHLIP®-mediated delivery of PEGylated liposomes to cancer cells,” *Journal of Controlled Release*, 167, 228-237, (2013).
3. L. Yao, **J. Daniels**, A. Moshnikova, S. Kuznetsov, A. Ahmed, D. M. Engelman, Y. K. Reshetnyak, O. A. Andreev, “pHLIP peptide targets nanogold particles to tumors,” *Proced. Natl. Acad. Sci U.S.A.*, 110, 465-470, (2013).

The final chapter is composed of research in preparation for publication:

4. **J.L. Daniels**, T.M. Crawford, O.A. Andreev, Y.K. Reshetnyak, “pHLIP® Coated Gold Nanoparticles,” *in preparation*.

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CHAPTER 1

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Family of pH (Low) Insertion Peptides for Tumor Targeting

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ABSTRACT

Cancer is a complex disease with a range of genetic and biochemical markers within and among tumors, but a general tumor characteristic is extracellular acidity, which is associated with tumor growth and development. Acidosis could be a universal marker for cancer imaging and the delivery of therapeutic molecules, but its promise as a cancer biomarker has not been fully realized in the clinic. We have discovered a unique approach for the targeting of acidic tissue using the pH-sensitive folding and transmembrane insertion of pH (Low) Insertion Peptide (pHLIP). The essence of the molecular mechanism has been elucidated, but the principles of design need to be understood for optimal clinical applications. Here, we report on a library of 16 rationally designed pHLIP variants. We show how the tuning of the biophysical properties of peptide–lipid bilayer interactions alters tumor targeting, distribution in organs, and blood clearance. Lead compounds for PET/single photon emission computed tomography and fluorescence imaging/MRI were identified, and targeting specificity was shown by use of noninserting variants. Finally, we present our current understanding of the main principles of pHLIP design.

INTRODUCTION

The promise of exploiting tumor acidosis as a cancer biomarker has not been fully realized in clinical practice, even though the acidity has been a known property since the work of Otto Warburg nearly a century ago. The problem has been to find a practical way to target acidity. While studying membrane protein folding, we discovered a peptide (pH (Low) Insertion Peptide called pHLIP®) that reversibly folds and inserts across membranes in response to pH changes, and this discovery has led to a novel way to target acidic tissue. Our biophysical studies have revealed the molecular mechanism of pHLIP action, which is based on the increase of hydrophobicity of carboxyl groups when they become protonated under mildly acidic conditions, leading to peptide insertion into a membrane (1-4). We have shown that pHLIP can target acidic tissue and selectively translocate polar, cell-impermeable molecules across cell membranes in response to low extracellular pH (1, 5-10). As noted in the Molecular Imaging and Contrast Agent Database (MICAD) at NCBI, a pHLIP labeled with a fluorescent dye or a PET-agent (⁶⁴Cu-DOTA) is a marker for *in vivo* acidity (1, 5, 6), and it has recently been reported that a pHLIP peptide labeled with SPECT (⁹⁹Tc) probe targets acidic tumors (7), as well as PET (¹⁸F) probe (Daumar et al., in press in Bioconjugate Chemistry). All prior studies *in vivo* were carried out with the WT-pHLIP sequence and showed that a good contrast and tumor to blood ratio can be achieved only more than 24 hours after pHLIP injection, when it has accumulated in the tumor and largely cleared from the blood (6, 7). However, for the use of pHLIP-based radioactive imaging agents in the clinic, a more rapid background signal reduction is absolutely essential. To address this important need, to

tune tumor targeting properties, and to broaden our understanding of the molecular mechanism of pHLIP action, we designed a set of 16 pHLIP variants based on chemical and physical principles. Comprehensive biophysical studies performed with non-labeled peptides were correlated with investigations of tumor targeting and organ distributions of fluorescent versions of the pHLIP variants. As a result, a set of design criteria has been established, and pHLIP candidates for imaging and therapeutic applications, including lead compounds for PET/SPECT and fluorescence/MR imaging, have been identified.

MATERIALS AND METHODS

Conjugation of pHLIP variants with fluorescent dyes

pHLIP variants were prepared by solid-phase peptide synthesis using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry and purified by reverse phase chromatography at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale. For cell and *in vivo* studies variants were conjugated with AlexaFluor®750-C₅-maleimide, Tetramethylrhodamine-5-maleimide, single isomer or BODIPY®-TMR-C₅-maleimide (Invitrogen). The details of peptides conjugation could be found in Supplementary data.

Steady-state fluorescence and circular dichroism measurements

Peptide intrinsic fluorescence and circular dichroism (CD) measurements were carried out on a PC1 ISS spectrofluorometer (ISS, Inc.) and a MOS-450 spectrometer (Biologic, Inc.), respectively, under temperature control at 25°C. Peptide fluorescence

spectra were recorded with the spectral widths of excitation and emission slits set at 4 and 2 nm, respectively, using excitation wavelengths of 295 or 280 nm. The concentrations of the peptides and POPC liposomes were 7 μ M and 1.5 mM, respectively. The method of liposome preparation and oriented circular dichroism measurements could be found in Supplementary data.

Titration experiments

Samples containing 5 μ M of peptides and varying concentrations of lipids were prepared using an electronic repeater pipette (Eppendorf). Different pHs were used for peptides with different pK_as of insertion; the pH of the solution was one pH unit below the apparent pK value for each peptide. The samples at high and low pHs were allowed to equilibrate at 4°C overnight or 2 hours, respectively, before the measurements. The peptide intrinsic fluorescence spectra were measured using 280 nm excitation at 25°C with the emission polarizer set at 90° to reduce the contribution of scattered light. A series of POPC blanks with the same concentrations of lipids at pH8 were measured with the same instrument settings and were subtracted from the corresponding fluorescence spectra of peptides in the presence of POPC. The areas under the emission spectra were calculated and the values were normalized to the first point (the emission of the peptide in the absence of POPC). The titration data were fitted by the peptide-membrane partition model to calculate the mole-fraction partition coefficient, K

$$F = F_0 + \Delta F \frac{K \cdot C_{lip}}{W + K \cdot C_{lip}}$$

where F_0 and ΔF are the fluorescence intensity at the beginning and fluorescence increase as a result of the titration (in our case F_0 is 1), C_{lipids} is the concentration of lipids; W is the molar concentration of water (55.3 M). Nonlinear least squares curve fitting procedures using Levenberg-Marquardt algorithm were implemented in Origin 8.5.0 SR1. The Gibbs free energy (ΔG) was calculated according to the equation:

$$\Delta G = -RT \cdot \ln(K)$$

Where R is the gas constant and T is the temperature in Kelvin.

pH-dependence

The pH-dependent partitioning of the peptides into lipid bilayers was investigated by the shift of the position of the peptide intrinsic fluorescence spectral maximum for the pHLIP variants induced by a drop of pH from 8 to 3 by addition of HCl in the presence of POPC liposomes. 3 μ M of the peptide was incubated overnight with 2 mM of 100 nm POPC liposomes, and the pH was decreased by the addition of aliquots of 4, 2, 1 and 0.1 M HCl. The resulting pHs were measured using a micro-electrode probe (Thermo Electron Corporation, Orion Ross Micro pH electrode). Fluorescence spectra were recorded at each pH value. The spectra were analyzed by decomposition algorithms using an on-line PFAST toolkit (Protein Fluorescence And Structural Toolkit: <http://pfast.phys.uri.edu/>) to obtain spectral maxima (λ_{max}). Finally, the positions of the fluorescence spectral maxima (λ_{max}) of the single component solutions were plotted versus pH and the Henderson–Hasselbalch equation was used to fit the data:

$$\lambda_{max} = \lambda_{max}^2 + \frac{(\lambda_{max}^1 - \lambda_{max}^2)}{1 + 10^{n \cdot (pH - pKa)}}$$

where λ_{max}^1 and λ_{max}^2 are the beginning and end of the transition, n is the cooperativity parameter, and the pKa is the midpoint of the transition. The details of assumptions made for the analysis of pH-dependence data could be found in Supplementary.

Stopped-flow fluorescence measurements

Stopped-flow fluorescence measurements were carried out on a SFM-300 mixing apparatus connected to a MOS-450 spectrometer (Biologic, Inc.) under temperature control. All solutions were degassed for several minutes under vacuum before loading into the syringes to minimize air bubbles. pHLIP variants (7 μ M) were pre-incubated with POPC (1.5 mM) at pH 8.0 to reach binding equilibrium, and insertion was induced by fast mixing (5 ms dead time) of equal volumes of pHLIP-POPC variants at pH 8.0 and appropriately diluted HCl, to obtain a drop of pH from 8 to the desired value. Each kinetic curve was recorded several times (~10) and then averaged, excluding the first 2-3 shots.

Cell lines

Human cervix adenocarcinoma (HeLa) and human lung carcinoma (A549) cells were acquired from the American Type Culture Collection. Human lung carcinoma and human cervix adenocarcinoma with stable expression of green fluorescent protein (GFP), A549-GFP and HeLa-GFP, respectively, were acquired from Cell Biolabs Inc.

Cells were authenticated, stored according to 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), 10 μ g/mL of ciprofloxacin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The pH5.9 medium was prepared by mixing 13.3 g of dry DMEM in 1 L of deionized water. Human cervical epithelial cells (HCvEpC), human mammary epithelial cells (HMEpC) and human bronchial epithelial cells (HBepC) were acquired from Cell Applications Inc. Cells were authenticated, stored according to supplier's instructions, and used within 2 months. HCvEpC, HMEpC and HBepC cells were cultured in cervical, mammary and bronchial epithelial cell growth medium, respectively, provided by Cell Applications Inc.

Fluorescence Microscopy

HeLa-GFP and A549-GFP cells were grown in 35-mm dishes with 14-mm glass-bottom windows coated with poly-d-lysine or collagen. Cells were washed with DMEM not containing FBS, pH 5.9 or 7.4 and then incubated with 2 μ M of Rho- or BODIPY-labeled Var-3, K-Var3, Var7 or K-Var7 peptides at pH 5.9 or pH 7.4. After 30 minutes of incubation, cells were washed 4-5 times. Fluorescent images were acquired with a Retiga CCD camera (Qimaging, Burnaby, BC, Canada) mounted to an inverted Olympus IX71 microscope (Olympus America, Inc., Center Valley, PA). The details of cellular uptake quantification could be found in Supplementary data.

Cytotoxicity Assay

HeLa, A549, HCvEpC, HMEpC or HMEpC cells were loaded in the wells of 96-well plates (~5,000 cells per well) and incubated overnight. The increasing amounts of pHLIP variants (1, 2, 4 and 8 μ M) were added to HCvEpC, HMEpC or HMEpC cells, or growth medium was replaced with the medium without FBS containing increasing amounts of pHLIP in case and after 3 hours of incubation an equal volume of the medium containing 20% of FBS was added in case of HeLa and A549 cells. After 24, 48 and 72 hours of incubation, a colorimetric reagent (CellTiter 96 AQueous One Solution Assay, Promega) was added for 1 hour followed by measuring absorbance at 490 nm to assess cell viability. All samples were prepared in triplicate. None of the tested pHLIP variants showed any cytotoxic effect.

Animal studies

Athymic female nude mice ranging in age from 4 to 6 weeks and weighing from 15 to 18 g were obtained from Harlan Laboratories (Indianapolis, IN). In total, 266 mice were used in the experiments, and the number of mice used to study each pHLIP variant can be found in Tables S2-S5 of the Supplementary Data. Mouse tumors were established by subcutaneous injection of HeLa-GFP or A549-GFP cells (10^6 cells/0.1 ml/flank) in the right flank of each mouse. When tumors reached 5-6 mm in diameter, tail vein injections of 100 μ l of Alexa750-pHLIPs (40 μ M) were performed. Animals were imaged at 4 and 24 hours post-injection on a FX Kodak in-vivo image station under gas anesthesia with supplemental heat provided to maintain core body

temperature. Animals were euthanized at 4 or 24 hours, and necropsy was performed immediately after euthanization. Tumors and major organs were collected for further imaging. The tumor/organ ratio was calculated according to the equation:

$$\frac{Tumor}{Organ} = \frac{F_{tumor} - F_{backg}}{F_{organ} - F_{backg}}$$

where F_{tumor} , F_{organ} and F_{backg} are the mean fluorescence intensities of tumor, organ, and background signal, respectively. The contrast-to-noise ratio (CNR) was calculated according to the equation:

$$CNR = \frac{F_{tumor} - F_{backg}}{SD_{backg}}$$

where SD_{backg} is the standard deviation of the background signal. Fluorescence intensity was obtained from image analysis using Kodak 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. All animal studies were conducted according to an approved University of Rhode Island animal protocol (AN07-01-015), in compliance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals.

RESULTS

Design of pHLIP variants

We have reported the basic molecular mechanism of the interaction of WT-pHLIP with lipid bilayers (1, 3, 11). In these studies we found three states of the peptide: State I, in solution as an unstructured monomer at neutral pH when no lipid membrane

is present; State II, bound at the surface of a lipid bilayer as a largely unstructured monomer at neutral pH, and State III, inserted across the bilayer as a monomeric helix at acidic pH. To broaden our understanding of the main principles of pHLIP peptide interactions with membranes and to select the best sequences for clinical use, we employed our knowledge and designed 16 variants of the WT-pHLIP (Var0) sequence:

Var0-WT	ACEQNPIY	<u>WARYADWLFPTPLLLLDLALLV</u>	DADEGT
Var1-2D1D	ACEDQNPY	<u>WARYADWLFPTPLLLLDLALLV</u>	DG
Var2-2D1D	ACEDQNPY	<u>WRAYADLFTPLTLLDLLALW</u>	DG
Var3-3D	ACDDQNP	<u>WRAYLDLLFPTDTLLLDLLW</u>	
Var4-3E	ACEEQNP	<u>WRAYLELLFPTETLLELLW</u>	
Var5-3Da	ACDDQNP	<u>WARYLDWLFPTDTLLLDL</u>	
Var6-3Db	CDNNNP	<u>WRAYLDLLFPTDTLLLDW</u>	
Var7-3Ea	ACEEQNP	<u>WARYLEWLFPTETLLEL</u>	
Var8-3Eb	CEEQQP	<u>WAQYLELLFPTETLLEW</u>	
Var9-3Ec	CEEQQP	<u>WRAYLELLFPTETLLEW</u>	
Var10-2D	ACEDQNP	<u>WARYADWLFPTTLLLD</u>	
Var11-2E	ACEEQNP	<u>WARYAEWLFPTTLLLE</u>	
Var12-1D	ACEDQNP	<u>WARYADLLFPTTLAW</u>	
Var13-1E	ACEEQNP	<u>WARYAELLFPTTLAW</u>	
Var14-Rev	Ac-TEDAD	<u>VLLALDLLLLPTTFLWDAYRAW</u>	YPNQECA-Am
Var15-2N	CDDDDNPNY	<u>WARYANWLFPTPLLLLNALLV</u>	EAEET

Var16-2P CDDDDNPNY WARYAPWLFTTPLLPGALLV EAEET

Biophysical studies

First, we studied the interaction of the pHLP variants with lipid bilayers, employing the fluorescence and CD spectroscopic techniques previously used (3). The intrinsic tryptophan fluorescence and CD spectra of non-labeled variants measured in solution at pH8 in the absence and presence of POPC liposomes and in the presence of liposomes at low pH (pH 4-5) indicate that each peptide interacts with a lipid bilayer in a pH-dependent manner and that the peptides form transmembrane helices in state III (Supplementary Table S1 and Figure S1).

The partitioning of pHLP variants into bilayers at pHs 8 and 4-5 was assessed in titration experiments by measuring changes of intrinsic peptide fluorescence (Figure 1). On average, the partitioning of the variants into the membrane at low pH is about 50 fold higher than at high pH. As expected, the truncated variants Var5-13 have lower affinities for the membrane at neutral pH compared to WT and other variants, since a number of hydrophobic residues were removed from the sequences. In general, variants with Glu residues have slightly higher affinities compared to the same variants with Asp (since Glu is more hydrophobic due to the additional methylene group). The highest affinity was observed for Var15, which forms a helical structure at neutral pH. At low pH, the lowest affinity for the lipid bilayer was observed for the most truncated versions Var10-13 and variants (Var15 and 16) with no protonatable residues in the TM, which reflects the reduced stability of the TM helix. The numbers

below are the differences between the Gibbs free energies of the interactions of variants with the membrane at low and high pHs (in kcal/mol): $\Delta\Delta G = \Delta G_{\text{pH4}} - \Delta G_{\text{pH8}}$

Var1	Var2	Var3	Var4	Var5	Var6	Var7	Var8	Var9	Var10	Var11	Var12
2.17	0.28	2.23	1.72	2.31	1.93	2.39	2.19	2.07	1.79	1.94	1.95
Var13	Var14	Var15	Var16								
1.15	1.78	0.82	1.47								

The above numbers reflect both the differences in binding affinities, and to some degree, the strength of the helical structure formation in the membrane. The largest differences are observed for Var3, Var5, and Var7. The smallest differences are found for Var2 and Var15 due to the strong interactions of these peptides with the bilayer in state II at pH8.

The pKa of peptide insertion is determined by the pKa of protonation of Asp/Glu residues in the TM and at the inserting end of the peptide. As charged carboxyl groups located at the transition zone between the aqueous and hydrophobic environments sink more deeply into the bilayer, the pKa is progressively raised by the reduced dielectric constant, and the pKa of Asp/Glu residues increases. Thus, all truncated variants (Var5-13), which demonstrated weaker interactions with lipid bilayer surfaces at neutral and high pHs, would have lower pKa's of insertion. The pKa of pHLIP variant insertion was measured by following the shift of the position of the intrinsic peptide emission maximum as the pH is changed from 8 to 2 (Figure 2). As expected, the shift of pKa to lower pHs correlates with the truncation of the pHLIP sequence. Thus, the

pKa of Var10, with a single Asp residue in its TM, is the lowest (pKa=4.5). All variants containing Glu residues (Var7-9, 11, 13) have higher pKa values compared to the related sequences with Asp residues (Var5-6, 10, 12), again as expected if the increased hydrophobicity of the Glu causes a deeper association with the bilayer, lowering the dielectric and raising the pK.

To this point, we have considered the equilibrium energies of progressive binding and insertion events, but it is also important to note that sequence variation will alter the kinetics of insertion. The barrier for insertion includes the resistance to the passage of the inserting terminal end of the peptide from the aqueous compartment outside to the aqueous compartment inside a cell or liposome. We have found in recent work that the kinetics of insertion correlates with the number of protonatable groups at the inserting end or the presence of polar cargo (12). The characteristic membrane insertion time for WT-pHLIP is about 30 sec, and slightly faster kinetics were observed for Var14, which is the reverse sequence of WT. Var14 has the same number of protonatable residues at its N-terminus as WT has at its C terminus, except for the free C-terminus itself. The data are consistent with the view that the acetylated N-terminus of Var14 inserts across the bilayer and the amidated C-terminus stays outside, reversing the direction of insertion. The characteristic time of Var1-2 insertion, which has fewer protonatable residues at its C-terminus than WT, was about 1-2 sec, which correlates well with our recent findings (12). Further truncation of the peptide inserting end, which reduces the number of protonatable residues, resulted in faster peptide insertion, completed within the first 30-100 msec for Var3-13. Var15-16 have no protonatable

residues in the TM, while they have the same number of Asp/Glu as WT at the inserting end, and as a result the time of insertion was reduced to 10-11 sec relative to WT. The kinetics data are shown in Figure 3.

Tumor targeting and distribution of Alexa750-pHLIPs in organs

We used a mouse xenograft model to assess the tumor targeting and organ distribution of the pHLIP variants. Tumors were created by subcutaneous injection of HeLa-GFP cancer cells in the right flanks of each mouse, and were grown to about 5 mm in diameter. To perform biodistribution studies, pHLIP variants were conjugated with Alexa750. We demonstrated that the kinetics of insertion of Alexa750-WT-pHLIP into lipid bilayers is similar to the kinetics of insertion of non-labeled peptide (Supplementary Figure S2). Fluorescently labeled pHLIPs were given as single intravenous injections, and the major organs and tumors were collected at 4 and 24 hours after fluorescent pHLIP administration. The GFP and NIR signals were monitored in tumors and organs after sacrificing the mice. The mean intensities of NIR fluorescence normalized to the signal in kidneys at 4 hours are shown on Supplementary Figure S3. The mean intensities of NIR fluorescence of all organs and the number of mice used in the study for each variant are given in Supplementary Table S2 and Table S3.

The pHLIP variants that show higher affinities for membranes at high pH have higher short-term liver uptake (Var 1, 2, 4, 14-16), however the liver is cleared at 24 h except for Var1. Var3 has high tumor targeting compared to kidney and compared to other

pHLIPs as well. Whole-body (data not shown) and organ imaging confirmed that the truncated variants Var5-13 have much faster blood clearance than WT, and at 4 hours the signal has accumulated in the tumor, while being washed out of the organs. Tumor targeting was minimal for the shortest pHLIP variants Var12-13. The tumor/muscle, tumor/kidney and tumor/liver ratios are given in Figure 4 for all pHLIP variants calculated at 4 and 24 h after injection. Variants 0, 3-4, 14-16 have the highest tumor/muscle ratio at 24 hours. The same pHLIP variants have the highest tumor/kidney ratios, while only Var3 shows significantly less accumulation in the liver. Among the truncated pHLIP variants, Var7 demonstrates the highest tumor targeting.

This body of work allows us to propose different pHLIP variants for different applications. We have selected Var3 and Var7 as the best candidates for future studies for fluorescence/MRI and SPECT/PET imaging, respectively, guided by the fact that the imaging time window for the stable fluorescence and MRI agents can be much wider than for the short half-life SPECT and PET agents. Var3 had the highest tumor/organ ratios, especially at 24 hours after construct administration. Var7 had a lower tumor/organ ratio, but showed much faster blood clearance. The cytotoxicity of the selected pHLIP variants was evaluated on human cervix adenocarcinoma cells (HeLa), human lung carcinoma (A549) cells as well as human cervical (HCvEpC), mammary (HMEpC) and bronchial (HBEPc) epithelial cells. Peptides at concentrations of up to 8 μ M were evaluated up to 72 hours incubation with cells, and no toxicity was observed.

pH-insensitive K-pHLIPs

As a control, we have previously used K-pHLIP-WT, where the two key Asp residues in the TM part of the peptide were replaced by Lys residues (1). K-pHLIP cannot interact with a membrane in a pH-dependent manner over the range of neutral and low pHs we are studying, and therefore should not target acidic tumors. Because of the potential clinical applications of Var3 and Var7 we synthesized and tested K-versions of each:

K-Var3   ACDDQNP WRAYLKLLFPTKTLLLKLLW

K-Var7   ACEEQNP WARYLKWLFPTKTLLLKL

Each of these peptides failed to interact with lipid bilayers in a pH-dependent fashion (Figure 5a-b). No shift in the position of the peptide fluorescence maximum was seen for either of the K-pHLIPs in the presence of lipid as a result of pH decrease, and the CD spectra do not change and do not exhibit the minima at 208 and 225 nm that are characteristic signals reporting alpha-helical conformations. The CD spectra reveal a negative band at 234 nm and a positive band at 221 nm, suggestive of an exciton split doublet (3, 13) possibly arising from peptide aggregation.

Var3 and Var7, as well as K-Var3 and K-Var7 were conjugated with two different fluorescent dyes: zwitterionic, tetramethylrhodamine (Rho), and non-charged, BODIPY to ensure that distribution and localization of fluorescent peptides is independent of the choice of dye. The fluorescence images demonstrating cellular uptake of Rho- and BODIPY-labeled Var3, Var7, K-Var3, and K-Var7 are shown in

Figure 5c-d and Supplementary Figure S4, respectively. The fluorescence signal of cells treated with fluorescent Var3 and Var7 at pH7.4 was weaker compared to cells treated with peptides at pH5.9. The cellular uptake of Var3 was 1.12 times higher at low pH compared to neutral pH, and the uptake of Var7 was 1.34 times higher at low pH. Our biophysical data show that the affinity of Var3 for a membrane at pH8 is higher than the affinity of Var7 at the same pH, therefore we see slightly higher cellular uptake of Var3 at pH7.4 compare to Var7 and a less pronounced difference in cellular uptake between neutral and low pHs for Var3. For the cases of K-Var3 and K-Var7, the fluorescence signal was very similar when cells were treated at either high or low pHs; moreover, the fluorescence signal at pH7.4 was slightly higher than at pH5.9. Also, the distributions of fluorescent signals for D- and E-variants and K-variants were different. K-Var3 and K-Var7 were distributed as cytoplasmic dots. In contrast to pHLIP variants, K-pHLIPs are positively charged and partially aggregated, and, most probably, might be taken up by endocytosis and trapped in endosomes in a pH-independent manner, while cellular uptake of Var3 and Var7 is pH-dependent. Biodistribution and tumor targeting of Alexa750-K-pHLIPs were also investigated using xenograft models in mice. In contrast to Var3 and Var7, K-pHLIPs did not show significant tumor targeting (Figure 5e-f and Supplementary Table S4). To demonstrate that tumor targeting by Var3 and Var7 and the lack of tumor targeting by K-Var3 and K-Var7 is independent of choice of tumor model and imaging fluorescent probe, we used BODYPI labeled Var3, Var7, K-var3 and K-Var7, which were tested on tumors created by subcutaneous injection of A549-GFP human lung carcinoma cells. The organs were collected at 4 hours and analyzed (Supplementary Table S5). The tumor

to muscle ratio at 4 hours for Var3 and Var7 labeled with BODIPY and Alexa750 was 6.0 ± 0.9 , while for K-Var3 and K-Var7 it was 2.6 ± 0.3 (Figure 5g). Thus, the replacement of the Asp/Glu residues by Lys in the TMs of Var3 and Var7 leads to the loss of both pH-dependent interactions with membranes and targeting of acidic tumors, supporting a central role for the carboxyl groups in targeting.

DISCUSSION

Rapid cell growth and an inadequate blood supply produce hypoxic conditions that cause a partial use of glycolysis in tumor cells, resulting in acidification of the cytosol, to which the cell adjusts by pumping protons into the external environment. But, hypoxia and low blood supply are not the only mechanisms responsible for the development of an acidic environment within solid tumors (14). Malignant cancers have an elevated glucose uptake even under normal oxygen conditions, overwhelming the mitochondrial capacity and using glycolysis for the overflow. This condition is known as “aerobic glycolysis” or the Warburg effect (15). Cells exhibiting a Warburg effect catabolize glucose at a high rate (16, 17), and the use of glycolysis results in a much higher level of production of H^+ and lactic acid, which are pumped across cell plasma membranes into the extracellular space, where they accumulate in poorly perfused regions (18-20). In addition to the lactic acid-output, intracellular titration of acid with bicarbonate and the engagement of the pentose phosphate shunt releases CO_2 from tumor cells. Expression of carbonic anhydrase 9 and 12 on the tumor cell surface catalyzes the extracellular trapping of acid by hydrating cell-generated CO_2

into HCO_3^- and H^+ (21, 22). These mechanisms combine to create an acidic extracellular milieu favoring tumor growth, invasion, and development.

The pHLIP peptides can exploit tumor acidity as a useful biomarker. Based on the results of our previous and current investigations, design principles can be formulated to set directions for different clinical uses:

- i) all pHLIP peptides have a membrane-binding sequence, which contains a number of hydrophobic residues essential for membrane targeting;
- ii) a pHLIP is stable across a lipid bilayer at low pH;
- iii) to achieve pH-dependent targeting, at least one protonatable group (Asp or Glu, or any other) needs to be in the sequence;
- iv) in addition to the membrane-binding sequence, a pHLIP peptide may contain a membrane inserting sequence (the C-terminus for most investigated pHLIPs or the acetylated N-terminus of the reverse pHLIP sequence), which crosses the lipid bilayer and emerges in the cytoplasm. The protonatable residues could be only in the inserting sequence or in both the TM and inserting parts of the peptide;
- v) the number of protonatable residues increases the cooperativity of the transition, narrowing the pH range over which insertion occurs;
- vi) the non-inserting flanking sequence may be used for modulation of peptide solubility, for example by adding polar and negatively charged residues at the non-inserting end (all pHLIP peptides are soluble in aqueous solution at neutral and high pHs).

The list of the major biophysical properties that affect tumor targeting by pHLIP peptides could be found in Supplement data.

Based on the studies reported here, and motivated by potential clinical applications, we selected two pHLIP variants for further development (in addition to WT-pHLIP): Var3 and Var7. For each of these variants we tested versions with the TM carboxyl groups replaced by amino groups (D to K and E to K), and showed that the K-peptides lose their abilities to interact with membranes in a pH-dependent manner and to target tumors *in vivo*. Var3 demonstrates high tumor to organ ratios at 24 hours (similar to WT) and could be well suited for optical imaging or MRI one day after construct administration. Var3 might also be suitable for fluorescence-guided surgery, marking a tumor with fluorescently-labeled pHLIP and performing surgery on the next day, when the contrast index is the highest. Var7 is one of the truncated versions of pHLIP, which could be appropriate for SPETC/PET imaging, since it demonstrates fast tumor targeting and blood clearance.

In this report, we show that the biophysical principles that guide pHLIP peptide interactions with lipid bilayers can be used to determine peptide performance *in vivo*, and we identify two pHLIP variants as appropriate candidates for uses in clinical imaging of acidic tissues, such as solid tumors.

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REFERENCES

1. Andreev OA, Dupuy AD, Segala M, Sandugu S, Serra DA, Chichester CO, et al. Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues in vivo. *Proc Natl Acad Sci U S A*. 2007;104:7893-8.
2. Musial-Siwiek M, Karabadzhak A, Andreev OA, Reshetnyak YK, Engelman DM. Tuning the insertion properties of pHLIP. *Biochim Biophys Acta*. 2010;1798:1041-6.
3. Reshetnyak YK, Segala M, Andreev OA, Engelman DM. A monomeric membrane peptide that lives in three worlds: in solution, attached to, and inserted across lipid bilayers. *Biophys J*. 2007;93:2363-72.
4. Barrera FN, Weerakkody D, Anderson M, Andreev OA, Reshetnyak YK, Engelman DM. Roles of carboxyl groups in the transmembrane insertion of peptides. *J Mol Biol*. 2011;413:359-71.
5. Reshetnyak YK, Yao L, Zheng S, Kuznetsov S, Engelman DM, Andreev OA. Measuring tumor aggressiveness and targeting metastatic lesions with fluorescent pHLIP. *Mol Imaging Biol*. 2011;13:1146-56.
6. Vavere AL, Biddlecombe GB, Spees WM, Garbow JR, Wijesinghe D, Andreev OA, et al. A novel technology for the imaging of acidic prostate tumors by positron emission tomography. *Cancer Res*. 2009;69:4510-6.
7. Macholl S, Morrison MS, Iveson P, Arbo BE, Andreev OA, Reshetnyak YK, et al. In Vivo pH Imaging with (99m)Tc-pHLIP. *Mol Imaging Biol*. 2012.

8. Reshetnyak YK, Andreev OA, Lehnert U, Engelman DM. Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix. *Proc Natl Acad Sci U S A*. 2006;103:6460-5.
9. An M, Wijesinghe D, Andreev OA, Reshetnyak YK, Engelman DM. pH-(low)-insertion-peptide (pHLIP) translocation of membrane impermeable phalloidin toxin inhibits cancer cell proliferation. *Proc Natl Acad Sci U S A*. 2010;107:20246-50.
10. Wijesinghe D, Engelman DM, Andreev OA, Reshetnyak YK. Tuning a polar molecule for selective cytoplasmic delivery by a pH (Low) insertion peptide. *Biochemistry*. 2011;50:10215-22.
11. Andreev OA, Karabadzhak AG, Weerakkody D, Andreev GO, Engelman DM, Reshetnyak YK. pH (low) insertion peptide (pHLIP) inserts across a lipid bilayer as a helix and exits by a different path. *Proc Natl Acad Sci U S A*. 2010;107:4081-6.
12. Karabadzhak Alexander G, Weerakkody D, Wijesinghe D, Thakur Mak S, Engelman Donald M, Andreev Oleg A, et al. Modulation of the pHLIP Transmembrane Helix Insertion Pathway. *Biophysical journal*. 2012;102:1846-55.
13. Roy RS, Gopi HN, Raghothama S, Gilardi RD, Karle IL, Balaram P. Peptide hairpins with strand segments containing alpha- and beta-amino acid residues: cross-strand aromatic interactions of facing Phe residues. *Biopolymers*. 2005;80:787-99.

14. Chiche J, Brahimi-Horn MC, Pouyssegur J. Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer. *J Cell Mol Med.* 2010;14:771-94.
15. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *J Gen Physiol.* 1927;8:519-30.
16. Gillies RJ, Robey I, Gatenby RA. Causes and consequences of increased glucose metabolism of cancers. *J Nucl Med.* 2008;49 Suppl 2:24S-42S.
17. Newell K, Franchi A, Pouyssegur J, Tannock I. Studies with glycolysis-deficient cells suggest that production of lactic acid is not the only cause of tumor acidity. *Proc Natl Acad Sci U S A.* 1993;90:1127-31.
18. Grillon E, Farion R, Fablet K, De Waard M, Tse CM, Donowitz M, et al. The spatial organization of proton and lactate transport in a rat brain tumor. *PLoS One.* 2011;6:e17416.
19. Rehncrona S. Brain acidosis. *Ann Emerg Med.* 1985;14:770-6.
20. Xiong ZG, Pignataro G, Li M, Chang SY, Simon RP. Acid-sensing ion channels (ASICs) as pharmacological targets for neurodegenerative diseases. *Curr Opin Pharmacol.* 2008;8:25-32.
21. Swietach P, Vaughan-Jones RD, Harris AL. Regulation of tumor pH and the role of carbonic anhydrase 9. *Cancer Metastasis Rev.* 2007;26:299-310.
22. Ihnatko R, Kubes M, Takacova M, Sedlakova O, Sedlak J, Pastorek J, et al. Extracellular acidosis elevates carbonic anhydrase IX in human glioblastoma cells via transcriptional modulation that does not depend on hypoxia. *Int J Oncol.* 2006;29:1025-33.

FIGURES

Figure 1

Figure 1. Titration of pHLIP variant binding to liposomes at high and low pHs.

The titration of the pHLIP variants with increasing concentration of POPC liposomes at pH8 (*a*) and pH4-5 (*b*) are followed using intrinsic peptide fluorescence changes. For comparison the mole-fraction partition coefficients for Var0 (WT-pHLIP) at pH8 and 5 are 4.87×10^4 and 1.54×10^6 , respectively, and the corresponding Gibbs free energies are 6.04 and 7.98 kcal/mol.

Figure 2

Figure 2. pH-dependent bilayer insertion of variants. Peptide intrinsic fluorescence changes are used to follow the insertion as a function of pH (transition from the state II to state III as the pH is lowered).

Figure 3

Figure 3. Kinetics of insertion. Representative kinetic curves for the insertion of variant peptides into the lipid bilayer are shown, the characteristic time of insertion of Var0 (WT-pHLIP) is about 40 sec.

Figure 4

Figure 4. Tumor to organ fluorescence ratios. Tumor to muscle, tumor to kidney, and tumor to liver ratios calculated at 4 and 24 hour time points are shown for all pHLIP variants.

Figure 5

Figure 5. Comparisons with control K-pHLIPs. The CD (*a*) and tryptophan fluorescence (*b*) spectra of K-Var3 and K-Var7 show that these variants lose the ability to interact with a lipid bilayer in a pH-dependent manner. (*c-d*) Cellular uptake at pH7.4 and 5.9 of Var3, K-Var3, Var7, and K-Var7 labeled with Rhodamine are shown. The cellular uptake of four peptides labeled with BODIPY are shown at both pHs in Supplementary Figure S4. (*e-f*) Whole-body NIR fluorescence images of mice obtained at 4 and 24 hours after *i.v.* administration of Var3, K-Var3 (*e*) and Var7, K-Var7 (*f*) conjugated with Alexa750. The tumor is indicated by arrows on the NIR images as reported by the GFP signal shown in adjacent images. Images obtained at different time points are presented with maximum contrast, the contrast-to-noise ratios are: Var3, 4h – 103; Var3, 24h – 186; Var7, 4h – 85; Var7, 24h – 360; K-Var3, 4h – 87; K-Var3, 24h – 54; K-Var7, 4h – 22; K-Var7, 24h – 16. (*g*) The A549-GFP tumor to muscle ratios calculated for 4 hours for BODIPY-pHLIPs and -K-pHLIPs and HeLa-GFP tumor to muscle ratios calculated for 4 and 24 hours time points for Alexa750-pHLIPs and -K-pHLIPs are shown. The mean fluorescence of HeLa-GFP and A549-GFP tumors and organs at 4 and 24 hours after Alexa750-K-pHLIPs and at 4 hours after BODIPY-pHLIPs and -K-pHLIPs administration are given in the Supplementary Table S4 and S5, respectively.

SUPPLEMENTARY DATA

MATERIALS AND METHODS

Conjugation of pHLIP variants with fluorescent dyes

The lyophilized powder of a peptide was dissolved in a solution containing 3M urea, and the peptide solution was transferred to buffer using a G-10 size-exclusion spin column, or was dissolved directly in phosphate buffer at pH8 for biophysical studies. The concentrations of the peptides were determined by absorbance (for Var0-2 and Var14-16: $\epsilon_{280} = 13,940 \text{ M}^{-1} \text{ cm}^{-1}$; and for Var3-13 and K-Var3, K-Var7: $\epsilon_{280} = 12,660 \text{ M}^{-1} \text{ cm}^{-1}$).

For studies on cultured cells and animals, each pHLIP variant was conjugated with AlexaFluor 750 C₅-maleimide (Invitrogen, catalog #A30459), and Var3, Var7, K-Var3, and K-Var7 were conjugated with Tetramethylrhodamine-5-maleimide, single isomer (Invitrogen, catalog #T6027) or BODIPY-TMR C₅-maleimide (Invitrogen, catalog #B30466) in DMF or DMSO at a ratio of 1:1.1 of dye:peptide and incubated at room temperature for about 6 hours and then at 4°C until the conjugation reaction was completed. The reaction progress was monitored by reverse phase HPLC. The purity of products was assessed by analytical HPLC and peak identity was confirmed by SELDI-TOF mass spectrometry.

Liposome preparation

Large unilamellar vesicles (LUVs) were prepared by extrusion. POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids, Inc.) dissolved in chloroform at a concentration of 1 mg/ml were desolvated on a rotary evaporator and dried under high vacuum for several hours. The phospholipid film was then rehydrated in 100 mM phosphate buffer, pH8.0, vortexed for 2 hours, and repeatedly extruded through membranes with 100 or 50 nm pore sizes to obtain LUVs.

Oriented circular dichroism measurements

Oriented circular dichroism (OCD) was measured from supported bilayers deposited on a stack of quartz slides with special polish for far UV measurements, with spacers of 0.2 mm thickness on one side of each slide (Starna). Quartz slides were cleaned by sonication for 10 min in cuvette cleaner solution (Decon Contrad 5% in water), 2-propanol, acetone, 2-propanol, and rinsed with deionized water. Then the slides were immersed in a mixture of concentrated sulfuric acid and hydrogen peroxide (ratio 3:1) for 5-10 min to completely remove any remaining organic material from the slides. Slides were then thoroughly rinsed with and stored in deionized water (Milli-Q purified water kept at 25 °C). A POPC lipid monolayer was deposited on the clean quartz substrate by the Langmuir-Blodgett (LB) method using a KSV minitrough. For the LB deposition, a POPC lipid solution in chloroform was spread on the subphase and allowed to evaporate chloroform for about 30 min, followed by monolayer compression to 32 mN/m. An initial layer was deposited by retrieving the slide from

the subphase at a rate of 15 mm/min. The second layer of the bilayer was created by fusion. For this step, the monolayer on the slide was incubated with a solution of POPC vesicles (50 nm in diameter obtained by extrusion) mixed with the peptide solution at pH4 (0.5 mM POPC and 10 μ M peptide). The fusion occurred during 6 hour incubation at 100% humidity. Then, excess vesicles were carefully removed and the slides were stacked to make a pile filled with the peptide solution (5 μ M) at pH4. Bilayers with the peptide solution were allowed an additional 6 hour equilibration. Measurements were taken at 3 steps during the process: when the monolayers were incubated with the excess of liposomes, soon after spaces between slides were filled with the peptide solution, and 6 hours after the second measurement. 14 slides (28 bilayers) were assembled and OCD spectra were recorded on a MOS-450 spectrometer with 2 s sampling time. Control measurements were carried out of the peptide between slides with and without supported bilayers and in the presence of an excess of POPC liposomes.

Assumptions made for the analysis of pH-dependence data

Peptide intrinsic fluorescence changes are used to follow the insertion as a function of pH (transition from the state II to state III as the pH is lowered). Decomposition of the fluorescence spectra measured at various pHs gives the positions of the maxima, and the single component solutions are shown. The Henderson-Hasselbalch equation with cooperativity was used to fit the experimental points, and the fitting curves and 95% confidence intervals are shown by the red and blue lines, respectively. Each graph notes the apparent pK of the peptide's insertion into the membrane obtained from the

fitting of the experimental data. The pKa of Var0 (WT-pHLIP) is 6.0. The pKa values obtained by fitting of the experimental data by the Henderson-Hasselbalch equation with a fixed cooperativity parameter ($n=1$) are very similar to the data shown in the figure: Var1=6.14; Var2=5.59; Var3=5.09; Var4=5.32; Var5=4.84; Var6=5.06; Var7=5.51; Var8=5.75; Var9=5.59; Var10=4.92; Var11=5.52; Var12=4.52; Var13=5.16; Var14=5.69; Var15=5.59; Var16=5.63. It is assumed that there is a linear relation between the position of the maximum and the contribution of state II (or state III) (state I is the peptide in solution at pH8; state II is the peptide in the presence of POPC liposomes at pH8; state III is the result of folding and insertion of the peptide with POPC when the pH is dropped from 8 to 3.6 by the addition of an aliquot of HCl). However, due to the fact that the quantum yields in states II and III are slightly different, there was a slight non-linearity, so we completed the analysis by estimating the contributions of the states for WT-pHLIP and found that the apparent pK shifts no more than on 0.05 pH units toward lower pHs. Since this shift is less than the experimental error, we present the pKa values for transitions from the state II to III based on the analysis of the positions of spectral maxima.

Quantification of cellular uptake

For quantification of cellular uptake of BODIPY-labeled Var3 and Var7 at pH7.4 and 5.9 we used A549-GFP cells in suspension. 2 μ M of fluorescently labeled peptides were incubated with cells in DMEM media at pH7.4 and 5.9 for 30 min at 37°C and 5% CO₂. Then, cells were pelleted by centrifugation (2000 rpm, 4 min) at room temperature, the supernatant was removed and the pellet was washed three times with

the same media and resuspended in 50 μ L, and the cell suspension solution was placed into cell counting chambers. The phase-contrast, GFP fluorescent, and BODIPY fluorescent signals were measured and analyzed using ImagePro Plus.

Biophysical properties, which affect tumor targeting

- The apparent pK of peptide insertion into membrane determines the proportion of available peptide molecules inserted into plasma membrane of cancer cells at various pHs. Currently we have pHLIP peptides with pKas varying over the range from 4.5 to 6.5 for insertion into POPC lipid bilayers. Various pHLIP variants might be used to probe the pHs of more or less acidic tumors. The insertion of pHLIP responds to the pH at the surfaces of cancer cells, not that of the surrounding fluid. Due to the transmembrane potential gradient, protons accumulate at the surfaces of cells in close vicinity of membrane, and the pH is lower than in the bulk extracellular environment or in blood. Current methods of measuring pH *in vivo* report the average value of extracellular pH in tumors, which can vary from 6.0 to 7.2, but the pH at the surfaces of cancer cells are likely to be lower.
- pHLIP is a relatively hydrophobic peptide, therefore significant initial association with a lipid bilayer or plasma membrane occurs at neutral pH. The strength of this interaction (state II) is determined by the balance of hydrophobic and polar/charged residues in the pHLIP sequence. Stronger partitioning of the peptides onto bilayers at neutral pH increases the time of circulation in the blood. A long circulation time is a disadvantage for SPECT/PET imaging, since the short half-life-time probes are used

and tumor targeting and blood clearance need to be very fast (at least within 4 hours). However, long circulation times of pHLIP variants in the blood could be very well suited for fluorescence or MR imaging, as well as for the delivery of therapeutic agents. We find that all pHLIP variants that have higher affinities for membranes at neutral pH show high tumor/organ ratios at 24 hours post-injection.

- An important parameter is the difference between the Gibbs free energy of peptide binding to membranes at low pH vs. high pH. This parameter reflects the difference in the affinity of the peptide to the membrane at low and neutral pH, as well as the strength of the formation of TM helix at low pH. A larger $\Delta\Delta G$ will ensure a greater differentiation between the inserted and non-inserted peptides as a function of pH.
- For applications *in vivo*, the kinetics of peptide insertion across the lipid bilayer are important for rapid equilibration with tissues and clearance from the blood. Based on insertion kinetics we can group all pHLIP peptides into: i) the peptides with protonatable residues both in the TM and in the inserting end show the slowest kinetics of insertion (minutes); ii) peptides that are truncated at the inserting end and have few or no protonatable residues at the inserting end, partition in bilayer much faster (seconds); iii) peptides that have protonatable residues at the inserting end but not in the TM show intermediate times of insertion (~20 s); and finally, peptides with only one protonatable residue in the TM have the fastest kinetics of insertion, which coincides with time of formation of helical structure (~100 msec).
- Exit kinetics should be different in cells and liposomes, since peptides that have protonatable residues that are translocated across a cell membrane move them to the

neutral pH of the cytoplasm. To exit the cell membrane, these groups must be protonated, which is much less likely in the cytoplasm, so these peptides become anchored in a cell. Such an “anchor” can significantly (by orders of magnitude) reduce the rate of peptide exit, and the peptide could stay in the plasma membrane for weeks. Such an effect would explain our observation that mouse tumors are stably labeled with fluorescent WT-pHLIP.

FIGURES

Figure 1

Figure S1. Three states of pHLIP variants. The pHLIP variants were studied for the presence of the three basic states of pHLIP: state I is the peptide in solution at pH8; state II is the peptide in the presence of POPC liposomes at pH8; state III is the folding and insertion of the peptide with POPC when the pH is dropped from 8 to 3.6 by the addition of an aliquot of HCl. The states are monitored by changes of the

steady-state tryptophan fluorescence at $\lambda_{\text{ex}}=295$ nm (*a*) and CD (*b*) spectroscopic signals. The concentrations of the peptides and POPC were 7 μM and 1.5 mM, respectively. **State I:** in aqueous solution at pH8 and in the absence of lipids, all variants except Var16 (and to some degree Var1) have long wavelength emission maxima and are largely unstructured. Further, the addition of several Asp residues to the N-terminus (Var15) significantly increases peptide solubility compared to a similar sequence investigated previously, which has no Asp at the N-terminus. **State II:** when liposomes are added to the solution, the majority of the peptide is bound at the surface of the membrane, as observed by a shift of the emission maxima to shorter wavelengths and an increase of fluorescence intensity. In the cases of Var4, 15, and 16, partial formation of alpha-helical structure is seen, while all other pHLIP variants are predominantly unstructured. **State III:** when the pH is lowered, we observe the formation of a helical structure and further propagation of tryptophan residues into the membrane, reflected by a further blue shift of the fluorescence maximum and a further increase of intensity relative to State II, with the single exception of Var4. There is some variation in the positions of the maxima and the increases of intensity in states II and III for different peptides, which could result from differences in the positioning of the tryptophan fluorophores as a result of the sequence changes. The strength of the helical signal is smallest for Var12 and 13 in State III, which most probably have less stable TM configurations given their lengths. For the variants 1, 2, 3, 7, 15, and 16, the oriented circular dichroism (OCD) signal was measured at low pH on supported bilayers (green curves). In each case the helical orientation was found to be TM.

Figure S2. Comparison of the kinetics of WT-pHLIP and Alexa750-WT-pHLIP insertion into the membrane. The changes of tryptophan fluorescence signal of Alexa750-WT-pHLIP in the presence of POPC liposome in a result of pH drop from 8 to 4-5 was recorded in a stopped-flow apparatus, multiplied by (-1), normalized, and compared with the kinetics of insertion of non-labeled WT.

Figure S3. Distribution of Alexa750-pHLIP in organs and tumors. Normalized mean NIR fluorescence of tumor and organs at 4 and 24 hours after construct administration are shown (the signals in kidney at 4 hours are taken as 100%). The non-normalized values of fluorescence are given in Supplementary Tables S2 and S3.

Figure S4. Cellular uptake of BODIPY labeled Var3, Var7, K-Var3, and K-Var7.

Fluorescence and phase-contrast images of cells treated with fluorescence peptides at pH7.4 and 5.9 for 30 min and washed 4-5 times of the same media are shown. Also, cell experiments were carried out with Alexa750-labeled Var3, Var7, K-Var3, and K-Var7. Despite the fact that the signal was low (due to the low NIR sensitivity of the Retiga CCD camera) the distribution of the signal in cells was the same as in the case of Rho and BODIPY-labeled peptides.

TABLES

Parameters	Name	State I	State II	State III	Name	State I	State II	State III
$\lambda_{\max}$ S $\theta_{225} \times 10^3$	Var1	349 1 -2.42	345 1.47 -3.85	339 1.81 -9.46	Var9	353 1 -2.39	350 1.28 -3.20	339 2.10 -7.90
	Var2	350 1 -2.74	345 2.08 -3.76	342 2.76 -8.64	Var10	350 1 -2.40	347 1.47 -2.46	340 2.86 -8.40
	Var3	353 1 -2.07	347 1.66 -3.94	338 2.08 -13.49	Var11	351 1 -2.14	345 2.60 -3.08	340 3.20 -9.02
	Var4	353 1 -2.32	340 2.66 -10.14	345 3.32 -15.28	Var12	353 1 -0.82	352 1.36 -0.96	341 2.90 -4.80
	Var5	351 1 -2.20	355 2.05 -3.38	340 3.52 -6.25	Var13	355 1 -1.76	349 1.72 -2.28	340 3.61 -6.14
	Var6	353 1 -2.12	350 0.96 -2.89	339 1.64 -6.95	Var14	351 1 -2.00	346 1.54 -3.79	344 2.94 -9.91
	Var7	352 1 -2.64	345 1.74 -4.12	340 3.08 -8.82	Var15	351 1 -4.30	339 2.14 -8.94	339 2.52 -10.08
	Var8	353 1 -0.89	353 1.19 -1.22	337 2.48 -9.43	Var16	346 1 -5.41	345 1.89 -7.03	339 2.14 -8.57

Table S1. The three states of each pHLIP variant. The spectral parameters of the pHLIP variants in states I, II, and III are presented. The parameters were obtained from analysis of the fluorescence and CD spectra shown in Figure 1. The maximum position of the fluorescence spectrum, $\lambda_{\max}$, in nm; S is the normalized area under the spectra (normalization was done on the area under the spectrum in State I); $\theta_{225} \times 10^3$, $\text{deg cm}^2 \text{dmol}^{-1}$ – the molar ellipticity at 225 nm.

	Var0-WT <i>n</i> = 9	Var1-2D1D <i>n</i> = 6	Var2-2D1D <i>n</i> = 5	Var3-3D <i>n</i> = 7	Var4-3E <i>n</i> = 4	Var5-3Da <i>n</i> = 5
Kidney	3630.6±485.6	4324.0±444.6	3456.3±585.4	6125.6±1004.1	6755.6±698.7	3097.0±528.3
Tumor	1373.8±334.5	1812.4±819.0	1145.3±737.5	3054.9±726.2	2439.3±443.2	559.4±95.6
Heart	621.4±152.3	752.9±398.4	554.6±421.2	855.0±331.6	980.5±187.3	334.0±43.4
Lungs	1003.6±297.5	1016.6±567.7	761.1±506.8	803.1±256.2	1077.3±84.2	405.4±34.5
Liver	1651.4±470.2	2309.7±1457.0	1432.4±950.8	1829.6±741.6	3615.3±1099.4	561.2±103.0
Spleen	546.4±131.4	757.5±445.0	539.3±372.3	675.6±244.6	987.3±320.8	432.2±63.0
Bladder	825.4±495.8	1030.5±646.3	665.3±486.4	1104.6±628.7	1198.8±719.3	452.4±47.6
Stomach	909.2±277.1	1249.6±783.1	1092.0±793.5	1029.6±355.0	1239.8±438.8	470.8±71.7
Intestine	884.2±260.5	1116.4±646.0	590.9±347.0	574.3±221.9	888.5±442.9	257.6±23.0
Muscle	476.8±110.1	766.5±285.3	413.7±248.9	541.9±180.3	629.8±97.9	250.6±17.9
Brain	270.1±46.3	303.1±113.3	233.5±72.9	350.6±95.6	372.3±13.9	193.2±13.5

	Var6-3Db <i>n</i> = 5	Var7-3Ea <i>n</i> = 16	Var8-3Eb <i>n</i> = 5	Var9-3Ec <i>n</i> = 5	Var10-2D <i>n</i> = 6	Var11-2E <i>n</i> = 6
Kidney	2536.1±297.2	3583.3±427.9	3729.2±749.0	3367.9±667.6	3575.0±540.4	4164.3±833.5
Tumor	461.6±71.6	970.1±449.1	910.6±180.1	1169.4±297.9	819.9±306.7	981.0±357.9
Heart	262.2±29.5	351.1±81.0	515.0±284.4	613.4±250.6	341.5±87.7	532.3±104.5
Lungs	266.4±23.7	413.3±135.6	526.0±62.2	958.0±367.2	412.8±42.4	560.2±42.2
Liver	414.8±31.8	695.1±244.2	938.2±79.2	1309.2±255.3	539.0±114.3	744.2±57.1
Spleen	316.0±50.6	479.3±310.6	443.8±86.8	629.2±96.1	423.4±29.3	495.7±46.5
Bladder	298.6±38.3	554.4±243.4	608.8±113.3	942.0±204.0	420.0±77.3	623.0±145.3
Stomach	268.6±31.2	605.6±205.6	563.4±93.3	934.2±251.3	513.1±70.2	763.0±107.8
Intestine	246.4±35.0	437.8±109.2	380.6±87.7	471.8±98.9	321.6±109.2	353.3±14.7
Muscle	220.8±30.0	277.8±67.2	339.0±50.7	450.8±97.8	281.5±70.0	330.2±22.2
Brain	180.2±18.7	206.3±36.0	230.2±18.4	222.0±16.7	217.4±26.4	241.5±24.7

	Var12-1D <i>n</i> = 7	Var13-1E <i>n</i> = 6	Var14-Rev <i>n</i> = 5	Var15-2N <i>n</i> = 5	Var16-2P <i>n</i> = 6
Kidney	2004.5±527.7	2653.7±357.1	4162.9±210.7	5657.0±843.8	3733.0±582.9
Tumor	614.5±186.7	459.0±99.3	1245.0±308.7	2075.8±252.2	1407.0±142.1
Heart	368.7±100.4	351.7±61.5	691.0±104.7	837.6±188.0	342.7±28.9
Lungs	423.8±122.0	349.7±78.9	808.2±116.3	1190.8±419.3	451.0±85.2
Liver	435.9±139.0	457.2±46.8	1863.0±312.6	1888.8±303.1	1344.0±153.2
Spleen	295.7±75.5	365.2±20.4	527.8±64.6	717.4±61.9	370.7±24.0
Bladder	287.2±51.5	414.7±102.9	648.6±235.5	743.6±59.8	525.7±121.2
Stomach	341.2±84.6	483.3±96.1	685.6±101.3	556.6±94.6	361.2±61.5
Intestine	227.5±59.0	277.8±31.1	839.8±196.9	720.4±52.9	403.3±67.3
Muscle	270.8±30.7	261.8±24.4	421.4±35.8	500.6±37.1	396.0±37.8
Brain	207.0±33.0	211.0±13.5	290.6±27.2	405.0±65.5	216.7±14.9

Table S2. Mean near infrared fluorescence with SD calculated for each organ collected at 4 h after Alexa750-pHLIP variant administration. The mean fluorescence values of organs were normalized to the signal in kidney for each mouse and then averaged for each variant; they are presented in Figure S3. *n* is number of animals.

	Var0-WT <i>n</i> = 9	Var1-2D1D <i>n</i> = 4	Var2-2D1D <i>n</i> = 9	Var3-3D <i>n</i> = 7	Var4-3E <i>n</i> = 3	Var5-3Da <i>n</i> = 5
Kidney	854.4±186.9	3349.5±2262.1	1525.2±472.4	1141.8±416.7	1151.4±262.4	431.0±121.5
Tumor	818.8±157.4	1366.1±776.7	881.4±560.6	950.9±434.1	1106.3±651.1	252.2±67.6
Heart	248.5±33.3	580.9±280.2	240.3±94.3	186.0±31.3	255.0±75.1	166.0±16.0
Lungs	355.7±38.8	758.3±422.8	351.9±190.9	190.4±31.8	287.7±93.6	198.6±15.3
Liver	602.2±104.4	2211.1±973.9	660.8±427.5	366.3±72.0	737.3±90.4	209.0±18.2
Spleen	226.1±13.0	626.3±368.0	285.3±170.8	199.0±25.5	249.3±30.7	184.0±14.5
Bladder	250.2±27.5	861.6±743.0	301.3±183.5	221.6±33.2	282.3±72.8	188.0±10.8
Stomach	248.9±33.1	884.0±619.8	353.2±178.4	198.4±18.2	257.0±28.0	201.8±16.3
Intestine	270.2±40.5	808.7±473.3	237.2±75.3	184.3±19.5	261.3±34.8	141.6±2.1
Muscle	197.6±31.0	555.4±320.9	213.5±88.9	158.7±21.6	185.3±45.1	141.4±5.1
Brain	166.3±10.8	253.5±73.8	164.8±30.8	147.7±8.1	163.0±24.3	136.8±3.2

	Var6-3Db <i>n</i> = 5	Var7-3Ea <i>n</i> = 17	Var8-3Eb <i>n</i> = 5	Var9-3Ec <i>n</i> = 5	Var10-2D <i>n</i> = 5	Var11-2E <i>n</i> = 4
Kidney	380.5±46.0	785.3±200.9	1287.1±307.6	1242.2±280.6	1013.5±104.4	828.6±129.1
Tumor	218.6±40.8	453.1±173.2	441.6±102.0	441.2±53.9	345.1±140.2	400.3±61.0
Heart	142.8±4.9	173.3±20.3	201.8±19.8	247.0±92.6	196.8±79.3	186.3±10.6
Lungs	148.8±8.4	181.6±24.2	330.2±76.0	270.6±58.6	198.4±17.3	207. ±3±8.7
Liver	220.2±5.7	299.8±51.7	455.6±76.9	536.0±101.4	338.3±47.5	285.5±12.4
Spleen	164. ±69.0	200.1±22.6	244.8±39.1	273.6±7.3	217.0±71.8	204.3±15.1
Bladder	156.0±10.8	187.2±23.5	236.8±37.7	264.2±30.6	207.4±60.2	214.3±18.2
Stomach	152.0±4.3	185.8±26.2	249.4±59.1	258.2±38.2	227.9±89.2	198.5±9.3
Intestine	141.4±3.8	207.8±28.9	192.6±14.2	233.0±19.8	194.4±73.3	173.0±9.5
Muscle	134.2±1.8	154.5±15.0	171.6±15.7	182.2±8.1	180.6±64.6	173.0±6.1
Brain	137.6±2.9	147.8±7.5	154.6±6.7	159.0±7.4	165.5±30.7	157.8±2.6

	Var12-1D <i>n</i> = 6	Var13-1E <i>n</i> = 3	Var14-Rev <i>n</i> = 5	Var15-2N <i>n</i> = 5	Var16-2P <i>n</i> = 6
Kidney	469.8±72.1	444.9±88.9	1157.4±147.5	1618.9±283.3	819.3±124.1
Tumor	323.7±93.8	218.3±15.1	867.4±286.1	1416.0±468.6	575.5±231.1
Heart	202.7±23.7	157.7±6.7	230.8±23.7	370.8±52.3	164.2±20.1
Lungs	221.1±30.7	184.7±8.3	285.4±67.0	473.2±68.9	171.3±29.5
Liver	252.3±32.9	224.0±21.9	567.2±130.6	742.8±50.2	421.5±52.7
Spleen	165.8±98.0	158.3±10.4	221.6±40.7	295.8±34.5	183.3±13.9
Bladder	242.7±26.6	188.0±16.6	274.6±36.8	417.6±26.6	166.7±11.2
Stomach	197.4±23.9	170.3±11.7	189.8±22.2	288.6±55.5	166.5±20.7
Intestine	166.7±20.0	152.7±12.1	289.4±72.0	296.0±36.8	168.3±7.1
Muscle	184.6±28.2	147.7±7.4	188.8±25.9	260.8±14.3	147.0±7.4
Brain	159.5±17.9	146.7±2.5	168.6±14.0	214.2±9.3	145.0±6.9

Table S3. Mean near infrared fluorescence with SD calculated for each organ collected at 24 h after administration of Alexa750-pHLIP variants. The mean fluorescence values of each organ were normalized to the signal in kidney at 4 h (Table S2) for each mouse and then averaged for each variant; they are presented in Figure S3. *n* is the number of animals.

	K-Var3, 4h <i>n</i> = 11	K-Var3, 24h <i>n</i> = 8	K-Var7, 4h <i>n</i> = 10	K-Var7, 24h <i>n</i> = 10
Kidney	921.6±306.2	254.5±91.9	852.5±345.0	235.2±15.5
Tumor	395.9±107.6	220.0±70.0	306.0±107.6	185.7±9.8
Heart	319.6±140.2	165.4±51.1	228.2±56.2	156.4±27.7
Lungs	296.1±66.9	154.1±16.0	225.0±80.0	136.2±10.7
Liver	2637. ±6446.4	684.2±138.5	1920.4±752.3	538.2±65.2
Spleen	1057.5±416.6	284.3±32.0	552.3±140.8	255.2±80.1
Bladder	331.1±100.1	176.5±26.7	265.4±70.9	155.3±11.0
Stomach	286.2±70.0	168.4±20.2	260.6±84.0	156.7±15.5
Intestine	298.7±46.0	173.9±14.5	289.7±84.9	166.9±9.7
Muscle	201.3±29.9	141.1±5.6	178.8±31.4	127.7±4.2
Brain	192.5±25.9	139.7±5.9	158.8±28.5	134.3±2.9

Table S4. Mean near infrared fluorescence with SD calculated for each organ collected at 4 and 24 h after Alexa750-K-pHLIP variant administration. *n* is the number of animals.

	Var3, 4h <i>n</i> = 4	K-Var3, 4h <i>n</i> = 4	Var7, 4h <i>n</i> = 4	K-Var7, 4h <i>n</i> = 4
Kidney	154.3±14.4	128.8±10.6	189.5±51.0	121.3±4.6
Tumor	325.0±11.4	151.5±16.7	308.0±115.8	136.3±9.3
Heart	128.3±8.1	114.5±6.0	124.0±13.1	111.5±3.5
Lungs	127.5±11.4	116.0±7.0	124.0±11.6	111.0±1.4
Liver	391.5±78.9	152.8±19.8	210.5±67.6	142.3±30.5
Spleen	122.0±3.9	118.3±4.5	125.0±10.4	113.0±4.2
Bladder	241.7±23.8	150.8±19.3	233.0±64.6	143.8±18.8
Stomach	257.0±66.2	233.0±75.2	269.8±76.6	212.5±26.6
Intestine	197.8±26.7	159.0±42.3	222.5±118.3	133.0±19.3
Muscle	150.5±6.8	126.8±3.0	141.0±20.5	119.3±4.8
Brain	142.0±13.0	135.3±11.8	131.5±23.5	130.5±7.9

Table S5. Mean near infrared fluorescence with SD calculated for each organ collected at 4 h after BODIPY-labeled Var3, K-Var3, Var7, and K-Var7 administration. *n* is the number of animals.

CHAPTER 2

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pHLIP®-Mediated Delivery of PEGylated Liposomes to Cancer Cells

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Key words: acidosis, membrane-associated folding, peptide, fusion, endocytosis, delivery of payload, ceramide, pH-sensitive

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ABSTRACT

We develop a method for pH-dependent fusion between liposomes and cellular membranes using pHLIP® (pH (Low) Insertion Peptide), which inserts into lipid bilayer of membrane only at low pH. Previously we establish the molecular mechanism of peptide action and show that pHLIP can target acidic diseased tissue. Here we investigate how coating of PEGylated liposomes with pHLIP might affect liposomal uptake by cells. The presence of pHLIP on the surface of PEGylated-liposomes enhanced membrane fusion and lipid exchange in a pH dependent fashion, leading to increase of cellular uptake and payload release, and inhibition of cell proliferation by liposomes containing ceramide. A novel type of pH-sensitive, “fusogenic” pHLIP-liposomes was developed, which could be used to selectively deliver various diagnostic and therapeutic agents to acidic diseased cells.

INTRODUCTION

Liposomes are widely used nanocarriers that allow encapsulation of various therapeutics and diagnostic agents [1]. Once internalized by cells, liposomes must disrupt or fuse with the endosomal membrane in order to release the payload into the cytoplasm. Endosomal trapping of liposomes (and their contents within) pose a significant challenge to cytoplasmic delivery of the drug payload. Thus, pH-sensitive functionalities have been incorporated into liposomes to promote the mixing of lipids (between the liposome and the endosomal membrane) under the acidic environment of the endosomes, which in turn facilitates payload release into the cytoplasm [2-8]. Meanwhile, to mask against opsonization and to increase circulation time in blood, liposomes are usually coated with poly(ethyleneglycol) (PEG) polymers [9-12]. Unfortunately, the presence of PEG on the surface of liposomes hinders drug release, even when liposomes have been internalized via receptor mediated endocytosis [13]. One approach to avoid this difficulty is to use pH-sensitive PEG polymers that disassemble in the endosomal low pH environment, thus exposing lipid bilayer membrane of the liposome for direct interaction with the membrane of the endosome [2, 6, 14].

Liposomes can be fashioned into targeted drug carriers. Current targeting strategies usually involve conjugation of liposomes with antibodies or other ligands. However, these methods have limitations due to the heterogeneous expression of the targeted receptors in diseased tissues. Alternatively, we have developed a nanotechnology platform to target the microenvironment of diseased tissues. One of the common

characteristics of diseased tissues (cancerous tumors, ischemia, inflammation, arthritis, atherosclerosis) is an acidic extracellular environment. We designed peptides that insert into cellular membranes under slightly acidic pH but not at normal pH. These peptides, called pHLIP® peptides (pH (Low) Insertion Peptides), demonstrated a high ability of targeting acidic tissues *in vivo*, (including tumors, arthritic sites, and ischemic myocardium)[15-19]. The targeting mechanism of pHLIP is based on its pH-triggered insertion into lipid bilayers that ultimately results in the formation of a transmembrane helix [20-23]. One promising application is to use pHLIP for targeting liposomes towards diseased tissues. Here, new formulations of pH-sensitive, “fusogenic,” PEGylated liposomes are developed by including pHLIP in the liposomal coating. We demonstrated that pHLIP-PEG-liposomes bind and fuse with cellular membranes at low pH, but not at normal pH, and promote cellular uptake.

MATERIALS AND METHODS

Materials

The N-capped pHLIP peptide,

Acetyl-NH-ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT-CO₂H, was prepared by standard solid phase synthesis and purified at the W.M. Keck Foundation Biotechnology Resources Laboratory at Yale. The lipids, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (**DOPE**), 1,2-dioleoyl-sn-glycero-3-phosphocholine (**DOPC**), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (**POPC**), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt)

(**DSPE-PEG₂₀₀₀-Maleimide**), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (**DSPE-PEG₂₀₀₀**), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl] (**Rhod-PE**), N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)750]} (**C8-PEG₇₅₀** ceramide) and N-hexanoyl-D-erythro-sphingosine (**C6** Ceramide), were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). The fluorescein labeled lipids, N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (**FITC-DHPE**), rhodamine labeled fatty acid, octadecyl rhodamine B chloride (**R18**), and fluorescent dyes: Wheat germ agglutinin Texas Red, ER-tracker green, mito-tracker deep red, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (**ANTS**), and *p*-xylene-bis-pyridinium bromide (**DPX**) were from Life Technologies (Carlsbad, CA) . The fatty acid conjugated with 1.4 nm gold nanoparticles, palmitoyl nanogold, and HQ Silver^{TM} Enhancement Kit were purchased from Nanoprobes, Inc (Yaphank, NY). DNA staining dye, propidium iodide (PI), was from Sigma-Aldrich Co, LLC (St. Louise, MO).

Synthesis of DSPE-PEG₂₀₀₀-pHLIP

DSPE-PEG₂₀₀₀-pHLIP was synthesized by the covalent conjugation of DSPE-PEG₂₀₀₀-maleimide with the single cysteine residue at the N-terminus of pHLIP. In a typical coupling reaction, 600 nmol of peptide was mixed with 500 nmol of DSPE-PEG₂₀₀₀-Maleimide in 200 μ L of methanol and left for overnight stirring at room temperature under argon. The existence of the product was ensured by SELDI-TOF mass-spectrometry analysis.

Liposome preparation

Liposomes were prepared by extrusion. A chloroform solution of the desired lipid mixture (1 μmol) including solution of DSPE-PEG₂₀₀₀-pHLIP in methanol was vortexed and evaporated using rotary evaporator, producing an even thin film. pHLIP is an unstructured peptide, which could be dissolved in organic solvents including methanol and chloroform. The lipid film was left under a vacuum for overnight to remove residuals of organic solvent. The hydration of the dry lipid film was accomplished by adding 1 mL of stock buffer solution (10 mM phosphate, 150 mM NaCl, pH 8) to the flask of lipids and agitating. Typically, a hydration time of 1 hr with vigorous vortexing was needed for pHLIP-containing liposome. After 10 freeze-thaw-vortex cycles, the resulting multilamellar liposome solution was extruded 15 or 31 times through 50, 100, or 200 nm polycarbonate filters and sterilized by filtering through 0.2 μm filter. The final lipid concentration was re-calculated by knowing the concentration of fluorescent lipids, which was obtained spectrophotometrically. Small amount of liposomes were diluted in methanol and absorbance of rhodamine ($\epsilon_{556}=125,000 \text{ M}^{-1}\text{cm}^{-1}$) or fluorescein ($\epsilon_{495}=88,000 \text{ M}^{-1}\text{cm}^{-1}$) was measured. When loading with propidium iodide (PI), ANTS, or DPX was required, PI (4 mM), ANTS (25 mM), or DPX (90 mM) were added as a component of the rehydration solution. After liposome extrusion, free PI, ANTS, or DPX were removed by dialysis using dialysis tube with MWCO of 8000 Da, followed by stirring in 1 L buffer solution for 24 hrs with 3 times buffer refreshing, or by fast spin semi-dry column using sephadex G25. In each case we ensure that ionic strength of solution within liposomes was the same as outside of liposomes. The ionic strength was regulated by addition of NaCl.

Cryo-electron microscopy

About 5 μ L droplet of liposome solution was spread on an 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 (Hillsboro, OR), 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 20,000x and 40,000x.

DLS measurements

The size of liposomes was measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS instrument with Malvern disposable cuvettes (Malvern USA, Southborough, MA) right after the extrusion of liposomes and in some cases, within a week after preparation. Samples were typically 200 nmol liposomes in 1 mL of aqueous buffer (10 mM phosphate, 150 mM NaCl, pH 8.0).

Inter-liposomal fusion assay

R18-labeled liposomes (50 μ L, 200 μ M) were mixed with an equal volume of unlabeled POPC vesicles (lipid concentration was varied from 0 to 6 mM) in an aqueous buffer (10 mM phosphate, 150 mM NaCl, pH 8.0). The R18 fluorescence intensity was monitored on an ISS spectrofluorometer (Champaign, IL) at excitation and emission wavelengths set at 556 nm, and 590 nm, respectively, as the pH was lowered from 8 to 4 (via addition of small amounts of HCl). A circulating water bath

was connected to cuvette holder to control sample temperature at 22°C. Complete fusion and mixing of lipids was achieved via 5 freeze-thaw cycles, the fluorescence intensity obtained afterwards was defined as “100% fusion.” The degree of liposome fusion in % was calculated according to the equation:

$$Fusion = 100 \times \frac{F - F_0}{F_{max} - F_0}$$

where F_0 is the initial fluorescent intensity of liposome mixture at pH8 (no fusion), F_{max} is the maximum fluorescent intensity of liposome mixture at pH 4 after 5 freeze-thaw cycles (100% fusion), and F is the fluorescent intensity of liposome mixture at pH 4 before freeze-thaw cycles.

In other fusion assay, 100 μ M of 50 nm DOPC liposomes of ‘5:5’ formulation containing 5% R18 and encapsulated with 25 mM of fluorescent dye ANTS were mixed with equal volume of 2 mM of 200 nm POPC liposomes encapsulated with 90 mM of DPX, which is a quencher of ANTS fluorescence, in the 10 mM phosphate buffer of pH 8 containing of 40 mM NaCl. The pH was reduced by addition of aliquot of concentrated HCl. The increase of R18 fluorescence signal and decrease of ANTS signal due to the liposome fusion and mixing of ANTS with DPX were observed on the same liposomes. ANTS fluorescence was excited at wavelength of 360 nm and observed at 530 nm. In addition, changes of scattering signal monitored at 650 nm with excitation at 650 nm were recorded.

Cell lines

Lung carcinoma A549 and human cervix adenocarcinoma HeLa cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were authenticated, stored according to supplier's instructions, and used within 3-4 months after frozen aliquots resuscitations. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 10 µg/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 medium (pH 6.5). The pH 6.5 media was prepared by mixing 13.5 g of dry DMEM powder with 0.2 g of sodium bicarbonate in 1 L of deionized water.

Cellular uptake of fluorescently labeled liposomes

Typically, a suspension of A549 cells was prepared by dissociating 4-6 days old monolayer grown in 75cm² flask with trypsin, EDTA (Gibco, Gaithersburg, MD). Trypsinized cells were counted using a hemacytometer and diluted to 1 x 10⁶ cells/mL in serum-free media (for 1 hr incubation) or PBS (for 15 min incubation) as stock solutions. The liposomes (20 nmol), containing fluorescent probe lipids or fatty acid, were incubated with 2 x 10⁵ cells in 1 mL of the desired solution for 1 hr or 15 min at 37°C or 4°C. In some studies, ATP-depleting media (DMEM + 20 mM NaN₃/ 50 mM 2-deoxy-glucose) was added for 1 hr at 37°C prior to liposome addition and was kept throughout the liposome incubation period. After incubation, the cells were pelleted by centrifugation (2000 rpm, 4 min) at room temperature (or 4°C). Then the supernatant

was removed and the pellet was resuspended in 1 mL of fresh serum-free media (or PBS) and centrifuged second time. The second pellet was resuspended in 100 μ L of the same media. Sequentially, 20 μ L of the cell suspension solution was loaded into a counting chamber and the fluorescent signal of individual cells was measured using Nexcelom Cellometer Vision (Lawrence, MA). For each measurement, fluorescent data of 2000-3000 cells were obtained and statistically analyzed. All samples were prepared at least in triplicate for each formulation of liposomes. The fluorescent signals of untreated cells were used as a common divisor for normalization of all the fluorescent data in each assay. Cell viability was confirmed by addition of trypan blue to cell sample. After cellometer counting, the residual cells were reseeded in collagen-coated cell dishes and viewed under the microscope after 24, 48, and 72 hrs.

Fluorescence microscopy

HeLa and A549 cells adapted for low pH growth (pH 6.5 and pH 6.2, respectively) were seeded in collagen-coated cell dishes (5,000 and 15,000 cells/dish, respectively). After 24 hr the culture medium was removed and cells were incubated with 6 nmol of fluorescein-labeled liposome, DOPC/DSPE-PEG₂₀₀₀-pHLIP/DSPE-PEG₂₀₀₀/fluorescein-DHPE (85:5:5:5) in 240 μ L of PBS (pH 6.3) for 1 hr at 37°C under 5% CO₂, followed by 3 times washing with sterile PBS (pH 7.4). Sequentially, wheat germ agglutinin Texas Red (10 μ g/mL) was used to stain the plasma membranes of live cells for co-localization studies. For the cells treated with R18 labeled liposome, ER-tracker green and Mito-tracker Deep Red FM were used to stain endoplasmic

reticulum (ER) and mitochondria of live cells. Fluorescent images were acquired with a Retiga CCD camera (Qimaging, Burnaby, BC, Canada) mounted to an inverted Olympus IX71 microscope (Olympus America, Inc., Center Valley, PA). Fluorescent images from different stains were obtained in the same field of view with corresponding filters.

Cellular uptake of nanogold labeled liposomes

A549 cells adapted for low pH media, grown to 80-90% confluence on 8-well chamber slide were incubated in 200 μ L serum-free DMEM (pH 6.5) with 12 nmol of nanogold labeled liposome for 15 min at 37°C under 5% CO₂. After incubation, the medium was removed and cells were washed three times with sterile PBS (pH7.4) and fixed in cold methanol for 10 min, followed by washing twice with sterile PBS (pH7.4) and once with distilled H₂O. After air-drying, cells were treated with silver enhancement solution (following manufacture's instruction) and viewed under the light microscope.

Delivery of PI

A549 cells adapted for low pH media were seeded in collagen-coated cell dishes (5,000 cells/dish). After 24 hrs, the culture medium was removed and cells were treated with 10 nmol of PI loaded liposome DOPE/pHLIP-PEG-DSPE/DSPE-PEG₂₀₀₀/fluorescein-DHPE (85:5:5:5 or 85:0:10:5) in 100 μ L of serum-free DMEM (pH 6.5) for 1 hr at 37°C under 5% CO₂. After incubation, the medium was removed

and cells were washed three times with sterile PBS (pH 7.4) and viewed under fluorescence microscope.

Cytotoxicity Assay

A549 cells grown in standard growth medium of pH 7.4 and A549 cells adapted for low pH (pH 6.2) growth medium were loaded in the wells of 96-well plates (~5,000 and 7,000 cells per well, respectively) and incubated overnight. Next day cells were treated with increasing concentrations (0, 10, 50, 100, 200, 400 μ M) of DOPE and DOPC/pHLIP-PEG-DSPE/DSPE-PEG₂₀₀₀ (85:5:5:5 or 85:0:10:5) for 2 hrs followed by the addition of equal volume of growth medium with 20% of serum of the same pH (to have 10% FBS of final concentration, since constructs were not removed). Cell viability was assessed after 1 and 2 days of incubation with constructs using the colorimetric reagent (CellTiter 96 AQueous One Solution Assay, Promega), which was added for 1 hr to cells followed by measuring absorbance at 490 nm. All samples were prepared in triplicate.

Inhibition of cell proliferation by liposomal ceramide

A549 cell suspension (160,000 cells) were incubated with 160 nmol of liposomal ceramide in 1 mL of 10 mM phosphate buffer (150 mM NaCl, 1 mM MgCl₂ and 1 mM CaCl₂) at pH 6.1 or pH 8 for 1 hr at 37°C under 5% CO₂. After incubation, cells were pelleted by centrifugation (2000 rpm, 4 min) at room temperature followed by the removal of supernatant. The pellet was resuspended in 1 mL of fresh serum-free

media and centrifuged second time. The second pellet was reseeded into 96-well tissue culture plates, 5,000 cells per well with 250 μ L medium. After 72 hrs, the medium was removed and Cell Titer 96 Aqueous One solution (Promega Co., Madison, WI) was added to each well. The viability of cells was measured by using a SpectraMax plate reader (Molecular Devices, Inc. Sunnyvale, CA) at 490 nm. Relative viability was calculated with cells treated only with medium alone as a control.

RESULTS

Liposome composition and characterization

The main goal of our investigation is to demonstrate that pHLIP can promote cellular uptake of PEGylated liposomes and enhance lipid mixing and fusion between liposomal and cellular membranes. It is well established that PE lipids (such as DOPE), which are minimally hydrated and have small headgroups, possess high fusogenic propensity [24]. In our study, we tested both fusogenic (DOPE-based) and non-fusogenic (DOPC-based) liposomes.

To introduce pHLIP in a liposome coat, we conjugated DSPE-PEG₂₀₀₀ maleimide lipid with Cys residue in the N-terminal region of pHLIP. Due to the fact that pHLIP is an unstructured polypeptide, it could be dissolved in organic solvent with no harm to its structure. The conjugation product was characterized by SELDI-TOF mass spectrometry (Figure 1). The narrow peak at 4154 Da corresponds to pHLIP, and the broad peak centered at ~7000 Da corresponds to the conjugation product DSPE-

PEG₂₀₀₀-pHLIP. DSPE-PEG₂₀₀₀-pHLIP was used for preparation of liposomes containing pHLIP. The detailed composition of liposomes used in the study is given in Table 1. The first number in the formulation code refers to the mole percentage of pHLIP-PEG lipids (DSPE-PEG₂₀₀₀-pHLIP), whereas the second number refers to the amount of PEG lipids (DSPE-PEG₂₀₀₀). The different formulations of liposomes were characterized by cryo-TEM and DLS. Special attention was given to the stability of DOPC/DOPE ‘5:5’ and ‘10:0’ formulations containing 5% and 10% DSPE-PEG₂₀₀₀-pHLIP, respectively. The average size of DOPC and DOPE liposomes of ‘5:5’ formulation was about 125 nm in diameter with PDI (polydispersity index) of about 0.06 and Zeta potential of about -16 mV. The measurements were performed within several days after liposomes preparations and numbers represent averaged values. The average size of DOPE and DOPC liposomes containing 10% DSPE-PEG₂₀₀₀-pHLIP was larger in size. The DOPE liposomes were stable for several days with average size of 150 nm, PDI of 0.06 and Zeta-potential of -19.5 mV. However, the DOPC ‘10:0’ liposome formulation was very unstable with increased size of liposomes of 200 nm. Figure 2 shows cryo-TEM images of DOPC/DOPE liposomes with and without pHLIP in liposome coat. These liposomes have formulations “0:10” (an example of a control liposome without pHLIP) and “5:5” (an example of a pHLIP-containing liposome) (Table 1). The TEM images illustrate that our liposome samples consist of a mixture of small unilamellar (<100 nm) and multilamellar vesicles, mostly with two bilayers (~100 nm). pHLIP-containing liposomes show more complex multilamellar structures, especially for liposomes consisting of fusogenic DOPE lipids (Figure 2B), which is most probably due to the pHLIP-induced membrane fusion. Since pHLIP has

high affinity to the lipid bilayer of membrane, fusion promoted by pHLIP results in formation of multilamellar structures rather than bigger liposomes. Such multilamellar structures allow interaction of pHLIP peptides with lipid bilayer of the same liposome.

Inter-liposomal fusion

First, we investigated pHLIP-induced membrane fusion on the model system of phospholipid vesicles. Inter-liposomal fusion was monitored by fluorescence increase of octadecyl rhodamine B (R18), which was part of liposome composition. When rhodamine dye molecules are in close proximity to each other, such as in the case of high concentration (7 mol % of R18) in two-dimensional membrane space, the self-quenching occurs [25]. The fluorescence increase is observed when liposomes containing R18 fuse with unlabeled POPC liposomes, which leads to the decrease of effective concentration of R18, that is, an increase of averaged distance between rhodamine dyes. Four types of R18-labeled liposomes were studied, i.e. DOPC- or DOPE-based PEG-liposomes with or without pHLIP (Figure 3).

Typical changes of R18 fluorescence are shown in Figure 3A. The fluorescence signal of solution containing unlabeled POPC liposomes (6 mM) mixed with an equal volume of R18-labeled liposomes (200 μ M) at pH 8 was taken as 0% of fusion. After equilibrating at pH 8 for $\sim$ 2 min, the pH was dropped from 8 to 4, and increase of fluorescence indicating on fusion was observed only for liposomes containing pHLIP in coat. Non-physiological pH 8 and pH 4 in this model assay on liposomes were used to ensure that all pHLIP molecules exist in either state II or state III, respectively.

Fluorescence signal measured after 5 freeze-thaw cycles was taken as 100% of fusion. Furthermore, we investigated dependence of fluorescence increase on amount of unlabeled POPC liposomes (Figure 3B). Mixing of R18-liposomes containing pHLIP with increasing concentrations of unlabeled POPC liposomes led to the enhancement of de-quenching, indicating that more inter-liposomal fusion had occurred. On the contrary, control liposomes (without pHLIP) showed no fluorescence change as the concentration of unlabeled POPC increased. Thus, inter-liposomal fusion is strongly dependent on the presence of pHLIP.

To confirm that increase of R18 fluorescence is associated with fusion, not just flip-flopping of R18 we performed another fusion assay. 50 nm DOPC liposomes of '5:5' formulation encapsulated with fluorescent dye ANTS were mixed with 200 nm POPC liposomes encapsulated with DPX, quencher of ANTS fluorescence. The fluorescence signal of ANTS was decreased only when pH was reduced from 8 to 4 (Figure 3C). The decrease of ANTS emission could occur only in the result of fusion of liposomes and mixing of ANTS with DPX.

pHLIP-mediated inter-liposomal association ('sticking') takes place at pH 8, since binding of pHLIP to lipid bilayer at pH8 (without insertion into membrane) is associated with energy release of about 6 kcal/mol [23]. At the end of the equilibration period of 2 min at pH 8, most of pHLIP peptides could be associated with an inter-liposomal POPC membrane, and the subsequent pH drop triggers membrane fusion, since pHLIP inserts into bilayer and adopts TM configuration within about 60 sec after

changes of pH [22]. The peptide inserted state is stabilized by release of additional 2 kcal/mol of energy [23]. Our data indicate that most of pHLIPs prefer the inter-liposomal POPC membrane surface over its own intra-liposomal DOPE or DOPC surface, which is perhaps because adequate level of PEGylation effectively shields the intra-liposomal membrane from association with pHLIP. However, we cannot exclude that some amount of peptides inserts in membranes of their own liposomes as well.

The effect of PEGylation level (1-10 mol %), pHLIP content (1-10 mol %), and liposome type (DOPC vs. DOPE) on the efficiency of inter-liposomal fusion was further investigated (Figure 3D). We established that 5 mol % of PEG₂₀₀₀ is an optimal level of PEGylation. This level of PEGylation roughly corresponds to the transition between the tight packing ‘brush’ conformation and the loose ‘mushroom’ conformation of the liposomal PEG shield [26]. A lower level of PEGylation at 1 mol % led to the reduction of fusion for both DOPC and DOPE pHLIP-liposomes. In this case, we suspect that the membrane surface is not adequately shielded by PEG and too many pHLIP molecules associate and insert into the intra-liposomal DOPC/DOPE membrane at pH 8 and 4, respectively. However, more PEGylation at 10 mol % also led to lesser inter-liposomal fusion, especially for DOPC pHLIP-liposomes, perhaps because shielding of the DOPC surface is too complete and membrane fusion becomes difficult. At 10 mol % PEGylation, DOPE-based liposomes show higher percentage of fusion than DOPC-based liposomes (35% vs. 12%), in agreement with the fusogenic nature of PE lipids. However, when the amount of PEGylation is reduced to 5 mol %, DOPC-based pHLIP-liposomes are just as adapt at membrane fusion as DOPE-based

liposomes. Given the same overall amount of PEGylated lipids, (1, 5, or 10 mol %), the presence of more pHLIP molecules always correlate with higher levels of membrane fusion, except of DOPC liposomes containing 10% of PEG-pHLIP (compare to DOPC liposomes containing just 5% of PEG-pHLIP), which most probably is associated with pure stability of this particular formulation. Therefore all experiments on cells were performed with liposomes containing no more than 5% of PEG-pHLIP.

Cellular uptake of liposomes

Cellular toxicity of liposomes with ('5:5') or without ('0:10') pHLIP was tested on A549 cells at both pH 6.2 and pH 7.4. Cells were treated with increasing concentrations of liposomes (up to 400 μ M of lipids) for 1 and 2 days followed by standard MTS assay to check cells viability. No cellular toxicity was observed (data not shown).

Membrane fusion between liposomes and cells was evaluated in a cell suspension assay. A549 cells in suspension were treated with fluorescently labeled pHLIP-liposomes ("DOPE+pHLIP" or "DOPC+pHLIP") or control-liposomes without pHLIP ("DOPE" or "DOPC") under various conditions. After washing, fluorescence of individual cells was counted using a cellometer. The average fluorescence of ~ 2000 treated cells was calculated and normalized to that of untreated cells. First, we investigated whether pHLIP can induce membrane fusion between liposomes and cells in a pH-dependent fashion. A549 cells were treated with R18-labeled DOPC or DOPE

liposomes with or without pHLIP at pH 7.4 or 6.5. Incubation with cells was either 15 min in PBS (Figure 4A) or 60 min in serum-free DMEM (Figure 4B). For DOPC liposomes at pH 7.4, no difference in fluorescence intensity was observed between cells treated with pHLIP-liposomes vs. cells treated with control liposomes. However, at pH 6.5, the presence of pHLIP increased the amount of lipid mixing between liposomes and cells. The difference in cellular uptake of DOPE-based liposomes with and without pHLIP was observed already at pH 7.4, while the effect became more pronounced at pH 6.5. The results support the idea that pHLIP insertion into cellular membrane at low pH can facilitate uptake of liposomes by cells. Due to the fusogenic nature of DOPE lipids, DOPE-based liposomes show much higher cellular uptake than DOPC-based liposomes in general, which is in agreement with the data of inter-liposomal fusion assay discussed previously. The different incubation time of 15 min vs. 60 min might reflect different pathways of liposome-cell interaction. During short incubation period, predominantly direct liposomal fusion with plasma membrane occurs; while during long incubation period, both fusion and cellular internalization through endocytosis (most probably, macropinocytosis) could happen. Our data show that the fluorescence signal is significantly higher at the longer incubation time, especially at low pH, suggesting that endocytosis plays an important role in pHLIP-mediated cellular uptake of liposomes.

Next, cell treatment was carried out at low temperature or in an ATP-depletion medium to reduce energy dependent endocytic uptake. The decrease of temperature significantly (~ 80%) reduced overall liposome uptake (Figure 4C). Regardless of the

route of entry (being endocytosis or direct interaction with plasma membrane), first, pHLIP needs to insert into the cellular membrane in an environment of low pH. Our kinetics studies indicate that the rate of insertion is reduced with decrease of temperature [22]. Therefore, we assume that experiments at low temperature would affect both fusion, and endocytotic pathways of liposomal uptake. Results from the experiments of cells treated at pH 6.5 for 60 min in the ATP depletion medium or serum-free DMEM (Figure 4D), indicate that endocytosis is responsible for ~ 50% of pHLIP-mediated cellular uptake of liposomes.

How does the amount of pHLIP and PEG present on liposome coat influence the level of cellular uptake of liposomes? To investigate this question, cells were incubated with DOPC-liposomes of various compositions in serum-free DMEM at pH 6.5 for 60 min. When the total amount of PEG-lipids was held constant, the presence of more pHLIP led to higher cellular uptake (Figure 4E, dark grey bars within the 10 mol % total PEG-lipid series). Furthermore, less amount of PEGylation was more favorable for membrane fusion, resulting in higher cellular uptake (Figure 4E, 1 mol % vs. 10 mol %). The results are consistent with the data obtained in inter-liposomal fusion assay.

The uptake of liposomes was assessed using the fluorescence of Rho-FA (R18). In place of R18, we also incorporated rhodamine-PE or fluorescein-DHPE into the liposomes as alternative fluorescent probes. Cells were incubated with DOPC-liposomes with or without pHLIP in serum-free DMEM at pH 6.5 for 60 min. A similar pattern of pHLIP-dependent cellular uptake of liposomes was observed

regardless of fluorescent lipid probe used (Figure 4F). Therefore, pH- and pHLIP-dependent cellular uptake of liposomes is not influenced by the choice of the fluorescent lipid.

Fluorescence microscopy

Fluorescent lipids incorporated in liposomes allow visualization of liposome-cell interactions using fluorescence microscopy. A549 cells in suspension were incubated with DOPE liposomes with or without pHLIP in serum-free DMEM medium at pH 6.5 for 1 hr. After washing, treated cells were grown on collagen-coated dishes and became adherent. The light (Figure 5A-a, -c) and fluorescent images (Figure 5A-b, -d) were taken 4 days after cell treatment with liposomes. Cells treated with pHLIP-containing liposomes show much stronger fluorescence than cells treated with liposomes containing no pHLIP. It is evident that R18 of liposomes containing pHLIP targeted specific cellular compartment. To figure out what organelle was marked by R18 fluorescence, cells treated with pHLIP-liposomes containing R18 (Figure 5B-b) were labeled with Mito-tracker and ER-tracker to stain mitochondria (Figure 5B-a) and endoplasmic reticulum (data not shown), respectively. The co-localization study reveals that the R18 (Figure 5B-b) was co-localized in mitochondria (Figure 5B-c, 5B-d). R18 contains a hydrophobic lipid tail and rhodamine ester, which is positively charged at physiological pH. It is well known that such amphiphilic molecules specifically target mitochondria in living cells [27-30]. It is important to outline that effective mitochondria targeting by R18 was observed mostly in case of pHLIP-mediated uptake of liposomes by cells.

Liposomes containing a different rhodamine lipid probe, Rhod-PE, were also investigated. In this case, the rhodamine portion of the molecule is zwitterionic and neutral, (while the overall molecule is -1 negatively charged due to the phosphate anion in the lipid headgroup). Adherent A549 cells were incubated with Rhod-PE labeled liposomes with or without pHLIP in PBS at pH 6.3 for 30 min, and distribution of fluorescent signal was monitored under microscope immediately after cell washing. Compared to cells treated with control liposomes without pHLIP (Figure 5C-b), cells treated with pHLIP-containing liposomes (Figure 5C-d) were more fluorescent and have different distribution of fluorescent signals. Unlike R18, Rhod-PE lipids do not stain mitochondria, but instead accumulate in perinuclear region and plasma membranes (Figure 5C-d). By contrast, punctate staining was observed for cells treated with liposomes without pHLIP (Figure 5C-b), indicating endosomal trapping of liposomes.

In addition, liposomes containing fluorescein-DHPE probes (5 mol %) were also tested. Adherent A549 cells were treated with fluorescein-labeled pHLIP-containing DOPC liposomes in PBS, pH 6.2, for 1 hr (Figure 5D-a) and the same cells were stained with wheat germ agglutinin conjugated with Texas Red to visualize plasma membrane (Figure 5D-b). It is clearly seen that the plasma membrane of cells treated with pHLIP-containing liposomes was stained with fluorescein lipids, revealing that pHLIP induced fusion between liposome and plasma membrane.

Finally, instead of fluorescent probes, nanogold particles (1.4 nm) conjugated to fatty acid, were introduced in liposome coat. Adherent A549 cells were treated with nanogold-containing DOPE liposomes with or without pHLIP in serum-free DMEM at pH 6.5, for 15 min, followed by fixation and silver enhancement to enlarge nanogold particles up to micron size particles and visualize them under light microscope. Liposomes containing pHLIP delivered more nanogold to cells (Figure 5E-b) than liposomes without pHLIP (Figure 6E-a). Similar to the localization of Rhod-PE probe, nanogold particles are clearly visible at perinuclear region and plasma membranes of treated cells.

Delivery of an encapsulated cargo

The main goal in effective use of liposomes, as a delivery agent, is to deliver and release polar payload into cells. Therefore, we investigated pHLIP-mediated liposomal delivery of an encapsulated cargo. Propidium iodide (PI), a membrane-impermeable DNA staining dye, was encapsulated in liposomes to serve as the model payload. Adherent A549 cells were incubated in serum free DMEM at pH 6.5, for 1 hr with fluorescein-labeled DOPE liposomes loaded with PI, with or without pHLIP in liposomal coating (Figure 6). Fluorescein (Figure 6A-B) and PI (Figure 6C-D) fluorescent images, taken after 1 hour treatment, evidently demonstrate that pHLIP-containing liposomes delivered much more PI to the nuclei than control liposomes. Further, more staining of cellular membranes was observed on cells treated with pHLIP-containing liposomes. According to the data presented on Figure 4, endocytosis is responsible only for ~ 50% of the pHLIP-mediated cellular uptake of

liposomes and the other 50% are due to the pHLIP-induced direct fusion with plasma membrane (Figure 4D). Fusion is much faster than endocytosis. Our study shows that the internalization of liposomes was observed even after 15 min treatment (Figure 4). Thus, we assume that the release of PI encapsulated in liposomes occurred as a result of fusion of liposomes with plasma membrane. These results are consistent with the notion that pHLIP can promote membrane fusion and lipids exchange, leading to enhancement of payload release.

Delivery of liposomal ceramide inhibites proliferation of cancer cells

Our data suggest that pHLIP can enhance cellular delivery of liposomes. Can this approach be applied toward the delivery of cytotoxic agents of therapeutic potential? It was demonstrated that PEGylated liposomes containing short chain C6 ceramide can induce apoptosis in human breast cancer cells [31-33]. We prepared pHLIP-containing liposomes with 30 mol % of C6 ceramide to investigate whether pHLIP can enhance the cellular delivery of this cytotoxic signaling lipid. Cryo-TEM images confirmed the integrity of the ceramide- and pHLIP-containing liposomes (Figure 7A, B). The size of the liposomes were measured immediately after extrusion (Figure 7C) and then monitored for 3 days (Figure 7D). Ceramide-containing liposomes with pHLIP were slightly larger (~ 125 nm in diameter) than liposomes without pHLIP (~ 95 nm in diameter). Nonetheless, aggregation of pHLIP-containing liposomes was not significant during the 3-day follow-up period. These data confirmed that the ceramide liposomes were uniform in size and stable in solution.

The cytotoxic effects of ceramide-containing liposomes were evaluated in anti-proliferation assay. First, A549 cells in suspension were incubated with ceramide-liposomes with or without pHLIP, at pH 8.0, or pH 6.1, for 60 min in a phosphate buffer. pH 8 was used to enhance pH-dependent effect of pHLIP-coated liposomes. After washing, treated cells were grown on 96-well plates (at ~ 5000 cells per well) for 3 days before viable cell populations were counted by MTS colorimetric assay. When the liposome treatment was carried out at pH 8, control liposomes exhibit some toxicity to the cells, while pHLIP-coated liposomes show practically no toxicity (Figure 7F). By contrast, when the treatment was carried out at pH 6.1, significant differences in cell viability was observed between cells treated with pHLIP- and ceramide-containing liposomes (< 10% of cell survival), and cells treated with control liposomes containing ceramide, but no pHLIP (~ 60% of cell survival) (Figure 7E). Compared to untreated cell population, the treatment with ceramide-containing liposomes at low pH alone inhibited <40% of cell proliferation, whereas the presence of pHLIP in liposome coat contributed to an additional 50% of inhibition, presumably by elevating the intracellular concentration of C6 ceramide. Thus, pHLIP enhances delivery of liposomal ceramide to cells at low pH, which induces cell death.

DISCUSSION

pH-sensitive liposomes were designed to mimic the highly efficient intracellular delivery systems utilized by viruses and pathogenic organisms. There are two main pathways of viruses entry into cells: direct fusion with plasma membrane and endocytotic uptake by host cells [34-41]. Membrane fusion also occurs in membrane trafficking, including neurotransmitter release in synaptic transmission, gamete

formation in sexual reproduction, and myotube formation in organ development [42]. In all these cases, two distinct membranes have to come close to each other, merge, mix, which leads to the fusion. This process is triggered and regulated by fusion proteins. The entire fusion machinery of viruses is a single fusion protein within their own membrane, while intracellular fusion machines consist of several proteins located in both membranes to be fused. Despite the differences between viral and intracellular fusion proteins, their main role is to bring two individual membranes together [34, 37, 42-45]. Only upon activation, often by low pH in endosomes, does the fusion peptides (which are a part of the fusion protein) become exposed and available for insertion into the target membrane [42]. The process of membrane fusion mediated by fusion proteins can be as fast as 8 ms [46].

Inspired by the action of fusion peptides, which insert into the target membrane to induce fusion, we decorated PEG-coated liposomes with pHLIP (Figure 8A). Our data indicate that pHLIP adopts random coil configuration in aqueous solution at normal and high pH [21]. We assume that on the surface of liposomes coated with PEG polymer, pHLIP would adopt coil configuration as well. Our estimation of volume occupied by single pHLIP coil according to the freely-jointed model of polymer physics and the surface area on the liposomes available for the coating indicates that about 50% of liposome surface is coated with pHLIP peptides in the case of '5:5' liposome formulation. At $\text{pH} < 7.0$ Asp/Glu residues are protonated, which increases peptide hydrophobicity and affinity to membrane and leads to the peptide insertion into lipid bilayer and formation of a stable transmembrane helix. The biophysical

studies indicate that process on model membrane could be completed within 60 sec [47]. Our biophysical inter-liposomal assays clearly demonstrates that indeed pHLIP induces fusion of both fusogenic (DOPE-based) and non-fusogenic (DOPC-based) PEG-liposomes with POPC vesicles at low pH. The content of liposomes is mixing in a result of the fusion. Experiments on cultured cells further confirmed pHLIP's ability to induce fusion and lipid mixing between PEG-liposomes and biological membranes, evidenced by the following: i) pHLIP-induced cellular uptake of DOPE- and DOPC-based PEG-liposomes are dependent on acidic extracellular pH, and ii) fluorescent lipid probes (or nanogold-lipid conjugates) brought in by the liposomes are distributed throughout the cell. It is important that lipid mixing occurs not in a result of flip-flopping of fluorescent lipids and fatty acids, but in a result of membrane fusion, which leads to the efficient cytoplasmic delivery of liposomal payload. In addition to delivering probes and model cargos we also showed that, at low extracellular pH (pH 6.0), pHLIP can enhance the biological effect of PEGylated liposomes containing short chain C6 ceramide, which induces apoptosis and cell death [48, 49]. We concluded that the presence of up to 5 mol % of PEG polymer in the liposomal coatings favors pHLIP interaction with the inter-membrane surface over its own intra-liposomal surface. The amount of pHLIP peptide in the liposome coat correlates with the degree of fusion: the more pHLIP, the more fusion.

Our data indicate that there might be several routes of liposome entry into the cell: i) direct fusion with plasma membrane, and ii) endocytotic uptake of liposomes, and then, fusion with endosomal membrane (Figure 8B). In both cases, the first event is

the insertion of pHLIP peptides into target membrane triggered by reduced pH, bringing liposomal and cellular membranes close to each other, which can induce fusion of liposome with cellular membrane. Since there are many pHLIP peptides on a liposome surface, their simultaneous insertion might lead to plasma membrane ruffling and blebbing, which may trigger internalization of liposomes. It is well documented that pHLIP shows direct spontaneous insertion into membrane of liposomes and cells *in vitro* and *in vivo*, and this process does not involve receptor interaction [19, 50, 51]. Therefore, we assume that plasma membrane ruffling and blebbing might promote macropinocytosis, which is an endocytic route of cellular internalization not associated with the activation of a particular receptor on the cellular surface [52-54]. More detailed investigation is needed to confirm that pHLIP-triggered endocytotic uptake of liposomes occur via macropinocytosis. When endosomes with encapsulated liposomes detach from the plasma membrane inside a cell, they undergo acidification. Low pH (5.0-5.5) inside endosomes would promote even more efficient insertion of pHLIP into the endosomal membrane, leading to lipid mixing and fusion. As a result, liposomal payload is released into the cytoplasm and liposomal lipids could be found in the membrane of various cellular compartments.

pHLIP-coated liposomes represent a novel type of pH-sensitive “fusogenic” liposomes. In pHLIP-coated liposomes, the fusogenic properties are determined not only by the choice of lipids, but also by the presence of membrane-inserting peptides on the liposome surface, as in some pathogens. The energy of membrane-associated folding of pHLIP is utilized to bring liposomal and cellular membranes close to each other to induce lipid mixing, or membrane blebbing and ruffling. Since extracellular

acidity is associated with the development of various pathological states, such as solid tumors, ischemic stroke, neurotrauma, epileptic seizure, inflammation, infection, wounds, cystic fibrosis, and others, pHLIP-coated liposomes could be used to deliver and release various diagnostic and therapeutic agents to these sites of disease.

CONCLUSIONS

We introduced new formulation of pHLIP-PEG-coated liposomes. pHLIP promotes cellular uptake of both fusogenic (DOPE-based) and non-fusogenic (DOPC-based) liposomes at low pH. Our data indicate that about 50% of cellular uptake of pHLIP-PEG-coated liposomes at low pH could be attributed to the direct fusion with plasma membrane and about 50% due to the endocytotic pathway of cellular entry. Lipids and fatty acids conjugated with various reporters (such as fluorescent dyes or gold nanoparticles), when incorporated into pHLIP-PEG-coated liposomes, were distributed to various cellular compartments, including mitochondrial, nuclear, and plasma membranes, at the same time, encapsulated in liposome polar cargo, such as propidium iodide, was delivered to nuclear DNA at low pH. We show that pHLIP-PEG-coated liposomes containing C6-ceramide induced cell death much more effectively (~90% inhibition) than PEG-liposomes containing no pHLIP in their coating (~40% inhibition) at low pH. Since low extracellular pH is a characteristic feature of many pathological states, we assume that pHLIP-PEG-coated liposomes could be used for selective delivery and cytoplasmic release of diagnostic and therapeutic agents.

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REFERENCES

- [1] V.P. Torchilin, Passive and active drug targeting: drug delivery to tumors as an example, *Handb Exp Pharmacol*, (2010) 3-53.
- [2] J.A. Boomer, M.M. Qualls, H.D. Inerowicz, R.H. Haynes, V.S. Patri, J.M. Kim, D.H. Thompson, Cytoplasmic delivery of liposomal contents mediated by an acid-labile cholesterol-vinyl ether-PEG conjugate, *Bioconjug Chem*, 20 (2009) 47-59.
- [3] E. Fattal, P. Couvreur, C. Dubernet, "Smart" delivery of antisense oligonucleotides by anionic pH-sensitive liposomes, *Adv Drug Deliv Rev*, 56 (2004) 931-946.
- [4] V. Budker, V. Gurevich, J.E. Hagstrom, F. Bortzov, J.A. Wolff, pH-sensitive, cationic liposomes: a new synthetic virus-like vector, *Nat Biotechnol*, 14 (1996) 760-764.
- [5] X. Guo, F.C. Szoka, Jr., Steric stabilization of fusogenic liposomes by a low-pH sensitive PEG--diortho ester--lipid conjugate, *Bioconjug Chem*, 12 (2001) 291-300.
- [6] S. Simoes, J.N. Moreira, C. Fonseca, N. Duzgunes, M.C. de Lima, On the formulation of pH-sensitive liposomes with long circulation times, *Adv Drug Deliv Rev*, 56 (2004) 947-965.
- [7] E. Yuba, C. Kojima, A. Harada, Tana, S. Watarai, K. Kono, pH-Sensitive fusogenic polymer-modified liposomes as a carrier of antigenic proteins for activation of cellular immunity, *Biomaterials*, 31 (2010) 943-951.
- [8] M. Sanchez, F.J. Aranda, J.A. Teruel, A. Ortiz, New pH-sensitive liposomes containing phosphatidylethanolamine and a bacterial dirhamnolipid, *Chem Phys Lipids*, 164 (2011) 16-23.

- [9] S.M. Moghimi, J. Szebeni, Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties, *Prog Lipid Res*, 42 (2003) 463-478.
- [10] A.L. Klibanov, K. Maruyama, A.M. Beckerleg, V.P. Torchilin, L. Huang, Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target, *Biochim Biophys Acta*, 1062 (1991) 142-148.
- [11] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes, *FEBS Lett*, 268 (1990) 235-237.
- [12] T.M. Allen, C. Hansen, Pharmacokinetics of stealth versus conventional liposomes: effect of dose, *Biochim Biophys Acta*, 1068 (1991) 133-141.
- [13] J.W. Park, D.B. Kirpotin, K. Hong, R. Shalaby, Y. Shao, U.B. Nielsen, J.D. Marks, D. Papahadjopoulos, C.C. Benz, Tumor targeting using anti-her2 immunoliposomes, *J Control Release*, 74 (2001) 95-113.
- [14] J.Y. Legendre, F.C. Szoka, Jr., Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes, *Pharm Res*, 9 (1992) 1235-1242.
- [15] E.A. Sosunov, E.P. Anyukhovsky, A.A. Sosunov, A. Moshnikova, D. Wijesinghe, D.M. Engelman, Y.K. Reshetnyak, O.A. Andreev, pHLP targets ischemic myocardium, *Proc Natl Acad Sci U S A*, (2012).
- [16] P. Daumar, C.A. Wanger-Baumann, N. Pillarsetty, L. Fabrizio, S.D. Carlin, O.A. Andreev, Y.K. Reshetnyak, J.S. Lewis, Efficient (18)F-Labeling of Large 37-Amino-

Acid pHLIP Peptide Analogues and Their Biological Evaluation, *Bioconjug Chem*, 23 (2012) 1557-1566.

[17] S. Macholl, M.S. Morrison, P. Iveson, B.E. Arbo, O.A. Andreev, Y.K. Reshetnyak, D.M. Engelman, E. Johannesen, In Vivo pH Imaging with (99m)Tc-pHLIP, *Mol Imaging Biol*, 14 (2012) 725-734.

[18] A.L. Vavere, G.B. Biddlecombe, W.M. Spees, J.R. Garbow, D. Wijesinghe, O.A. Andreev, D.M. Engelman, Y.K. Reshetnyak, J.S. Lewis, A novel technology for the imaging of acidic prostate tumors by positron emission tomography, *Cancer Res*, 69 (2009) 4510-4516.

[19] Y.K. Reshetnyak, L. Yao, S. Zheng, S. Kuznetsov, D.M. Engelman, O.A. Andreev, Measuring tumor aggressiveness and targeting metastatic lesions with fluorescent pHLIP, *Mol Imaging Biol*, 13 (2011) 1146-1156.

[20] O.A. Andreev, A.D. Dupuy, M. Segala, S. Sandugu, D.A. Serra, C.O. Chichester, D.M. Engelman, Y.K. Reshetnyak, Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues in vivo, *Proc Natl Acad Sci U S A*, 104 (2007) 7893-7898.

[21] Y.K. Reshetnyak, M. Segala, O.A. Andreev, D.M. Engelman, A monomeric membrane peptide that lives in three worlds: in solution, attached to, and inserted across lipid bilayers, *Biophys J*, 93 (2007) 2363-2372.

[22] O.A. Andreev, A.G. Karabadzhak, D. Weerakkody, G.O. Andreev, D.M. Engelman, Y.K. Reshetnyak, pH (low) insertion peptide (pHLIP) inserts across a lipid bilayer as a helix and exits by a different path, *Proc Natl Acad Sci U S A*, 107 (2010) 4081-4086.

- [23] Y.K. Reshetnyak, O.A. Andreev, M. Segala, V.S. Markin, D.M. Engelman, Energetics of peptide (pHLIP) binding to and folding across a lipid bilayer membrane, *Proc Natl Acad Sci U S A*, 105 (2008) 15340-15345.
- [24] I.M. Hafez, P.R. Cullis, Roles of lipid polymorphism in intracellular delivery, *Adv Drug Deliv Rev*, 47 (2001) 139-148.
- [25] J. Arnhold, D. Wiegel, O. Hussler, K. Arnold, Quenching and dequenching of octadecyl Rhodamine B chloride fluorescence in Ca^{2+} -induced fusion of phosphatidylserine vesicles: effects of poly(ethylene glycol), *Biochim Biophys Acta*, 1191 (1994) 375-383.
- [26] S.D. Li, L. Huang, Stealth nanoparticles: high density but sheddable PEG is a key for tumor targeting, *J Control Release*, 145 (2010) 178-181.
- [27] L.V. Johnson, M.L. Walsh, L.B. Chen, Localization of mitochondria in living cells with rhodamine 123, *Proc Natl Acad Sci U S A*, 77 (1980) 990-994.
- [28] V. Weissig, S.V. Boddapati, S.M. Cheng, G.G. D'Souza, Liposomes and liposome-like vesicles for drug and DNA delivery to mitochondria, *J Liposome Res*, 16 (2006) 249-264.
- [29] S.V. Boddapati, P. Tongcharoensirikul, R.N. Hanson, G.G. D'Souza, V.P. Torchilin, V. Weissig, Mitochondriotropic liposomes, *J Liposome Res*, 15 (2005) 49-58.
- [30] S. Biswas, N.S. Dodwadkar, R.R. Sawant, A. Koshkaryev, V.P. Torchilin, Surface modification of liposomes with rhodamine-123-conjugated polymer results in enhanced mitochondrial targeting, *J Drug Target*, 19 (2011) 552-561.

- [31] T. Stover, M. Kester, Liposomal delivery enhances short-chain ceramide-induced apoptosis of breast cancer cells, *J Pharmacol Exp Ther*, 307 (2003) 468-475.
- [32] T.C. Stover, A. Sharma, G.P. Robertson, M. Kester, Systemic delivery of liposomal short-chain ceramide limits solid tumor growth in murine models of breast adenocarcinoma, *Clin Cancer Res*, 11 (2005) 3465-3474.
- [33] J.A. Shabbits, L.D. Mayer, Intracellular delivery of ceramide lipids via liposomes enhances apoptosis in vitro, *Biochim Biophys Acta*, 1612 (2003) 98-106.
- [34] M. Luo, Influenza virus entry, *Adv Exp Med Biol*, 726 (2012) 201-221.
- [35] J.J. Skehel, D.C. Wiley, Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin, *Annu Rev Biochem*, 69 (2000) 531-569.
- [36] S.P. Yates, R. Jorgensen, G.R. Andersen, A.R. Merrill, Stealth and mimicry by deadly bacterial toxins, *Trends Biochem Sci*, 31 (2006) 123-133.
- [37] Y.A. Chen, R.H. Scheller, SNARE-mediated membrane fusion, *Nat Rev Mol Cell Biol*, 2 (2001) 98-106.
- [38] R.J. Eisenberg, D. Atanasiu, T.M. Cairns, J.R. Gallagher, C. Krummenacher, G.H. Cohen, Herpes virus fusion and entry: a story with many characters, *Viruses*, 4 (2012) 800-832.
- [39] C.B. Wilen, J.C. Tilton, R.W. Doms, Molecular mechanisms of HIV entry, *Adv Exp Med Biol*, 726 (2012) 223-242.
- [40] J. Mercer, A. Helenius, Gulping rather than sipping: macropinocytosis as a way of virus entry, *Curr Opin Microbiol*, 15 (2012) 490-499.
- [41] M. Kielian, S. Jungerwirth, Mechanisms of enveloped virus entry into cells, *Mol Biol Med*, 7 (1990) 17-31.

- [42] L.K. Tamm, J. Crane, V. Kiessling, Membrane fusion: a structural perspective on the interplay of lipids and proteins, *Curr Opin Struct Biol*, 13 (2003) 453-466.
- [43] B.C. Poschner, K. Fischer, J.R. Herrmann, M.W. Hofmann, D. Langosch, Structural features of fusogenic model transmembrane domains that differentially regulate inner and outer leaflet mixing in membrane fusion, *Mol Membr Biol*, 27 (2010) 1-11.
- [44] F. Li, F. Pincet, E. Perez, W.S. Eng, T.J. Melia, J.E. Rothman, D. Tareste, Energetics and dynamics of SNAREpin folding across lipid bilayers, *Nat Struct Mol Biol*, 14 (2007) 890-896.
- [45] V.S. Markin, J.P. Albanesi, Membrane fusion: stalk model revisited, *Biophys J*, 82 (2002) 693-712.
- [46] M.K. Domanska, V. Kiessling, A. Stein, D. Fasshauer, L.K. Tamm, Single vesicle millisecond fusion kinetics reveals number of SNARE complexes optimal for fast SNARE-mediated membrane fusion, *J Biol Chem*, 284 (2009) 32158-32166.
- [47] A.G. Karabadzhak, D. Weerakkody, D. Wijesinghe, M.S. Thakur, D.M. Engelman, O.A. Andreev, V.S. Markin, Y.K. Reshetnyak, Modulation of the pHLIP Transmembrane Helix Insertion Pathway, *Biophys J*, 102 (2012) 1846-1855.
- [48] Y.A. Hannun, C. Luberto, Ceramide in the eukaryotic stress response, *Trends Cell Biol*, 10 (2000) 73-80.
- [49] Y.A. Hannun, L.M. Obeid, Ceramide and the eukaryotic stress response, *Biochem Soc Trans*, 25 (1997) 1171-1175.

- [50] Y.K. Reshetnyak, O.A. Andreev, U. Lehnert, D.M. Engelman, Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix, *Proc Natl Acad Sci U S A*, 103 (2006) 6460-6465.
- [51] O.A. Andreev, D.M. Engelman, Y.K. Reshetnyak, pH-sensitive membrane peptides (pHLIPs) as a novel class of delivery agents, *Mol Membr Biol*, 27 (2010) 341-352.
- [52] J. Mercer, A. Helenius, Virus entry by macropinocytosis, *Nat Cell Biol*, 11 (2009) 510-520.
- [53] J.A. Swanson, C. Watts, Macropinocytosis, *Trends Cell Biol*, 5 (1995) 424-428.
- [54] S.D. Conner, S.L. Schmid, Regulated portals of entry into the cell, *Nature*, 422 (2003) 37-44.

FIGURES

Figure 1. SELDI-TOF mass spectrum of DSPE-PEG₂₀₀₀-pHLIP. The narrow peak at 4154 corresponds to the pHLIP peptide. The broad peak centered at ~7000 Da corresponds to the conjugated product DSPE-PEG₂₀₀₀-pHLIP. The individual spikes on the broad peak differ from each other by the repeating unit(s) of PEG (mass: 44 Da). The marked mass at 7029 corresponds to the expected mass of the product minus one unit of PEG ($7074 - 44 = 7030$ Da).

Figure 2. Cryo-TEM images of R18-labeled liposomes. The control liposomes without pHLP are shown on panels (A) (DOPE/DSPE-PEG₂₀₀₀/R18, 83:10:7) and (C) (DOPC/DSPE-PEG₂₀₀₀/R18, 83:10:7); and the pHLP coated liposomes are shown on panels (B) (DOPE/DSPE-PEG₂₀₀₀/DSPE-PEG₂₀₀₀-pHLP/R18, 83:5:5:7) and (D) (DOPC/DSPE-PEG₂₀₀₀/DSPE-PEG₂₀₀₀-pHLP/R18, 83:5:5:7). The images were obtained using JEOL 2100 TEM with an accelerating voltage of 200 kV. The total lipid concentration of samples shown is 1 mM.

Figure 3. Inter-liposomal fusion assays. (A) Increase of rhodamine fluorescence reflects fusion of membranes. The fluorescence changes were monitored in the solution of R18-labeled DOPC or DOPE liposomes with or without pHLIP in liposome coat (200 μ M total lipid concentration) mixed with equal volume of 6 mM POPC at pH 8. The addition of POPC liposomes to the solution of R18-labeled DOPC or DOPE liposomes at pH 8 leads to the fluorescence decrease due to the dilution (data not shown). Spectral signal at pH 8 was taken as “0% fusion,” the fluorescence was monitored several minutes after pH drop to pH 4, followed by 5 freeze-thaw cycles performed to induce complete fusion (“100% fusion”). Liposome formulations are ‘5:0’ (with pHLIP) and ‘0:5’ (without pHLIP). (B) Dependence of fluorescence intensity changes of rhodamine on concentration of POPC liposomes when mixed with 200 μ M (total lipid concentration) of R18-labeled DOPC or DOPE liposomes with or

without pHLIP in liposome coat. Liposome formulations are '10:0' (with pHLIP) and '0:10' (without pHLIP). **(C)** To confirm that increase of rhodamine fluorescence occurs due to fusion of liposomes, another assay was carried. 50 nm DOPC liposomes of '5:5' formulation with entrapped ANTS at pH 8 were mixed with 200 nm POPC with entrapped DPX, a quencher of ANTS fluorescence. The DOPC liposomes also contained 5% of R18. The increase of rhodamine fluorescence in a result of pH drop from pH 8 to pH 4 (red line) was accompanied by the decrease of ANTS fluorescence (black line) in a result of liposomes fusion and mixing of ANTS and DPX. **(D)** The efficiency of pHLIP-mediated inter-liposomal fusion depends on the amount of PEGylated lipids (with 5 mol % of PEG-lipids as the optimal). The detailed formulation of liposome composition is presented in Table 1.

Figure 4. Cellular uptake of liposomes. A549 cells in suspension were treated with fluorescently labeled liposomes (DOPC or DOPE) with or without pHLIP in liposome coat under various conditions. Cellular uptake of fluorescent liposomes was assessed by counting of fluorescent cells on a cellometer. All data were normalized to the fluorescence intensity of untreated cells (dotted line). **(A-B)** pH- and pHLIP-dependent cellular uptake of liposomes is shown. Cells were incubated with R18-labeled liposomes with ('5:5') or without ('0:10') pHLIP in liposome coat at pH 7.4 or 6.5 in PBS for 15 min **(A)** or in serum-free DMEM for 60 min **(B)**. **(C-D)** Cells were incubated with R-18 labeled liposomes at pH 6.5 in PBS at 4°C or 37°C for 15 min **(C)** and in serum-free DMEM or ATP depletion medium at 37°C for 60 min **(D)**. Low temperature and ATP depletion medium are used to reduce endocytotic uptake. **(E)** Cellular uptake of DOPC liposomes (pH 6.5, DMEM, 37°C, 60 min) depends on amount of pHLIP and PEG in liposome coat. **(F)** The pH- and pHLIP-dependent cellular uptake does not depend on choice of fluorescent lipids. Liposomes ('5:5')

DOPC) containing different fluorescent lipids—Rho-FA (R18), Rhodamine-PE (Rho-lipid), or Fluorescein-DHPE (FITC-lipid)—were incubated with cells (pH 6.5, DMEM, 37°C, 60 min), and pHLIP-dependent fluorescence increase was observed in each case.

Figure 5. Cellular localization of liposomes. (A) A549 cells in suspension were incubated with R18 DOPE liposomes with (c & d: ‘5:5’) or without pHLIP (a & b: ‘0:10’) in liposome coat in serum-free DMEM medium at pH 6.5 for 1 hr. The light (a, c) and fluorescent images (b, d) were taken 4 days after cell treatment with liposomes.

(B) Mito-tracker fluorescent dye was used to stain mitochondria of cells treated with R18 DOPE liposomes containing pHLIP **(a)**. pHLIP mediated uptake of R18 liposomes show specific staining of mitochondria **(b)**. An overlay of **(a)** & **(b)** is shown in panel **(c)** to be certain that the staining pattern of R18 is truly due to mitochondrial localization. On the panel **(d)** a zoom-in picture of mitochondria stained with R18 liposome is shown. **(C)** Adherent A549 cells were treated with Rho-PE DOPE liposomes with **(c & d**: '5:5' formulation) or without pHLIP (**a & b**: '0:10') in PBS at pH 6.3 for 30 min. Both light **(a, c)** and fluorescent images **(b, d)** were taken immediately after washing. **(D)** Adherent A549 cells were treated with fluorescein DOPC liposomes with 5 mol % of pHLIP-PEG-lipids (PBS, pH 6.2, 1 hr). Staining of plasma membrane by FITC-lipids of liposomes shown on panel **(a)** is confirmed by staining of the plasma membrane of the same cells by Texas Red-labeled wheat germ agglutinin, as shown on panel **(b)**. **(E)** Adherent A549 cells were treated with nanogold-containing DOPE liposomes with or without pHLIP in serum-free DMEM (pH 6.5, 15 min). After fixation and silver enhancement, cells were visualized under the light microscope **(a**: no pHLIP; **b**: with pHLIP). Liposomes containing 10 mol % of pHLIP delivered more nanogold to cells (image **b**) than control liposomes without pHLIP (image **a**). The scale bar is 20 μ m in all images.

Figure 6. pHLIP-mediated liposomal delivery of propidium iodide (PI). Adherent A549 cells were incubated in serum free DMEM (pH 6.5, 1 hr) with fluorescein-labeled DOPE liposomes loaded with PI, with or without pHLIP ('5:5' or '0:10' formulation) in liposomal coating. Fluorescent images of cells treated with pHLIP-containing liposomes (**A** & **C**) or control liposomes without pHLIP (**B** & **D**) are shown side by side. The fluorescent images of the same cells were taken with two filter settings: green - fluorescein fluorescence; red - PI fluorescence. The exposure time is 172 ms for **A** & **B** and 80 ms for **C** & **D**. The scale bar is 20 μm .

Figure 7. pHLP-mediated delivery of liposomal ceramide inhibits proliferation of cancer cells at low pH. (A-B) Liposomes containing 30 mol % of C-6 ceramide were characterized using Cryo-TEM: control liposomes without pHLP and liposomes containing pHLP are shown on panels A and B, respectively. (C) DLS was used to investigate the changes of liposome size with time. The size of the liposomes was measured immediately after liposome extrusion and monitored for 3 days. (D) A549 cells in suspension were incubated with ceramide liposomes (with or without pHLP) at pH 8.0 or 6.1 for 60 min in a phosphate buffer. After washing, treated cells were grown in 96-well plates for 3 days before viable cell populations were estimated via MTS assays.

Figure 8. Schematic presentation of pHLIP/PEG coated liposomes (A) and their interaction with cellular membranes (B). The encapsulated model payload molecules and the head-groups of fluorescent lipids are shown in red.

TABLES

Formulations of fluorescently labeled liposomes

Liposome code ^a	'0:10'	'1:9'	'5:5'	'10:0'	'0:5'	'1:4'	'5:0'	'0:1'	'1:0'
DOPC (or DOPE)	83% ^b	83%	83%	83%	88%	88%	88%	92%	92%
DSPE-PEG ₂₀₀₀ -pHLIP	0%	1%	5%	10%	0%	1%	5%	0%	1%
DSPE-PEG ₂₀₀₀	10%	9%	5%	0%	5%	4%	0%	1%	0%
Fluorescent lipids ^c	7%	7%	7%	7%	7%	7%	7%	7%	7%

Formulations of nanogold labeled liposomes

	Control-liposome	pHLIP-liposome
DOPE	86%	86%
DSPE-PEG ₂₀₀₀	10%	0%
DSPE-PEG ₂₀₀₀ -pHLIP	0%	10%
Palmitoyl Nanogold	2.5%	2.5%
R18	1.5%	1.5%

Formulations of ceramide liposome

	Control-liposome	pHLIP-liposome
DOPC	37%	37%
DOPE	17.5%	17.5%
DSPE-PEG ₂₀₀₀	7.5%	2.5%
DSPE-PEG ₂₀₀₀ -pHLIP	0%	5%
C8-PEG ₇₅₀ ceramide	7.5%	7.5%
C6 ceramide	30%	30%
R18	0.5%	0.5%

Notes:

- Ratio in liposome code refers to molar ratio between pHLIP-PEG-lipids and PEG-lipids.
- Percentages indicate mole fractions.
- Fluorescent lipids are R18, Rho-PE, or fluorescein-DHPE.

Table 1. Formulation of liposomes used in various experiments

CHAPTER 3

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pHLIP peptide targets nanogold particles to tumors

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Key words: gold nanoparticles, tumor targeting, acidity, targeted delivery

Authors Contribution: LY, JD, AM, SK, and AA performed experimental work; YRK and OAA designed research; LY, JD, and OAA processed and analyzed data; DME, YKR, and OAA wrote the manuscript.

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ABSTRACT

Progress in nanomedicine depends on the development of nanomaterials and targeted delivery methods. In this work, we describe a novel method for the preferential accumulation of gold nanoparticles in tumors. The method is based on the use of the pH (Low) Insertion Peptide (pHLIP®), which targets various imaging agents to acidic tumors. We compare tumor targeting by non-functionalized nanogold particles with nanogold-pHLIP conjugates, where nanogold is covalently attached to the N-terminus of pHLIP. We observed pH-dependent, pHLIP-mediated uptake of gold nanoparticles by cultured cells. Our most important finding is that intratumoral and intravenous administrations both demonstrated a significant enhancement of tumor uptake of gold nanoparticles conjugated with pHLIP. Statistically significant reduction of gold accumulation was observed in acidic tumors and kidney when pH-nonsensitive K-pHLIP was used as a vehicle, suggesting an important role of pH in the pHLIP-mediated targeting of gold nanoparticles. The pHLIP technology can substantially improve the delivery of gold nanoparticles to tumors by providing specificity of targeting, enhancing local concentration in tumors, and allowing staining throughout the entire tumor mass for an extended period of time (several days), which is beneficial for radiation oncology and imaging. The technology also could be applied to the delivery of other types of nanoparticles to diseased tissue.

INTRODUCTION

Targeting of therapeutic nanoparticles could reduce side effects in treating cancer, as most antitumor drugs are toxic, killing not only tumor cells but also causing serious damage to healthy cells. One significant difference between cancerous and normal tissues is that the former exhibits a significantly acidic extracellular environment (1). Acidosis is a hallmark of tumor development both at very early and advanced stages (2, 3), as a consequence of anaerobic metabolism (the Pasteur effect) (4), the activity of carbonic anhydrases IX and XII (5), and the “aerobic glycolysis” known as the Warburg effect (6). Thus, the targeting of most solid tumors might be achieved by using pH-sensitive drugs and delivery systems.

We have been developing a novel approach for targeting acidic tissue, which is based on the family of pH (Low) Insertion Peptides (pHLIPs). pHLIPs are water-soluble, moderately hydrophobic polypeptides originally derived from the bacteriorhodopsin C helix. A pHLIP is triggered by acidity to fold and insert across a membrane to form a stable transmembrane alpha helix (7). At neutral pH, pHLIP is in equilibrium between soluble and membrane-bound unstructured forms, while in a low pH environment, the protonation of negatively charged residues (Asp or Glu) (8, 9) enhances peptide hydrophobicity, increasing the affinity of the peptide for the lipid bilayer and triggering peptide folding and subsequent membrane insertion (8, 10). The Gibbs free energy of pHLIP binding to a POPC liposome surface at 37°C is about -7 kcal/mol near neutral pH and the additional free energy of folding and insertion across a lipid bilayer at low pH is nearly -2 kcal/mol (11). Thus the affinity of the peptide for a

membrane at low pH is several times higher than at neutral pH, allowing pHLIP to distinguish and mark acidic diseased tissue (8, 10, 12, 13). We show that the N-terminus of pHLIP stays outside of the bilayer, while the C-terminus inserts across the lipid bilayer at low pH (14, 15), and small molecules (mostly imaging agents) covalently conjugated with the N-terminus of pHLIP can be delivered to tumors and tethered to the surfaces of cancer cells in a pH-dependent manner (12, 13, 16). As a control for pH-targeting *in vivo* we used K-pHLIP, where Asp residues in the transmembrane part of pHLIP were replaced by Lys residues. As a result, pHLIP loses its pH-dependent ability to insert into liposome phospholipid bilayers *in vitro* or cell membranes *in vitro* or *in vivo* (12, 13, 16). K-pHLIP labeled with fluorescent dyes, PET and SPECT agents show no targeting of tumors compared to the WT-pHLIP (12, 13, 16, 17). pHLIP peptides conjugated with optical and PET imaging probes are listed as pH targeting agents in the Molecular Imaging and Contrast Agent Database (MICAD), NCBI. The results of the study demonstrating tumor targeting by SPECT-pHLIP are currently in press (17).

Davies et al. recently reported the pHLIP-mediated, pH-controlled delivery of 13 nm water soluble gold nanoparticles coated with luminescent europium into human platelets *in vitro* (18). In this related work, we show here that pHLIP not only induces the binding of 1.4 nm gold nanoparticles to cancer cells at acidic pH *in vitro*, but also that it stabilizes binding in tumors established in mice. Gold is an inert and non-toxic material with unique properties suitable for many applications such as cancer diagnosis and treatment (19-21). For example, the irradiation of gold atoms at their k-

edge excitation energy (soft x-rays) will generate Auger electrons or highly reactive species that may produce a clinically achievable dose enhancement of as much as 10 fold, capable of local inactivation of biological molecules (22, 23). The targeted delivery of gold to diseased tissue that we demonstrate here could lead to the successful application of gold nanoparticles in the diagnosis and treatment of tumors.

RESULTS

Biophysical studies

Changes of tryptophan fluorescence and circular dichroism (CD) spectral signals can be used to monitor pHLIP binding to a membrane lipid bilayer at neutral pH and insertion at lower pH (7). We found that nanogold attached to pHLIP significantly quenches tryptophan fluorescence, making it unreliable for use in monitoring pHLIP insertion into a membrane (nevertheless, a wavelength shift of the tryptophan fluorescence maximum was observed. Since gold nanoparticles are achiral in the UV range (24) and do not interfere with the CD signals of a peptide, we measured the CD spectra of gold-pHLIP to study peptide-bilayer interactions. Our data indicate that pHLIP was predominantly unstructured at pH8 in the absence or presence of liposomes, whereas at low pH in the presence of POPC liposomes, we observed helix formation (Figure 1). Thus, we conclude that the attachment of gold nanoparticles to the N-terminus of pHLIP did not interfere with the ability of the peptide to form a helical structure interacting with the membrane at low pH. We interpret these data to indicate that the pHLIP inserted into the membrane at low pH when it formed a helix.

Nanogold uptake by cultured cells

We compared the binding of gold-pHLIP or gold nanoparticles to cultured cells at neutral and low pHs. Nanoparticles (2 μ M, 200 μ L) were incubated with HeLa-GFP cells at pH7.4 or 6.5. After 1 hour, cells were washed, fixed, and stained with silver enhancement solution, resulting in the deposition of silver on gold nanoparticles to form micrometer sized particles, which were visualized under a light microscope (Figure 2). The images on Figure 2 were analyzed according to **equation 2** (see **Methods**) to establish the ratios of gold (and silver) concentrations between the cells treated with gold-pHLIP or gold nanoparticles and the untreated cells at pH7.4 and 6.5. Cells treated with gold-pHLIP nanoparticles at both pHs (Figure 2c, g) showed higher uptake of gold (3 fold at pH7.4 and 6.4 fold at pH6.5) compared to cells treated with unmodified gold nanoparticles (Figure 2b, f). Cellular uptake of gold-pHLIP nanoparticles at pH6.5 (Figure 2g, h) was 1.6 times higher than that at pH7.4 (Figure 2c, d). These data demonstrated the pH-dependent, pHLIP mediated uptake of gold nanoparticles by cells.

Animal studies

When subcutaneous HeLa tumors reached 5-8 mm in diameter, they were intratumorally or intravenously (IV) injected with gold nanoparticles, gold-pHLIP, and gold-K-PHLIP conjugates. At 24 h after intratumoral administration of 15 μ g gold or gold-pHLIP nanoparticles, mice were euthanized, and the tumors and major organs were harvested. The amount of gold in the tissues and organs was established by ICP-

MS analysis (Figure 3a and Table 1 supporting information). These results indicated that up to 8 μg (i.e. 54% id/g) of gold per gram of tumor was delivered by pHLIP and remained in the tumor after 24 h, compared to 0.7 $\mu\text{g/g}$ (8% id/g) in the case of non-functionalized gold nanoparticles. Accumulation of gold was also observed in kidneys (about 2.1 μg per gram). Since small particles (1.4 nm) were used in this study, it is likely that clearance was predominantly urinary, as has been observed with fluorescent-, PET- and SPECT-labeled pHLIP constructs (13, 25). We have previously observed that the pHLIP can accumulate in kidneys due to the low pH in the tubules (16, 26).

I.V. administration was given as two consecutive injections within 24 h. Split dosing was employed to avoid high-concentration mediated aggregation. Each injection contained 150 μL of 20 μM gold-pHLIP, gold-K-pHLIP, or non-functionalized gold particles (i.e. 45 μg). Necropsies were performed 24 h after the last injection. These data showed that 1.4 μg of gold per gram (1.5% id/g) of tissue was delivered by pHLIP to the tumors (Figure 3b and Table 1 supporting information). When pH-nonsensitive K-pHLIP was used as a delivery vehicle, tumor targeting was reduced 3 fold (0.47 $\mu\text{g/g}$ or 0.5%). The uptake of gold-K-pHLIP was also reduced 3 fold in kidneys, and the distribution in other organs was similar to gold-pHLIP.

To assess distribution of these gold nanoparticles within tissues, tumors, kidneys, and livers were collected and sectioned 4 h and 24 h after gold-pHLIP or gold nanoparticle administration. Silver enhancement was used to visualize the gold nanoparticles in

tissue sections. The same conditions of silver enhancement were used for all slides shown in Figure 4. The amount of gold/silver correlated with the darkness of the tissue sections. These data show that gold distribution within the tumors, kidney, and liver were higher and uniform at both time points for gold-pHLIP injection than for unmodified gold administration.

The sections were further analyzed under a microscope with x10 (Figure 5) and x100 (Figure 6) objectives. The gold distribution in tumors and livers (Figure 5d, l) was homogeneous, in contrast to the distribution in kidneys (Figure 5h), where the gold was mostly accumulated in the cortex. Cellular localization was confirmed by staining of cell nuclei with DAPI (Figure 6). The images of the tumor sections demonstrated that pHLIP was distributed throughout the entire tumor mass, penetrating to the cells in the tumor interior and labeling the extracellular space and cellular membrane (Figure 6a-c).

DISCUSSION

Gold nanoparticles are of interest as potential diagnostic and therapeutic agents *in vivo*, since they can be used as X-ray contrast agents (27), radiation enhancers (28), and laser (29, 30) and radiofrequency (31) thermotherapy enhancers. If tumors can be loaded with gold, this would lead to a higher dose to the cancerous tissue compared with the dose received by normal tissue during a radiotherapy treatment. Calculations indicate that the dose enhancement can be significant, as much as 200% or greater (28). Gold nanoparticles are not toxic: the LD₅₀ of this material is approximately 3.2 g

Au kg⁻¹ (27). Thus, the targeted delivery of gold nanoparticles could improve the treatment of cancers. Specific delivery can be accomplished by conjugating gold or any other nanoparticles to antibodies or ligands that target proteins overexpressed on cancer cell surfaces, and this approach has been actively explored for many years for delivery of small molecules. However, several recent studies have raised serious questions on the efficacy of targeting ligands on the nanoparticle accumulation in tumor tissues. Multiple reports have shown that targeted nanoparticles did not lead to increased tumor accumulation over non-targeted controls, although increased cellular uptake was observed in each case (32-34). In addition, histological studies showed that antibodies conjugated with gold nanoparticles do not penetrate deeply into tumors, but mostly stain peripheral tumor regions (35). The direct injection of micron-sized gold particles does not lead to tumor targeting, as particles stayed only at the injection site and were not able to diffuse even within a tumor, hindering tumor coverage (22).

In the present work, we report the promising finding that pHLIP shows pH-dependent uptake of small gold nanoparticles by cultured cells, and, the most important, pHLIP can enhance IV delivery of gold nanoparticles to tumors by 6 fold. On the other hand, nano-sized gold particles with no conjugated pHLIP were washed out quickly from tumors and other organs. Targeting with the pH-insensitive K-pHLIP (wherein two Asp residues were replaced by Lys) resulted in statistically significant 3 fold reductions of gold accumulation. We interpret these data to indicate that the preferential distribution of pHLIP-targeted gold was mediated by the acidosis present

in tumors and kidney. The direct injection of gold-pHLIP into tumors resulted in nearly uniform labeling of cancer cells with gold nanoparticles throughout the entire tumor. Direct injection resulted in accumulation of 8.1 $\mu\text{g/g}$ (54% id/g) in the tumor after 24 h. An N-fold increase in diameter gives N^3 increase in number of gold atoms per particle, so with gold particles of 14 nm it might be possible to increase the amount of gold 1000-fold and to reach about 1% of gold of tumor mass, which is the amount that would be needed for a significant enhancement of the radiation therapeutic effect.

This work demonstrated that pHLIP can mediate targeted delivery of nanoparticles to tumors. Further improvements of nanoparticle coatings may be able to improve bioavailability, enhance tumor targeting and reduce accumulation in non target organs, pHLIP technology can substantially improve the delivery of gold nanoparticles to primary tumors (and possibly metastatic lesions) by providing specificity of targeting, enhancing local concentrations, and improved retention in the tumor mass for an extended period of time (several days).

METHODS

Peptide conjugation with gold nanoparticles

The pHLIP (ACEQNPIYWARYADWLFTTPLLLLDLALLVDADET) and K-pHLIP (ACEQNPIYWARYAKWLFTTPLLLKLALLVDADET) peptides were prepared by Dr. James I. Elliott at the W.M. Keck Foundation Biotechnology Resources

Laboratory at Yale University (New Haven, CT). Monomaleimido Nanogold® and non-functionalized particles (1.4 nm) were from Nanoprobes, Yaphank, NY. A pHLIP peptide containing a single cysteine residue near its N-terminus was conjugated with the single maleimide group on each nanogold particle. The progress of the coupling reaction was monitored by reversed phase HPLC. The concentration of peptide and nanogold was determined by absorbance at 280 nm ($\epsilon=13,940 \text{ M}^{-1} \text{ cm}^{-1}$) and 420 nm ($\epsilon=155,000 \text{ M}^{-1} \text{ cm}^{-1}$), respectively.

Circular dichroism measurements

Circular dichroism (CD) measurements were carried out on a MOS-450 spectrometer (BioLogic; Montrose, CO) at 25°C. Nonfunctionalized nanogold particles or nanogold-pHLIP conjugates were pre-incubated with 200 mol excess of POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine from Avanti Polar Lipids, Alabaster, AL) liposomes in 20 mM phosphate buffer, pH8.0. Liposomes with 50 nm diameter were prepared by extrusion. To induce the folding/insertion of peptide into the membrane lipid bilayer, HCl was added to lower the pH value from 8 to 4. CD signals of gold nanoparticles were taken as baseline signals and were subtracted from the corresponding signals of the nanogold-pHLIP conjugates.

Cell lines

HeLa cells (human cervix adenocarcinoma, ATCC; Manassas, VA) without and with stable GFP expression, were cultured in Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 10% fetal bovine serum, 10 µg/mL of ciprofloxacin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Some cells were adapted by serial passages to improve growth in low pH medium (pH6.5). The pH 6.5 medium was prepared by mixing 13.5 g of dry DMEM with 0.2 g of sodium bicarbonate in 1 L of deionized water.

Experiments on cultured cells

HeLa-GFP cells grown in pH6.5 or 7.4 media were seeded in an 8-chamber slide (Lab-Tek™, Thermo Scientific; Rochester, NY). At 80% confluency, cells were treated at pH 6.5 or 7.4 with 2 µM of nanogold or nanogold-pHLIP in 200 µL of serum-free DMEM at 37°C under 5% CO₂. Cells not treated with nanogold were used as negative controls. After incubation for 1 h, the treatment solution was removed and cells were washed twice with serum-free DMEM (pH 7.4), and once with sterile PBS (pH7.4). Subsequently, the cells were fixed with cold methanol at -20°C for 15 min, and then washed twice with sterile PBS (pH7.4) and once with deionized water. After air drying, the chamber walls were removed and the cell slides were developed with freshly prepared HQ SILVER™ reagent (Nanoprobes; Yaphank, NY). The reagent nucleates on the nanogold particles, resulting in the precipitation of metallic silver and the formation of micrometer sized particles with low background. The developing time was varied until optimal conditions were found (about 20 min). Subsequently, the cell slides were rinsed twice with deionized water, and viewed under a light microscope.

Estimation of cellular uptake of gold/silver based on the analysis of cell images

After silver staining, the brightfield images of cells were analyzed with the ImageJ program. For cells treated with nanogold, the mean intensities of the fields with cells (I_i) and without cells ($I_{0,i}$, background) were calculated. The concentration of gold/silver (c_i) can be represented as a mean intensity according to the **Beer–Lambert law**:

$$c_i = \frac{A_i}{\varepsilon d} = \frac{1}{\varepsilon d} \ln \frac{I_{0,i}}{I_i} \quad (1)$$

$$\frac{c_i}{c_{nt}} = \frac{\ln \left(I_{0,i} / I_i \right)}{\ln \left(I_{0,nt} / I_{nt} \right)} \quad (2)$$

where A_i is the absorbance; ε is the extinction coefficient and d is the thickness of the sample. For cells not treated with nanogold, c_{nt} , I_{nt} , and $I_{0,nt}$ correspond to the concentration of gold/silver, measured as the mean intensities of the fields with and without cells, respectively. We assume that the extinction coefficient and thickness are the same for all slides, so that the ratio of concentrations can be calculated according to equation (2) by knowing the mean intensities.

Tumor targeting in mice

Athymic female nude mice ranging in age from 4 to 6 weeks and weighing from 15 to 18 g were obtained from Harlan Laboratories (Indianapolis, IN), 38 mice were used in

the study. Mouse tumors were established by subcutaneous injection of HeLa cells (10^6 cells/0.1 ml/flank) in the right flank of each mouse. When tumors reached 5-8 mm in diameter, intratumoral or tail vein injections of nanogold samples were performed. For intratumoral injection, 50 μ L of 20 μ M nanogold or nanogold-pHLIP in sterile PBS (pH7.4) was given at each of three different spots in each tumor. For tail vein injection, two consecutive injections of 150 μ L of 20 μ M nanogold-pHLIP or non-functionalized nanogold and nanogold-K-pHLIP as controls were given within 24 h. Animals were euthanized at 4 or 24 h after the last injection. Necropsy was performed immediately after euthanasia. Tumors and major organs were collected for further histological analysis and study using inductively coupled plasma mass spectroscopy (ICP-MS). Non-injected mice with similar-sized tumors were used as negative controls.

All animal studies were conducted according to an approved animal protocol (AN07-01-015), in compliance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals.

Histological analysis of tumors and organs

The excised tumors and organs were fixed in 4% formalin in PBS solution (pH 7.4) for 24 h at 4°C. Tissues were then rinsed with sterile PBS (pH7.4), blotted dry, and placed in 30% sucrose in PBS solution for at least 24 h at 4°C. Samples were mounted in HistoPrep® frozen tissue embedding medium (Fisher Scientific; Pittsburgh, PA) and

frozen in the quick freezer chamber of a cryostat (Vibratome UltraPro5000, GMI; Ramsey, MN) at -80°C . Samples were frozen only one time, to minimize tissue damage. When the temperature of mounted frozen samples was equilibrated with the working chamber temperature (-12°C), the tissue was sectioned at a thickness of 10–20 μm . Sections were mounted on microscope slides coated with poly-lysine, dried in air, and washed with deionized water. Subsequently, silver enhancement of gold nanoparticles was carried out (developing time: 10 min). In some cases, further staining with 4',6-diamidino-2-phenylindole (DAPI) was performed to stain cell nuclei. The stained section was covered with a drop of mounting medium (Permount®, Fisher Scientific; Pittsburgh, PA) and then a cover slide was placed over the medium. The slides were examined with an inverted fluorescence microscope (IX71 Olympus).

ICP-MS analysis

Mouse tissue samples were dissolved in aqua regia, freshly prepared by mixing concentrated nitric and hydrochloric acids in a volume ratio of 1:3. If necessary, sonication or heating was used to facilitate the digestion of tissue samples. Then the concentrated sample solutions were diluted to 10 mL to give 2% nitric acid and analyzed via ICP-MS (Thermo scientific X7 series) against calibration standards IMS 103 (UltraScientific; N.Kingston, RI).

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REFERENCES

1. Gatenby RA & Gillies RJ (2008) A microenvironmental model of carcinogenesis. *Nat Rev Cancer* 8(1):56-61 .
2. Gillies RJ, Liu Z, & Bhujwalla Z (1994) ³¹P-MRS measurements of extracellular pH of tumors using 3-aminopropylphosphonate. *Am J Physiol* 267(1 Pt 1):C195-203 .
3. Newell K, Franchi A, Pouyssegur J, & Tannock I (1993) Studies with glycolysis-deficient cells suggest that production of lactic acid is not the only cause of tumor acidity. *Proc Natl Acad Sci U S A* 90(3):1127-1131 .
4. Krebs HA (1972) The Pasteur effect and the relations between respiration and fermentation. *Essays Biochem* 8:1-34.
5. Swietach P, Hulikova A, Vaughan-Jones RD, & Harris AL (2010) New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation. *Oncogene* 29(50):6509-6521 .
6. Warburg O, Wind F, & Negelein E (1927) The metabolism of tumors in the body. *J Gen Physiol* 8(6):519-530.
7. Hunt JF, Rath P, Rothschild KJ, & Engelman DM (1997) Spontaneous, pH-dependent membrane insertion of a transbilayer alpha-helix. *Biochemistry* 36(49):15177-15192 .
8. Andreev OA, Engelman DM, & Reshetnyak YK (2009) Targeting acidic diseased tissue: New technology based on use of the pH (Low) Insertion Peptide (pHLIP). *Chim Oggi* 27(2):34-37 .

9. Musial-Siwek M, Karabadzhak A, Andreev OA, Reshetnyak YK, & Engelman DM (2010) Tuning the insertion properties of pHLIP. *Biochim Biophys Acta* 1798(6):1041-1046 .
10. Andreev OA, *et al.* (2010) pH (low) insertion peptide (pHLIP) inserts across a lipid bilayer as a helix and exits by a different path. *Proc Natl Acad Sci U S A* 107(9):4081-4086 .
11. Reshetnyak YK, Andreev OA, Segala M, Markin VS, & Engelman DM (2008) Energetics of peptide (pHLIP) binding to and folding across a lipid bilayer membrane. *Proc Natl Acad Sci U S A* 105(40):15340-15345 .
12. Andreev OA, Engelman DM, & Reshetnyak YK (2010) pH-sensitive membrane peptides (pHLIPs) as a novel class of delivery agents. *Mol Membr Biol* 27(7):341-352 .
13. Vavere AL, *et al.* (2009) A novel technology for the imaging of acidic prostate tumors by positron emission tomography. *Cancer Res* 69(10):4510-4516 .
14. Reshetnyak YK, Segala M, Andreev OA, & Engelman DM (2007) A monomeric membrane peptide that lives in three worlds: in solution, attached to, and inserted across lipid bilayers. *Biophys J* 93(7):2363-2372 .
15. Reshetnyak YK, Andreev OA, Lehnert U, & Engelman DM (2006) Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix. *Proc Natl Acad Sci U S A* 103(17):6460-6465 .
16. Reshetnyak YK, *et al.* (2011) Measuring tumor aggressiveness and targeting metastatic lesions with fluorescent pHLIP. *Mol Imaging Biol* 13(6):1146-1156 .

17. Macholl S, Morrison, M.S., Iveson, P., Arbo, B.E., Andreev, O.A., Reshetnyak, Y.K., Engelman, D.M., Johannesen, E. (2012) In vivo pH imaging with ^{99m}Tc-pHLIP
Mol Imaging Biol.
18. Davies A, Lewis DJ, Watson SP, Thomas SG, & Pikramenou Z (2012) pH-controlled delivery of luminescent europium coated nanoparticles into platelets. *Proc Natl Acad Sci U S A* 109(6):1862-1867 .
19. Huang X, Jain PK, El-Sayed IH, & El-Sayed MA (2007) Gold nanoparticles: interesting optical properties and recent applications in cancer diagnostics and therapy. *Nanomedicine (Lond)* 2(5):681-693 .
20. Jain PK & El-Sayed MA (2007) Universal scaling of plasmon coupling in metal nanostructures: extension from particle pairs to nanoshells. *Nano Lett* 7(9):2854-2858 .
21. von Maltzahn G, *et al.* (2009) Computationally guided photothermal tumor therapy using long-circulating gold nanorod antennas. *Cancer Res* 69(9):3892-3900 .
22. Herold DM, Das IJ, Stobbe CC, Iyer RV, & Chapman JD (2000) Gold microspheres: a selective technique for producing biologically effective dose enhancement. *Int J Radiat Biol* 76(10):1357-1364 .
23. Hainfeld JF, Slatkin DN, & Smilowitz HM (2004) The use of gold nanoparticles to enhance radiotherapy in mice. *Phys Med Biol* 49(18):N309-315 .

24. Slocik JM, Govorov AO, & Naik RR (2011) Plasmonic circular dichroism of Peptide-functionalized gold nanoparticles. *Nano Lett* 11(2):701-705 .
25. Macholl S, *et al.* (2012) In Vivo pH Imaging with (99m)Tc-pHLIP. *Mol Imaging Biol* .
26. Andreev OA, *et al.* (2007) Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues in vivo. *Proc Natl Acad Sci U S A* 104(19):7893-7898 .
27. Hainfeld JF, Slatkin DN, Focella TM, & Smilowitz HM (2006) Gold nanoparticles: a new X-ray contrast agent. *Br J Radiol* 79(939):248-253 .
28. Hainfeld JF, Dilmanian FA, Slatkin DN, & Smilowitz HM (2008) Radiotherapy enhancement with gold nanoparticles. *J Pharm Pharmacol* 60(8):977-985 .
29. Mahmoud MA & El-Sayed MA (2010) Gold nanoframes: very high surface plasmon fields and excellent near-infrared sensors. *J Am Chem Soc* 132(36):12704-12710 .
30. Black KC, *et al.* (2008) Gold nanorods targeted to delta opioid receptor: plasmon-resonant contrast and photothermal agents. *Mol Imaging* 7(1):50-57 .
31. Glazer ES, *et al.* (2010) Noninvasive radiofrequency field destruction of pancreatic adenocarcinoma xenografts treated with targeted gold nanoparticles. *Clin Cancer Res* 16(23):5712-5721 .
32. Sonvico F, *et al.* (2005) Folate-conjugated iron oxide nanoparticles for solid tumor targeting as potential specific magnetic hyperthermia mediators:

synthesis, physicochemical characterization, and in vitro experiments. *Bioconjug Chem* 16(5):1181-1188 .

33. Kirpotin DB, *et al.* (2006) Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Res* 66(13):6732-6740 .
34. Huang X, *et al.* (2010) A reexamination of active and passive tumor targeting by using rod-shaped gold nanocrystals and covalently conjugated peptide ligands. *ACS Nano* 4(10):5887-5896 .
35. Hainfeld JF, *et al.* (2011) Micro-CT enables microlocalisation and quantification of Her2-targeted gold nanoparticles within tumour regions. *Br J Radiol* 84(1002):526-533 .

FIGURES

Figure 1. Circular dichroism spectra of gold-pHLIP in buffer pH 8.0 (state I), and in the presence of POPC liposomes at pH 8.0 (state II), and pH 4.0 (state III).

Figure 2. Cellular uptake of gold-pHLIP and gold nanoparticles. Nanoparticles (2 μ M, 200 μ L) were incubated with HeLa-GFP cells at pH7.4 or 6.5. After 1 hour, cells were washed, fixed, and stained with silver enhancement solution, resulting in the deposition of silver on gold nanoparticles to form micrometer sized particles, which were visualized under a light microscope. The images *a-g* and *d-h* were taken with x10 and x40 objectives, respectively.

Figure 3. ICP-MS analysis of the amount of gold in the excised tissues. Either a single intratumoral injection of gold-pHLIP or gold nanoparticles (a; 20 μ M, 50 μ L) or two IV injections of gold-pHLIP, gold-K-pHLIP and gold (b; 20 μ M, 150 μ L each) were given to mice bearing subcutaneous tumors. Organs were collected at 24 h after the last injection. Organ uptakes of gold via intratumoral and IV injections are shown in (a) and (b), respectively. The amount of gold per gram of tissue is given in Table S1, supporting information. Mean $\pm$ SEM values are shown; * p <0.0001 vs respective value of tumor uptake of gold targeted by gold-pHLIP.

Figure 4. Accumulation of gold-pHLIP and gold nanoparticles in tumor, kidney, and liver. The slices indicated by * were not treated with silver enhancement solution.

Figure 5. Photomicrographs (x10) of gold nanoparticles in tumor, kidney, and liver sections after silver staining.

Figure 6. Distributions of gold-pHLIP enhanced by silver in tumor, kidney, and liver. Slices were visualized under an inverted optical microscope with a x100 objective. The nuclei were stained with DAPI (blue color). Bright field (*a, d, g*) and fluorescent (*b, e, h*) images of the same sections and their overlays (*c, f, i*) of tumor, kidney, and liver slices are presented.

SUPPORTING INFORMATION

	Au-pHLIP, IT <i>n</i>=5	Au, IT <i>n</i>=4	Au-pHLIP, IV <i>n</i>=10	Au-K- pHLIP, IV <i>n</i>=7	Au, IV <i>n</i>=4
Blood	0.10±0.25	0.12±0.06	0.63±0.04	0.58±0.09	0.14±0.05
Tumor	8.05±1.69	0.72±0.35	1.35±0.24	0.47±0.11	0.22±0.02
Heart	0.49±0.29	0.05±0.02	0.62±0.04	0.39±0.03	0.07±0.01
Lung	0.64±0.49	0.09±0.04	0.90±0.14	0.66±0.10	0.13±0.01
Liver	1.29±0.57	0.21±0.09	19.27±1.24	23.99±1.82	0.35±0.01
Kidney	2.14±0.37	0.94±0.39	14.76±0.52	5.35±0.80	1.35±0.16
Spleen	0.87±0.55	0.11±0.05	8.24±0.93	13.23±1.91	0.14±0.04
Bladder	0.54±0.68	0.24±0.11	1.56±0.44	1.09±0.10	0.16±0.10
Stomach	0.02±0.07	0.05±0.02	0.50±0.03	0.30±0.03	0.09±0.01
Intestine	0.12±0.04	0.08±0.04	0.78±0.11	0.30±0.03	0.10±0.02
Muscle	0.47±0.41	0.17±0.06	0.29±0.02	0.20±0.03	0.06±0.01
Brain	0.02±0.07	0.19±0.09	0.08±0.02	0.06±0.02	0.01±0.01

Table 1. The average amount of gold calculated in micro grams per gram of tissue obtained from ICP-MS analysis of the excised tissues after intratumoral injection of 15 µg of gold and IV administration of 90 µg of gold. Mean ± SEM values are shown, and *n* is the number of animals used in experiment for each construct.

CHAPTER 4

In preparation for publication

pHLIP® Coated Gold Nanoparticles

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ABSTRACT

Gold nanoparticles find a wide range of applications in medical sciences. We are particularly interested in the use of gold nanoparticles as radio- and thermo-synthesizers for the enhancement of radiation effects and photo-induced hyperthermia. One of the most important aspects of success is the enhancement of gold nanoparticle uptake by cancer cells. Therefore, we introduced novel approaches in the synthesis of gold nanoparticles coated with tumor-targeting pH (Low) Insertion Peptide (pHLIP®) and polyethylene glycol (PEG). The presence of pHLIP® peptide in the nanoparticle coating enhances the stability of particles and promotes pH-dependent cellular uptake. The spherical particles with a metallic core diameter of ~6 nm and a mean hydrodynamic diameter of ~43 nm were prepared with citrate as a reducing agent. When the particles were injected into tumors in mice (21 µg of gold), they were nearly homogeneously distributed within tumor masses and did not stain the adjacent muscle tissue. One hour after injection, tumors retained up to ~30% of the injected gold. This opens the opportunity to use pHLIP® and PEG coated spherical gold nanoparticles for the enhancement of radiation therapy. The spiked gold nanoparticles with a metallic core diameter of ~148 nm and a mean hydrodynamic diameter of ~161 nm were prepared using ascorbic acid as a reducing agent in the presence of disk-like bicelles. Only the presence of bicelles ensured the appearance of resonance in the near infrared region. The illumination of spiked gold nanoparticles by an 805 nm laser led to the time- and concentration-dependent increase of temperature. The temperature was raised from 26°C to 40°C within 5 min. This allows the possibility to use pHLIP® and PEG coated spiked gold nanoparticles for photo-induced hyperthermia.

INTRODUCTION

Gold nanoparticles (ranging in size from 1 to 200 nm) have numerous applications in medicine, including the enhancement of x-ray radiation damage to tumor tissues ¹⁻⁴ and the induction of localized heating in photothermal therapy ⁵⁻⁷. The latter is achieved when gold nanoparticles are exposed to light and impart heat energy to their local environment due to the temporary existence and decay of localized surface plasmon polaritons. Whenever gold nanoparticles are used, not only should the particles be stable, but they should preferentially target diseased tissue such as a cancerous tumor.

Many contemporary therapeutic and imaging approaches to cancer therapy rely on tumor-targeting biomarkers such as antibody technologies or vitamin binding ⁸⁻¹². Although there are significant successes, the inter- and intra-heterogeneity of tumors, their complexity, and their ability to adapt to new conditions often create parts of a tumor that may not express enough of the biomarker to be targeted. This results in the regrowth of resistant tumors and poor therapeutic outcomes where the average improvements in survival are measured in months. At the same time, there is an emerging body of evidence indicating that the acidity of the tumor tissue plays a key role in determining cancer cell invasiveness and resistance to therapies. Tumors exhibit marked heterogeneity due to genetic alterations, which lead to the modification of biochemical pathways. These modifications, which result from metabolic alterations, lead to a surge in extracellular acidity. Tumor cells adapt to this acid-

induced toxic environment by stimulating proteins that regulate intracellular homeostasis ^{13, 14}.

Among pH-targeting agents, the family of pH (Low) Insertion Peptides (pHLIP®) find a broad range of applications in biomedical sciences. pHLIP® peptides insert across cellular membranes in a pH-dependent manner, with one terminus exposed to the extracellular space and the other terminus in the cytoplasm ¹⁵⁻¹⁸. The molecular mechanism behind a pHLIP® peptide's action is based on the protonation of Asp/Glu residues, which enhances the peptide's hydrophobicity and promotes its membrane-associated folding and insertion as a transmembrane helix ^{19, 20}. It was shown that pHLIP® peptides have excellent targeting of acidic tumors and allows the specific delivery of imaging and therapeutic agents to cancer cells within tumors ^{16, 21-23}. The number and variety of pHLIP® based nanomedicines continues to grow. For example, coating lipid-based, polymeric, and metallic nanoparticles with pHLIP® peptides enhances the targeting of acidic tumors and the number particles internalization within cancer cells ²⁴⁻²⁹.

We have shown the specific tumor accumulation of gold nanoclusters of 1.4 nm diameter conjugated to a pHLIP® peptide ³⁰, and an enhancement of radiation effects observed for cancer cells treated with these pHLIP® gold nanoclusters ³¹. As well, novel photo-induced pHLIP® coated hollow gold nanospheres containing chlorin e6 were introduced recently ^{27, 28}. The nanospheres experience hyperthermia within 5 min of laser exposure leading to the release of photosensitizers due to the reduction of

electrostatic interaction. While the obtained results are very interesting and promising, there are aspects which require improvement and justify further work: i) the coating of gold nanoclusters conjugated with pHLIP® peptide should be improved to avoid particle aggregation; and ii) a greater amount of gold might be needed in tumors to observe significant radiation enhancement effects. Therefore, here we introduce novel approaches for the synthesis of pHLIP® and PEG coated spherical and spiked gold nanoparticles for the enhancement of radiation treatment and near infrared (NIR) thermal therapy.

MATERIALS AND METHODS

Materials for Synthesis of pHLIP® and PEG Coated Gold Nanoparticles

Gold III chloride, 5% aqueous solution, was purchased from Salt Lake Metals (Salt Lake City, UT). Sodium citrate tribasic, L-ascorbic acid, and urea (98+ %) dehydrate were purchased from Sigma-Aldrich Co, LLC (St. Louis, MO). The wild type (WT) pHLIP® peptide:

ACEQNPIYWARYADWLFTTPLLLLDLALLVDADET

was synthesized and purified by C.S. Bio Co. (Menlo Park, CA), and the concentration of the peptide was determined by absorbance at 280 nm ($\epsilon=13,940 \text{ M}^{-1} \text{ cm}^{-1}$). m-polyethylene glycol-SH (mPEG-SH), about 5 kDa in mass, was purchased from Creative PEGworks (Chapel Hill, NC). The lipids, 1,2-dimyristoyl-**sn**-glycero-3-phosphocholine (DMPC) and 1,2-diheptanoyl-**sn**-glycero-3-phosphocholine (DHPC, D7PC), were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Tris-(2-

carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Thermo Fisher Scientific (Waltham, MA). The procedures for spherical and spiked pHLIP® and PEG coated gold nanoparticle synthesis are described in the Results section.

Bicelle Preparation

Bicelles were prepared by thin-film method: a chloroform solution of DMPC and D7PC (DHPC) lipids were dried using a rotary evaporator to produce an even thin film, followed by the additional overnight evaporation under a high vacuum to remove traces of organic solvents. The lipid layer was re-suspended in 10 mM phosphate buffer, pH 6.5, and underwent ten freeze-thaw-vortex cycles using liquid nitrogen and a warm bath (40°C).

Purification of Gold Nanoparticles

To remove large particles (in the case of spherical gold nanoparticles) and excess reducing agents, the final solution of particles underwent two forms of filtration. Large particles were removed using centrifugation (5 min at 10,000 relative centrifugal force (RCF). Excess reducing agents were removed by size-exclusion chromatography using Sephadex™ G25 fast spin column. 500 µL of particles was added to 5 mL of Sephadex™ G25 gel filtration beads (Sigma-Aldrich Co, LLC) (the beads were hydrated in the desired buffer) and centrifuged for 0.5 min increments at roughly 36 RCF. Amicon® Ultra-0.5, Ultracel-30 membrane, 30 kDa filters (EMD Millipore) were used according to manufacturer's instructions to concentrate particles for 21 µg

injections *in vivo*. The final spherical nanoparticle solutions were passed through a 0.2 μm sterile Acrodisk® syringe filter with HT Tuffryn® membrane (Pall Life Sciences) for sterilization.

UV-visible Absorption Spectroscopy

The absorbance of gold nanoparticles was recorded on a Genesys 10S UV-Vis Spectrophotometer to establish the wavelength range of resonance.

Particle Size

The sizes of bicelles and gold nanoparticles in solution were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern) instrument and a nanoparticle tracking system, Nanosight (NS300, Malvern). Bicelle size histograms determined by DLS were fitted with a Gaussian function and the peak size was reported. Transmission electron microscopy (TEM) (JEOL 2100) with an accelerating voltage of 200 kV at magnifications in the range of 10,000x to 150,000x was used to establish the shape, size, and homogeneity of the gold nanoparticles. Samples were prepared for TEM by drying 5 μL of the nanoparticle solution on a carbon type-B, 300 mesh, copper grid (Ted Pella, Inc).

ICP-MS Analysis

The investigated samples (cells, tumor tissue, or nanoparticles in solution) were dissolved in a concentrated solution of aqua regia (1:3 volume ratio of nitric acid and

hydrochloric acid) and sonicated. The final solutions were diluted to 2% (wt/vol) nitric acid with distilled water and centrifuged to remove organic tissue. The gold content was quantified using inductively coupled plasma mass spectrometry (ICP-MS) (Thermo scientific X7 series, and Thermo X-Series 2 quadrupole) by using calibration standards IMS 103 (UltraScientific).

Heating

In a PMMA cuvette, 300 μ L of solution was illuminated by an 805 nm temperature controlled laser diode with 500 mW output (Thorlabs TCLDM9). The sample was irradiated through the cuvette window of 4.5 mm in width and 12.5 mm in depth, where 300 μ L of the sample reached ~8 mm in height. The solution temperature was measured every minute with an immobilized FLIR E6 thermal imager (FLIR Systems, Inc.) from above to avoid obstruction by the cuvette walls. A sample of phosphate-buffered saline (PBS), pH 7.4, was illuminated and used as a reference.

Cancer Cells

JC murine mammary-gland adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and 10 μ g/mL ciprofloxacin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Cytotoxicity Assay

JC cancer cells were seeded in a 96-well plate (3,000-3,500 cells per well in 100 μ L RMPI medium) and incubated overnight. The next day, 100 μ L of gold nanoparticles in PBS, pH 7.4, supplemented with 10 mM of D-glucose was added to the cells at two concentrations and incubated for 48 hours. Final gold concentrations after the addition of particles to cells were 0.025 g/L and 0.0125 g/L. Cell viability was assessed by a colorimetric reagent (CellTiter 96 AQueous One Solution Assay, Promega), which was added to cells for one hour (without removing treatment solutions), followed by the measurement of absorbance at 490 nm by an iMark^{TM} Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.). The readings were corrected for the absorbance of gold nanoparticles at 490 nm. All samples were prepared in triplicate.

Cellular Uptake of Gold Nanoparticles

Various amounts of JC cancer cells (from 100,000 to 1 million cells in different experiments) were treated in suspension with gold nanoparticles (typically the final concentration of gold with cells was 0.06 g/L) for three hours at 37°C in serum-free Leibovitz's L-15 medium at pH 6.0 or pH 7.4 (final treatment volume was 500 μ L). In another experiment, JC cells were pre-treated with gold nanoparticles in serum-free L-15 medium at pH 7.4 for 20 min, and then media (with different pHs) were added to give a total volume of 500 μ L and a final solution pH of 7.4 or 6.0-6.2. After treatment, samples were pelleted (5 min at 600 RCF) and washed with PBS, pH 7.4, three times. The amount of gold in each pellet was then quantified using ICP-MS. The

amount of gold not taken up by cells was quantified in the supernatant by absorbance measurements. Control samples of gold nanoparticles without cells and cells without particles were investigated at both neutral and low pH as well. All samples were prepared in triplicate.

Uptake and Distribution of Gold in Tumors

All animal studies were conducted according to the animal protocol AN07-01-015 approved by the Institutional Animal Care and Use Committee, University of Rhode Island, in compliance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals. Female Balb/C mice ranging in age from 4 to 6 weeks and weighing from 18 to 22 g were obtained from Envigo RMS, Inc; 16 mice were used in the study. Tumors were established in the right flank by subcutaneous injection of JC cancer cells (10^6 cells in 100 μ L). After about two weeks, 50 μ L of 0.11 g/L gold particles (5.5 μ g of gold), or 0.42 g/L gold particles (21 μ g of gold) in PBS, pH 7.4, supplemented with 10 mM D-glucose were injected intra-tumorally. An hour after injection, mice were euthanized and the tumors were immediately collected for histological and gold-quantification analysis. The amount of gold in each tumor was quantified using ICP-MS. A non-injected mouse with a similar-sized tumor was used as a negative control. The distribution of gold within the tumor was accessed by white light imaging of the tumor center.

Histological Staining and Imaging of Tumor Sections

Immediately after tumors were collected from the mice, they were frozen in tissue-tek OCT compound using liquid nitrogen and stored at -80 °C until sectioned using a cryostat at -25°C (Vibratome UltraPro5000, GMI). When the temperature of the mounted frozen samples was equilibrated with the working chamber temperature (-25°C), the tissue was sectioned at a thickness of 20 µm. Tumor sections were then fixed in a 4% formaldehyde solution (Sigma-Aldrich Co.), washed three times with deionized water, permeated with 0.3% Triton X-100 (Sigma-Aldrich, Co.) in PBS, pH 7.4, for one hour, washed two times with deionized water, and stained with HQ Silver^{TM} silver enhancement solution (Nanoprobes, Inc) for 15 min followed by a final washing. The tumor sections were finally stained with 3 µM of 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Thermo Fisher Scientific) for two and a half hours to mark the cell nuclei. Adjacent tumor slides were fixed in 4% formaldehyde and stained with hematoxylin and eosin (H&E) (Thermo Fisher Scientific and Poly Scientific R & D Corp). The stained section was covered with a drop of mounting medium (Permount®, Fisher Scientific) and then a cover slide was placed over the medium. The slides were examined under an inverted fluorescence microscope (IX71 Olympus).

RESULTS

We have synthesized and investigated gold nanoparticles coated with pHLIP and PEG. In our study we used WT pHLIP® peptide (4.1 kDa) with a single Cys residue at the N-terminus and PEG polymer (about 5 kDa) with a free SH group. The pHLIP® and

PEG were used in a mixture with reducing agents, tri-sodium citrate or ascorbic acid to prepare spherical or spiked gold nanoparticles, respectively. The pHLIP® peptide was dissolved in a solution of 2.7 M urea and mixed (in equal parts) with degassed, deionized water containing ten times molar-excess of TCEP, which prevents the formation of disulfide bonds. The PEG polymer was dissolved in a degassed, deionized water containing ten times molar-excess of TCEP.

Synthesis and Characterization of Spherical pHLIP® and PEG Coated Gold Nanoparticles

To obtain pHLIP® and PEG coated spherical gold nanoparticles, the solution of gold III chloride was reduced using tri-sodium citrate mixed with pHLIP® peptide and PEG polymer. The solution containing gold III chloride, citrate, pHLIP®, and PEG was blown with argon (to avoid oxidation of pHLIP® and PEG) and sealed for incubation at room temperature. Within 15-20 min a change in color of the solution was observed (Supplementary Figure 1S) and particles were left overnight at room temperature to ensure the complete reduction of the gold and the coating of nanoparticles by pHLIP® and PEG.

In the course of this study we varied the amount of citrate, pHLIP®, and PEG, then monitored changes in absorbance of the nanoparticle solutions for the appearance of a resonance peak at 525 nm (Figure 1). The absence of pHLIP® and PEG leads to the aggregation and precipitation of particles within a couple of hours after their preparation (Figure 1a and Supplementary Figure 1S). The best resonance was

observed when gold nanoparticles were coated with both pHLIP® and PEG. The amount of sodium citrate was varied from 0.42% to 2.8% wt/vol, where the concentration of citrate is reported as a final concentration in the nanoparticle solution (Figure 1b). A low amount of citrate (0.42% wt/vol) in solution results in the shift and broadening of the resonance peak, as well as the appearance of large particles, which was confirmed by the nanoparticle tracking system (data not shown). At the same time, particle sizes did not change when the amount of citrate was varied from 0.83% wt/vol to 2.8% wt/vol. The best results were obtained when citrate levels were varied between 0.83% wt/vol and 1.7% wt/vol. The amount of pHLIP® peptide and PEG polymer was altered as well. An increased percentage of pHLIP® and decreased percentage of PEG leads to a broadening and shift (by 6-8 nm) of resonance to longer wavelengths (Figure 1c). The amounts of 10% pHLIP® and 90% PEG were chosen as an optimal configuration. The final selected composition of reagents (0.83% citrate and 10% pHLIP-90% PEG) comprised the following molar parts: 0.5 parts of 10 mM gold III chloride; 0.075 parts of tri-sodium citrate (10% wt/vol); 0.1125 parts of 0.5 mM PEG with 5 mM TCEP; 0.0125 parts 0.5 mM pHLIP® peptide with 5 mM TCEP and 1.35 M urea, and 0.3 parts of degassed deionized water.

After synthesis, the solution of nanoparticles was purified to remove large particles and excess reducing agents. The large particles were removed by centrifugation. The excess reducing agents were removed when the nanoparticles were transferred to a PBS, pH 7.4, solution using size-exclusion chromatography.

Spherical gold nanoparticles coated with pHLIP® and PEG were stable for at least 36 days at 4°C in PBS, pH 7.4. The stability of gold nanoparticles was assessed at two gold concentrations: 0.1 g/L and 0.02 g/L. A high stability profile was obtained for both concentrations (Figure 1d). On the other hand, when particles were synthesized using a citrate reducing agent containing only PEG, or without both PEG and pHLIP®, the stability was seriously compromised; particles settled over time forming large visible aggregates and the resonance peak was shifted and broadened. Thus, the presence of pHLIP® peptide in gold nanoparticle coating is essential to maintain colloidal stability over time when using the methods described here.

TEM imaging was employed to analyze the size and shape distributions of gold nanoparticles (Figure 1e, f). The nanoparticles were homogeneous and round. Fitting the histogram of the particle size distribution by a log-normal function (Figure 1g) revealed that the metallic core was about 6.0 ± 2.2 nm in diameter. The mean hydrodynamic size (diameter), determined by nanoparticle tracking system, was 43.0 ± 1.5 nm.

Templated Synthesis and Characterization of pHLIP® and PEG Coated Spiked Gold Nanoparticles

Another approach we explored in the preparation of pHLIP® and PEG coated gold nanoparticles was based on use of bicelles as a template. Bicelles are disk-like micelles, which consist of long-chain phospholipids, such as dimyristoylphosphatidylcholine (DMPC), and short-chain phospholipids, such as

dihexanoylphosphatidylcholine (DHPC, D7PC) (Figure 2a). We chose to work with D7PC lipids, which have a low critical micelle concentration (CMC). Assuming the DMPC and DHPC head groups occupy the same areas, the molar ratio of long-chain to short-chain lipids is expressed via q -values, which reflects different aspect ratios of disk-like bicelles ^{32, 33}:

$$q = \frac{[DMPC]}{[DHPC]} = \frac{2\pi R^2}{\pi h(\pi R + h)}$$

where R is the radius and h is the height of the bicelle (Figure 2a). The height of the bicelles in our study was 4.2 nm and the radius was varied depending on ratio of DMPC to DHPC lipids. The diameters of the bicelles of different asymmetry, $d(q)$, calculated according to the above equation (without accounting for the DHPC capping) are the following: $d(0.2)=4.2$ nm; $d(0.3)=6.0$ nm; $d(0.5)=8.6$ nm; $d(0.75)=12$ nm; and $d(1.0)=15$ nm. To investigate the integrity of the DMPC-D7PC bicelle structures of various aspect ratios, we monitored their change in size at concentrations of 1.0, 0.5 and 0.1% wt/vol (Figure 2b). Because we were only concerned with the change in size upon dilution, no attempts were made to account for asymmetric particle geometries in DLS measurements. A slight increase in size was observed at low concentrations (0.1% wt/vol) for bicelles with q -values less than 0.75. On the other hand, highly asymmetric bicelles with a q -value equal to 1 lose their structural integrity as a result of dilution. Therefore, all other experiments were performed with bicelles of q -values of 0.75 and lower.

The templated synthesis of pHLIP® and PEG coated spiked gold nanoparticles was always initiated shortly after bicelle preparation. We ensured that the addition of the gold chloride solution and reducing agents does not lead to the dilution of the bicelle solution to lipid concentrations less than 0.1% wt/vol. The following steps were performed in the coating of bicelles: i) a solution of gold III chloride was added to the solution of bicelles and mixed; ii) next, the reducing agent, ascorbic acid, was added and mixed; and iii) upon color change, the solution containing pHLIP® and PEG was immediately added to complete the coating process. The appearance of NIR absorbance was indicative of the formation of spiked gold nanoparticles. In the absence of bicelles, the resonance was shifted to 525 nm (Figure 2c) reflecting the formation of solid spherical gold nanoparticles, which was also confirmed by TEM. Interestingly, the use of citrate as a reducer instead of ascorbic acid shifts the equilibrium towards the formation of solid spherical gold particles rather than templated synthesis of gold spiked nanoparticles (data not shown). At the concentrations of gold used in this study, the change of bicelle asymmetry (the increase of q -values from 0.3 to 0.75) does not affect NIR resonance (Figure 2c). Variation in the amount of gold affects the resonance peak just slightly (Figure 2d). The normalized absorbance of nanoparticles does not change upon dilution or the addition of detergent that disrupts lipid structures, Triton X-100, indicating that the nanoparticles have stability and integrity after synthesis (data not shown). The presence of pHLIP® and PEG resulted in the formation of particles that were more stable in aqueous solutions compared to the particles without coating, which were

precipitated shortly after their preparation. The final composition of reagents was the following molar parts: 0.05 part of 15 mM of lipids arranged into bicelles (> 0.1% wt/vol), 0.15 parts of 5 mM gold III chloride; 0.0225 parts of 250 mM ascorbic acid; 0.414 parts of 0.5 mM PEG with 5 mM TCEP; 0.046 parts 0.5 mM pHLIP® peptide with 5 mM TCEP and 1.35 M urea; and 0.3175 parts of degassed, deionized water.

TEM images of filtered pHLIP® and PEG coated gold nanoparticles demonstrated the presence of irregular, spiked gold nanoparticles (Figure 2e). The structures were the same for bicelles with different q -values. Conversely, spiked structures were not observed in absence of bicelles. The mean size (diameter) of spiked nanoparticles established by TEM was 148.0 ± 50.0 nm (Figure 2d). The mean hydrodynamic diameter, determined by nanoparticle tracking system, was 160.7 ± 29.0 nm.

Interaction of Spherical pHLIP® and PEG Coated Gold Nanoparticles with Cancer Cells

First, we have tested the cytotoxicity of spherical pHLIP® and PEG coated gold nanoparticles. JC murine mammary-gland adenocarcinoma cells were treated with gold nanoparticles (0.025 and 0.0125 g/L of gold) for 48 hours. The results indicate that gold nanoparticles do not exhibit any cellular toxicity.

Next, we evaluated the pH-dependent cellular uptake of spherical pHLIP® and PEG coated gold nanoparticles. The experiment was performed several times in different ways, such that the treatment of cells with gold nanoparticles was done at neutral and

low pH, or the pre-treatment of cells with gold nanoparticles was done at neutral pH followed by the lowering of pH with media. All obtained data indicate that the uptake of spherical pHLIP® and PEG coated gold nanoparticles by JC cancer cells at pH 6.0-6.2 was about 3 times higher than the uptake at neutral pH. The results confirm pH-dependent cellular uptake of gold nanoparticles coated with pHLIP® and PEG.

Distribution of pHLIP® and PEG Coated Gold Nanoparticles within Tumors

JC cancer cells were inoculated into the right flank of mice by subcutaneous injection. Spherical pHLIP® and PEG coated gold nanoparticles were administered as a single intra-tumoral injection at quantities of 5.5 and 21 µg gold. One hour after injection, animals were sacrificed so that tumors could be collected and processed to establish the amount of gold within and the distribution of particles throughout a tumor. Among all of the mice, there were animals with different tumor weights that were separated into 3 groups: 0.1, 0.3, and 0.4 g. The percentage of injected dose accumulated within the tumors increased with the increase of tumor weight: 16.2% for 0.1 g tumor; 18.0% for 0.3 g, and 29.1% for 0.4 g of tumor (Supplementary Figure 2S). The mean tumor uptake of gold (calculated for 8 animals) was 6.8 µg of gold per gram of tumor (with a standard error of 1.6) when a total of 5.5 µg of gold was injected. The mean tumor uptake of gold (calculated for 6 animals) was 24.5 µg/g (with standard error of 8.5) when 21 µg of gold was injected. The highest accumulation of gold ~47 µg/g was found in the smallest tumors (85.0±2.3 mg) injected with 21 µg of gold.

Also, we investigated the distribution of gold within a tumor mass. White light images were taken from the center of the tumor mass (Figure 3a). Then, the frozen tumor samples were sectioned and stained with hematoxylin and eosin (H&E) (Figure 3b). The gold was distributed nearly homogeneously within the tumor mass except the right edge of the tumor and there was no gold accumulation in the surrounding muscle. Muscle tissue adjacent to tumor is indicated by arrows on Figure 3a and 3b, and a magnified image of the border of the section containing both cancer cells and muscle is shown on Figure 3c. Adjacent sections were treated with Triton to permeabilize cellular membranes and stained with silver enhancement solution and DAPI. The representative images of gold enhanced by silver and stained nucleus are shown on Figure 3d-f.

Heating of pHLIP® and PEG Coated Spiked Gold Nanoparticles

Three concentrations of pHLIP® and PEG coated spiked gold nanoparticles were illuminated in solution by an 805 nm laser diode with 500 mW output. The laser radiant exposure was $\sim 400 \text{ J/cm}^2$. For particles at a concentration of 0.06 g/L gold, a temperature increase of 14 degree units (from 26°C to 40°C) was observed within 5 minutes (Figure 4), which is $\sim 18 \text{ J}$ imparted to water or $\sim 12 \%$ energy converted to heat. More concentrated particles showed greater temperature changes at all time points compared to less concentrated particles.

DISCUSSION

The motivation for our work was based on a desire to introduce pHLIP® and PEG coated gold nanoparticles. We developed methods for the preparation of pHLIP® and PEG coated spherical gold nanoparticles that might find application in radiation therapy as enhancement agents, as well as pHLIP® and PEG coated spiked gold nanoparticles for NIR thermal therapy. In both cases, pHLIP® peptide and PEG polymer were used as components of the reducing solution (sodium citrate reducer in the case of spherical nanoparticles and ascorbic acid in the case of spiked nanoparticles). The optimal combination of pHLIP® and PEG was selected to be 10% and 90%, respectively. A decreased percentage of pHLIP® in the nanoparticle coating might lead to the reduction of particle stability (resulting from the reduction of the overall negative charge due to the presence of pHLIP® peptide on particle surfaces) and the loss of pH-dependent properties. An increase in the percentage of pHLIP® peptide might be advantageous; but it comes with an increased cost of nanoparticle production. Previously, we investigated and showed the utility of liposomes coated with different ratios of pHLIP® and PEG ^{24, 29}. As well, employing a pHLIP® PEG conjugate instead of directly coating the surface with pHLIP could possibly further optimization. Spherical gold nanoparticles coated with pHLIP® and PEG exhibited high stability in solution over a one month time period, opposed to the particles with no coating or just PEG coating. The size of the metallic core of the particles was in the range of 4 to 8 nm in diameter and the hydrodynamic size (diameter) of coated particles was in the range of 38 to 48 nm. The nanoparticles showed pH-dependent uptake by cancer cells with no signs of cytotoxicity. We performed intra-tumoral

injections of spherical gold nanoparticle solutions and investigated gold uptake and distribution within tumors at one hour after injection. We focused on intratumoral injections because it does not seem like there is any clinical utility to perform a systemic administration of particles, since radiation therapy will be delivered locally and the location of the tumor would be known from prior imaging. Also, there is a requirement to have as much gold as possible within a tumor (which could be achieved only by local administration of the particles) to induce radiation enhancement effects. To have radiation delivered within a one hour time frame of particle administration is expected to be in line with clinical practice. Our results indicate that gold nanoparticles (21 μg of injected gold) were distributed within the tumor mass very well and with an overall uptake of 24.5 μg of gold per gram of tumor mass, reaching values of 47 $\mu\text{g/g}$ in tumors with masses of 1 gram and less. This is a higher uptake compared to that of pHLIP® gold nanoclusters ³⁰. We believe that the presence of pHLIP® peptide in the coating of gold nanoparticles was crucial. The peptide allowed particles to stay within the tumor and promoted the uptake of particles by cancer cells at low extracellular pH, as we have shown previously ^{30, 31}. On the other hand, particles were able to diffuse within a tumor as opposed to the direct injection of micron-sized gold particles that hindered tumor coverage by staying only at the injection site ³. Also, we would like to outline that the systemic administration of nanoparticles or antibodies conjugated with gold nanoparticles results in the staining of only peripheral tumor regions with limited penetration inside the tumor mass ³⁴. We did not observe accumulation of gold within muscle tissue adjacent to the tumors. The amount of injected gold in our study was much less than the amount of

gold used in previous radiation therapy studies on mice (see review and references within it ³⁵). However, we believe that the targeting of gold nanoparticles directed by pHLIP® peptide to the vital cellular structures such as plasma and nuclear membranes (as was shown previously ³¹) could lead to the enhancement of radiation effects even at lower gold concentrations.

The synthesis of spiked gold nanoparticles was performed using phospholipid disk-like bicelles as a template to create nanoparticles with resonance around 800 nm for NIR laser thermotherapy. Previously, platinum nanowheels were synthesized by the reduction of aqueous platinum with ascorbic acid in the presence of bicelles ^{36, 37}. As well, other phospholipid structures like spherical liposomes were used as a template for gold deposition to create biodegradable plasmon resonant nanoshells, which were implemented in the laser-induced release of encapsulated molecules ³⁸⁻⁴⁰. Another approach was based on the assembly of liposomes and micelles, called metallosomes, by using lipids conjugated to gold nanoclusters ⁴¹. Bicelles represent a class of model membranes for nuclear magnetic resonance (NMR) structural studies of membrane biomolecules ^{33, 42-44}, but they have found additional applications in biomedical sciences as well ⁴⁵⁻⁴⁷. Bicelles are discoidal phospholipid nanostructures at high lipid concentrations composed of long- and short-chain lipids or lipid-detergent mixtures. At higher lipid-to-detergent *q*-values (lipid-to-detergent molar ratios) bicelle edges are stabilized by the detergent, forming disk-like structures. The limitation in working with bicelles is typically the high CMC of lipids and detergents, which restricts the degree of bicelle dilution without the lipid phase transforming to vesicles. For

example, the CMC of the widely used dihexanoylphosphatidylcholine (D6PC) lipids is 14 mM. Thus, when the best characterized system of DMPC–D6PC bicelles are diluted below CMC, they are expected to be converted into a mixture of free D6PC lipids and DMPC lipid bilayer liposomes. The D7PC lipids have a 10 times lower CMC (1.4 mM), and it was shown that DMPC–D7PC mixtures retain bicelle properties at concentrations even as low as 0.1% wt/vol ⁴⁸. Therefore, we selected the DMPC-D7PC lipid system and ensured that the addition of aqueous gold and reducing solutions does not lead to the dilution of lipids below 0.1% wt/vol. The presence of bicelles induced the formation of spiked gold nanoparticles with plasmon resonance in the NIR range. The absence of bicelles leads to the formation of spherical gold nanoparticles with absorbance at 525 nm. The size of gold spiked nanoparticles established by TEM imaging and nanoparticle tracking system was in the range of 100 to 170 nm and 130 to 200 nm, respectively. The heating of pHLIP® and PEG coated spiked gold nanoparticles in solution by an 805 nm laser diode led to the increase of solution temperature by 14 degrees within 5 min. The temperature increase was concentration dependent. Our next step would be an evaluation of the effectiveness of pHLIP® and PEG coated spiked gold nanoparticles in combination with NIR laser treatment for photo-induced hyperthermia. Our goal is to employ lower power NIR laser radiation systems, which is currently available for indocyanine green (ICG) imaging in clinical endoscopy and laparoscopy (see table 4 for the description of ICG clinical imaging systems in the review article ⁴⁹).

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REFERENCES

- [1] Hainfeld, J. F., Dilmanian, F. A., Slatkin, D. N., and Smilowitz, H. M. (2008) Radiotherapy enhancement with gold nanoparticles, *J Pharm Pharmacol* 60, 977-985.
- [2] Hainfeld, J. F., Slatkin, D. N., and Smilowitz, H. M. (2004) The use of gold nanoparticles to enhance radiotherapy in mice, *Phys Med Biol* 49, N309-315.
- [3] Herold, D. M., Das, I. J., Stobbe, C. C., Iyer, R. V., and Chapman, J. D. (2000) Gold microspheres: a selective technique for producing biologically effective dose enhancement, *Int J Radiat Biol* 76, 1357-1364.
- [4] Retif, P., Pinel, S., Toussaint, M., Frochot, C., Chouikrat, R., Bastogne, T., and Barberi-Heyob, M. (2015) Nanoparticles for Radiation Therapy Enhancement: the Key Parameters, *Theranostics* 5, 1030-1044.
- [5] O'Neal, D. P., Hirsch, L. R., Halas, N. J., Payne, J. D., and West, J. L. (2004) Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles, *Cancer Lett* 209, 171-176.
- [6] von Maltzahn, G., Park, J. H., Agrawal, A., Bandaru, N. K., Das, S. K., Sailor, M. J., and Bhatia, S. N. (2009) Computationally guided photothermal tumor therapy using long-circulating gold nanorod antennas, *Cancer Res* 69, 3892-3900.
- [7] Dickerson, E. B., Dreaden, E. C., Huang, X., El-Sayed, I. H., Chu, H., Pushpanketh, S., McDonald, J. F., and El-Sayed, M. A. (2008) Gold nanorod assisted near-infrared plasmonic photothermal therapy (PPTT) of squamous cell carcinoma in mice, *Cancer Lett* 269, 57-66.

- [8] Srinivas, P. R., Kramer, B. S., and Srivastava, S. (2001) Trends in biomarker research for cancer detection, *Lancet Oncol* 2, 698-704.
- [9] Janssens, J. P., Verlinden, I., Gungor, N., Raus, J., and Michiels, L. (2004) Protein biomarkers for breast cancer prevention, *Eur J Cancer Prev* 13, 307-317.
- [10] Hanke, J. H., Webster, K. R., and Ronco, L. V. (2004) Protein biomarkers and drug design for cancer treatments, *Eur J Cancer Prev* 13, 297-305.
- [11] Goldsmith, S. J. (1997) Receptor imaging: competitive or complementary to antibody imaging?, *Semin Nucl Med* 27, 85-93.
- [12] Freimark, B., Clark, D., Pernasetti, F., Nickel, J., Myszka, D., Baeuerle, P. A., and Van Epps, D. (2007) Targeting of humanized antibody D93 to sites of angiogenesis and tumor growth by binding to multiple epitopes on denatured collagens, *Mol Immunol* 44, 3741-3750.
- [13] Gerweck, L. E., and Seetharaman, K. (1996) Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer, *Cancer Res* 56, 1194-1198.
- [14] Raghunand, N., Altbach, M. I., van Sluis, R., Baggett, B., Taylor, C. W., Bhujwalla, Z. M., and Gillies, R. J. (1999) Plasmalemmal pH-gradients in drug-sensitive and drug-resistant MCF-7 human breast carcinoma xenografts measured by ³¹P magnetic resonance spectroscopy, *Biochem Pharmacol* 57, 309-312.
- [15] Weerakkody, D., Moshnikova, A., Thakur, M. S., Moshnikova, V., Daniels, J., Engelman, D. M., Andreev, O. A., and Reshetnyak, Y. K. (2013) Family of pH

- (low) insertion peptides for tumor targeting, *Proc Natl Acad Sci U S A* 110, 5834-5839.
- [16] Andreev, O. A., Engelman, D. M., and Reshetnyak, Y. K. (2014) Targeting diseased tissues by pHLIP insertion at low cell surface pH, *Front Physiol* 5, 97.
- [17] Reshetnyak, Y. K., Andreev, O. A., Lehnert, U., and Engelman, D. M. (2006) Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix, *Proc Natl Acad Sci U S A* 103, 6460-6465.
- [18] Reshetnyak, Y. K., Segala, M., Andreev, O. A., and Engelman, D. M. (2007) A monomeric membrane peptide that lives in three worlds: in solution, attached to, and inserted across lipid bilayers, *Biophys J* 93, 2363-2372.
- [19] Andreev, O. A., Dupuy, A. D., Segala, M., Sandugu, S., Serra, D. A., Chichester, C. O., Engelman, D. M., and Reshetnyak, Y. K. (2007) Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues in vivo, *Proc Natl Acad Sci U S A* 104, 7893-7898.
- [20] Karabadzhak, A. G., Weerakkody, D., Wijesinghe, D., Thakur, M. S., Engelman, D. M., Andreev, O. A., Markin, V. S., and Reshetnyak, Y. K. (2012) Modulation of the pHLIP transmembrane helix insertion pathway, *Biophys J* 102, 1846-1855.
- [21] Adochite, R. C., Moshnikova, A., Carlin, S. D., Guerrieri, R. A., Andreev, O. A., Lewis, J. S., and Reshetnyak, Y. K. (2014) Targeting breast tumors with pH (low) insertion peptides, *Mol Pharm* 11, 2896-2905.

- [22] Cruz-Monserrate, Z., Roland, C. L., Deng, D., Arumugam, T., Moshnikova, A., Andreev, O. A., Reshetnyak, Y. K., and Logsdon, C. D. (2014) Targeting pancreatic ductal adenocarcinoma acidic microenvironment, *Sci Rep* 4, 4410.
- [23] Karabadzhak, A. G., An, M., Yao, L., Langenbacher, R., Moshnikova, A., Adochite, R. C., Andreev, O. A., Reshetnyak, Y. K., and Engelman, D. M. (2014) pHLIP-FIRE, a cell insertion-triggered fluorescent probe for imaging tumors demonstrates targeted cargo delivery in vivo, *ACS Chem Biol* 9, 2545-2553.
- [24] Yao, L., Daniels, J., Wijesinghe, D., Andreev, O. A., and Reshetnyak, Y. K. (2013) pHLIP(R)-mediated delivery of PEGylated liposomes to cancer cells, *J Control Release* 167, 228-237.
- [25] Emmetiere, F., Irwin, C., Viola-Villegas, N. T., Longo, V., Cheal, S. M., Zanzonico, P., Pillarsetty, N., Weber, W. A., Lewis, J. S., and Reiner, T. (2013) (18)F-labeled-bioorthogonal liposomes for in vivo targeting, *Bioconjug Chem* 24, 1784-1789.
- [26] Han, L., Ma, H., Guo, Y., Kuang, Y., He, X., and Jiang, C. (2013) pH-Controlled Delivery of Nanoparticles into Tumor Cells, *Adv Healthc Mater*.
- [27] Yu, M., Guo, F., Wang, J., Tan, F., and Li, N. (2016) A pH-Driven and photoresponsive nanocarrier: Remotely-controlled by near-infrared light for stepwise antitumor treatment, *Biomaterials* 79, 25-35.
- [28] Yu, M., Guo, F., Wang, J., Tan, F., and Li, N. (2015) Photosensitizer-Loaded pH-Responsive Hollow Gold Nanospheres for Single Light-Induced

Photothermal/Photodynamic Therapy, *ACS Appl Mater Interfaces* 7, 17592-17597.

- [29] Wijesinghe, D., Arachchige, M. C., Lu, A., Reshetnyak, Y. K., and Andreev, O. A. (2013) pH dependent transfer of nano-pores into membrane of cancer cells to induce apoptosis, *Sci Rep* 3, 3560.
- [30] Yao, L., Daniels, J., Moshnikova, A., Kuznetsov, S., Ahmed, A., Engelman, D. M., Reshetnyak, Y. K., and Andreev, O. A. (2013) pHLIP peptide targets nanogold particles to tumors, *Proc Natl Acad Sci U S A* 110, 465-470.
- [31] Antosh, M. P., Wijesinghe, D. D., Shrestha, S., Lanou, R., Huang, Y. H., Hasselbacher, T., Fox, D., Neretti, N., Sun, S., Katenka, N., Cooper, L. N., Andreev, O. A., and Reshetnyak, Y. K. (2015) Enhancement of radiation effect on cancer cells by gold-pHLIP, *Proc Natl Acad Sci U S A* 112, 5372-5376.
- [32] Luchette, P. A., Vetman, T. N., Prosser, R. S., Hancock, R. E., Nieh, M. P., Glinka, C. J., Krueger, S., and Katsaras, J. (2001) Morphology of fast-tumbling bicelles: a small angle neutron scattering and NMR study, *Biochim Biophys Acta* 1513, 83-94.
- [33] Prosser, R. S., Hwang, J. S., and Vold, R. R. (1998) Magnetically aligned phospholipid bilayers with positive ordering: a new model membrane system, *Biophys J* 74, 2405-2418.
- [34] Hainfeld, J. F., O'Connor, M. J., Dilmanian, F. A., Slatkin, D. N., Adams, D. J., and Smilowitz, H. M. (2011) Micro-CT enables microlocalisation and quantification of Her2-targeted gold nanoparticles within tumour regions, *Br J Radiol* 84, 526-533.

- [35] Shrestha, S., Cooper, L. N., Andreev, O. A., Reshetnyak, Y. K., and Antosh, M. P. (2016) Gold Nanoparticles for Radiation Enhancement in Vivo, *J J Rad Oncology* 3, 026.
- [36] Song, Y., Dorin, R. M., Garcia, R. M., Jiang, Y. B., Wang, H., Li, P., Qiu, Y., van Swol, F., Miller, J. E., and Shelnutt, J. A. (2008) Synthesis of platinum nanowheels using a bicellar template, *J Am Chem Soc* 130, 12602-12603.
- [37] Garcia, R. M., Song, Y., Dorin, R. M., Wang, H., Moreno, A. M., Jiang, Y. B., Tian, Y., Qiu, Y., Medforth, C. J., Coker, E. N., van Swol, F., Miller, J. E., and Shelnutt, J. A. (2011) Templated growth of platinum nanowheels using the inhomogeneous reaction environment of bicelles, *Phys Chem Chem Phys* 13, 4846-4852.
- [38] Troutman, T. S., Barton, J. K., and Romanowski, M. (2008) Biodegradable Plasmon Resonant Nanoshells, *Adv Mater* 20, 2604-2608.
- [39] Leung, S. J., and Romanowski, M. (2012) NIR-activated content release from plasmon resonant liposomes for probing single-cell responses, *ACS Nano* 6, 9383-9391.
- [40] Leung, S. J., and Romanowski, M. (2012) Light-activated content release from liposomes, *Theranostics* 2, 1020-1036.
- [41] Hainfeld, J. F., Furuya, F. R., and Powell, R. D. (1999) Metallosomes, *J Struct Biol* 127, 152-160.
- [42] De Angelis, A. A., and Opella, S. J. (2007) Bicelle samples for solid-state NMR of membrane proteins, *Nat Protoc* 2, 2332-2338.

- [43] Raschle, T., Hiller, S., Etzkorn, M., and Wagner, G. (2010) Nonmicellar systems for solution NMR spectroscopy of membrane proteins, *Curr Opin Struct Biol* 20, 471-479.
- [44] Sanders, C. R., and Prosser, R. S. (1998) Bicelles: a model membrane system for all seasons?, *Structure* 6, 1227-1234.
- [45] Rodriguez, G., Soria, G., Coll, E., Rubio, L., Barbosa-Barros, L., Lopez-Iglesias, C., Planas, A. M., Estelrich, J., de la Maza, A., and Lopez, O. (2010) Bicosomes: bicelles in dilute systems, *Biophys J* 99, 480-488.
- [46] Barbosa-Barros, L., Rodriguez, G., Barba, C., Cocera, M., Rubio, L., Estelrich, J., Lopez-Iglesias, C., de la Maza, A., and Lopez, O. (2012) Bicelles: lipid nanostructured platforms with potential dermal applications, *Small* 8, 807-818.
- [47] Barbosa-Barros, L., de la Maza, A., Estelrich, J., Linares, A. M., Feliz, M., Walther, P., Pons, R., and Lopez, O. (2008) Penetration and growth of DPPC/DHPC bicelles inside the stratum corneum of the skin, *Langmuir* 24, 5700-5706.
- [48] Lu, Z., Van Horn, W. D., Chen, J., Mathew, S., Zent, R., and Sanders, C. R. (2012) Bicelles at low concentrations, *Mol Pharm* 9, 752-761.
- [49] Alander, J. T., Kaartinen, I., Laakso, A., Patila, T., Spillmann, T., Tuchin, V. V., Venermo, M., and Valisuo, P. (2012) A review of indocyanine green fluorescent imaging in surgery, *Int J Biomed Imaging* 2012, 940585.

FIGURES

Figure 1. Characterization of Spherical Gold Nanoparticles. The absorbance spectra of spherical gold nanoparticle solutions synthesized *a*) in absence and presence of pHLIP® peptide and/or PEG polymer; *b*) with various amounts of sodium citrate, where the percentage of citrate is reported as the final wt/vol concentration (10% pHLIP® and 90% PEG); *c*) with various amounts of pHLIP® and PEG (1.7% wt/vol citrate). *d*) White light images of particles at the beginning and end of stability studies. The representative TEM images of pHLIP® and PEG coated spherical gold

nanoparticles obtained at *e)* 20,000x and *f)* 40,000x magnifications, and *g)* the corresponding histogram of particle sizes.

Figure 2. Characterization of Spiked Gold Nanoparticles. *a)* The disk-like shape of a bicelle with height, h , and radius, R , composed of DMPC and D7PC lipids. *b)* Size dependence of bicelles, established by DLS, on their q -value (which is the ratio of long- to short-chain lipids that reflect the asymmetry of a bicelle). *c)* The absorbance spectra of gold nanoparticles obtained by gold reduced by ascorbic acid, pHLP®, and PEG in the absence and presence of bicelles of different asymmetry. *d)* The

absorbance spectra of gold nanoparticles obtained by gold reduced by ascorbic acid, pHLIP® and PEG in presence of bicelles of $q=0.75$, where an increasing amount of gold was used and the ratios represent different lipid:gold molar amounts. *e*) The representative TEM image of pHLIP® and PEG coated spiked gold nanoparticles obtained at 12,000x magnification, and *f*) the corresponding histogram of particle sizes.

Figure 3. Tumor Imaging of Spherical Gold Nanoparticle Uptake. *a*) The white light image of a tumor center showing gold distribution (after a 21 μ g of intra-tumoral injection of pHLIP® and PEG coated spherical gold nanoparticles); *b*) a section from tumor center stained with H&E; and *c*) the magnified image of the tumor section outlined by a square in panel *b*, which shows tumor (T) and muscle (M). The arrows on panels *a* and *b* indicate muscle tissue. *d*) The bright field image of a tumor section stained with silver enhancement solution, *e*) the fluorescent image of the same tumor section stained with DAPI to visualize cell nuclei, and *f*) an overlay of the bright field and fluorescent images.

Figure 4. Heating of Spiked Gold Nanoparticles. The change in temperature over time measured in solutions containing pHLIP® and PEG coated spiked gold nanoparticles of different concentration irradiated by an 805 nm laser.

SUPPLEMENTARY MATERIALS

Figure S1. Reduction Time Points of Spherical Gold Nanoparticle Synthesis. The time progression of gold reduction by a solution of citric acid (1.7% wt/vol) to induce the formation of gold particles. GNP1 is a sample without pHLIP® and PEG; GNP2 is a sample containing pHLIP® but no PEG; GNP3 is a sample containing PEG but no pHLIP®; and GNP4 is a sample containing both pHLIP® and PEG in the reducing solution.

Figure S2. Tumor Uptake of Spherical Gold Nanoparticles Differentiated by Tumor Mass. The percentage of injected dose of gold accumulated within tumors of different masses.