2016

Design and Development of pH Sensitive Nanomedicine for Targeted Drug Delivery

Mohan C. Pereira
University of Rhode Island, mohancma@uri.edu

Follow this and additional works at: https://digitalcommons.uri.edu/oa_diss

Recommended Citation

Pereira, Mohan C., "Design and Development of pH Sensitive Nanomedicine for Targeted Drug Delivery" (2016). *Open Access Dissertations*. Paper 477.
https://digitalcommons.uri.edu/oa_diss/477

This Dissertation is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.

**DESIGN AND DEVELOPMENT OF pH SENSITIVE NANOMEDICINE FOR
TARGETED DRUG DELIVERY**

BY

MOHAN C PEREIRA

**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF**

DOCTOR OF PHILOSOPHY

IN

BIOLOGICAL PHYSICS

UNIVERSITY OF RHODE ISLAND

2016

DOCTOR OF PHILOSOPHY DISSERTATION

OF

MOHAN C PEREIRA

APPROVED:

Dissertation Committee:

Major Professor: Oleg A. Andreev

Yana K. Reshetnyak

Geoffry Bothun

Nasser H. Zawia

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2016

ABSTRACT

Targeted drug delivery has been the major topic in drug formulation and delivery. As nanomedicine emerges to create nano-scale therapeutics and diagnostics, it is still essential to embed targeting capability to these novel systems to make them useful. Design of robust nanomedicine with universal targeting capabilities could provide effective solutions to the ever increasing demand of curing and preventing major diseases.

Diseases like carcinoma & ischemia are accompanied with hypoxia and acidosis. Because of the elevated glycolysis and acidosis in the diseased cells, they show a lower pH in the surface of the cells which promotes the appearance of aggressive malignant cells with enhanced multidrug resistance. Therefore, the extracellular acidity can be used as a hallmark to target and deliver nanotheranostics. Thus coating nanoparticles with **pH (Low) Insertion Peptides (pHLIPs)** increase efficiency of targeting acidic diseased tissues. This novel method could be used as a promising method to create future nanotheranostics for cancer and other diseases which are dominating the present world.

The main goal of the work presented here is to

- Review the field of advanced targeted nanomedicine and its future trends;
- Design pHLIP® coated lipid & surfactant based nanovesicles to deliver therapeutic and diagnostic agents;
- Use different drugs and dyes for imaging and therapy ;
- Assess the cytotoxicity, targeting capabilities and the cellular interaction of these nanovesicles in *in-vitro* and *in-vivo*.

ACKNOWLEDGMENTS

I want to express here my extreme gratitude to my mentor Dr. Oleg A Andreev, for his precious advice and guidance during the elaboration of this thesis and my entire research work. I would like to convey my special thanks to Dr. Yana K. Reshetnyak for introducing me systematically to the field of biological physics through course work and supporting me in many ways to complete this thesis. I have immensely benefited from their broad knowledge and unparalleled experience of the field.

I would like to thank the rest of my graduate program committee members Dr Geoffrey Bothun , Dr Gerhard Müller, Dr David Heskett and Dr Clinton Chichester III for serving on my committee and being very supportive throughout this process.

I am so grateful for all my teachers who inspired me to become who I am today.

And I thank all my colleagues in our research group for their help with my experiments and their constructive critique. I thank my parents and my siblings for their support, understanding and sacrifice as I was completing this work, thousand miles away from them. I want to thank my forever-family for their sacrificial love and care for me in my journey.

PREFACE

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

The results of my studies presented here were published in three papers:

1. **Pereira, Mohan C**, Yana K. Reshetnyak, and Oleg A. Andreev. "Advanced targeted nanomedicine." *Journal of biotechnology* 202 (2015):
2. Wijesinghe, D., **Pereira, Mohan C.**, Lu, A., Reshetnyak, Y. K., & Andreev, O. A. “pH dependent transfer of nano-pores into membrane of cancer cells to induce apoptosis.” *Scientific reports*, 3 (2013):
3. **Pereira, M.C.**, Monica, P., Carafa, M., Andreev O.A., Reshetnyak, Y.K. Novel pHLIP coated niasomes for diagnostic and therapy, *in preparation*

Work was presented on the following 3 Meetings/Conferences:

“Formulation and Delivery of Lipophilic Drugs to Cancer Cells by pHLIP® coated Liposomes”, *World Medical Nanotechnology Congress & Expo*, Dallas, TX (2016)

“pH dependent transfer of nano-pores into membrane of cancer cells to induce apoptosis”, *CHI's Biologics Formulation & Delivery Summit*, Boston, MA (2014).

“pH dependent transfer of nano-pores into membrane of cancer cells to induce apoptosis”, *American Physical Society – New England section*, Bridgewater, MA (2013).

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	iii
PREFACE	iv
TABLE OF CONTENTS	v
LIST OF TABLES	vi
LIST OF FIGURES	vii
CHAPTER 1	1
Advanced Targeted Nanomedicine.....	1
CHAPTER 2	39
pH dependent transfer of nano-pores into membrane of cancer cells to induce apoptosis.....	39
CHAPTER 3	70
Novel pHLIP coated niasomes for diagnostic and therapy.....	70

LIST OF TABLES

CHAPTER 1	PAGE
Table 1. Current pHLIP applications in nanotechnology	37
CHAPTER 3	
Table 1. Niosome Characterization Data.....	87
Table S1. p-values for Cellular uptake after adding trypan blue for Fig 2 (e,f)	96
Table S2. In-Vivo Data for 24 h post injection	96
Table S3. p-values for Cellular uptake after adding trypan blue for Fig 2 (e,f)	97

LIST OF FIGURES

CHAPTER 1	PAGE
Figure 1. Different applications of pHLP for targeting of acidic tissues.....	38
 CHAPTER 2	
Figure 1. Schematic presentation of lipid bilayer of the pHLP-coated liposomes containing gramicidin A (gA) ion-conductive channels	61
Figure 2. Biophysical characterization of liposomes containing gramicidin A	62
Figure 3. Inhibition of cell proliferation	63
Figure 4. Monitoring changes of intracellular pH.....	64
Figure 5. Monitoring changes of intracellular concentration of Na ⁺	65
Figure 6. Mitochondria depolarization assay.....	66
Figure 7. Schematic presentation of interactions of lipid bilayer of the pHLP-coated liposomes containing gA with plasma membrane of a cell ns	67
Figure S1. Size and Zeta Potential of pHLP coated gA liposomes	68
Figure S2. Control experiments	69
 CHAPTER 3	
Figure 1. Cryo -TEM	88
Figure 2. Cellular Uptake of R18 Niosomes.....	89
Figure 3. In-Vivo Kinetics.....	90
Figure 4. In-Vivo Imaging 24 hrs.....	91
Figure 5. Tumor Sections	92
Figure 5. Delivery of Ca ²⁺ into Cancer Cells using Pyr-WT coated niosomes	93

Figure 5. ECIS Assay	94
Figure 5. Delivery of Ca ²⁺ into tumors using Pyr-WT coated niosomes.....	95
Figure S1. Diameter and zeta potential of the pHLIP-coated niosomes.	98
Figure S2. Cyto-toxicity Assays.....	99
Figure S3. Cellular uptake of R-18 niosomes.....	100

CHAPTER 1

Published in Proceedings of Journal of Biotechnology on

20th of January, 2015

Advanced Targeted Nanomedicine

Mohan C Pereira , Yana Reshetnyak, Oleg A. Andreev

Physics Department, University of Rhode Island, 2 Lippitt Rd., Kingston, RI, 02881,
USA

Corresponding author:

Oleg Andreev, Physics Department, University of Rhode Island, 2 Lippitt Rd.,
Kingston, RI, 02881, USA, Phone: (401)-874-2054, Fax: (401) 874-2380, E-mail:
andreev@mail.uri.edu

Abstract

Targeted drug delivery has been the major topic in drug formulation and delivery. As nanomedicine emerges to create nano scale therapeutics and diagnostics, it is still essential to embed targeting capability to these novel systems to make them useful. Here I discuss various targeting approaches for delivery of therapeutic and diagnostic nano materials in view of search for more universal methods to target diseased tissues. Many diseases are accompanied with hypoxia and acidosis. Coating nanoparticles with pH Low Insertion Peptides (pHLIPs) increases efficiency of targeting acidic diseased tissues. It has been showing promising results to create future nanotheranostics for cancer and other diseases which are dominating in the present world.

Keywords: pHLIP, nanomedicine, passive targeting, active targeting, tissue acidity, environmental targeting

Highlights

- Nanomaterials are used to target both imaging and therapeutic agents
- Targeting a tumor environment might better address issue of tumor heterogeneity
- pHLIP senses acidity on the surface of cells
- pHLIP targets nanomaterials to acidic diseased tissue
- pHLIP promotes cytoplasmic delivery of nanomaterial's payloads

Abbreviations

bFGF, basic fibroblast growth factor; EGFR, epidermal growth factor receptor; DGLs, dendrigraft poly-L-lysines ; DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine; EuL, europium luminescent; HER, human epidermal receptors; HIF, Hypoxia-inducible factor; LbL, Layer-by-Layer MSP, mesoporous silica nanoparticles; PEO/PPO, poly(ethylene oxide)/poly(propylene oxide); PET, positron emission tomography; pHILIP, pH Low Insertion Peptide ; SPECT, single photon emission computed tomography; Tz, tetrazine

Acknowledgments

The work was supported by the NIH grants CA133890, CA174413 and GM073857 to OAA and YKR.

1. Introduction

For a long time people have been working on developing therapeutic agents so that they can tune pharmacological and pharmacokinetic properties to treat diseases and get desired results. In their continuous quest for food and survival, our ancestors must have experienced the effects of natural sources of pharmacologically active chemical substances produced by organic and inorganic materials such as plants, fungi, insects, animal excreta, reptiles and mineral ores for better or for worse. They must have learnt to extract active ingredients from natural resources using crude methods and enhanced to use as pain killers or to heal wounds or to treat all types of diseases known to them. Ebers papyrus, dated back to ~ 1500 BC provides a detailed description of medical treatments used by ancient Egyptians [1, 2]. In early as 7 century AD, metal, mineral and herbal based particles called *Bhashma* has been used in Ayurvedic medicine in Indian sub-continent. Modern analysis showed that these formulations contained Fe_2O_3 , FeS_2 , CuS and SiO_2 and also particle sizes were regulated in the range of 1-2 μm [3, 4]. Undoubtedly, these ancient knowledge had laid the foundation for modern drug formulation and delivery and made a huge breakthrough in this field as the chemical analysis became first available to us in 19th century [5].

While the conventional drugs are still being widely used, the innovation of therapeutic nanoparticles has been radically changing the future of drug formulation and delivery [6-11]. Nanoparticles are becoming more popular due to their unique tunable physicochemical properties. They have shown promising results in delivery of variety of molecules improving the therapeutic index of drugs by enhancing their efficacy and/or increasing their tolerability in the body. Nano-carriers could also improve the

bioavailability of water-insoluble drugs, carry large payloads, protect the therapeutic agents from physiological barriers, as well as enable the development of novel classes of bioactive macromolecules [12].

2. Targeted delivery of nanoparticles

Almost a century ago, Paul Ehrlich introduced the concept of targeted drug delivery. It was considered as a hypothetical ‘magic bullet’ as an entity consisting of two components — the first one should recognize and bind the target, while the second should provide a therapeutic action in this target. Currently, the concept of ‘magic bullet’ includes a coordinated behavior of three components – drug, targeting moiety and pharmaceutical carrier [13]. Nanoparticles can be designed to have all three properties of the revised version of Ehrlich’s “magic bullet”, and they could be used as therapeutics and/or diagnostics. When designing the nano-drugs it is essential to understand the target region. Target regions could be whole organs (heart, lung, brain, liver and etc), tissues (muscle), cells (nerve, dendrite and etc), disease specific structures (tumor cells) or cellular components. The efficacy of the therapeutics, effectiveness of the diagnostics, safety, affordability and access will measure the final success of nanoparticles in medicine in regard to its applied value to the patients.

3. Three major ways of delivery of nanoparticles

Nanoparticle drug delivery systems use the characteristics of disease tissues to selectively target their payloads, either by passive, active or physical targeting [14, 15].

3.1 Passive targeting

When nanoparticles localize into specific organs or site of disease via biological mechanisms, such as RES (reticuloendothelial system) or EPR (enhanced permeability and retention) effects, they are known as 'passive targeting agents' [16].

RES also called macrophage system or mononuclear phagocyte system, is a class of cells which are part of the body's defense mechanisms. If nanoparticles are not protected against RES, they will end up in liver, spleen or lymph nodes very soon. Even though this seems to be a disadvantage, RES mechanism can be successfully used for mapping liver [16-21], spleen [18, 19, 22-25] and lymph nodes [17, 18, 22]. The information gain from mapping of lymph nodes gives vital indications for cancer staging and metastatic potential of tumor, which could prevent unnecessary dissection surgery.

The EPR effect is very common for most of the solid tumors and has been exploiting as a passive mechanism to deliver therapeutic agents. As tumor grows its architecture of vasculature become quite abnormal, showing lack of lymphatic drainage and leaky blood vessels. This allows the long circulating nanoparticles to accumulate in tumor site overtime at higher levels compared to other organs [26-34].

3.2 Active Targeting

Active targeting uses peripherally conjugated specific targeting moieties for enhanced delivery of nanoparticles. The targeting moieties are various ligands including antibodies, peptides, aptamers or small molecules that possess high affinity toward unique molecular signatures found in diseased tissue to achieve active targeting [35].

Three general categories of active targeting methods are i) angiogenesis-associated targeting, ii) uncontrolled cell proliferation targeting and iii) tumor cell targeting.

Chemical stimulation for angiogenesis is caused by variety of proangiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). They are the key ingredients of this very complex biological mechanism which is essential for life. Pathological angiogenesis or abnormally rapid proliferation of blood vessels is common for growth of solid tumors. Therefore the targeting of angiogenesis has become a focus for cancer therapeutics [34, 36-42]. Thus, the growth factors are attacked by inhibitors of angiogenesis to regulate the tumor progression [43, 44].

Another significant target for cancer cells are the cell proliferation markers. These markers are not unique to cancer cells but they are overexpressed in certain cancer cells.

The human epidermal receptors (HER), transferrin receptors and folate receptors are widely being employed. Actively targeting nanoparticles have been using the monoclonal antibodies to target overexpressed cell proliferation receptors [35, 45-48].

According to the American Cancer Society statistics, in US, it is estimated that more than 1.6 million new cases of cancer will be diagnosed and more than 0.5 million cancer deaths will occur in 2014. The four most common types of cancers that will be diagnosed in 2014 in the United States are breast in women and prostate in men, lung, and colorectal. Therefore targeting of specific tumor cells is becoming another popular area. FDA has already approved several monoclonal antibodies for the treatment of specific types of cancers. Trastuzumab, a humanized monoclonal antibody against HER-2 which is overexpressed in human breast cancer, for the treatment of HER-2-positive metastatic breast cancer was introduced in 1998. Panitumumab, a human

antibody against EGFR produced in transgenic mice (XenoMouse), for the treatment of EGFR-positive colorectal cancer was approved in 2006. Avastin, a humanized antibody against VEGF, for the treatment of metastatic colorectal cancer was introduced in 2004. Cetuximab, a chimeric antibody directed to EGFR (HER-1) for the treatment of advanced colorectal cancer appeared in 2004. Avastin® (Genentech, South San Francisco, CA) is a recombinant humanized anti-VEGF monoclonal antibody for the treatment of non-small cell lung cancer, metastatic colorectal cancer and metastatic breast cancer were approved [35].

The major advantage of the active targeting over the passive targeting is a selective delivery of nanoparticles to the specific tumors/pathogenic tissues, which remain in the site of disease for an extended period of time, thereby increasing the local accumulation of the nanoparticles in the sites of interest. [49-52]. However, the heterogeneity and adaptability of cancers are difficult to overcome, which makes it problematic to create a common cure based on active targeting. Therefore, it is important to identify a universal hallmark for majority of cancers or consider use of external physical stimuli as a targeting strategy.

3.3 Physical Targeting

In many cases, pathological area differs from normal tissues in certain physical properties, such as temperature, lack of oxygen and pH. These natural properties are common for majority of cancers independent of origin, and could be exploited as targeting approaches. The principle behind use of nanoparticles for physical targeting is that a stimulus, which may be applied externally or originate within the pathological site, induces either a physical change in the structure of the nanoparticle itself, thereby

causing the eradication of the target or modulating the rate at which an embedded drug is released. pH, lack of oxygen, temperature, ultrasound, electromagnetic radiation and mechanical forces serve as stimuli for physical targeting.

3.3.1 Magnetic-sensitive systems

Use of magnetic sensitive nanoparticles was first proposed by Widder, Senyi and their colleagues in late 1970s [53, 54]. These nanoparticles are primarily fabricated in such way that therapeutic agents are attached to, or encapsulated within, a magnetic core or shell by giving capability of functionalizing their surfaces. Once functionalized, the nanoparticles are injected into the bloodstream, often using a catheter to position the injection site near the target. Powerful rare earth magnets are focused over the target site to apply magnetic fields with high-gradient and the forces affecting the particles. So, the field permits nanoparticles to be captured and released at the target. Clinical trials done by Koda et al. to deliver doxorubicin hydrochloride showed great deal of success for hepatocellular carcinoma [55]. This method may be effective for targets close to the body's surface, but as the magnetic field strength falls off rapidly with distance, sites deeper within the body become more difficult to target [56]. Several groups recently proposed a way to overcome this hurdle by implanting magnets near the target site, within the body [57, 58]. However, this method is invasive and not systematic, which apply significant restrictions for clinical use. Moreover this method would not target metastases, which make cancer a lethal disease.

Key advantages of magnetic NPs are they can be (i) visualized (super paramagnetic NPs are used in MRI);[59, 60] (ii) guided or held in place by means of a magnetic field; (iii)

heated in a magnetic field to trigger drug release or to produce hyperthermia/ablation of tissue; (iv) targeted specific locations in the body; (v) reduced the quantity of drug needed to attain a particular concentration in the vicinity of the target; (vi) reduced of the concentration of the drug at non target sites minimizing severe side effects and (vii) used to perform magnetic guided surgeries for surgically impossible tumors either due to too hemorrhagic or localized in tissues with high risk of healthy tissue injury.

Despite of these advantages they also inherit limitations. MNPs accumulate throughout the cross section from the external source to the depth marking the effective field limit which is a drawback. Magnetic agglomeration at the absence of magnetic field is tackled by superparamagnetic NPs. Creation of smaller NPs, is essential to achieve superparamagnetism. As the size becomes smaller, particles' response to the magnetic field is remarkably reduced. As a result of that it makes difficult to direct particles and keep them in the proximity of the target while withstanding the drag of blood flow.

3.3.2 Ultrasound-sensitive systems

When nanoparticles are used to target solid tumors, homogeneous distribution of most of them in tumor is always questionable. Although nanoparticles can extravasate from the blood to the extracellular matrix, they do not always travel far away from the blood vessels. Thus, only a small population of cancer cells located close to the blood vessels will be exposed to the cytotoxic drugs thus minimizing it's therapeutic index. As nanoparticles stay for longer periods of time, they could target and bring harm to normal tissues. Researchers have explored ways to use ultrasound to enhance the cellular uptake of nanoparticles in the target sites by increasing the permeability of the capillary walls,

pushing them through the extracellular matrix, enhancing the release of the drug from the nanoparticles and improving the cellular uptake.

Escoffre and his colleagues showed on 2D and 3D *in vitro* models that the focused-ultrasound not only helps nanoparticles to penetrate into the interior of the tumor, it also helps to release drugs to the intercellular space without altering chemical properties [61-64]. The use of phenomenon of cavitation, which is the formation of bubbles in a medium exposed to intense focused ultrasound to disrupt the targeted tissue, is another application of this kind. Microbubble-based drug-delivery vehicles can flow through the vasculature into the ultrasound focal zone within the tumor region and they can release payloads upon the rupture. These bubbles can be also used as imaging contrast agents to improve diagnosis and detection of cancer [65-69].

Non-invasiveness, the absence of ionizing radiations, and the facile regulation of tissue penetration depth by tuning frequency, duty cycles and time of exposure can be identified as the main advantages of using ultrasound sensitive systems [70]. Ultrasound can trigger release of drugs from various nanocarriers by destabilization. Ultrasound triggered delivery systems has the ability to release drugs into cytosol as result of pore formation in the cell membrane. Therefore they can bypass degenerative endocytotic pathway which is especially useful in DNA transfection [71]. Low frequency ultrasounds can be used to promote the delivery of some liposomal based drugs through the skin to inhibit progression of melanocytic lesions[72]. However, the increased vessel permeability through ultrasound can also impact negatively by promoting metastatic dissemination. Possible metastatic dissemination can be minimized by using NPs containing agents, which are able to efficiently interact with

ultrasonic waves and decrease the threshold frequencies of ultrasound waves. Effectiveness of microbubbles based drug delivery for tissue targeting may be limited by their short lifespan and absence of extravasation. The development of perfluorocarbon (PFC) nanoemulsions and PFC droplets functionalized with aptamers, which can be converted into microbubbles under therapeutic ultrasounds have significantly improved cellular uptake and/or release of drugs in tumor sites[73].

3.3.3 Temperature-sensitive systems

Above we gave examples of external stimuli applied to diseased area to enhance delivery of nanoparticles and/or release of imaging/therapeutic moieties. Another approach relays on exploration of natural properties or microenvironment in diseased tissue. It is observed that certain types of malignant cancer (bladder, prostate and etc) [74] and some other disease conditions show difference in local temperature compared to normal tissues. Understanding of the differences of local temperature in pathological sites paved the path to temperature-sensitive delivery systems. Long circulating, thermo-sensitive nanocarriers can be fabricated and used to exploit micro-environmental temperature differences in diseased sites. For example, in 1978, Yatvin *et al.* first time suggested a method to use temperature-sensitive liposomes to target mild hyperthermia in disease sites. Active or passive targeting methods have to be first employed to accumulate thermo-sensitive nanocarriers in targeted sites. As the nanocarriers accumulate in target sites, carried drugs are released in response to the micro-environmental temperature in more controlled manner.

In general, the thermo-sensitive polymers have the ability to swell and de-swell, depending on temperature. The two basic types of thermo-sensitive materials are: i)

positive temperature-sensitive hydrogels that are swollen and hydrated at higher temperatures and contract on cooling below the upper critical solution temperature (UCST) and ii) negative temperature-sensitive hydrogels that are swollen at lower temperatures and contract on heating above the lower critical solution temperature (LCST). Water solubility of the system decreases as temperature increases. The latest type of system dominates the literature on drug release applications [75]. Upon the introduction of UCST/LCST polymers to nanocarrier systems, they gained the ability to actively respond to the temperature changes in the microenvironment. As they respond to the environmental temperature these systems remarkably change their features such as conformation, solubility and hydrophilic/hydrophobic balance to release therapeutic agents in those sites [76].

Injectable thermo-sensitive hydrogel systems are also considered as potential thermo-sensitive nano-carriers owe to their number of advantages, including simplicity of drug formulation, protective environment for drugs, prolonged and localized drug delivery, and ease of application. Chitosan and related derivatives, poly(N-isopropylacrylamide)-based (PNIPAAm) copolymers, poly(ethylene oxide)/poly(propylene oxide) (PEO/PPO) copolymers and its derivatives, and poly(ethylene glycol)/ biodegradable polyester copolymers are widely used by researchers to construct injectable thermo-sensitive hydrogels [74, 77-80].

DPPE/DSPC are the most common lipids used in making thermo sensitive liposomes. With the right lipid composition temperature- dependent fusion of liposomes can be achieved at mild hyperthermia region (39 °C- 42 °C) enhancing increased level of drug available at the diseased site [75]. Early *in vivo* work done over a range of tumor

models, including various carcinomas, sarcomas, and lymph node metastases, and over a range of drugs, including adriamycin, methotrexate, bleomycin and cisplatin showed the benefits of temperature-sensitive liposomes when use with mild hyperthermia [81-87]. Temperature sensitive nano-systems offer numerous advantages such as elimination of invasive surgical procedures, the ability to bypass physiological barriers, and allowing drugs to reach hard-to-access sites in the body, ability to stealth the thermo sensitive systems against immune system and environmental degradation and ability to combine this with induced hyperthermia. Owe to extensive studies done in these systems over past three decades that gives the capability for wide variety of choices to design nanocarriers of desired architectures. Limitations of thermo-sensitive nanocarriers can be overcome by combining them with additional stimuli-responsive materials.

3.3.4 Targeting hypoxia

Hypoxia (low oxygen concentrations) plays a vital role in many tumors by contributing to chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness, resistance to cell death, altered metabolism and genomic instability. The hypoxic regions often lie surrounding areas of necrosis in solid tumors [88-90]. Hypoxia-inducible factor (HIF), which is a transcriptional complex, acts as the hypoxia sensor in a cell. Overexpression of HIF- α , the regulatory subunit of HIF is a measure of increased vascular density, severity of tumor grade, failure of conventional treatment and prognosis. Therefore HIF has become an attractive, direct and indirect therapeutic target in recent years [91-93]. Thambi *et al.* reported about self-assembled nanoparticles called hypoxia-responsive nanoparticles, which can selectively release hydrophobic agents

under hypoxic conditions [94]. *In vivo* studies showed selective accumulation of nanoparticles at the hypoxic regions in tumors compared to normal cells.

Development of hypoxia targeting nanoparticles opens new paths to overcome drug delivery impediments mentioned earlier. For instance, the brain which demands high amount of oxygen is vulnerable to hypoxia as a result of irregularity of the blood flow induced by either cardiac failure, traumatic brain injury, brain cancer or subcortical vascular disease. Neuronal dysfunction and cell death in major neurodegenerative diseases also have direct and indirect links to hypoxia. Since HIF involved in virtually all aspects of the response to hypoxia, HIF based nanocarriers have the potential advantage of simultaneously regulating the expression of a large number of genes thus treatment of diseases. However, for HIF related therapies it is vital to discriminate between various HIF isoforms [95, 96]. Another potential advantage of hypoxia targeting NPs is the ability to treat inaccessible brain tumors, which cannot be achieved through conventional surgeries.

3.3.5 Targeting acidity

It's well known that the cancer cells undergo glycolysis even in the presence of oxygen which has very significant importance to cancer cells survival and proliferation. As a result, high glycolytic activity and production of carbonic and lactic acids, which are intensively pumped out cells to keep intracellular pH near neutral, are common characteristics of solid tumors. It leads to extracellular acidification of tumor microenvironment, which promotes cancer invasiveness and aggressiveness [97-100]. This acidic microenvironment is common even for several other diseased tissues, such

as ischemia, inflammation, arthritis and atherosclerosis. Thus, extracellular acidity might serve as a general marker for detecting and targeting of diseased tissue.

pH-sensitive nanoparticle systems are the one of the well-studied delivery systems. These systems contain pH sensitive polymers, lipids or peptides such as pHLIPs (pH Low Insertion Peptides). Key principle of making pH sensitive fusogenic liposomes is to identify a lipid which is stable at physiological pH but destabilized upon acidification following cellular internalization, thereby, promoting the release of their contents into the cytosol. First generation of such liposomes are designed using the cone shape of phosphatidylethanolamine (PE) lipids [101]. Cytoplasmic delivery of membrane-impermeable therapeutic agents has been improved by the DOPE-based pH sensitive liposomes [102-104]. The layer-by-layer (LbL) nanoparticle assembling technique is developed to prepare pH-sensitive nanoparticles. They contain a pH-sensitive outer stealth layer which allows targeting and retaining of nanoparticles in acidic tumor regions [105].

pH sensitive systems can be also achieved by the self-assembled surfactants. They are mostly known as niosomes and analogues to liposomes. For example, Di Marzio and colleagues reported that the use of non-ionic surfactants polysorbate 20 and polysorbate 21 together with cholesterol can form highly stable pH sensitive nano-vesicles which have the potential to deliver both hydrophilic and hydrophobic therapeutic agents to target sites [106]. pH sensitive systems also show great success for delivering drugs through oral drug administration due to vast environmental pH differences in gastrointestinal system [107-109]. The development of highly pH sensitive NPs to target

acidity helps not only for treatments but also for diagnosis of wide array of carcinomas and many other pathological diseases.

4. pHLIP-technology

The pHLIP technology, which was introduced several years ago, has been showing success in targeted drug delivery for cancer and other pathogenic conditions. pHLIP, being a membrane peptide, has affinity to cellular membranes and it targets extracellular acidity. In contrast to other pH-sensitive systems, it “senses” acidity at the surface of cancer cells, where pH is the lowest due to the reversed pH gradient compared to cells in healthy tissue [110].

4.1. Molecular mechanism of pHLIP action

The original pHLIP composed of 36 amino acids is an isolated C-helix of bacteriorhodopsin. The water-soluble pHLIP peptide has been observed to behave differently at different pHs when it is in the vicinity of the lipid bilayer. The pHLIP binds to lipid bilayer surfaces as an unstructured monomer above pH 7, and it spontaneously inserts as an α -helix across lipid bilayers at low pH, with a pK_{app} of 6 [111]. The insertion of pHLIP into membrane, is reversible and unidirectional, in most cases the C-terminus is translocated across the membrane whereas the N-terminus stays outside of the membrane [112, 113]. The opposite occurs for reverse sequence [114].

The insertion mechanism is based on the protonation of Asp/Glu residues located in the membrane inserting parts of the pHLIP [111, 115-117]. The thermodynamics and kinetics of pHLIPs membrane-associated folding and insertion has been extensively investigated. Peptide's three major forms (in solution, attached to, and inserted across lipid bilayers) are monomeric at peptide concentration less than $\sim 7 \mu\text{M}$ [112]. The

process of insertion and folding (helix formation) is fast and varies from milliseconds to minute depending on peptide sequence (Andreev et al., 2010; Karabadzhak et al., 2012). The energy of peptide's association with bilayer is about 6-7 kcal/mol [113]. As pHLIP inserts into the membrane and folds it releases additional energy (about 2 kcal/mol) [113], which could be utilized to tether cargo molecules to the surface of cancer cells (Fig 1a), or move cell impermeable cargo molecules across the membrane into a cell (Fig. 1b). It has been shown tumor targeting of fluorescent, PET and SPECT probes attached to the N-terminus of pHLIP [118-123]. As biological membrane remains the main barrier for cytoplasmic drug delivery it was demonstrated that the energy of membrane-associated folding of pHLIP can be used to successfully deliver into cells polar and membrane-impermeable molecules including cyclic peptides, toxins, peptide nucleic acids (PNAs) linked to pHLIP's C-terminus via cleavable links [124-128]. Over a decade the original pHLIP peptide has given birth to more than 20 pHLIP variants and available to use as probing and delivery agent [114]. The ability of pHLIP to probe extracellular acidic environment, which is associated with tumors and many other pathological conditions has opened new avenues for future drug delivery.

4.2. pHLIP based nanomedicine

Roughly a half a century ago, a British biophysicist and a medical scientist, Alec Bangham made a remarkable observation that phospholipids in aqueous systems can form closed bilayered structures and named them Bangasomes. Today we call them liposomes. After Bangham and his collaborators established the existence of liposomes, the liposomes have moved a long journey from being just another exotic entity of biophysical and colloidal science research and became a pharmaceutical carrier of

choice for various real-world applications, as we outline in previous sections. As a result of that, now liposomal drugs have been approved for clinical applications or they are undergoing clinical evaluation [129].

When pHLIP is introduced to the conventional liposomes as a coating moiety (Fig. 1c), it allows to enhance the therapeutic value of the liposomes. It was shown that 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 (Fig. 1d), and inhibition of cell proliferation by liposomes containing ceramide [130]. For liposomal drug production and storage the controlling of their size becomes a major issue. When liposomes are in storage they could fuse with each other and give a poly-disperse liposomal mixture and diminish the desirable properties as nano-carriers. The same study [131] reveals that liposomes containing just 5% of pHLIP lipid composition minimize inter-liposomal fusion and make them stable for several days if stored at neutral pH, since pHLIP brings overall negative charge to the surface of liposomes at neutral and high pHs. At the same time, drop of a pH leads to the protonation of Asp/Glu residues, reduces overall negative charge at the surface of liposomes and enhances interaction of pHLIP-coated liposomes with cellular membranes. It was studied the interaction between pHLIP-coated liposomes and cells as a function of incubation time of 15 min vs. 60 min and it was shown existence of different pathways of liposome-cell interaction. Predominantly direct liposomal fusion with plasma membrane occurs during short incubation period; while during long incubation period, both fusion and cellular internalization through endocytosis (most probably, macro pinocytosis) could happen. Since pegylation helps to prevent

opsonization, it promotes longer circulation of liposomes in blood, and enhances probability for liposomes to interact with cell membrane in a pH dependent manner. Thus, the pH-sensitive, "fusogenic" pHLIP-coated liposomes could be used to selectively deliver various diagnostic and therapeutic agents to acidic diseased cells. Emmetiere *et al.* [132] (Emmetiere et al., 2013) introduced dual-delivery approach using pHLIP to tether liposomes to cancer cells in tumor [132]. First, pHLIP was conjugated to the tetrazine (Tz), which is one of the bioorthogonally reactive small molecules to form pHLIP-Tz conjugate. pHLIP-Tz was injected into mice to selectively label the surface of cancer cells. Then, bioorthogonally reactive trans-cyclooctenes coated liposomes containing ^{18}F PET isotope were given as a second injection. The long circulating radiolabeled liposomes were accumulated in tumor sites mainly via EPR effect, followed the click reaction between tetrazine-pHLIP and trans-cyclooctenes in liposome coat resulting in covalent conjugation and tethering of liposomes to cancer cells. This *in vivo* click reaction allows achieving high signal/noise ratio, higher accumulation of radiolabeled liposomes in tumor site, low nonspecific binding and reduced toxicity to kidneys and bone marrow.

According to the World Health Organization statistics the ischemic heart diseases are the top leading cause of death, affecting millions of men and women worldwide. Several techniques for passive and active imaging and as well as drug delivery to the diseased cardiac tissue have been developed during past few years [133, 134], which including different types of nanoparticles for drug delivery such as liposomes, drug-polymer conjugates, polymeric micelles, dendrimers, nanoshells, and nucleic acid-based carriers [129, 135, 136]. A study was done to evaluate ability of pHLIP-coated liposomes to

target ischemic myocardium using two murine ischemia models: regional ischemia induced by coronary artery occlusion and global low-flow ischemia in isolated hearts [137]. In both models, two candidates from the pHLIP family (WT and Var7) were chosen along with pH insensitive kVar7 (K-pHLIP), which has Lys instead of key Asp/Glu residues and cannot insert into membrane at any pH [114]. It was shown that pHLIP-coated liposomes bind to ischemic regions but not to normal myocardium while kVar7 and liposomes coated just with PEG (no pHLIP) do not show any targeting [137]. The study ensures that pHLIP-coated liposomes have the ability to target ischemic regions with therapeutic agents as well as to mark disease regions to assist pHLIP-fluorescent-aided cardiac surgeries.

Another novel approach of pHLIP-coated-liposomes is the delivery of nano-pores to induce apoptosis in cancer cells [138]. Proper ion balance between intracellular and extracellular media is crucial for normal cell functioning. Any alterations in the conductance of membranes for ions will lead to cell death. To change cellular ion balance the pore-forming gramicidin A (10 mol%) was delivered to cellular membrane using pHLIP-coated DOPC liposomes. Liposomes were stable for more than a month in 4 °C. Hydrophobic gramicidin A monomers were introduced into pHLIP-coated-liposomes, where it makes beta-helices and forms transmembrane pore with diameter of 4-5 Å that can transfer monovalent cations through the membrane at rate of 10^7 cations per second [139]. As we outlined above, when pHLIP-coated liposomes target cancer cells, they either fuse with the plasma membrane or are up-taken by endocytosis and then fuse with lysosomal membranes. It leads to delivery of gramicidin A pores (nano-pores) to cellular membranes of cancer cells. These nano-pores acidify the

intracellular space and eliminate the vital Na^+/K^+ ion balance. This method not only opens pathways to treat acidic solid tumors, but also give opportunity to deliver various membrane peptides and proteins to the cells, widening the applications in biotechnology and nano-medicine.

Nano-gold particles have also caught attention in pHLIP-nanotechnology. Beneficial properties of gold in medicine has been considered for centuries [140]. And gold has been used in modern science as a contrast agent in electron microscopy. There are well-established methods to obtain uniform stable gold nano-structures in the range of few nanometers up to couple hundred of nanometers [141]. Tuning size and shape of nano-gold structures allows to obtain unique physical properties, which can be very useful for multiple applications across both therapeutics and diagnostics [142]. Functionalized nano-gold particles are valuable for wide variety of nanomedicinal uses and now they are undergoing through evaluations of toxicity [141], stability [143], pharmacokinetics [144], cellular trafficking [145-148], efficacy for gene regulation [149] and drug delivery [147], and use in photo-thermal therapies [150, 151].

Lan et al. showed that nano-gold particles which are 1.4 nm size in diameter conjugated to N-terminus of pHLIP can successfully target cancer cells at low pH [131]. *In vivo* studies done using mice-tumor models demonstrated high tumor uptake in both intravenous and intra-tumoral administration compared to non-functionalized nano-gold particles. The beauty of pHLIP-nano gold technology is in its capability of providing the specific targeting, enhancing local concentration in tumor mass in homogeneous way while remaining in cells for an extended period (several days), by allowing applications of radiation therapy and imaging.

Davies and colleagues employed pHLIP to deliver luminescent europium coated nanoparticles into platelets [152]. The 13 nm gold nanoparticles were co-coated with a europium luminescent, EuL and the pHLIP to give pHLIP.EuL.Au. Human platelets are vulnerable to transfection or microinjection. But with this method authors could deliver nanoparticles which have roughly 640 lanthanide probes per particle. The result shows that the internalization of nanoparticles into the platelets happened only at low pH and not at normal pH. The significance of this research is that pHLIP can translocate multimodal nanoparticles in a pH dependent manner.

Non-viral vectors are also a hot topic in gene therapy due to their advantages over viral vectors such as simplicity of use, ease of large-scale production and lack of specific immune response [153]. Recently it was reported a novel use of pHLIP to deliver pDNA to tumor cells [154]. DGL-PEG-pHLIP were made by conjugating the surface of dendrigraft poly-L-lysines (DGLs, generation 3 with 123 amino groups per molecule) to the N-terminus of pHLIP followed by electrostatic interactions between negatively charged pDNA and positively charged DGL head group of DGL-PEG-pHLIP to create DGL-PEG-pHLIP/pDNA nanoparticles. Results of *in vitro* studies showed that higher cellular uptake of nanoparticles occurs at low pH (pH 6.0) compared to normal pH (pH 7.4) and nanoparticles enter cells mainly by adsorptive mediated endocytosis. The *in vivo* studies also followed the same pattern by showing high tumor uptake of DGL-PEG-pHLIP/pDNA nanoparticles. It was clear that pHLIP enhanced the pH-controlled localization of the nanoparticles in tumors.

Zhao and colleagues (2013) show that pHLIP peptide not only targets acidic tumor micro environment but also it can release nanoparticles in a controlled manner to the

intracellular space. They chose mesoporous silica nanoparticles (MSN), in particular MCN-41, to load with doxorubicin. MSN has the attributes of high homogeneous porosity, inertness, biocompatibility, high payload capacity and easy surface functionalization capability. The MSN particles were attached to the C-terminus of pHLIP by disulfide bond. At low pH (< 6.5), doxorubicin-loaded pHLIPss-MSN rapidly inserted into the cell membrane and translocated MSN into cytoplasm. The disulfide bond was cleaved in cytoplasm to release doxorubicin.

5. Conclusion

There are several challenges for targeted nano drug delivery systems to overcome. Still most of these drug systems undergo *in vitro* and *in vivo* testing using animal models. Therefore their relevancy to the real patients has to be evaluated extensively. Each nano drug platform is distinctive and need to be assessed experimentally as new system, which is strenuous. Nanoparticles stability, size uniformity, controlled drug release rate, sterile preparations in large scale and manufacturing cost have to be addressed in order to make them available to the market. But with recent scientific advances, next ten years it is expected to see large number of targeted drug delivery systems based on nanoparticles in the market. pHLIP nanotechnology has been showing its potential capability to address most of the challenges mentioned above. pHLIP technology used alone or combined with other approaches might lead to new formulations translatable to clinics.

References

1. Shadlen, K.C., *early drug discovery and the rise of pharmaceutical chemistry*. 2011.
2. Jones, A.W., *Early drug discovery and the rise of pharmaceutical chemistry*. Drug testing and analysis, 2011. **3**(6): p. 337-344.
3. Pal, D., C.K. Sahu, and A. Haldar, *Bhasma: The ancient Indian nanomedicine*. Journal of advanced pharmaceutical technology & research, 2014. **5**(1): p. 4.
4. Mohaptra, S. and C. Jha, *Physicochemical characterization of Ayurvedic bhasma (Swarna makshika bhasma): An approach to standardization*. International journal of Ayurveda research, 2010. **1**(2): p. 82.
5. Ansari, S. and M. Farha Islam, *Influence of nanotechnology on herbal drugs: A Review*. Journal of advanced pharmaceutical technology & research, 2012. **3**(3): p. 142.
6. Shi, J., et al., *Nanotechnology in drug delivery and tissue engineering: from discovery to applications*. Nano letters, 2010. **10**(9): p. 3223-3230.
7. Cai, W. and X. Chen, *Nanoplatfoms for targeted molecular imaging in living subjects*. Small, 2007. **3**(11): p. 1840-1854.
8. Gao, X., et al., *< i> In vivo</i> molecular and cellular imaging with quantum dots*. Current Opinion in Biotechnology, 2005. **16**(1): p. 63-72.
9. Davis, M.E., *Nanoparticle therapeutics: an emerging treatment modality for cancer*. Nature reviews Drug discovery, 2008. **7**(9): p. 771-782.
10. Zhang, L., et al., *Nanoparticles in medicine: therapeutic applications and developments*. Clinical Pharmacology & Therapeutics, 2007. **83**(5): p. 761-769.
11. Heath, T.D., R.T. Fraley, and D. Papahdjopoulos, *Antibody targeting of liposomes: cell specificity obtained by conjugation of F (ab') 2 to vesicle surface*. Science, 1980. **210**(4469): p. 539-541.
12. Swami, A., et al., *Nanoparticles for targeted and temporally controlled drug delivery*. Multifunctional Nanoparticles for Drug Delivery Applications, 2012: p. 9-29.
13. Torchilin, V.P., *Drug targeting*. European Journal of Pharmaceutical Sciences, 2000. **11**: p. S81-S91.

14. Egusquiaguirre, S.P., et al., *Nanoparticle delivery systems for cancer therapy: advances in clinical and preclinical research*. Clinical and Translational Oncology, 2012. **14**(2): p. 83-93.
15. Petros, R.A. and J.M. DeSimone, *Strategies in the design of nanoparticles for therapeutic applications*. Nature Reviews Drug Discovery, 2010. **9**(8): p. 615-627.
16. Shilo, M., et al., *Nanoparticles as computed tomography contrast agents: current status and future perspectives*. Nanomedicine, 2012. **7**(2): p. 257-269.
17. Aviv, H., et al., *Radiopaque iodinated copolymeric nanoparticles for X-ray imaging applications*. Biomaterials, 2009. **30**(29): p. 5610-5616.
18. Rabin, O., et al., *An X-ray computed tomography imaging agent based on long-circulating bismuth sulphide nanoparticles*. Nature materials, 2006. **5**(2): p. 118-122.
19. Hainfeld, J., et al., *Gold nanoparticles: a new X-ray contrast agent*. 2014.
20. Kojima, C., et al., *X-ray computed tomography contrast agents prepared by seeded growth of gold nanoparticles in PEGylated dendrimer*. Nanotechnology, 2010. **21**(24): p. 245104.
21. Kim, D., et al., *Antibiofouling polymer-coated gold nanoparticles as a contrast agent for in vivo X-ray computed tomography imaging*. Journal of the American Chemical Society, 2007. **129**(24): p. 7661-7665.
22. Oh, M.H., et al., *Large-scale synthesis of bioinert tantalum oxide nanoparticles for X-ray computed tomography imaging and bimodal image-guided sentinel lymph node mapping*. Journal of the American Chemical Society, 2011. **133**(14): p. 5508-5515.
23. Xiao, M., et al., *Gold nanotags for combined multi-colored Raman spectroscopy and x-ray computed tomography*. Nanotechnology, 2010. **21**(3): p. 035101.
24. Boote, E., et al., *Gold nanoparticle contrast in a phantom and juvenile swine: models for molecular imaging of human organs using x-ray computed tomography*. Academic radiology, 2010. **17**(4): p. 410-417.
25. Sun, I.C., et al., *Heparin-Coated Gold Nanoparticles for Liver-Specific CT Imaging*. Chemistry-A European Journal, 2009. **15**(48): p. 13341-13347.
26. Greish, K., *Enhanced permeability and retention of macromolecular drugs in solid tumors: A royal gate for targeted anticancer nanomedicines*. Journal of Drug Targeting, 2007. **15**(7-8): p. 457-464.

27. Torchilin, V.P., *Passive and active drug targeting: drug delivery to tumors as an example*, in *Drug delivery*. 2010, Springer. p. 3-53.
28. Acharya, S. and S.K. Sahoo, *PLGA nanoparticles containing various anticancer agents and tumour delivery by EPR effect*. *Advanced Drug Delivery Reviews*, 2011. **63**(3): p. 170-183.
29. Maruyama, K., *Intracellular targeting delivery of liposomal drugs to solid tumors based on EPR effects*. *Advanced drug delivery reviews*, 2011. **63**(3): p. 161-169.
30. Wang, A.Z., R. Langer, and O.C. Farokhzad, *Nanoparticle Delivery of Cancer Drugs*. *Annual Review of Medicine*, 2012. **63**(1): p. 185-198.
31. Li, W. and F. Szoka, Jr., *Lipid-based Nanoparticles for Nucleic Acid Delivery*. *Pharmaceutical Research*, 2007. **24**(3): p. 438-449.
32. Rasmussen, J.W., et al., *Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications*. *Expert Opinion on Drug Delivery*, 2010. **7**(9): p. 1063-1077.
33. Huang, K., et al., *Size-Dependent Localization and Penetration of Ultrasmall Gold Nanoparticles in Cancer Cells, Multicellular Spheroids, and Tumors in Vivo*. *ACS Nano*, 2012. **6**(5): p. 4483-4493.
34. Khalid, M., et al., *Long Circulating Poly(Ethylene Glycol)-Decorated Lipid Nanocapsules Deliver Docetaxel to Solid Tumors*. *Pharmaceutical Research*, 2006. **23**(4): p. 752-758.
35. Byrne, J.D., T. Betancourt, and L. Brannon-Peppas, *Active targeting schemes for nanoparticle systems in cancer therapeutics*. *Advanced drug delivery reviews*, 2008. **60**(15): p. 1615-1626.
36. Hicklin, D.J. and L.M. Ellis, *Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis*. *Journal of Clinical Oncology*, 2005. **23**(5): p. 1011-1027.
37. John, A. and G. Tuszynski, *The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis*. *Pathology Oncology Research*, 2001. **7**(1): p. 14-23.
38. Goth, M.I., et al., *Physiological and pathological angiogenesis in the endocrine system*. *Microscopy research and technique*, 2003. **60**(1): p. 98-106.
39. Jain, R.K., *Tumor angiogenesis and accessibility: Role of vascular endothelial growth factor*. *Seminars in Oncology*, 2002. **29**(6, Supplement 16): p. 3-9.

40. Seaman, S., et al., *Genes that Distinguish Physiological and Pathological Angiogenesis*. Cancer Cell, 2007. **11**(6): p. 539-554.
41. Chung, A.S. and N. Ferrara, *Developmental and pathological angiogenesis*. Annual review of cell and developmental biology, 2011. **27**: p. 563-584.
42. Folkman, J. *Role of angiogenesis in tumor growth and metastasis*. in *Seminars in oncology*. 2002. Elsevier.
43. Folkman, J., *Fighting cancer by attacking its blood supply*. Scientific American, 1996. **275**(3): p. 150-156.
44. Mousa, S.A., *Angiogenesis Inhibitors and Stimulators: Potential Therapeutic Implications*. 2000.
45. Sudimack, J. and R.J. Lee, *Targeted drug delivery via the folate receptor*. Advanced Drug Delivery Reviews, 2000. **41**(2): p. 147-162.
46. Qian, Z.M., et al., *Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway*. Pharmacological reviews, 2002. **54**(4): p. 561-587.
47. Mamot, C., et al., *Epidermal growth factor receptor (EGFR)-targeted immunoliposomes mediate specific and efficient drug delivery to EGFR-and EGFRvIII-overexpressing tumor cells*. Cancer research, 2003. **63**(12): p. 3154-3161.
48. Gullotti, E. and Y. Yeo, *Extracellularly Activated Nanocarriers: A New Paradigm of Tumor Targeted Drug Delivery*. Molecular Pharmaceutics, 2009. **6**(4): p. 1041-1051.
49. Baldini, M., et al., *Patterns of failure after trimodality therapy for malignant pleural mesothelioma*. The Annals of thoracic surgery, 1997. **63**(2): p. 334-338.
50. Kaufman, D., *Challenges in the treatment of bladder cancer*. Annals of Oncology, 2006. **17**(suppl 5): p. v106-v112.
51. Meacham, C.E. and S.J. Morrison, *Tumour heterogeneity and cancer cell plasticity*. Nature, 2013. **501**(7467): p. 328-337.
52. Gonzalez-Angulo, A.M., F. Morales-Vasquez, and G.N. Hortobagyi, *Overview of resistance to systemic therapy in patients with breast cancer*, in *Breast Cancer Chemosensitivity*. 2007, Springer. p. 1-22.
53. Senyei, A., K. Widder, and G. Czerlinski, *Magnetic guidance of drug-carrying microspheres*. Journal of Applied Physics, 1978. **49**(6): p. 3578-3583.

54. Widder, K.J., A.E. Senyei, and D.G. Scarpelli, *Magnetic microspheres: a model system for site specific drug delivery in vivo*. Experimental Biology and Medicine, 1978. **158**(2): p. 141-146.
55. Koda, J., et al. *A multicenter, phase I/II trial of hepatic intra-arterial delivery of doxorubicin hydrochloride adsorbed to magnetic targeted carriers in patients with hepatocellular carcinoma*. in *European Journal of Cancer*. 2002. PERGAMON-ELSEVIER SCIENCE LTD THE BOULEVARD, LANGFORD LANE, KIDLINGTON, OXFORD OX5 1GB, ENGLAND.
56. McBain, S.C., H.H. Yiu, and J. Dobson, *Magnetic nanoparticles for gene and drug delivery*. International journal of nanomedicine, 2008. **3**(2): p. 169.
57. Kubo, T., et al., *Targeted delivery of anticancer drugs with intravenously administered magnetic liposomes in osteosarcoma-bearing hamsters*. International journal of oncology, 2000. **17**(2): p. 309-324.
58. Yellen, B.B., et al., *Targeted drug delivery to magnetic implants for therapeutic applications*. Journal of Magnetism and Magnetic Materials, 2005. **293**(1): p. 647-654.
59. Thorek, D.J., et al., *Superparamagnetic Iron Oxide Nanoparticle Probes for Molecular Imaging*. Annals of Biomedical Engineering, 2006. **34**(1): p. 23-38.
60. Andreas, K., et al., *Highly efficient magnetic stem cell labeling with citrate-coated superparamagnetic iron oxide nanoparticles for MRI tracking*. Biomaterials, 2012. **33**(18): p. 4515-4525.
61. Escoffre, J., et al., *Focused ultrasound mediated drug delivery from temperature-sensitive liposomes: in-vitro characterization and validation*. Physics in medicine and biology, 2013. **58**(22): p. 8135.
62. Dalecki, D., *Mechanical bioeffects of ultrasound*. Annu. Rev. Biomed. Eng., 2004. **6**: p. 229-248.
63. Hagtvet, E., et al., *Ultrasound enhanced antitumor activity of liposomal doxorubicin in mice*. Journal of Drug Targeting, 2011. **19**(8): p. 701-708.
64. Pitt, W.G., G.A. Hussein, and B.J. Staples, *Ultrasonic drug delivery – a general review*. Expert Opinion on Drug Delivery, 2004. **1**(1): p. 37-56.
65. Unger, E.C., et al., *Local drug and gene delivery through microbubbles*. Progress in cardiovascular diseases, 2001. **44**(1): p. 45-54.
66. Lindner, J.R., *Evolving applications for contrast ultrasound*. The American Journal of Cardiology, 2002. **90**(10, Supplement 1): p. 72-80.

67. Ferrara, K., R. Pollard, and M. Borden, *Ultrasound microbubble contrast agents: fundamentals and application to gene and drug delivery*. Biomedical Engineering, 2007. **9**.
68. Dijkmans, P.A., et al., *Microbubbles and ultrasound: from diagnosis to therapy*. European Journal of Echocardiography, 2004. **5**(4): p. 245-246.
69. Ibsen, S., C.E. Schutt, and S. Esener, *Microbubble-mediated ultrasound therapy: a review of its potential in cancer treatment*. Drug design, development and therapy, 2013. **7**: p. 375.
70. Mura, S., J. Nicolas, and P. Couvreur, *Stimuli-responsive nanocarriers for drug delivery*. Nat Mater, 2013. **12**(11): p. 991-1003.
71. Pitt, W.G., G.A. Hussein, and B.J. Staples, *Ultrasonic drug delivery-a general review*. Expert opinion on drug delivery, 2004. **1**(1): p. 37-56.
72. Tran, M.A., et al., *Targeting V600EB-Raf and Akt3 using nanoliposomal-small interfering RNA inhibits cutaneous melanocytic lesion development*. Cancer research, 2008. **68**(18): p. 7638-7649.
73. Rapoport, N.Y., et al., *Controlled and targeted tumor chemotherapy by ultrasound-activated nanoemulsions/microbubbles*. Journal of Controlled Release, 2009. **138**(3): p. 268-276.
74. Stefanadis, C., et al., *Increased temperature of malignant urinary bladder tumors in vivo: the application of a new method based on a catheter technique*. Journal of clinical oncology, 2001. **19**(3): p. 676-681.
75. Ta, T. and T.M. Porter, *Thermosensitive liposomes for localized delivery and triggered release of chemotherapy*. Journal of Controlled Release, 2013. **169**(1): p. 112-125.
76. Fitzpatrick, S.D., et al., *Temperature-sensitive polymers for drug delivery*. 2012.
77. Yatvin, M.B., et al., *Design of liposomes for enhanced local release of drugs by hyperthermia*. Science, 1978. **202**(4374): p. 1290-1293.
78. McCoy, C.P., et al., *Triggered drug delivery from biomaterials*. Expert opinion on drug delivery, 2010. **7**(5): p. 605-616.
79. Lindner, L.H., et al., *Novel temperature-sensitive liposomes with prolonged circulation time*. Clinical Cancer Research, 2004. **10**(6): p. 2168-2178.
80. Gong, C., et al., *Thermosensitive polymeric hydrogels as drug delivery systems*. Current medicinal chemistry, 2013. **20**(1): p. 79-94.

81. Yatvin, M., et al., *Selective delivery of liposome-associated cis-dichlorodiammineplatinum (II) by heat and its influence on tumor drug uptake and growth*. Cancer research, 1981. **41**(5): p. 1602-1607.
82. Zou, Y., et al., *Targeting behavior of hepatic artery injected temperature sensitive liposomal adriamycin on tumor-bearing rats*. Selective cancer therapeutics, 1990. **6**(3): p. 119-127.
83. Weinstein, J., et al., *Liposomes and local hyperthermia: selective delivery of methotrexate to heated tumors*. Science, 1979. **204**(4389): p. 188-191.
84. Tacker, J. and R. Anderson, *Delivery of antitumor drug to bladder cancer by use of phase transition liposomes and hyperthermia*. The Journal of urology, 1982. **127**(6): p. 1211-1214.
85. Maekawa, S., K. Sugimachi, and M. Kitamura, *Selective treatment of metastatic lymph nodes with combination of local hyperthermia and temperature-sensitive liposomes containing bleomycin*. Cancer treatment reports, 1987. **71**(11): p. 1053-1059.
86. Nishimura, Y., et al., *Treatment of murine SCC VII tumors with localized hyperthermia and temperature-sensitive liposomes containing cisplatin*. Radiation research, 1990. **122**(2): p. 161-167.
87. Iga, K., et al., *Enhanced antitumor activity in mice after administration of thermosensitive liposome encapsulating cisplatin with hyperthermia*. Journal of Pharmacology and Experimental Therapeutics, 1991. **257**(3): p. 1203-1207.
88. Wilson, W.R. and M.P. Hay, *Targeting hypoxia in cancer therapy*. Nature Reviews Cancer, 2011. **11**(6): p. 393-410.
89. Brown, J.M. and W.R. Wilson, *Exploiting tumour hypoxia in cancer treatment*. Nature Reviews Cancer, 2004. **4**(6): p. 437-447.
90. Brahimi-Horn, M.C., J. Chiche, and J. Pouyssegur, *Hypoxia and cancer*. Journal of Molecular Medicine, 2007. **85**(12): p. 1301-1307.
91. Carroll, V.A. and M. Ashcroft, *Role of hypoxia-inducible factor (HIF)-1 α versus HIF-2 α in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway*. Cancer research, 2006. **66**(12): p. 6264-6270.
92. Poon, E., A.L. Harris, and M. Ashcroft, *Targeting the hypoxia-inducible factor (HIF) pathway in cancer*. Expert reviews in molecular medicine, 2009. **11**: p. e26.
93. Harris, A.L., *Hypoxia—a key regulatory factor in tumour growth*. Nature Reviews Cancer, 2002. **2**(1): p. 38-47.

94. Thambi, T., et al., *Hypoxia-responsive polymeric nanoparticles for tumor-targeted drug delivery*. Biomaterials, 2014. **35**(5): p. 1735-1743.
95. Freeman, R., *Targeting Hypoxia-Inducible Factor (HIF) as a Therapeutic Strategy for CNS Disorders - See more at: <http://www.eurekaselect.com/90699/article/targeting-hypoxia-inducible-factor-hif-therapeutic-strategy-cns-disorders#sthash.UaBqvECI.dpuf>*. Current Drug Target -CNS & Neurological Disorders, 2005. **4**(1): p. 85-92.
96. Rapisarda, A. and G. Melillo, *Overcoming disappointing results with antiangiogenic therapy by targeting hypoxia*. Nature Reviews Clinical Oncology, 2012. **9**(7): p. 378-390.
97. Wojtkowiak, J.W., et al., *Drug resistance and cellular adaptation to tumor acidic pH microenvironment*. Molecular pharmaceutics, 2011. **8**(6): p. 2032-2038.
98. Mahoney, B.P., et al., *Tumor acidity, ion trapping and chemotherapeutics: I. Acid pH affects the distribution of chemotherapeutic agents in vitro*. Biochemical pharmacology, 2003. **66**(7): p. 1207-1218.
99. Gatenby, R.A. and R.J. Gillies, *A microenvironmental model of carcinogenesis*. Nature Reviews Cancer, 2008. **8**(1): p. 56-61.
100. LaMonte, G., et al., *Acidosis induces reprogramming of cellular metabolism to mitigate oxidative stress*. Cancer & metabolism, 2013. **1**(1): p. 23.
101. Karanth, H. and R. Murthy, *pH-Sensitive liposomes-principle and application in cancer therapy*. Journal of pharmacy and pharmacology, 2007. **59**(4): p. 469-483.
102. Chu, C.-J., et al., *Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture*. Pharmaceutical research, 1990. **7**(8): p. 824-834.
103. Lee, R., et al., *The effects of pH and intraliposomal buffer strength on the rate of liposome content release and intracellular drug delivery*. Bioscience reports, 1998. **18**: p. 69-78.
104. Subbarao, N.K., et al., *The pH-dependent bilayer destabilization by an amphipathic peptide*. Biochemistry, 1987. **26**(11): p. 2964-2972.
105. Poon, Z., et al., *Layer-by-layer nanoparticles with a pH-sheddable layer for in vivo targeting of tumor hypoxia*. ACS nano, 2011. **5**(6): p. 4284-4292.
106. Di Marzio, L., et al., *Novel pH-sensitive non-ionic surfactant vesicles: comparison between Tween 21 and Tween 20*. Colloids and Surfaces B: Biointerfaces, 2011. **82**(1): p. 18-24.

107. Sonaje, K., et al., *Self-assembled pH-sensitive nanoparticles: a platform for oral delivery of protein drugs*. Advanced Functional Materials, 2010. **20**(21): p. 3695-3700.
108. Makhlof, A., Y. Tozuka, and H. Takeuchi, *Design and evaluation of novel pH-sensitive chitosan nanoparticles for oral insulin delivery*. European Journal of Pharmaceutical Sciences, 2011. **42**(5): p. 445-451.
109. Makhlof, A., Y. Tozuka, and H. Takeuchi, *pH-Sensitive nanospheres for colon-specific drug delivery in experimentally induced colitis rat model*. European Journal of Pharmaceutics and Biopharmaceutics, 2009. **72**(1): p. 1-8.
110. Chiche, J., M.C. Brahimi-Horn, and J. Pouyssegur, *Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer*. J Cell Mol Med, 2010. **14**(4): p. 771-94.
111. Hunt, J.F., et al., *Spontaneous, pH-dependent membrane insertion of a transbilayer α -helix*. Biochemistry, 1997. **36**(49): p. 15177-15192.
112. Reshetnyak, Y.K., et al., *A monomeric membrane peptide that lives in three worlds: in solution, attached to, and inserted across lipid bilayers*. Biophysical journal, 2007. **93**(7): p. 2363-2372.
113. Reshetnyak, Y.K., et al., *Energetics of peptide (pHLIP) binding to and folding across a lipid bilayer membrane*. Proceedings of the National Academy of Sciences, 2008. **105**(40): p. 15340-15345.
114. Weerakkody, D., et al., *Family of pH (low) insertion peptides for tumor targeting*. Proceedings of the National Academy of Sciences, 2013. **110**(15): p. 5834-5839.
115. Andreev, O.A., D.M. Engelman, and Y.K. Reshetnyak, *Targeting diseased tissues by pHLIP insertion at low cell surface pH*. Frontiers in physiology, 2014. **5**.
116. Karabadzhak, A.G., et al., *Modulation of the pHLIP transmembrane helix insertion pathway*. Biophys J, 2012. **102**(8): p. 1846-55.
117. Barrera, F.N., et al., *Roles of carboxyl groups in the transmembrane insertion of peptides*. J Mol Biol, 2011. **413**(2): p. 359-71.
118. Viola-Villegas, N.T., et al., *Understanding the pharmacological properties of a metabolic PET tracer in prostate cancer*. Proc Natl Acad Sci U S A, 2014. **111**(20): p. 7254-9.
119. Cruz-Monserrate, Z., et al., *Targeting pancreatic ductal adenocarcinoma acidic microenvironment*. Sci Rep, 2014. **4**: p. 4410.

120. Macholl, S., et al., *In vivo pH imaging with (99m)Tc-pHLIP*. Mol Imaging Biol, 2012. **14**(6): p. 725-34.
121. Daumar, P., et al., *Efficient (18)F-Labeling of Large 37-Amino-Acid pHLIP Peptide Analogues and Their Biological Evaluation*. Bioconjug Chem, 2012. **23**(8): p. 1557-66.
122. Reshetnyak, Y., et al., *Measuring Tumor Aggressiveness and Targeting Metastatic Lesions with Fluorescent pHLIP*. Molecular Imaging and Biology, 2011. **13**(6): p. 1146-1156.
123. Vavere, A.L., et al., *A novel technology for the imaging of acidic prostate tumors by positron emission tomography*. Cancer Res, 2009. **69**(10): p. 4510-6.
124. Thévenin, D., M. An, and D.M. Engelman, *pHLIP-mediated translocation of membrane-impermeable molecules into cells*. Chemistry & biology, 2009. **16**(7): p. 754-762.
125. Wijesinghe, D., et al., *Tuning a polar molecule for selective cytoplasmic delivery by a pH (Low) insertion peptide*. Biochemistry, 2011. **50**(47): p. 10215-22.
126. Moshnikova, A., et al., *Antiproliferative effect of pHLIP-amanitin*. Biochemistry, 2013. **52**(7): p. 1171-8.
127. Reshetnyak, Y.K., et al., *Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix*. Proceedings of the National Academy of Sciences, 2006. **103**(17): p. 6460-6465.
128. An, M., et al., *pH-(low)-insertion-peptide (pHLIP) translocation of membrane impermeable phalloidin toxin inhibits cancer cell proliferation*. Proceedings of the National Academy of Sciences, 2010. **107**(47): p. 20246-20250.
129. Torchilin, V.P., *Recent advances with liposomes as pharmaceutical carriers*. Nature reviews Drug discovery, 2005. **4**(2): p. 145-160.
130. Yao, L., et al., *pHLIP(R)-mediated delivery of PEGylated liposomes to cancer cells*. J Control Release, 2013. **167**(3): p. 228-37.
131. Yao, L., et al., *pHLIP peptide targets nanogold particles to tumors*. Proceedings of the National Academy of Sciences, 2013. **110**(2): p. 465-470.
132. Emmetiere, F., et al., *(18)F-labeled-bioorthogonal liposomes for in vivo targeting*. Bioconjug Chem, 2013. **24**(11): p. 1784-9.
133. Galagudza, M.M., et al., *Targeted drug delivery into reversibly injured myocardium with silica nanoparticles: surface functionalization, natural biodistribution, and acute toxicity*. International journal of nanomedicine, 2010. **5**: p. 231.

134. Scott, R.C., et al., *Aiming for the heart: targeted delivery of drugs to diseased cardiac tissue*. 2008.
135. Wang, A.Z., et al., *Biofunctionalized targeted nanoparticles for therapeutic applications*. 2008.
136. Verma, D.D., et al., *ATP-loaded liposomes effectively protect mechanical functions of the myocardium from global ischemia in an isolated rat heart model*. Journal of Controlled Release, 2005. **108**(2–3): p. 460-471.
137. Sosunov, E.A., et al., *pH (low) insertion peptide (pHLIP) targets ischemic myocardium*. Proceedings of the National Academy of Sciences, 2013. **110**(1): p. 82-86.
138. Wijesinghe, D., et al., *pH dependent transfer of nano-pores into membrane of cancer cells to induce apoptosis*. Scientific reports, 2013. **3**.
139. Hladky, S.B. and D.A. Haydon, *Ion transfer across lipid membranes in the presence of gramicidin A: I. Studies of the unit conductance channel*. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1972. **274**(2): p. 294-312.
140. Wang, G., et al., *Gold-peptide nanoconjugate cellular uptake is modulated by serum proteins*. Nanomedicine : nanotechnology, biology, and medicine, 2012. **8**(6): p. 822-832.
141. Papasani, M.R., G. Wang, and R.A. Hill, *Gold nanoparticles: the importance of physiological principles to devise strategies for targeted drug delivery*. Nanomedicine : nanotechnology, biology, and medicine, 2012. **8**(6): p. 804-814.
142. Brullot, W., V.K. Valev, and T. Verbiest, *Magnetic-plasmonic nanoparticles for the life sciences: calculated optical properties of hybrid structures*. Nanomedicine : nanotechnology, biology, and medicine, 2012. **8**(5): p. 559-568.
143. Gao, J., et al., *Colloidal stability of gold nanoparticles modified with thiol compounds: bioconjugation and application in cancer cell imaging*. Langmuir, 2012. **28**(9): p. 4464-4471.
144. Simpson, C.A., et al., *In vivo toxicity, biodistribution, and clearance of glutathione-coated gold nanoparticles*. Nanomedicine : nanotechnology, biology, and medicine, 2013. **9**(2): p. 257-263.
145. Sadauskas, E., et al., *Protracted elimination of gold nanoparticles from mouse liver*. Nanomedicine : nanotechnology, biology, and medicine, 2009. **5**(2): p. 162-169.

146. Lin, I.C., et al., *Cellular transport pathways of polymer coated gold nanoparticles*. Nanomedicine : nanotechnology, biology, and medicine, 2012. **8**(1): p. 8-11.
147. Gu, Y.-J., et al., *Gold-doxorubicin nanoconjugates for overcoming multidrug resistance*. Nanomedicine : nanotechnology, biology, and medicine, 2012. **8**(2): p. 204-211.
148. Wang, G., et al., *KDEL peptide gold nanoconstructs: promising nanoplatforms for drug delivery*. Nanomedicine : nanotechnology, biology, and medicine, 2013. **9**(3): p. 366-374.
149. Sharma, A., et al., *Polyethylenimine-conjugated gold nanoparticles: Gene transfer potential and low toxicity in the cornea*. Nanomedicine : nanotechnology, biology, and medicine, 2011. **7**(4): p. 505-513.
150. Letfullin, R.R., C.B. Iversen, and T.F. George, *Modeling nanophotothermal therapy: kinetics of thermal ablation of healthy and cancerous cell organelles and gold nanoparticles*. Nanomedicine : nanotechnology, biology, and medicine, 2011. **7**(2): p. 137-145.
151. Raoof, M., et al., *Stability of antibody-conjugated gold nanoparticles in the endolysosomal nanoenvironment: implications for noninvasive radiofrequency-based cancer therapy*. Nanomedicine : nanotechnology, biology, and medicine, 2012. **8**(7): p. 1096-1105.
152. Davies, A., et al., *pH-controlled delivery of luminescent europium coated nanoparticles into platelets*. Proceedings of the National Academy of Sciences, 2012. **109**(6): p. 1862-1867.
153. Niidome, T. and L. Huang, *Gene therapy progress and prospects: nonviral vectors*. Gene therapy, 2002. **9**(24): p. 1647-1652.
154. Han, L., et al., *pH-Controlled Delivery of Nanoparticles into Tumor Cells*. Adv Healthc Mater, 2013.
155. Yao, L., et al., *pHLIP®-mediated delivery of PEGylated liposomes to cancer cells*. Journal of Controlled Release, 2013. **167**(3): p. 228-237.
156. Han, L., et al., *pH-Controlled Delivery of Nanoparticles into Tumor Cells*. Advanced healthcare materials, 2013. **2**(11): p. 1435-1439.
157. Zhao, Z., et al., *A Controlled-Release Nanocarrier with Extracellular pH Value Driven Tumor Targeting and Translocation for Drug Delivery*. Angewandte Chemie International Edition, 2013. **52**(29): p. 7487-7491.

Tables

pHLIP nanotechnology	How it works	Administration	Authors
PEGylated-liposomes containing ceramide	Increase ceramide levels in cancer tissues promote cell death and tumor inhibition	In-vitro , In-vivo	[155]
pHLIP-Tz conjugates and trans-cyclooctenes coated liposomes containing ¹⁸ F PET isotopes	Tetrazine(Tz) labeled cancer cells are targeted by trans-cyclooctenes coated liposomes to radio label the tumor site	In-vivo	[132]
WT and Var7 coated liposomes	pHLIP coated liposomes binds to ischemic regions and assist pHLIP- fluorescent - aided cardiac surgeries	In-vivo	[137]
pHLIP liposomal nano-pores	Hydrophobic gA is delivered to cancer cells to form channels in cell membrane to destroy ion balance and induce apoptosis	In-vitro	[138]
pHLIP nano-gold particles	Homogeneous accumulation of gold in tumor sites allow radiation therapy and imaging	In-vivo	[131]
Eu coated pHLIP nanoparticles	At low pH, more lanthanide probes can be internalized to human platelets breaking the targeting barrier	In-vitro	[152]
pDNA nanoparticles	High uptake of DGL-PEG-pHLIP/pDNA in tumors	In-vivo, In-vitro	[156]
dox MSNs	Dox are loaded to porous silica nanoparticles and deliver to cancer cells using pHLIP	In-vitro	[157]

Table 1. Current pHLIP applications in nanotechnology

Figures

Figure1. Different applications of pHLIP for targeting of acidic tissues. Tethering of cargo molecules to the surface of cells (a). Cytoplasmic delivery of cargo molecules and it's release by break of cleavable link (shown in blue) (b). Assembly of multifunctional pHLIP-coated liposomes containing polar (green) and hydrophobic (yellow) payloads (c). Schematic presentation of interactions of lipid bilayer of the pHLIP-coated multifunctional liposomes with plasma membrane of a cell (presentation of liposome in the endosome is schematic and not in a scale) (d).

CHAPTER 2

Published in Scientific Report of Nature on

20th of December, 2013

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

Dayanjali Wijesinghe*, Mohan C. Pereira*, Andrew Lu, Yana K. Reshetnyak & Oleg A. Andreev

Physics Department, University of Rhode Island, 2 Lippitt Rd., Kingston, RI, 02881, USA

* First co-authors

Corresponding author:

Oleg Andreev, Physics Department, University of Rhode Island, 2 Lippitt Rd., Kingston, RI, 02881, USA, Phone: (401)-874-2054, Fax: (401) 874-2380, E-mail: andreev@mail.uri.edu

Abstract

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

Introduction

The control of intracellular pH and balance of ions in a cell are essential for a number of important physiological processes, such as protein, DNA and RNA synthesis and control of the cell cycle[1]. In cancerous tissue the balance of protons is disturbed, which leads to acidification of the extracellular milieu and reversing of the transmembrane pH gradient compared with normal tissue: the intracellular pH (pHi) in tumors is higher than the extracellular pH (pHe)[2, 3]. The equilibration of the extracellular and intracellular pHs as well as disruption of balance of other ions will lead to cell death. We propose to transfer in a pH-

dependent manner nano-pores to the plasma membrane of cancer cells to disrupt intracellular and extracellular ion balance and induce cytosol acidification. Nano-pores will be formed by linear hydrophobic peptide, gramicidin A (gA), consisting of alternating L- and D-amino acids, which forms cation-conductive β -helix in the membrane[4-6]. The diameter of gA β -helix channel is about 4-5 Å, which can conduct about 10^7 cations per second[5]. The high conductance is observed

for the protons, sodium and potassium ions. The gA channels formed in the liposomes will be delivered to the membrane of cancer cells by fusion. The fusion between the lipid bilayer of liposome and cancer cells will be promoted by the pH (Low) Insertion Peptide (pHLIP®). The pHLIP inserts into the lipid bilayer and forms transmembrane helix at pH < 7 [7, 8], and therefore is able to bring in close contact liposome and cellular membranes only at low pH. We have demonstrated that the pHLIP-based optical, PET and SPECT imaging agents can target acidic diseased tissue[9-13], and we proved that pHLIP technology can substantially improve delivery of nanoparticles and liposomes to the acidic diseased tissue[14, 15]. The presence of pHLIP on the surface of PEGylated-liposomes enhanced membrane fusion and lipid exchange in a pH dependent fashion, leading to the increase of cellular uptake and payload release[16]. In the present study we demonstrate that the pHLIP-coated liposomes could be used to transfer nano-pores (peptide channels) into the membrane of cancer cells, disrupt transmembrane pH gradient and balance of sodium ions, which leads to the apoptosis of cancer cells.

Materials and Methods

Materials

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

Synthesis of DSPE-pHLIP

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

Liposome preparation

Liposomes were prepared by extrusion: a chloroform solution of the 80–98 mol% of DOPC lipids mixed with 5% pHLIP conjugated lipids (pHLIP-DSPE), 2–5% fluorescent labeled lipids (FITC-DHPE) and 0–10% gA were evaporated using rotary evaporator producing an even thin film, followed by the additional overnight evaporation under the high vacuum to remove traces of organic solvents. This lipid layer was re-suspended in 1 mL 10 mM phosphate, 150 mM NaCl buffer and went through 10 freeze-thaw-vortex cycles. The resulting liposome solution was extruded 31 times through 50 or 100 nm filters, which were used for biophysical measurements and experiments on cultured cells, respectively.

Liposomes were sterilized by filtering through 0.2 μm filter before addition to cells. Solution of liposomes was kept at 4°C overnight before use to ensure gA channels formation. The concentration of lipids was calculated by measuring absorbance of FITC at 496 nm ($\epsilon = 89,000 \text{ M}^{-1} \text{ cm}^{-1}$) or gA and pHLIP at 280 nm ($\epsilon = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$ for gA and $\epsilon = 13,940 \text{ M}^{-1} \text{ cm}^{-1}$ for pHLIP) of the liposomes dissolved in methanol.

DLS and Zeta potential measurements

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

Cryo-electron microscopy

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

Steady-state fluorescence and circular dichroism measurements

Tryptophan fluorescence and circular dichroism (CD) measurements were carried out on a PC1 ISS spectrofluorometer (ISS, Inc.) and a MOS-450 spectrometer (Bio-Logic Inc.), respectively. All measurements were performed at 25°C. DOPC liposomes containing 0, 2% or 10% of gA were used in the study in phosphate buffer, pH 7.4. Fluorescence spectra of gA at excitation of 280 nm was recorded from 310 nm to 400 nm with the spectral widths of excitation and emission slits set at 4 and 2 nm, respectively. CD spectra were recorded from 200 to 260 nm with 0.5 nm increment using a cuvette with an optical path length of 0.5 cm.

Kinetics of FITC quenching

DOPC liposomes containing 2% and 10% gA, 2% FITC-DHPE and liposomes containing no gA were used in the kinetics studies. All solutions were degassed several minutes under a vacuum to minimize air bubbles before loading into syringes of the stopped-flow apparatus (Bio-Logic, Inc.). Liposomes of 1.5 mM lipid concentration in 10 mM phosphate buffer pH 8 (or pH 3) with 150 mM NaCl

were rapidly mixed (within 5 ms – dead time of the set up we used) with an equal volume of HCl (or NaOH) to have final pH of 3 (or pH 8). Changes of the fluorescence intensity of FITC at 519 nm excited at 496 nm were monitored in a real time. Baseline was measured by mixing the liposomes with equal volume of the buffer.

Cell lines

A549 (lung carcinoma), HeLa (human cervix adenocarcinoma) and M4A4 (human breast ductal carcinoma) cell lines were obtained from American Tissue Culture Collection (ATCC). Cells were authenticated, stored according to the supplier's instructions, and used within 3 months after frozen aliquot resuscitations. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1 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 media (pH 6.2). The pH media was prepared by mixing 13.5 g of dry DMEM powder with 0.1 g of sodium bicarbonate in 1 L of deionized water supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1 µg/mL of ciprofloxacin.

Proliferation assay

About 3,000–4,000 of A549 cells were seeded in 96 well-plates at pH 7.4 and 6.2. After 24 hrs, cells were treated for 3 hrs with various concentrations of pHLIP-coated DOPC liposomes in DMEM at pH 7.4 and 6.0 followed by addition of an equal volume of DMEM with 20% FBS. In other experiments, cells were treated with

liposomes for 18 hrs followed by the removal of the constructs and replacement of the media with DMEM containing 10% FBS. For the control experiments cells were treated at both normal and low pHs with the DOPC liposomes containing no gA or no pHLIP. Cells were grown until non-treated cells in control reached 80–90% confluence. Cell viability was assessed by the colorimetric reagent (CellTiter 96 AQueous One Solution Assay, Promega) of the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, which was added for 1 hour to cells followed by measuring absorbance at 490 nm. All samples were prepared in triplicate. Each experiment was repeated several times.

ECIS assay

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

Intracellular pH measurements

First, pH calibration curve was established: about 40,000 A549 cells were seeded in collagen coated glass bottom dishes (MatTek, Corp.) at pH 6.2. Next day cells were incubated with 20 mM of SNARF-5F in L-15 phenol red free media at pH 6.2 for 1 hr followed by washing three times with 50 mM phosphate/ citric acid solution at pH 5.5, 6.0, 6.6, 7.2 or 7.5. Next, 25 μ l of 0.4 μ g/ml nigericin was added to cells and incubated for 15 min to equilibrate the intracellular pH with the pH of buffer. Fluorescent spectra of SNARF-5F before and after addition of nigericin to the cells were recorded in the range of 520–800 nm on a fluorescent inverted microscope IX71 (Olympus America, Inc.) with an attached Shamrock 303 (AndorTechnology, Inc.) spectrograph equipped with a Newton EMCCD (electron multiplied charged couple device) camera (Andor Technology, Inc.) using 480 ± 10 nm excitation an interference filter and a long pass emission filter with 520 cutoff wavelength. Ratio of SNARF-5F fluorescent signal at 595 nm/640 nm were calculated for each pH and plotted against the respective pH, and fitted with a linear function using Origin9 software (Origin Lab, Corp.) to obtain calibration curve. In separate experiment A549 cells were treated at pH 7.4 and 6.0 with 250 μ M of the DOPC pHLIP-coated liposomes containing 0 or 10% gA and 5% pHLIP for 3 hrs followed by the addition of an equal volume of 20% FBS in DMEM and incubated overnight. At various days after the treatment cells were incubated with 60 μ M SNARF-5F in 200 ml of L-15 pH 6.2 for 1 hr, followed by washing with L-15 of pH 6.2 and recording SNARF-5F emission spectra. Next, nigericin was added to the cells to equilibrate pH and emission spectra of SNARF-5F were recorded once again. The SNARF-5F

emissions signal ratio of 595/640 nm was calculated and the corresponding intracellular pH was established using pH calibration curve.

Intracellular Na⁺ measurements

About 30,000 A549 cells of pH 7.4 and 50,000 A549 cells adapted to pH 6.2 were seeded in the collagen coated glass bottom dishes. Next day, cells were treated with 1 mL of 250 μ M lipid concentration of the DOPC liposomes with 10% gramicidin and 5% pHLIP (control – equal volume of PBS 7.4) in pH 6.2 and pH 7.4 DMEM without FBS and incubated for three hours. After 3 hours 1 ml of 20% FBS in DMEM medium at pH 6.2 and pH 7.4, respectively, were added and incubated until the experiments were performed. Next day, the treatment solution from the control and treated dishes was removed and 100 μ l of 100 μ M CoroNaTM Green AM solution in L-15 at pH 7.4 was added and incubated for 45 min. After 45 mins, cells were washed three times using PBS and imaged in L-15 at pH 6.2 or pH 7.4 using green channel of the fluorescent microscope. The same procedure was repeated with the treated and control cells on 3rd day after the treatment.

Quantification of the fluorescent signals from the cells was performed using an ImageProPlus software. Statistical analysis of the data was performed using the “Statistica 5.0” package. The p-level was computed based on the two-tailed test.

Mitochondria depolarization assay

About 30,000 A549 cells were seeded in the collagen coated glass bottom dishes. Next day, cells were treated at pH 7.4 and 6.2 with 1 mL of 250 μ M lipid concentration the DOPC pHLIP-coated liposomes containing 10% gA and 5%

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

Results

Preparation of liposomes and biophysical studies

We introduced pH-sensitive fusogenic liposomes containing 80 mol% of DOPC lipids, 5% of DSPE-maleimide lipids conjugated with Cys residue at the N-terminus of pHLIP peptide (ACEQNPIYWARYADWLFTTPLLALLVDADEGT), 5% of fluorescein (FITC) labeled lipids and 10% of gramicidin (gA: formyl-(L)W-(D)G-(L)A-(D)L-(L)A-(D)V-(L)V-(D)V-(L)W-(D)L-(L)W-(D)L-(L)W-(D)L-(L)W-ethanolamine) (Figure 1a). The DOPC pHLIP-coated liposomes containing gA formed single lamellar structures of the average size of about 120 nm in diameter (the polydispersity 0.07 ± 0.01 and zeta potential -230.7 ± 1.8 mV), which were stable within a month if kept at 4°C (Figure 1a, b SI). The liposome

surface was stabilized by the negatively charged pHLIP peptides. Our previous data indicate that one pHLIP molecule in its extended configuration can occupy about 120 lipids at pH 8[17]. Thus, 5% of pHLIP in liposome (about 3% on the outer surface) should fully cover liposome surface. Control liposomes containing gA, but no pHLIP and containing pHLIP, but no gA were also tested. Gramicidin A is a linear peptide spanning just half bilayer and capable of adopting different configurations. Single stranded helical form, when two monomers of gA located within inner and outer leaflets of bilayer form head-to-head dimer, corresponds to the channel (pore) configuration of 4-5 Å in the diameter (Figure 1b). Gramicidin in the channel configuration exhibits characteristic circular dichroism (CD) signal and short wavelength shift of the tryptophan fluorescence spectrum[18], monitored by us and shown on Figure 2a and Figure 2b, respectively. To demonstrate conductance of protons by the gA channel we performed kinetics measurements on liposomes containing 2% DHPE lipids head groups of which were labeled with FITC. The FITC carries two negative charges at pH 9. As the pH is lowered the carboxyl groups of FITC are protonated and FITC is transferred to the non-fluorescent non-charged form. Thus, decrease of pH from 9 to 3 is associated with the decreased of fluorescence. The pH 8 → 3 transition was induced by fast mixing (5 ms dead time) of the DOPC-FITC liposomes and HCl in the stopped-flow apparatus. The sudden decrease of about 75% of fluorescent signal was associated with the quenching of emission of FITC on the outer leaflet of bilayer. The quenching was completed within a dead time (5 ms) followed by

the slow kinetics reflected penetration of the protons across a bilayer. It led to the quenching of fluorescence of FITC located at the inner leaflet of bilayer (red line on Figure 2c). The data are in an accordance with the previously published results[19, 20]. At the same time, when 2% or 10% of gA was incorporated into liposomes the decrease of FITC fluorescence was completed within less than 5 ms due to the conductance of protons by the gA channel (blue line on Figure 2c). We also followed pH equilibration after the addition of base to induce pH 3 $\rightarrow$ 8 transition, which leads to the increase of FITC fluorescence. For the liposomes containing gA the changes of fluorescence were completed within less than 5 ms. At the same time, the slow kinetics was observed for the liposomes containing no gA (Figure 2d).

Inhibition of cell proliferation

Biophysical studies indicated that gA is adopting conductive channel form in the liposomes. The main question we proposed to answer if the pHLIP-coated liposomes will be able to transfer gA channel to the cellular membranes of cancer cells, destroy ion balance and induce cell death. All experiments were carried out on the cancer cells growing in standard DMEM media pH7.4 and on the cells adapted for low pH growth of 6.2 in DMEM. First, we tested ability of the pHLIP-coated liposomes containing 5 and 10% gA to inhibit proliferation of A549 lung carcinoma cells adapted for low pH growth (Figure 2a SI). Cells were treated at pH 6.2 with the constructs for four days. The liposomes containing 10% gA were more effective in inhibition of cell proliferation, since increase of the gA concentration in membrane significantly raise the probability of assembling of

conductive channel, when both monomers come together and form head-to-head b-helix. The liposome composition containing 10% gA was selected for further experiments. A549 (human lung carcinoma), HeLa (human cervix adenocarcinoma) and M4A4 (human breast ductal carcinoma) cell lines were treated with the pHLIP-coated liposomes containing 10% gA and 5% pHLIP for 18 hrs at pH 7.4 or pH 6.2 in DMEM followed by the replacement of media with DMEM containing 10% FBS. MTS assay was performed on 4-th day after the treatment to establish number of viable cells (Figure 3a–c). More than 80% of cells were dead in the result of treatment with the liposomes of 10 mM of total lipid concentration at pH 6.2. At the same time, the treatment of cells with the same construct at pH 7.4 did not affect cell proliferation, except of M4A4 cells at the highest concentration of the construct. We also treated A549 cells for 1 day in DMEM of pH6.2 and then transferred cells into the pH7.4 DMEM with 10% FBS (Figure 3c). When cells treated at low pH were transferred to normal pH the cytotoxic effect was significantly less. In a control experiment, the same cell lines were treated at the same conditions with the pHLIP- coated liposomes with no gA at concentrations up to 100 μ M at both pHs and no any effect on cell proliferation was observed (Figure 2c–d SI).

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

kinetics of inhibition of proliferation of A549 cells treated with the pHLP-coated liposomes containing 10% gA at pH 6.2 was monitored by the changes of capacitance measured at AC frequencies using the electric cell-substrate impedance sensing (ECIS) technique (Figure 3d). The capacitance varies in a linear fashion with the fractional cell coverage of the bottom of special chamber coated with the thin film gold electrodes connect to the ECIS electronics[21]. When cells were shrinking and dying after the treatment with the construct the capacitance was increasing. The cell death happens couple days after the treatment.

Acidification of intracellular space

We assumed that the pHLP-coated liposomes fuse with the cellular membrane and deliver gA channels to the plasma membrane. The efficient conductance of protons of gA channel from the extracellular space to cytoplasm leads to the acidification of intracellular space. The pH-sensitive fluorescent dye SNARF was used to measure pH in a cell[22]. SNARF in the ester form can freely diffuse across the membrane into cytoplasm, where in the result of esterification it is transferred to the cell-impermeable fluorescent carboxyl acid. Fluorescence of carboxy SNARF at 595 nm increases with pH changes from 8 to 5. Fluorescent spectra from the cells (Figure 4a) were recorded using an Andor spectrometer coupled with a fluorescent microscope. Nigericin, K^+/H^+ ionophore, was used to disrupt balance of the protons and equilibrate pH between the extracellular and intracellular spaces, which was needed for the calibration of the fluorescent signal of SNARF in cells[23]. Ratio of SNARF fluorescent signals at 595 nm to 640 nm

were calculated for each pH and plotted against the respective pH, which allowed constructing the calibration curve (Figure 4b). Then, fluorescent signal of SNARF was measured in A549 cells treated with the pHLP-coated liposomes containing 10% gA and non-treated cells. First day after the treatment, the pH inside the non-treated (control) and treated cells was 7.08 and 7.02, respectively (Figure 4b, blue and magenta circles). However, on the second day after the cells treatment the intracellular pH was dropped to 6.53 (Figure 4b, red circle), while the intracellular pH in the control group was around 7.23 (Figure 4b, green circle). After each measurement of intracellular pH, the nigericin was added to the cells, and the measured pH was found to be around 6.2–6.3 in each case as expected, as a pH of L-15 media used in the experiment.

Disruption of cellular balance of Na⁺ ions

It is known that gramicidin pore conducts well not only protons, but all monovalent cations including sodium and potassium ions. We used sodium ion indicator, CoroNa Green dye, that exhibits an increase in green fluorescence upon binding Na⁺ in presence of physiological concentrations of other monovalent ions[24, 25]. A549 cells non-treated and treated with the pHLP-coated liposomes containing 10% gA at both 7.4 and 6.2 pHs were loaded at different time points with the cell-permeable CoroNa Green AM, washed and imaged. We observed enhancement of the green fluorescence of cells treated with the liposomes at low pH on day 1 and even more significant increase of fluorescence on day3 (Figures 5). It indicates the equilibration of concentration

of sodium ions in the extracellular and intracellular spaces in the result of transfer of sodium ions into the cytoplasm via the gramicidin channel.

Mitochondria depolarization assay

Disturbance of the balance of monovalent ions and acidification of the intracellular space induces apoptosis, which was monitored by the depolarization of mitochondria. Membrane-permeable dye, JC-9, exhibits potential- dependent accumulation in the mitochondria monitored by the fluorescence emission shift from green (~ 530 nm) to orange (~ 590 nm)[26]. Consequently, the mitochondrial depolarization is reflected by increase in the green/orange fluorescence intensity ratio (Figures 6). The ratio of green/orange fluorescence of JC-9 dye was measured on the A549 cells at different time points after cells treatment at both pHs of 7.4 and 6.2 with the pHLIP-coated liposomes containing 10% gA and on non-treated cells. Only cells treated with the liposomes at low pH demonstrate increase of green/ orange fluorescence ratio at 3-rd and 4-th days after the treatment indicating to the mitochondria depolarization and cell apoptosis.

Discussion

The pHLIP-coated liposomes mimic highly efficient intracellular delivery systems utilized by the viruses and pathogenic organisms[27-29], since pHLIP at low pH performs function of the fusogenic peptides that brings membrane of the liposome and a cell in close contact[16]. There are two case scenarios: i) the liposome membrane fuse with the cellular membrane and transfer gramicidin

conductive channel to the plasma membrane, or ii) the liposome enters a cell via endocytosis, most probably, macropinocytosis, in the result of simultaneous insertion of several pHLIP peptides into the plasma membrane, which can lead to the plasma membrane ruffling and blebbing and can trigger internalization of the liposomes (Figure 7). In the latter case, the pHLIP-coated liposome will fuse with the membrane of endosome at low pH. In both case scenarios, the gramicidin channels will disrupt the balance of monovalent cations. At the same time, proton pumps and sodium/potassium ion channels actively pump protons and sodium ions from the cytoplasm to the extracellular space and potassium ions to the cytoplasm. It takes time for complete equilibration of ions. Disturbance of ion balance leads to the mitochondria depolarization, which is a signal of early apoptosis. Interestingly, cells treated for 1 day at low pH with the pHLIP-coated liposomes containing gramicidin, but then transferred to the media of normal pH, demonstrated much less cytotoxicity compare with the cells growing at low pH all the time.

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

Author contributions

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

Acknowledgments

The work was supported by the NIH grants CA133890, CA174413 and GM073857 to O.A.A. and Y.K.R

References

1. Casey, J.R., S. Grinstein, and J. Orlowski, *Sensors and regulators of intracellular pH*. Nature reviews Molecular cell biology, 2010. **11**(1): p. 50-61.
2. Gerweck, L.E. and K. Seetharaman, *Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer*. Cancer research, 1996. **56**(6): p. 1194-1198.
3. Raghunand, N., et al., *Plasmalemmal pH-gradients in drug-sensitive and drug-resistant MCF-7 human breast carcinoma xenografts measured by ³¹P magnetic resonance spectroscopy*. Biochemical pharmacology, 1999. **57**(3): p. 309-312.
4. Kelkar, D.A. and A. Chattopadhyay, *The gramicidin ion channel: a model membrane protein*. Biochimica et Biophysica Acta (BBA)-Biomembranes, 2007. **1768**(9): p. 2011-2025.
5. Hladky, S. and D. Haydon, *Ion transfer across lipid membranes in the presence of gramicidin A: I. Studies of the unit conductance channel*. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1972. **274**(2): p. 294-312.
6. Langs, D., et al., *Monoclinic uncomplexed double-stranded, antiparallel, left-handed beta 5.6-helix (increases decreases beta 5.6) structure of gramicidin A: alternate patterns of helical association and deformation*. Proceedings of the National Academy of Sciences, 1991. **88**(12): p. 5345-5349.
7. Andreev, O.A., D.M. Engelman, and Y.K. Reshetnyak, *Targeting acidic diseased tissue: New technology based on use of the pH (Low) Insertion Peptide (pHLIP)*. Chimica oggi, 2009. **27**(2): p. 34.
8. Reshetnyak, Y.K., et al., *A monomeric membrane peptide that lives in three worlds: in solution, attached to, and inserted across lipid bilayers*. Biophysical journal, 2007. **93**(7): p. 2363-2372.
9. Macholl, S., et al., *In vivo pH imaging with ^{99m}Tc-pHLIP*. Molecular Imaging and Biology, 2012. **14**(6): p. 725-734.
10. Andreev, O.A., et al., *Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues in vivo*. Proceedings of the National Academy of Sciences, 2007. **104**(19): p. 7893-7898.
11. Reshetnyak, Y.K., et al., *Measuring tumor aggressiveness and targeting metastatic lesions with fluorescent pHLIP*. Molecular Imaging and Biology, 2011. **13**(6): p. 1146-1156.
12. Daumar, P., et al., *Efficient ¹⁸F-labeling of large 37-amino-acid pHLIP peptide analogues and their biological evaluation*. Bioconjugate chemistry, 2012. **23**(8): p. 1557-1566.

13. Vāvere, A.L., et al., *A novel technology for the imaging of acidic prostate tumors by positron emission tomography*. Cancer research, 2009. **69**(10): p. 4510-4516.
14. Yao, L., et al., *pHLIP peptide targets nanogold particles to tumors*. Proceedings of the National Academy of Sciences, 2013. **110**(2): p. 465-470.
15. Sosunov, E.A., et al., *pH (low) insertion peptide (pHLIP) targets ischemic myocardium*. Proceedings of the National Academy of Sciences, 2013. **110**(1): p. 82-86.
16. Yao, L., et al., *pHLIP®-mediated delivery of PEGylated liposomes to cancer cells*. Journal of Controlled Release, 2013. **167**(3): p. 228-237.
17. Reshetnyak, Y.K., et al., *Energetics of peptide (pHLIP) binding to and folding across a lipid bilayer membrane*. Proceedings of the National Academy of Sciences, 2008. **105**(40): p. 15340-15345.
18. Rawat, S.S., D.A. Kelkar, and A. Chattopadhyay, *Monitoring gramicidin conformations in membranes: a fluorescence approach*. Biophysical journal, 2004. **87**(2): p. 831-843.
19. Karabadzhak, A.G., et al., *Modulation of the pHLIP transmembrane helix insertion pathway*. Biophysical journal, 2012. **102**(8): p. 1846-1855.
20. Kuyper, C.L., et al., *Proton permeation into single vesicles occurs via a sequential two-step mechanism and is heterogeneous*. Journal of the American Chemical Society, 2006. **128**(10): p. 3233-3240.
21. Wegener, J., C.R. Keese, and I. Giaever, *Electric cell-substrate impedance sensing (ECIS) as a noninvasive means to monitor the kinetics of cell spreading to artificial surfaces*. Experimental cell research, 2000. **259**(1): p. 158-166.
22. Han, J. and K. Burgess, *Fluorescent indicators for intracellular pH*. Chemical reviews, 2009. **110**(5): p. 2709-2728.
23. Hilderbrand, S.A., et al., *Near infrared fluorescence-based bacteriophage particles for ratiometric pH imaging*. Bioconjugate chemistry, 2008. **19**(8): p. 1635-1639.
24. Martin, V.V., A. Rothe, and K.R. Gee, *Fluorescent metal ion indicators based on benzoannelated crown systems: a green fluorescent indicator for intracellular sodium ions*. Bioorganic & medicinal chemistry letters, 2005. **15**(7): p. 1851-1855.
25. Meier, S.D., Y. Kovalchuk, and C.R. Rose, *Properties of the new fluorescent Na⁺ indicator CoroNa Green: comparison with SBFI and confocal Na⁺ imaging*. Journal of neuroscience methods, 2006. **155**(2): p. 251-259.

26. Smyth, P.G., S.A. Berman, and S. Bursztajn, *Markers of apoptosis: methods for elucidating the mechanism of apoptotic cell death from the nervous system*. Biotechniques, 2002. **32**(3): p. 648-665.
27. Hua, Y. and R.H. Scheller, *Three SNARE complexes cooperate to mediate membrane fusion*. Proceedings of the National Academy of Sciences, 2001. **98**(14): p. 8065-8070.
28. Eisenberg, R.J., et al., *Herpes virus fusion and entry: a story with many characters*. Viruses, 2012. **4**(5): p. 800-832.
29. Wilen, C.B., J.C. Tilton, and R.W. Doms, *Molecular mechanisms of HIV entry*, in *Viral Molecular Machines*. 2012, Springer. p. 223-242.

Figures

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

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

Figure 3. Inhibition of cell proliferation. HeLa, human cervix adenocarcinoma, (a), M4A4, human breast ductal carcinoma, (b), A549, human lung carcinoma (c) cells grown in standard media of pH 7.4 and adapted for low pH growth of 6.2 were treated with the DOPC pHLIP- coated liposomes containing 10% gA and 5% pHLIP for 18 hrs at pH 7.4 or 6.2 in DMEM followed by the replacement of media with DMEM containing 10% FBS of the same pH as treatment or DMEM of pH 7.4, 10% FBS (blue columns). MTS assay was performed on 4-th day after the treatment. (d) About 25,000 of A549 cells were seeded in an 8W10E 1 ECIS array in DMEM at pH 6.2, next day cells were treated for 3 hrs with different concentrations of the DOPC pHLIP-coated liposomes containing 10% gA and 5% pHLIP liposomes in DMEM at pH 6.0 followed by addition an equal volume of the DMEM containing 20% FBS at pH 6.2. The kinetics of inhibition of cell proliferation was monitored by changes of capacitance within 5 days.

Figure 4. Monitoring changes of intracellular pH. First, calibration curve was established: A549 cells grown at pH6.2 were incubated with SNARF-5F followed by washing with 50 mM phosphate/citric acid solution of pH 5.5, 6.0, 6.6, 7.2 and 7.5. To equilibrate extracellular and intracellular pH, the ionophore nigericin was added to cells and the fluorescence spectra of SNARF-5F were recorded (a). Ratio (595/640 nm) of SNARF-5F fluorescent signal was plotted for each respective pH (black dots) and the linear fit was performed to establish calibration curve (b). The fluorescence spectra of SNARF-5F was obtained on A549 cells non-treated and treated with the DOPC pHLIP-coated liposomes containing 10% gA and 5% pHLIP at pH 7.4 or 6.2 for 3 hrs followed by addition an equal volume of the DMEM containing 20% FBS. The SNARF-5F emission ratio of 595/640 nm was calculated and the corresponding intracellular pH was obtained from the pH calibration curve (color dots on panel b).

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

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

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

Supporting Information

Figure 1 SI. a) Size and Zeta Potential of pHLIP coated gA liposomes. Cryo-TEM image of the DOPC pHLIP-coated liposomes containing 10% gA at 40,000x magnification. **b)** Diameter and zeta potential of the DOPC pHLIP-coated liposomes containing 10% gA, which were kept at 4°C in 10 mM phosphate, 150 mM NaCl, pH 7.4 buffer, were monitored within 30 days.

Figure 2 SI. Control experiments **a)** A549 cells adapted for low pH growth were treated for 3 hrs with the DOPC pHLIP-coated liposomes containing 5% pHLIP and 0-10% gA in DMEM at pH 6.2 followed by the addition of an equal volume of the DMEM with 20% FBS. MTS assay was performed on 4-th day after the treatment. **b)** about 3,000-4,000 A549 cells grown at pH 7.4 and 6.2, respectively, were treated with the DOPC liposomes containing 10% gA, but no pHLIP for 18 hrs in DMEM at pH 7.4 or 6.2 followed by the replacement of media with DMEM containing 10% FBS. MTS assay was performed on 4-th day after the treatment. **c-d)** about 3,00-4,000 A549 cells grown at pH 7.4 and 6.2, respectively, were treated with the DOPC pHLIP-coated liposomes containing 5% pHLIP, but no gA for 18 hrs in DMEM at pH 7.4 or 6.2 followed by the replacement of media with the DMEM containing 10% FBS. MTS assay was performed on 4-th day after the treatment.

CHAPTER 3

Manuscript in Preparation 2016

Novel pHLIP® coated niasomes for diagnostic and therapy

Mohan C Pereira¹, Monica Pianella², Maria Carafa², Carlotta Marianecchi², Oleg A Andreev¹, Yana K. Reshetnyak^{1*}

¹Physics Department, University of Rhode Island, 2 Lippitt Rd., Kingston, RI, 02881, USA

² Department of Drug Chemistry and Technologies, Sapienza University of Rome, Pzz.le, Aldo Moro 5, 00185, Rome, Italy

Corresponding author:

*Yana K. Reshetnyak, Physics Department, University of Rhode Island, 2 Lippitt Rd., Kingston, RI, 02881, USA, Phone: (401)-874-2054, Fax: (401) 874-2060, E-mail: reshetnyak@uri.edu

Abstract

Currently, nanomedicine is becoming very popular over conventional methods due to the tunable physico-chemical properties used as successful therapeutic and diagnostic agents to treat cancer and many other pathogenic conditions. However, the success of these nanomedicines primarily relies on how specifically and efficiently they can target pathological sites by minimizing undesirable effects. Here, we propose a novel class of targeted nano- drug delivery system, which can be used as an effective nano-theranostic for cancer. We formulated small, unilamellar niosomes using non-ionic surfactants (SPAN20), cholesterol and pH(Low) Insertion Peptide (pHLIP), which is our targeting moiety. pHLIP coated niosomes can sense the extracellular acidity of cancerous cells and at low pH they can insert into the cellular membrane and successfully deliver niosomes carrying hydrophobic or hydrophilic agents into the cells. Intravenous injection of pHLIP-coated, niosome constructs show remarkable accumulation in mice tumors compared to their pH- insensitive niosome constructs. Long circulation time and uniform bio-distribution throughout the entire tumor and cells make them better delivery systems for future clinical applications.

Keywords: pHLIP, niosomes, drug delivery, tumor acidity, targeted therapy, nanomedicine

Introduction

At present, scientists from various disciplines all over the world are involved in the study of vesicles and other colloidal structures as carriers for passive or active targeting to improve cancer therapy. However, most of the currently approved nanomedicinal products for cancer therapy are non-targeted nanovectors that accumulate in tumor tissues purely through the enhanced permeability and retention (EPR) effect and release their payload in the extravascular tumor tissues for antitumor efficacy [1]. It is nearly impossible to find a universal active targeting moiety for cancer as it is essentially heterogeneous in nature[2, 3]. However, the extracellular acidity of tumor cells has been identified as a general property of cancerous cells and thus it can be used as a hallmark to target tumors[4, 5]. Since a little over a decade ago when pH(Low) Insertion Peptide (pHLIP) was discovered, this remarkable peptide has become a perfect candidate for this purpose [6, 7]. This pH sensitive peptide stays in monomeric and unstructured form in solution at normal pH in the absence of the lipid membrane. At the presence of the lipid membrane, it binds to the surface, and, mainly, it stays as an unstructured monomer. But at low pH, the Asp and Glu residues of the peptide protonate and enhance its hydrophobicity causing it to insert into the cellular membrane by forming an α -helix [8, 9]. By appropriately modifying its N- or C- terminus, pHLIP can be used as a bio-marker or it can be used to deliver membrane impermeable cargo into the cell by utilizing the energy released during its insertion process[10-12]. It has also shed new light on targeted nanomedicine since pHLIP has repeatedly proven its ability to sense extracellular acidity over a wide variety of applications. Therefore, since the discovery of pHLIP technology, various research groups have been applying this technology to

form pH-sensitive nanoparticles based on lipid and inorganic materials[13-17]. In this study we show how we can use pHLIP technology to formulate pH-sensitive, fusogenic niosomes for cancer therapy.

Niosomes are closed bilayer structures formed by the self-assembly of non-ionic amphiphiles in aqueous media. They were initially developed and patented by the cosmetic company L'Oreal in the late 1970s [18], and the first commercial product of “Niosome” antiaging cream was introduced to the market in the 1980s. They are analogous to liposomes; like liposomes, niosomes can be multilamellar or unilamellar vesicles, able to encapsulate both hydrophilic and lipophilic drugs. Non-ionic surfactant vesicles are osmotically active, chemically stable, and have long shelf lives compared to liposomes. Different types of surfactants have been proposed as starting materials such as the Span® series, Tween® series and the Brij® series to prepare niosomes[19]. Physical and chemical properties of these surfactant families can be utilized to achieve stable vesicular carriers, which have influence on fluidity, microviscosity and polarity of bilayers [20-23].

Sorbitan esters (Spans®) are FDA approved for use in food processing and pharmaceuticals [24]. They are safe, amphiphilic compounds derived from sorbitol (i.e., a synthetic sweetener). *Sorbitan esters* are prepared by *controlled* dehydration of *sorbitol* followed by lipase-catalyzed *esterification* of the resulting *sorbitan*. Vesicles prepared by Span-series surfactants have been reported to be viable drug carriers for different diseases and for different routes of administration (about 100 articles published in the last 5 years – data retrieved from Scopus).

Span 20 consists of a fully saturated short alkyl chain (lauryl - C12). The single carbon bonds in Span 20 allow the alkyl chain to pack tightly, resulting in a rigid membrane for the assembled niosomes. According to this, Span 20 seems to assemble into smaller and most stable niosomes compared to Span 40 and Span 60, which bear long saturated acyl chains [palmityl (C-16) and stearyl (C-18) chains respectively] [25]. It was reported that the length of alkyl chain is a crucial factor of permeability [26]. Furthermore, Span 40 and 60 are in a gel state, compared with a liquid surfactant at room temperature. Such features render Span 40- and Span 60-based niosomes less likely to leak the encapsulated water-soluble drug molecules into their surrounding solution. In contrast, Span 20-based niosomes are fluid at room temperature [27].

Materials and Methods

Materials

Sorbitan monolaurate (SPAN20), Cholesterol $\geq 99\%$, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) $\geq 99.5\%$ (titration) & N-(1-Pyrenyl)maleimide (Pyr-maleimide) were purchased from Sigma-Aldrich Inc (Atlanta, GA). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (sodium salt) (DSPE-maleimide), were obtained from Avanti Polar Lipids (Alabaster, AL). Octadecyl Rhodamine B Chloride (R18) was purchased from Thermo Fisher Scientific Inc. The wild type pH(Low) Insertion Peptide (ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT) was prepared by solid-phase peptide synthesis at C.S.Bio, CA and hereafter it will be referred as WT. All other chemicals used were of the highest purity available and solvents were of spectroscopic

grade. Water was purified through a Millipore(Bedford, MA) Milli-Q system and used for all experiments.

Synthesis of DSPE-WT pHLIP & Pyr- WT pHLIP

DSPE-WT and Pyr-WT were synthesized by the covalent conjugation of maleimide head group of DSPE and Pyrene with the single cysteine residue at the N-terminus of WT . The reaction was done with peptide to maleimide molar ratio 1.1:1 in Methanol and left for overnight stirring at room temperature under argon. The existence of the product was ensured by SELDI-TOF mass-spectrometry analysis.

SPAN-20 niosome preparation

10 mM stock solutions of SPAN20 and cholesterol were prepared by dissolving the products in chloroform & methanol mixture of 3:1 (v/v). Solutions of the 43–50 mol% of SPAN20, 50 mol% cholesterol mixed with 5 mol% DSPE-WT or Pyr-WT and 2 mol% fluorescent dye (R-18) were evaporated using a rotary evaporator at 60 °C producing an even thin film for 1 hour, followed by the additional one hour evaporation under a high vacuum to remove traces of organic solvents. This layer was hydrated in 2 mL ,10 mM, pH 7.4 HEPES buffer solution with or without 5 mM Ca^{2+} . The resulting solution was sonicated using the probe sonicator for 5 minutes at 60 degrees Celsius and extruded 31 times through 200 nm filters. Niosomes were sterilized by filtering through 0.2 μm filter before the addition to cells. The concentration of SPAN20 was calculated by measuring absorbance of R18 at 546 nm ($\epsilon = 104,126 \text{ M}^{-1} \text{ cm}^{-1}$) or pHLIP at 280 nm ($\epsilon = 13,940 \text{ M}^{-1} \text{ cm}^{-1}$) of the niosomes dissolved in methanol. Niosomes were stored at 4°C.

DLS and Zeta potential measurements

The size distribution of niosomes was measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS instrument with Malvern disposable cuvettes (Malvern USA). The shelf life was monitored for a one month period in 10 mM HEPES buffer at pH 7.4. In addition, size measurements were taken for 20 hours in 5% (v/v) Serum medium at pH 7.4. The zeta potential was measured on the same instrument using folded capillary cells from Malvern.

Cryo-electron microscopy

About 5 μ L droplet of niosome solution was spread on a Lacey Formar/Carbon electron microscopy grid and preserved in a frozen-hydrated state by a rapid freezing in liquid ethane. The vitrification process was performed via FEI Vitrobot system with the setting of a single blot of 3 sec, an offset of 1, and drain and wait time of 1 sec. Samples were imaged using JEOL 2100 TEM with an accelerating voltage of 200 kV at magnifications of 15,000 $\times$ and 25,000 $\times$.

Cell lines

Lung carcinoma A549 and mouse mammary metastatic cancer 4T1 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) and Roswell Park Memorial Institute Medium (RPMI) supplemented with 10% (v/v) fetal bovine serum, 0.1% (v/v) of 10 μ g/mL of ciprofloxacin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Cell proliferation assays

About 3,000 of A549 and 4T1 cells were seeded in 96 well-plates at pH 7.4. The following day, cells were treated for 2 hrs with various concentrations of pHLIP-coated niosomes in respective serum free media at pH 7.4 and followed by the addition of an equal volume of respective medium with 20% (v/v) FBS. For another set, the treatment was completely removed after 2 hours and incubated again after adding fresh 10% (v/v) FBS medium. Cells were grown until non-treated cells in control reached 80–90% confluence. Cell viability was assessed by the colorimetric reagent (CellTiter 96 AQueous One Solution Assay, Promega) of the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, which was added for 1 hour to cells followed by measuring absorbance at 490 nm. All samples were prepared in triplicate. Each experiment was repeated several times.

Measurement of cellular uptake

A549 and 4T1 cells in suspension were treated with R-18 labelled niosomes at low & high pH for about 1 hour. After washing, cellular uptake of fluorescent niosomes was assessed by the counting of fluorescent cells on a cellometer before and after adding trypan blue. All data were normalized to the fluorescent intensity of 4T1 cells treated at pH 7.8 with DSPE-pHLIP. After washing, another set of suspended cells were seeded on collagen-coated dishes and the following day the light and fluorescent images were taken for low pH treated cells for qualitative purpose.

Tumor Mouse Models

All animal studies were conducted according to the animal protocol **AN04-12-011** approved by the Institutional Animal Care and Use Committee at the University of Rhode Island, in compliance with the principles and procedures outlined by NIH for the Care and Use of Animals. 4T1 mammary tumors were established by subcutaneous injection of 4T1 cells (1×10^6 cells/mL, 0.1 mL/flank) in the right flank of adult female BALB/c mice (about 19–22 g weight) obtained from Harlan Laboratories.

Fluorescence Imaging of Organs

When 4T1 tumors reached 6–9 mm in diameter, they were intravenously injected with different constructs of niosomes contained R18 (100 μ L of 50 μ M) via the tail vein. At 4 h, 24 h & 48 h from post injection the mice were euthanized and organs were harvested and imaged using FX Kodak image station and mean fluorescence intensities of tumor and organs was calculated using ImageJ software.

Imaging of Tumor Sections

Frozen tumor tissues were sectioned at a thickness of 5 μ m using a Vibratome UltraPro 5000 Cryostat. Sections were mounted on microscope slides, dried in air, and washed with deionized water. Tumor sections with the R18 contained pHLIP niosomes were analyzed without further processing using Zeiss LSM 700 confocal module under DAPI and Rhodamine channel at 20x magnification. Following fluorescence imaging, the adjacent sections were then stained with hematoxylin and eosin (H&E) and imaged 20x magnifications.

Ca²⁺ Experiments: Cytotoxicity Assays and ECIS

About 3,000 4T1 cells were seeded in 96 well-plates at pH 7.4. The following day, cells were treated for 3 hrs with various concentrations of Pyr-WT-coated niosomes containing 5 mM Ca²⁺ in RPMI serum free media at pH 7.4 and pH 6.5. After 3h, the treatment was completely removed and incubated again after adding fresh 10% (v/v) FBS medium at pH 7.4. Cells were grown until non-treated cells in control reached 80–90% confluence. Control assay was run with the treatment niosomes without Pyr-WT. Cell viability was assessed by the colorimetric reagent (CellTiter 96 AQueousOne Solution Assay, Promega) of the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, which was added for 1 hour to cells followed by measuring absorbance at 490 nm. All samples were prepared in triplicate. Each experiment was repeated several times.

The kinetics of inhibition of proliferation of 4T1 cells treated with pyr-WT coated niosomes containing 5 mM Ca²⁺ HEPES physiological medium was monitored by changes of capacitance measured at AC frequencies using the ECIS (electric cell-substrate impedance sensing) technique. The capacitance varies in a linear fashion with the fractional cell coverage of the bottom of a special chamber coated with thin film gold electrodes connect to the ECIS electronics[28]. When cells were shrinking and dying after treatment with the construct the capacitance was increasing. The cell death starts within 30 minutes after the treatment.

Ca²⁺ Experiments: In Vivo Studies

4T1 mammary tumors were established by subcutaneous injection of 4T1 cells (1×10^6 cells/mL, 0.1 mL/flank) in both flanks for the IT injection group and only in the right flank for the IV injection group of adult female BALB/c mice (about 19–22 g weight) obtained from Harlan Laboratories. When tumors became visible, 50 μ L and 150 μ L, 2.5 mM Pyr-WT niosomes with 5 mM Ca²⁺ were injected into the mice directly into the tumor and via the tail vein respectively. The injections were performed for three consecutive days for both IT and IV groups. The control was not given any injection. On the 5th day from the initial injection, mice were euthanized and tumors were harvested and weighed.

Results

We were able to formulate very stable niosomes with the average hydrodynamic size of ~80 nm (Fig. 1, Table 1) and they showed a prolonged shelf life at 4 °C in 10 mM HEPES buffer at pH7.4 (SI .Fig 1). At this pH, anionic pHLIP adds electrostatic repulsion to the niosomes. In addition to that, 5% pHLIP, which is more than enough to cover the niosome surface completely, could act as grafted polymers, sticking out into the solution. When the particles come close enough to overlap, the local increase of polymer concentration leads to an osmotic pressure, causing a repulsive force which further minimizes inter-niosomal fusion. Due to these effects, we observed high zeta potential for the niosomes showing high stability over an extended period at 4 °C.

Cell proliferation assays done with 4T1 & A549 cells confirmed that DSPE-WT pHLIP and Pyr-WT pHLIP-coated niosomes do not show any toxicity for the surfactant

concentration up to 1 mM (SI Fig 2). The cellular uptake of pHLIP-coated niosomes were monitored using R18 fluorescence. It is evident that at low pH, significantly high cellular uptake occurs compared to cells treated at high pH (Fig.a, b, c & d) . After adding trypan blue, the fluorescent signal of R18 was remeasured for each pH and cell line to ensure the signal came only from the R18 within the cell. This data also confirms that at low pH there is higher uptake of pHLIP labelled niosomes (Fig 2, SI.Fig 3). Fluorescent images taken at low pH for both constructs and for both cell lines clearly show the distribution of R18-labeled niosomes over the cellular membranes and throughout the cell (Fig.2 a,b,c & d). This data confirms that the pH-dependent and mediated cellular uptake of pHLIP coated niosomes.

In-vivo kinetics (Fig 3) show a higher accumulation of both the DSPE-WT and Pyr-WT niosomes in tumor sites 24 hours post injection compared to other organs. The R18 intensity profile for the same organs (Fig 4 a,b) were analyzed after 24 hours from I.V. administration of DSPE-PEG, DSPE-WT pHLIP, DSPE-6K, Pyr-WT and Pyr-6K constructs. Here, 6K stands for the pH independent counter-part of WT pHLIP where all D(Asp) and E(Glu) residues were replaced by K(Lys). DSPE-PEG, DSPE- 6K, and Pyr- 6K show accumulation in the tumor site purely due to the EPR effect. However, DSPE- WT and Pyr- WT show about a two-fold accumulation compared to the other three constructs. Tumor sections taken from the interior of the tumors (Fig. 5) clearly show that both niosomes coated with DSPE –WT and Pyr-WT distributed throughout the tumor and fused with extra and intra cellular membranes more evenly.

We were able to use Pyr-WT coated niosomes to deliver 5 mM Ca^{2+} ions to 4T1 cancer cells and tumors as a novel therapeutic agent. Cyto-toxicity assays done with niosomes

with and without Pyr-WT shows that at low pH only the Pyr-WT coated niosomes can inhibit the cell proliferation in a concentration dependent manner (Fig.6). Kinetics data (ECIS) show that this cell death starts within 30 minutes of treatment(Fig.7).

After 3 consecutive days of intra-tumoral injections of Pyr-WT niosomes containing 5 mM Ca^{2+} , it was seen to significantly suppress the growth of tumors compared to its control on the 5th day. The IV injections also show that Pyr-WT coated niosomes with 5 mM Ca^{2+} could suppress tumor growth (Fig 8).

Discussion

The DSPE-WT and Pyr- WT coated niosomes, follow a closely similar mechanism utilized by viruses and pathogenic organisms to enter into cells [14, 15, 29, 30] . At low pH, pHLIP behaves as a fusogenic peptide to bring the niosome membrane and the extracellular membrane in close contact. There are two main possibilities for this cellular entry. Either the niosomes fuse directly with the extracellular membrane or they can be taken up into the cell via an endocytotic path, or more favorably, via micropinocytosis due to several simultaneous pHLIPs inserted into the membrane. Niosomes, which are internalized into the cell via endocytosis, will further fuse with the endosome membrane at low pH. In either case, niosomes modified with lipophilic or hydrophilic drugs can effectively enter into the cells in a pH-dependent manner.

In-vitro studies confirm that niosomes distribute throughout the cell and the cellular uptake is governed by the extracellular acidity of the cancerous cells. In vivo results show that the pHLIP coated niosomes possess long circulation half-life because of their stability, small size, and stealth properties which help niosomes to accumulate more in

tumor sites through the EPR effect. The tumor sections also show that the niosomes can distribute throughout the tumor. In addition to this, low toxicity, prolonged shelf life, and low manufacturing cost also make niosomes ideal targeted, drug delivery systems for carrying therapeutics and diagnostics to cancer.

Acknowledgement

The work was supported by the NIH grant GM073857 to O.A.A. and Y.K.R

References

1. Dawidczyk, C.M., L.M. Russell, and P.C. Searson, *Nanomedicines for cancer therapy: state-of-the-art and limitations to pre-clinical studies that hinder future developments*. Front Chem, 2014. **2**: p. 69.
2. Stingl, J. and C. Caldas, *Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis*. Nature Reviews Cancer, 2007. **7**(10): p. 791-799.
3. Weigelt, B., J.L. Peterse, and L.J. Van't Veer, *Breast cancer metastasis: markers and models*. Nature reviews cancer, 2005. **5**(8): p. 591-602.
4. Stubbs, M., et al., *Causes and consequences of tumour acidity and implications for treatment*. Molecular medicine today, 2000. **6**(1): p. 15-19.
5. Calorini, L., S. Peppicelli, and F. Bianchini, *Extracellular acidity as favouring factor of tumor progression and metastatic dissemination*. Exp Oncol, 2012. **34**(2): p. 79-84.
6. Andreev, O.A., D.M. Engelman, and Y.K. Reshetnyak, *Targeting diseased tissues by pHLIP insertion at low cell surface pH*. Frontiers in physiology, 2014. **5**.
7. Weerakkody, D., et al., *Family of pH (low) insertion peptides for tumor targeting*. Proceedings of the National Academy of Sciences, 2013. **110**(15): p. 5834-5839.
8. Reshetnyak, Y.K., et al., *Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix*. Proceedings of the National Academy of Sciences, 2006. **103**(17): p. 6460-6465.
9. Reshetnyak, Y.K., et al., *A monomeric membrane peptide that lives in three worlds: in solution, attached to, and inserted across lipid bilayers*. Biophysical journal, 2007. **93**(7): p. 2363-2372.
10. Thévenin, D., M. An, and D.M. Engelman, *pHLIP-mediated translocation of membrane-impermeable molecules into cells*. Chemistry & biology, 2009. **16**(7): p. 754-762.
11. Reshetnyak, Y.K., et al., *Energetics of peptide (pHLIP) binding to and folding across a lipid bilayer membrane*. Proceedings of the National Academy of Sciences, 2008. **105**(40): p. 15340-15345.
12. Andreev, O.A., D.M. Engelman, and Y.K. Reshetnyak, *pH-sensitive membrane peptides (pHLIPs) as a novel class of delivery agents*. Molecular membrane biology, 2010. **27**(7): p. 341-352.

13. Du, J.-Z., et al., *Tumor extracellular acidity-activated nanoparticles as drug delivery systems for enhanced cancer therapy*. Biotechnology advances, 2014. **32**(4): p. 789-803.
14. Wijesinghe, D., et al., *pH dependent transfer of nano-pores into membrane of cancer cells to induce apoptosis*. Scientific reports, 2013. **3**.
15. Yao, L., et al., *pHLIP®-mediated delivery of PEGylated liposomes to cancer cells*. Journal of Controlled Release, 2013. **167**(3): p. 228-237.
16. Arachchige, M.C., Y.K. Reshetnyak, and O.A. Andreev, *Advanced targeted nanomedicine*. Journal of biotechnology, 2015. **202**: p. 88-97.
17. Yao, L., et al., *pHLIP peptide targets nanogold particles to tumors*. Proceedings of the National Academy of Sciences, 2013. **110**(2): p. 465-470.
18. HANDJANI-VILA, R., et al., *Dispersions of lamellar phases of non-ionic lipids in cosmetic products*. International journal of cosmetic Science, 1979. **1**(5): p. 303-314.
19. Rajera, R., et al., *Niosomes: a controlled and novel drug delivery system*. Biological and Pharmaceutical Bulletin, 2011. **34**(7): p. 945-953.
20. Moghassemi, S. and A. Hadjizadeh, *Nano-niosomes as nanoscale drug delivery systems: an illustrated review*. Journal of Controlled Release, 2014. **185**: p. 22-36.
21. Marianecchi, C., et al., *Niosomes from 80s to present: the state of the art*. Advances in colloid and interface science, 2014. **205**: p. 187-206.
22. Lohumi, A., *A novel drug delivery system: niosomes review*. Journal of drug delivery and therapeutics, 2012. **2**(5).
23. Karim, K.M., et al., *Niosome: a future of targeted drug delivery systems*. Journal of advanced pharmaceutical technology & research, 2010. **1**(4): p. 374.
24. Cottrell, T. and J. van Peij, *7 Sorbitan esters and polysorbates*. Emulsifiers in food technology, 2004: p. 162.
25. Lo, C.T., et al., *Controlled self-assembly of monodisperse niosomes by microfluidic hydrodynamic focusing*. Langmuir, 2010. **26**(11): p. 8559-8566.
26. Hao, Y., et al., *Studies on a high encapsulation of colchicine by a niosome system*. International journal of pharmaceutics, 2002. **244**(1): p. 73-80.
27. Israelachvili, J., S. Marčelja, and R. Horn, *Physical principles of membrane organization*. Quarterly reviews of biophysics, 1980. **13**(02): p. 121-200.

28. Wegener, J., C.R. Keese, and I. Giaever, *Electric cell-substrate impedance sensing (ECIS) as a noninvasive means to monitor the kinetics of cell spreading to artificial surfaces*. Exp Cell Res, 2000. **259**(1): p. 158-66.
29. Chen, Y.A. and R.H. Scheller, *SNARE-mediated membrane fusion*. Nature reviews Molecular cell biology, 2001. **2**(2): p. 98-106.
30. Eisenberg, R.J., et al., *Herpes virus fusion and entry: a story with many characters*. Viruses, 2012. **4**(5): p. 800-832.

Tables

Construct	Avg. Diameter (nm)	Zeta Potential (mV)	PDI
45% SPAN20+5% DSPE- WT+50% Cholesterol	84.3 ± 0.9	-35.4 ± 2.3	0.19 ± 0.01
45% SPAN20+5% Pyr- WT+50% Cholesterol	78.5 ± 0.5	-31.3 ± 1.7	0.25 ± 0.01

Table 1. Niosome Characterization Data Average hydrodynamic diameter and zeta potential of the SPAN20 niosomes containing 5% of DSPE-WT & Pyr-WT coating at pH 7.4 HEPES buffer.

Figures

Figure 1: Cryo -TEM Cryogenic TEM image of the a.) DSPE-WT and b.) Pyr-WT coated SPAN20 niosomes, The images are taken at HV=200.0 kV and at 25,000x magnification

Figure 2. Cellular Uptake of R18 Niosomes Fig. 2 a-d : 4T1 and A549 cells uptake of DSPE-WT and Pyr-WT coated SPAN20 niosomes at low pH at 40x magnification Fig. 2 e & f: R18 fluorescent intensity per cell at high and low pH for two cell lines for before and after adding trypan blue. The normalization is done by the intensity of 4T1 cells at pH 7.8, treated with DSPE-WT (before adding trypan).

Figure 3: In-Vivo Kinetics Time-dependent distribution of DSPE-WT and Pyr-WT coated R18 SPAN20 niosomes in tumor, muscle, kidney and liver at 4 h , 24 h and 48 h from post-injection. The net, absolute fluorescent intensity of R18 is shown.

Figure 4: In-Vivo Imaging 24 h Fig.4 a: Absolute net mean R18 fluorescent intensity by tumor, muscle, kidney and liver for five constructs after 24 h from I.V. injection. DSPE-6K and Pyr-6K constructs are the controls. * $p \leq 0.05$ and ** $p \leq 0.005$

Fig.4 b: Distribution of different pHLIP niosomes over tumor, muscle, kidney and liver.

Figure 5: Tumor Sections Tumor sections with the R18 contained pHLIP niosomes were analyzed without further processing using Zeiss LSM 700 confocal module under DAPI and Rhodamine channel at 20x magnification.

Figure 6: Delivery of Ca²⁺ into Cancer Cells using Pyr-WT coated niosomes.

4T1 cells grown in standard media of pH 7.4 were treated with the pyr-pHLIP coated niosomes and niosome alone containing 5 mM Ca²⁺ HEPES buffer for 3 hours at pH 7.4 and 6.5. After 2 hours the treatment was completely removed and the replacement of media with RPMI medium containing 10% FBS of the pH 7.4. MTS assay was performed on 2nd day after the treatment.

Figure 7. ECIS Assay. About 25,000 of 4T1 cells were seeded in an 8W10E 1 ECIS array in RPMI at pH 7.4, next day cells were treated for 3 hrs with pyr-pHLIP-coated niosomes containing 5 mM Ca^{2+} HEPES physiological medium. Control was treated with the 5 mM Ca^{2+} HEPES physiological medium. After 3 hours the treatment was completely removed and fresh RPMI medium at 7.4 was added, The kinetics of inhibition of cell proliferation was monitored by changes of capacitance within 2 days.

Figure 8. Delivery of Ca^{2+} into tumors using Pyr-WT coated niosomes. 50 μL and 150 μL , 2.5 mM Pyr-WT niosomes with 5 mM Ca^{2+} were injected to mice directly into the tumor and via tail vein respectively. The injections were performed three consecutive days for both IT and IV groups. Control was not given any injection. The 5th day from the initial injection, mice were euthanized and tumors were harvested and weighed.

Supplementary Information

Construct		4T1		A549	
		DSPE-WT pH 7.8	Pyr-WT pH 7.8	DSPE-WT pH 7.8	Pyr-WT pH 7.8
DSPE-WT	pH 5.5	5.72405E-17	-	4.4094E-118	-
Pyr-WT	pH 5.5	-	3.188E-131	-	2.158E-176

Table 1 SI. p-values for Cellular uptake after adding trypan blue for Fig 2 (e,f)

	DSPE-PEG n=3	DSPE-WT n=3	DSPE-6K n=3	Pyr-WT n=3	Pyr-6K n=3
Tumor	1108 ± 28	2015 ± 96	859 ± 104	2409 ± 334	762 ± 102
Muscle	209 ± 29	363 ± 25	63 ± 11	1173 ± 384	82 ± 17
Kidney	233 ± 22	399 ± 38	115 ± 16	255 ± 22	1145 ± 15
Liver	449 ± 42	1100 ± 141	315 ± 47	893 ± 195	379 ± 134

Table 2 SI. In-Vivo Data for 24 h post injection. R-18 Fluorescence Intensities for different constructs of SPAN20 niosomes for 24 hour post injection time point

a.) Tumor

Construct	DSPE-PEG	DSPE-WT	DSPE-6K	Pyr-WT
DSPE-WT	0.011110833	-	-	-
DSPE-6K	0.004084324	0.005930916	-	-
Pyr-WT	0.085369064	0.440380217	0.054168579	-
Pyr-6K	0.14182076	0.002074218	0.951904631	0.053471726

b.) Muscle

Construct	DSPE-PEG	DSPE-WT	DSPE-6K	Pyr-WT
DSPE-WT	0.031788716	-	-	-
DSPE-6K	0.041197513	0.004241393	-	-
Pyr-WT	0.176471875	0.227173591	0.142288157	-
Pyr-6K	0.062865506	0.002848675	0.3722053	0.148297054

c.) Kidney

Construct	DSPE-PEG	DSPE-WT	DSPE-6K	Pyr-WT
DSPE-WT	0.048451793	-	-	-
DSPE-6K	0.028163836	0.01483796	-	-
Pyr-WT	0.593468874	0.069538949	0.018131454	-
Pyr-6K	0.035691411	0.015410281	0.766032839	0.022063672

d.) Liver

Construct	DSPE-PEG	DSPE-WT	DSPE-6K	Pyr-WT
DSPE-WT	0.05391268	-	-	-
DSPE-6K	0.15888622	0.034671751	-	-
Pyr-WT	0.200977834	0.526664862	0.130616957	-
Pyr-6K	0.950470866	0.070237338	0.625925234	0.221457553

Table 3 SI. p-values corresponds to Table 2

Figure 1 SI. Diameter and zeta potential of the pHLIP-coated nosomes containing, which were kept at 4 °C in HEPES pH 7.4 buffer and HEPES buffer with 5% FBS

Figure 2 SI.Cyto-toxicity Assays. 3000 cells at normal pHs were seeded in 96 well plates. Following day, plates were treated with different concentrations of niosome formulations in serum free media and incubated for 2 hours. For each treatment, after 2 hours the medium was removed and added fresh medium. For another set equal volume of medium with 20% FBS added. When cell confluency reached 80-90% MTS assay was done.

Figure 3 SI. Cellular uptake of R-18 niosomes. 4T1 & A549 cells in suspension were treated with R-18 labelled niosomes at low & high pH. Cellular uptake of fluorescent niosomes was assessed by counting of fluorescent cells on a cellometer.