Title
Nanoparticle formulations to improve gene therapy

Permalink
https://escholarship.org/uc/item/6r18x5hs

Author
Yong, Gen

Publication Date
2014

Peer reviewed|Thesis/dissertation
Nanoparticle formulations to improve gene therapy

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Gen Yong

Committee in charge:

Professor Michael Heller, Chair
Professor Sadik Esener, Co-Chair
Professor Andrew C. Kummel
Professor Robert F. Mattrey
Professor Shyni Varghese

2014
Copyright

Gen Yong, 2014

All rights reserved.
The Dissertation of Gen Yong is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2014
# TABLE OF CONTENTS

Signature Page ........................................................................................................ iii

Table of Contents ....................................................................................................... iv

List of Figures ............................................................................................................. v

Acknowledgements ..................................................................................................... vii

Vita ................................................................................................................................. viii

Abstract of the Dissertation ....................................................................................... ix

Chapter 1. Introduction .............................................................................................. 1

Chapter 2. Ultrasound Triggered Release of Dna Transfection Complexes ......... 9

Chapter 3. Silica Coating Adenoviruses Protects From Neutralizing Antibodies and Expands Viral Tropism ........................................................................... 27

Chapter 4. Inorganic Adenovirus Complexes For Ultrasound-Directed Viral Delivery ............................................................................................................. 54

Chapter 5. Conclusions and Future Directions ..................................................... 74

References .................................................................................................................. 80
LIST OF FIGURES

Chapter 2
Figure 2.1. Ultrasound release mechanism for liposomes .................................. 17
Figure 2.2. Transfection efficiency via ultrasound mediated DNA release ..... 18
Figure 2.3. Transfection efficiency via FU mediated DNA release ................. 20
Figure 2.4. Fluorescent microscopy of EGFP transfected cells ...................... 22
Figure 2.5. FACs analysis of pEGFP-N1 transfected cells ............................. 23

Chapter 3
Figure 3.1. Silica coating of adenoviruses .................................................... 36
Figure 3.2. Particle size distribution ............................................................... 38
Figure 3.3. Serum stability of viral particles .................................................. 39
Figure 3.4. Transduction efficiency of Ad-RFP on PANC-1 cells after coating with silica .......................................................... 41
Figure 3.5. Fluorescent microscopy of PANC-1 cells transduced with Ad-RFP (MOI 50) .......................................................... 43
Figure 3.6. Neutralization assay in the presence of antibodies ....................... 44
Figure 3.7. Transduction of CAR-negative cell lines .................................... 45
Figure 3.8. In vivo neutralization of Ad-Luc .................................................. 46

Chapter 4
Figure 4.1. Silica coating of Ad with nanoemulsions ................................... 61
Figure 4.2. Size and zeta potential of bare Ad, NE and silica coated Ad-NE complexes. ................................................................. 62

Figure 4.3. Proposed release mechanism for SiAd-NE viral particles .......... 64

Figure 4.4. Effects of US exposure on particle size (A) and zeta potential (B) of SiAd-NE........................................................................................................................................... 64

Figure 4.5. Transduction efficiency of SiAd-NE on PANC-1 cells before and after exposure to US ................................................................................................................. 65

Figure 4.6. Fluorescent microscopy of PANC-1 cells transduced with Ad-RFP (MOI 50) ................................................................................................................................................. 67

Figure 4.7. Neutralization assay in the presence of antibodies ..................... 68
ACKNOWLEDGEMENTS

I would like to acknowledge Professor Sadik Esener for his role as my mentor during my PhD studies. His guidance and advice has proved to be invaluable in advancing my research.

Chapter 2 in part is currently being prepared for submission for publication of the material. Benchimol, Michael; Barback, Christopher; Esener, Sadik. The dissertation author was the primary investigator and author of this material.

Chapter 3 in part is currently being prepared for submission for publication of the material. Esener, Sadik. The dissertation author was the primary investigator and author of this material.

Chapter 4 in part is currently being prepared for submission for publication of the material. Benchimol, Michael; Barback, Christopher; Esener, Sadik. The dissertation author was the primary investigator and author of this material.
VITA

2007 Bachelor of Science, Duke
2007-2008 Research Assistant, Institute of Bioengineering and Nanotechnology
2008-2009 Teaching Assistant, Department of Bioengineering, University of California, San Diego
2009-2013 Research Assistant, University of California, San Diego
2014 Doctor of Philosophy, University of California, San Diego

PUBLICATIONS

“Efficient Catalytic System for the Selective Production of 5-Hydroxymethylfurfural from Glucose and Fructose”, Angewandte Chemie 120;9485-9488

FIELDS OF STUDY

Major Field: Bioengineering (Nanoparticle Drug Delivery)

Studies in Nanoparticle Drug Delivery
Professor Sadik Esener
ABSTRACT OF THE DISSERTATION

Nanoparticle formulations to improve gene therapy

by

Gen Yong

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2014

Professor Michael Heller, Chair
Professor Sadik Esener, Co-Chair

In vivo systemic gene therapy approaches remain largely limited by short circulation time of genetic vectors and poor target specificity with techniques made to address one concern usually leading to a tradeoff for the other. This dissertation explores the use of nanoparticle delivery vehicles to
overcome the above two constraints for both non-viral and viral vectors for gene therapy. In chapter two, we demonstrate an ultrasound sensitized liposomal gene delivery vehicle that can be activated using clinically relevant focused ultrasound. We were able to trigger a 7 fold increase in gene transfection as measured via a luciferase reporter system by exposing the liposomes to focused ultrasound. In chapter three, we explored a novel coating method for viral vectors that retains viral activity while shielding the coated viruses from immune neutralization. Using this technique we were able to achieve viral transfection in passively immunized mice with our coated viruses in contrast to bare viruses. In chapter four, we demonstrated the ability to induce ultrasound triggered release of viral particles by introducing an ultrasound sensitizing agent in the form of a perfluorocarbon nanoemulsion into our coated viral nanoparticles. These ultrasound sensitized viral nanoparticles remain in an inactive state till triggered by ultrasound upon which they regain infectious capability. Released viral particles retain protection against neutralizing antibodies and can be precisely targeted using ultrasound.
CHAPTER 1. INTRODUCTION
Gene therapy was first conceptualized some 50 years ago as a means to treat diseases of aberrant genetics. Despite the initial wellspring of exuberance, gene therapy has largely failed to live up to its initial vision. In the 50 years since, promises of cancer cures and eradication of genetic based diseases have come and gone with limited concomitant progress in the clinic. The promise of gene therapy has been difficult to realize in part due to our limited understanding of the underlying biology, safety and efficacy of the vectors used and limitations in the delivery systems that result in off target effects and limited vector uptake.

Gene therapy can be broadly classified into 2 categories, non-viral and viral vectors. Non-viral vectors are cheap and easy to mass produce and have excellent safety profiles with low immunogenicity. Non-viral vectors however suffer from in-efficient gene transfer, transient transgene expression, rapid systemic clearance and off target effects/toxicities. Recent developments in vector design involving minicircle DNA technology have yielded marked improvements in vector performance giving greatly improved gene transfer and sustained transgene expression. However, despite multiple attempts to address the issues of delivery and clearance of non-viral vectors, these remain two major limitations to be overcome for any systemic approaches using non-viral vectors to be successful. Cationic lipoplexes and polyplexes that transfecct well in vitro are rapidly cleared in vivo and are associated with significant cytotoxic profiles. Other systemic approaches including liposomal encapsulation show marked improvements in circulation times without
addressing issues of target selectivity\textsuperscript{10–12}. Nanoparticles, through the use of specific ligands, may have the potential to effectively treat cell types which present unique surface receptors\textsuperscript{13,14}. However, in the absence of such uniqueness, nonspecific delivery remains an issue. The twin issues of circulation time and target specificity is a balancing act whereby we often have to sacrifice one aspect to improve the other.

In chapter 1, we present our unique take on a delivery vehicle for non-viral vectors that we believe espouses the best of both worlds; long circulation time with excellent target specificity. In our platform, therapeutic DNA complexes are co-encapsulated with an external triggering mechanism into a liposomal construct. The external triggering mechanism consists of a perfluorocarbon nanoemulsion that sensitizes the encapsulating liposome to ultrasonic rupture by lowering the cavitation threshold. Encapsulation of DNA into liposomes protects the DNA from degradation and clearance issues that plague current lipoplex approaches, while incorporation of an externally activated triggering mechanism allows us to only release DNA complexes in regions of interest while maintaining the bulk of the DNA in an inactive encapsulated state for the rest of the body.

Viral vectors while difficult to generate and test and expensive to produce, benefit from a long evolutionary program that enables them efficient access to cells, sustained high levels of transgene expression and the ability to propagate themselves \textit{in vivo}. Much of the early controversy regarding viral
vectors revolved around their safety profile and the idea of therapeutic viruses gone rogue, with the unfortunate death of a patient, Jesse Gelsinger, in a 1999 OTC trial conducted at U. Pennsylvania serving to drive home the very real danger that can entail when using viral vectors without sufficient understanding of their biology or the safety parameters of which\textsuperscript{15}. Much of the focus of recent work has been in the development of new generations of vectors that have improved specificity for diseased conditions while sparing normal tissue. While most of the work in the field has been focused on development of conditionally replicative vectors which exploit characteristic deficiencies in disease biology to achieve conditional replication\textsuperscript{16}, recent efforts have also explored the use of non-human pathogens, mainly as oncolytic agents, with limited tropism for healthy tissue but having natural affinity for tumor cells\textsuperscript{17}. Additionally, 10 years of clinical trials since have informed on safe margins of viral vectors for dosing in humans. Despite the many advances seen, immune response to viral therapy and issues of host cell tropism remain two major stumbling blocks to the widespread adoption of oncolytic viral therapy. The issue of immune activation following viral therapy has been widely documented. It has been shown in animal models that prior exposure to a given virus limits therapeutic outcome in mice\textsuperscript{18,19}. Clinical trials of viral therapy in humans have noted the induction of high titers of neutralizing antibodies and elevated cytokine levels, which would limit the possibilities for their use in vivo. Viral vectors currently in clinical trials are derivative of common human pathogens that we are exposed to on a regular
basis. Prior exposure to these viruses complicates interpretation of the clinical outcome and negates the possibility of repeat therapy to clear residual disease. A secondary concern arising from immune activation is the short persistence time in blood and bioavailability of therapeutic viruses. The speed at which viruses are cleared from the immune system precludes general systemic delivery of viral therapy. Most clinical trials currently underway are focused on intratumoral, intralesional or intra-arterial delivery of oncolytic viruses for regional therapy\textsuperscript{20,21}. The inability to achieve systemic delivery precludes the ability to properly treat disseminated disease which is the primary stage upon presentation in the clinic. Disseminated disease is implicated in adverse outcomes to conventional therapy and poor response and survival rates. The limitations on repeat dosing and lack of a true systemic therapy option from immune activation is the first and paramount hurdle that needs to be crossed for greater clinical uptake of oncolytic viral therapy.

In chapter 2, we will introduce a novel coating material/technique for viruses developed to enable coated viruses to evade immune detection and response. As a test platform, human adenoviruses serotype 5 (Ad5) were coated with silica gel through a template driven process. Silane chemistry has been shown to be compatible with physiological conditions. We have been able to manipulate silane chemistry to achieve coating of viruses into silica nanoparticles without any consequent loss of biological activity of the viruses while retaining the possibility of further downstream functionalization of the virus-silica nanoparticle through silane chemistry. By coating the virus in silica,
we are essentially changing the interaction surface the virus presents to the host preventing the host immune system from generating an effective immune response to the virus.

Despite the newer generation of viral vectors with improved safety tolerances towards healthy tissues, off target toxicities remain one of the major drivers of adverse events from virotherapy in the clinic\textsuperscript{22}. Efforts to improve disease specificity have largely focused on passive accumulation in diseased tissues\textsuperscript{11,23} or intrinsic active targeting strategies\textsuperscript{24–26}. However these techniques do little to address issues like liver/splenic accumulation\textsuperscript{21}, receptor ligand promiscuity and ubiquity of disease markers\textsuperscript{13,14,27}. For a systemic delivery approach to be effective, it must allow the virus to circulate for ample time to evade the immune system on its indirect path to the target. However, immune evasion should not negatively impact the ability to infect the cell target. Therefore, a triggering mechanism is necessary to convert the virus from a long circulating inactive state to an active infective state, whether it be physical, chemical, or biochemical (receptor-mediated). Activatable drug complexes that rely on external stimuli for drug release make for attractive alternatives to traditional targeting modalities\textsuperscript{28}. The ability to physically direct virus exposure to specific tissues is a key differentiator from biochemical targeting approaches, which depend on the presence of overexpressed surface receptors. Although physically-directed methods require knowledge of the location of the target, they do not require knowledge of the target phenotype. This is especially important in cancer, where there is great heterogeneity
between patient tumors, between different tumors in the same patient, and even within a single tumor\textsuperscript{29,30} Even if a patient is assessed to present a targetable receptor, it may be only present on a fraction of the patient’s tumor.

Ultrasound is a safe energy which can treat specific volumes precisely and noninvasively. Many approaches are being developed to locally enhance virus delivery with focused ultrasound. Gas-filled microbubbles are often used to sensitize tissues, and porate nearby cells\textsuperscript{31,32}. Unfortunately, few of these approaches have successfully hidden the viruses in a stealth layer. Those that have encapsulated the viruses have often used processes that impair the bioactivity of the virus or do not account for the short circulating half-lives of microbubbles and nanoemulsions.

Chapter 3 further builds upon the concepts introduced in chapter 2, and showcase an application developed using our platform that can have potential utility in the clinic. For this application, an externally activated ultrasound trigger was added to the virus-silica nanoparticle. The unique nature of silane chemistry and our manufacturing process allows us to introduce a variety of functional moieties to our virus-silica construct. Incorporation of nanoemulsions into the nanoparticle formulation has the advantages of improved stability of and spatial co-localization of nanoemulsions with viral particles. Nanoemulsions have been widely studied as ultrasound therapeutic and/or diagnostic agents, allowing for us to explore the possibility of developing an ultrasound triggered viral release mechanism. Under the action
of ultrasound, NE encapsulated within a carrier particle may nucleate a rapid liquid-to-vapor transition known as acoustic cavitation. This highly energetic and localized process results in the disruption of the NE’s encapsulating matrix (silica) and consequent exposure of virus. Ultrasound has the advantages of using a non-ionizing energy source with deep tissue penetration and narrow focus, allowing for pinpoint activation and release of our viral particles from an inactive silica encapsulated state to an active burst phase using an ultrasonic trigger.
CHAPTER 2. ULTRASOUND TRIGGERED RELEASE OF DNA TRANSFECTION COMPLEXES
Abstract

In vivo systemic gene delivery approaches remain largely limited by short circulation time of transfection complexes and poor target specificity with techniques made to address one concern usually leading to a tradeoff for the other. In our demonstration, we combined the known benefits of long circulation time with liposomes with an externally triggered transfection mechanism for target specificity. By encapsulating per-fluorocarbon nanoemulsions (NE) and transfection complexes carrying luciferase reporter plasmids in liposomes, we have demonstrated the ability to activate transfection events using an external ultrasound trigger. Liposomes carrying transfection complexes exposed to ultrasound show 8 fold greater luciferase activity over non-ultrasound exposed liposomes. When using focused ultrasound (2.3MPa) as a triggering mechanism, liposomes bearing NE show an 8 fold increase over background. Liposomes without NE showed no change in luciferase activity upon exposure to focused ultrasound (2.3MPa). We conclude that we have shown selective ultrasound activation of transfection events using NE as an ultrasound triggered transfection mechanism.
2.1 Introduction

Gene therapy was first conceptualized some 50 years ago. In the 50 years since, promises of cancer cures and eradication of genetic based diseases have come and gone with limited concomitant progress in the clinic. The promise of gene therapy has been difficult to realize in part due to the limitations of the delivery methods. Viral vectors can leverage the mechanisms that viruses have evolved to introduce their own genetic material for expression in the host cell, but they carry a number of limitations including cellular tropism that limits their use for some cell types, integration concerns, and immune responses to the virus. The latter severely limits the opportunity to use repeated administrations of a particular therapy largely limiting the efficacy of a given therapeutic regime. Viral vectors can also be difficult to generate and test and expensive to produce. Non-viral methods that shed these limitations are attractive, but to date have succeeded mostly in vivo in superficial targets. Two major limitations for any systemic approaches are the circulation time of the transfection complexes and target specificity. Cationic lipoplexes\textsuperscript{6,7} and polyplexes\textsuperscript{8,9} that transfect well in vitro are rapidly cleared in vivo and are associated with significant cytotoxic profiles\textsuperscript{1–4}. Other systemic approaches including liposomal encapsulation show marked improvements in circulation times without addressing issues of target selectivity\textsuperscript{10–12}. Nanoparticles, through the use of specific ligands, may have the potential to effectively treat cell types which present unique surface receptors\textsuperscript{13,14}. 
However, in the absence of such uniqueness, nonspecific delivery remains an issue.

Localized ultrasonic modulation of transfection efficiency has been explored as a triggering mechanism and a means to improve specificity. Pre-exposure of an area of interest to focused ultrasound has been shown to boost subsequent transfection efficiency in the exposed area with minimal resultant tissue damage. However, results are largely superficial and there remains significant room for improvement of transfection efficiencies. Microbubbles have also been used in conjunction with ultrasound to increase transfection efficiency of free floating DNA within the exposed area through sonoporation effects. Free floating DNA is also subject to rapid degradation and clearance preventing true systemic application of therapeutics. Microbubbles are limited by their short circulation times on the order of minutes. Longer circulating perfluorocarbon (PFC) nanoemulsions have been used as an ultrasound responsive carrier.

To address these most vital issues of gene delivery, we chose to encapsulate non-viral vectors in a recently developed ultrasound-ruptured liposome. Use of focused ultrasound to improve transfection efficiency was particularly attractive for its ability to spatially modulate transfection events. The aim of this study then is to package DNA into a coherent framework which provides protection and control over biodistribution to achieve tissue-selective transfection. Perfluorononane nanoemulsions and BP-fectin-plasmid
complexes coding for eGFP or firefly luciferase were encapsulated into PEGylated liposomes then subsequently released via exposure to focused ultrasound or probe sonication. Transfection efficiency was evaluated by luminescence readings and flow cytometry.

2.2. Materials and methods

Plasmids were obtained from the following sources; pEGFP-N1 was obtained from Clontech (USA), pGL3-CMV was obtained from Promega (USA), pCMV-RedFluc was obtained from Targeting Systems (USA). Zonyl FSE and perfluorononane were purchased from Sigma Aldrich (USA) and used without further purification. DSPE-PEG-5k was obtained from Laysan Bio Inc. (USA). Egg-PC and cholesterol were obtained from Avanti Polar Lipids (USA).

2.2.1 Preparation of nanoemulsions and liposomes

1050µl of PBS was heated to 95°C. 120µl of Zonyl FSE (14% w/v) was added to the heated PBS and briefly vortexed to ensure even mixing. 30µl of 99% PFN was next added to the bottom of the mixture which was then immediately placed into an ice bath, giving a final concentration of 1% Zonyl FSE and 2.475% PFN v/v in PBS. Mixture was then sonicated on ice using a Misonix XL-2000 (Farmingdale, USA) probe type sonicator operated at level 20 for 30 minutes until an evenly distributed PFN nanoemulsion (NE) was obtained. The NE was then dialyzed against DPBS using the Fast
SpinDialyzer (Harvard Apparatus, USA) for 12 hours, which allowed the use of large pore membranes for the efficient removal of free surfactants. Nanoparticle size and measurements were determined using a Zetasizer Nano ZS90 (Malvern).

Liposomes were prepared by a modified version of the reverse-phase evaporation technique developed by Papahadjopoulos et al. 6 mg DSPE-PEG-5K (Laysan Bio, Inc., Arab, AL) dissolved in chloroform was added to a 1.5 mL microcentrifuge tube. The chloroform was evaporated by applying a gentle argon stream while vortexing the open vial, leaving a lipid film on the walls. To this tube, 800 µL PBS (GIBCO, Bethesda, MD) was added, and the tube was vortexed for 1 minute. To break up the lipids into small micelles, a Misonix XL-2000 (Farmingdale, NY) probe type sonicator was operated at level 10 in the liquid, near the bottom of the tube for 30 0.5 sec pulses. This solution was set aside. 8.11 mg Egg-PC and 3.22 mg Cholesterol (Avanti Polar Lipids, Inc., Alabaster, AL) dissolved in chloroform were combined in a 4 mL glass flat-bottom vial. Chloroform was added to make up 1 mL and the vial was vortexed gently. 300 µL of an aqueous solution containing the material to be entrapped was added to the bottom of the vial. The aqueous solutions used were always adjusted to a pH 7.4 and isotonic with PBS to prevent osmotic shock. A fisherbrand homogenizer tip was lowered to just above the bottom of the vial, and was operated on high speed for 1 minute. This created a stable water-in-chloroform emulsion. Chloroform was removed with a weak stream of argon. After 20 minutes, the solution began to gel as the chloroform was almost
completely removed. To the emulsion gel, the PBS solution containing DSPE-PEG-5K was added, and the vial was vortexed on the lowest speed for 1 minute. The vial was then placed under an argon stream for 30 minutes to remove most of the remaining ether and agitate the solution. This separated the closely. 6 mg DSPE-PEG-5K (Laysan Bio, Inc., packed emulsions and completed the liposome formation. The resulting liposomes were about 0.4 µm in size. The liposomes were then dialyzed against DPBS using the Fast SpinDialyzer (Harvard Apparatus, South Natick, MA) for 12 hours, which allowed the use of large pore membranes for the efficient removal of the free nanoparticles.

2.2.2 Cell culture

In vitro assays were performed on a human pancreatic line (Panc-1). The cell line Panc-1 was maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin/Glutamine (PSG) (MEM-complete). Cells were incubated at 37°C at 5% CO₂ and passaged every 3-4 days.

Analysis using dual luciferase reporter

For transfections using luciferase reporters, Panc-1 cells were seeded on a 96 well plate with 8x10³ cells/well and left to attach overnight. 0.1-0.4ug of DNA complexed to BP-fectin (dose matched in the liposome samples) was added to each well and left to incubate for 4h at 37°C in MEM-complete. 1:50 dilution of renilla luciferase was also added to each well as a secondary
reporter. Cells were washed with 150ul PBS with 100ul MEM-complete added to each well and left to incubate another 44 hours. Dual-Luciferase® Reporter Assay System from Promega was used to analyze luciferase activity after 48 hours post transfection with the results read out on a TECAN plate reader.

2.2.3 Analysis using flow cytometry

For transfections with eGFP reporters, Panc-1 cells were seeded on a 6 well plate with \(2 \times 10^5\) cells/well and left to attach overnight. 2ug of DNA complexed to BP-fectin (dose matched in the liposome samples) was added to each well and left to incubate for 4h at 37\(^\circ\)c in MEM-complete. Cells were washed with 2ml PBS with 2ml MEM-complete added to each well and left to incubate another 44 hours. Cells were washed with PBS and harvested with trypsin at 48 hours post transfection. Suspended cells were centrifuged for 10 minutes at 1000 rpm then washed with PBS and fixed in 4% paraformaldehyde in PBS. Cells were analyzed using flow cytometry to determine the GFP expressing population. Ten thousand cells were analyzed for each sample.
2.3. Results

2.3.1 Ultrasound release mechanism

Perfluorocarbon nanoemulsions are known to reduce the cavitation threshold of the surrounding media. While the phenomenon is postulated to proceed through a droplet-to-bubble transition, much of the actual mechanism remains an unknown. In the current setup, a perfluorononane (PFN) nanoemulsion (NE) is co-encapsulated with DNA transfection complexes within a liposomal construct. Within the ultrasound focal zone, the PFN NE may nucleate a rapid liquid-to-vapor transition known as acoustic cavitation. This highly energetic and localized process results in the disruption of the NE’s encapsulating liposome and consequent release of DNA transfection complexes into the surrounding media (Figure 2.1). The resolution and precise control over the spatial orientation of the ultrasound focal zone prevents unwanted release of DNA transfection complexes in non-target areas.

Figure 2.1. Liposome encapsulating ultrasound sensitizing nanoparticle and plasmid complex renders liposome sensitive to ultrasound mediated rupture of the liposome to release plasmid complexes at site of ultrasound focus, triggering release of plasmid complexes for cellular uptake. A) Resolution and precise spatial orientation of focal zone prevents unwanted release of plasmid complexes in non-ultrasound exposed regions. B)
2.3.2 Liposome characterization.

Mean liposome diameter was 400 ± 75 nm. Using spectrophotometric techniques, liposomal loading efficiency was determined to be 30% with the remaining DNA recovered from the aqueous fraction.

2.3.3 In vitro transfection

![Figure 2.2](image-url)  
*Figure 2.2. Transfection efficiency via ultrasound mediated DNA release. Liposomes exposed to probe sonication showed 6 fold increased luminescence over background. Transfection efficiency of liposomal DNA-complexes was observed to be 20% of Bare DNA complexes.*

Transfection complexes of BP-fectin/DNA and nanoemulsions were packaged into liposomes and were alternatively exposed to probe sonication, FU or left untreated then plated onto PANC-1 cells. Transfection efficiency was determined via a dual luciferase reporter system (pGL3/pRL-CMV) and normalized to bare BP-fectin/DNA. Adding untreated liposomes to PANC-1 cells resulted in a negligible luminescence signal following a 48 hour incubation period. This suggests that liposomal encapsulation is successful in preventing the release of BP-fectin/DNA complexes in the absence of external
stimuli. The minimal luminescence signal observed could be attributed to incomplete cleanup of unencapsulated BP-fectin/DNA complexes and is close enough to baseline levels to be negligible. Exposure of the same liposomes to probe sonication (5 short pulses at low intensities) resulted in an 8x increase in luminescence over untreated liposome samples indicating the release of DNA upon ultrasound exposure. Increasing the number of number of pulses or intensities of sonication did not yield a further increase in luminescence levels suggesting complete liposomal release of DNA complexes. Subjecting bare BP-fectin/DNA complexes to a similar sonication protocol resulted in no noticeable change in luminosity indicating that the DNA complexes are robust and can withstand exposure to probe sonication. Untreated and pRL controls gave no background luminescence (Figure 2.2). Here we establish the ability to conditionally trigger the release of BP-fectin/DNA complexes using low intensities of probe sonication.

2.3.4 FU exposure

In order to duplicate the same effect in vivo, there is a need to move to a more clinically relevant model for ultrasound based activation. BP-fectin/DNA with and without nanoemulsions were encapsulated within liposomes and subsequently exposed to focused ultrasound (FU) at 2.3MPa or probe sonication. Liposomes were dialyzed overnight prior to use to remove any unencapsulated material. Nanoemulsions are used as an ultrasound
sensitizing material to reduce the cavitation threshold when exposed to ultrasound.

Figure 2.3. Transfection efficiency via FU mediated DNA release. DNA complexes and nanoemulsions were encapsulated into liposomes and alternatively subjected to probe sonication, exposure to FU (2.3 MPa) or left as-is prior to being plated onto the cells. Setup was repeated with liposomes encapsulating only DNA complexes without nanoemulsions to determine the effect of nanoemulsions on liposomal release via FU. Liposomal release of DNA complexes via FU was only observed when co-encapsulated with nanoemulsions (4x higher luminescence). Probe sonication proved sufficient to release DNA complexes from liposomal encapsulation though some form of ultrasound ablation of DNA complexes was observed with probe sonication in the presence of nanoemulsions.

Transfection efficiency was determined via a dual luciferase reporter system (pGL3/pRL-CMV) and normalized to bare BP-fectin/DNA. Adding untreated liposomes to PANC-1 cells resulted in a low background level of luminescence. For liposomes encapsulating DNA complexes and nanoemulsions, exposure to probe sonication or FU resulted in an increase of
luminescence over the background of 3x and 6.5x respectively (Figure 2.3). Probe sonication in the presence of nanoemulsions could have resulted in more violent cavitation events that would have damaged the DNA complexes resulting in decreased transfection efficiency for the probe sonicated sample over the FU exposed sample. Subjecting liposomes carrying only DNA complexes to the same ultrasound exposure of probe sonication and FU only yielded an increase in luminescence for the probe sonicated sample indicating that liposomal release of DNA complexes via exposure to FU is contingent on the presence of nanoemulsions. This is due to ultrasound intensities applied during FU being insufficient to cause cavitation on its own, giving no consequent liposomal release of DNA complexes. Here we show that liposomal release of DNA complexes via FU is contingent upon the presence of nanoemulsions to potentiate liposomal release to FU exposure by lowering the threshold ultrasound intensity required for cavitation events.

2.3.5 Fluorescent microscopy and FACS analysis

To determine if exposure of the DNA complexes to ultrasound resulted in a fundamental change in the way DNA complexes transfect cells, we packaged an EGFP reporter into liposomes and alternatively left them as-is or exposed to probe sonication. Samples were then plated onto PANC-1 cells and left to incubate for 48 hours. Cells were analyzed using fluorescent microscopy and FACs analysis. Population profiles of transfected cells using either the bare DNA complexes or from liposomal release via ultrasound
showed similar population distribution profiles under fluorescent microscopy indicating that the exposure of DNA complexes to ultrasound for liposomal release was not fundamentally changing the behavior of transfection complexes post liposomal release (Figure 2.4).

Figure 2.4. Fluorescent microscopy of EGFP transfected cells. Bare and ultrasound exposed liposome DNA complexes showed EGFP expression under fluorescent microscopy. EGFP expression was well distributed in both setups. Untreated and non-ultrasound exposed liposomal DNA complexes gave little to no fluorescent signal.

FACs analysis showed the same distribution data as that obtained by fluorescent microscopy with similar population distribution profiles of fluorescent cells seen under FACs (Figure 2.5). Transfection efficiency of liposomal released DNA complexes was 10 fold lower than bare DNA
complexes. This can be accounted for by factoring in the loading efficiency of DNA complexes into liposomes.

2.4. Discussion

Two major limitations for any systemic approaches to gene therapy are the circulation time and target specificity of the therapeutic vector. However, despite multiple attempts to address the issues of delivery and clearance of
non-viral vectors, these remain two major limitations to be overcome for any systemic approaches using non-viral vectors to be successful. In this study, we formulated a novel liposomal construct with DNA transfection complexes and PFN NE both co-encapsulated within the liposome. PFN NE have the ability to mediate inertial cavitation events when exposed to ultrasound through a rapid droplet to gas transition. Incorporation of NE into the liposomal formulation would then allow for conditional release of DNA transfection complexes, subject to an external ultrasound stimuli. Using current fabrication techniques, liposomes averaging 400nm in diameter were obtained for the above mentioned formulation. While a 400nm diameter is suboptimal for purposes of extended circulation time, we were still able to evaluate for NE-liposomes for ultrasound triggered release of DNA transfection complexes. Using focused ultrasound (2.3MPa) at 2.25MHz, we were able to trigger release of DNA transfection complexes from the encapsulating liposome as quantified by a dual luciferase reporter system. The ability to trigger release of DNA transfection complexes at 2.25MHz and 2.3MPa is of particular significance as these are parameters that can be easily achieved with current ultrasound diagnostic equipment readily available within the clinical setting. Exposure of liposomes in the absence of NE to the same intensity and frequency of focused ultrasound gives no changes in luciferase activity indicating that the release of DNA transfection complexes is specific to NE.

One concern of exposing DNA transfection complexes to an energetically disruptive event such as cavitation, would be potential damage
and changes to the DNA payload. FACS analysis of cells transfected with US released DNA showed population dynamics similar to that of bare DNA transfection complexes as quantified with a GFP reporter. However we note a 10 fold decrease in transfection efficacy which can be attributed to the loading efficiency of DNA transfection complexes into liposomes and marks an area of improvement for further development of this technology. A move to an alternative transfection agent or smaller payload in the form of siRNA or miRNA might benefit loading efficiency by reducing the size of the payload to be encapsulated in liposomes.

2.5. Conclusions

We have previously shown that high boiling point PFC nanoemulsions nucleate acoustic cavitation and can replace some of the functionalities of microbubbles. Use of nanoemulsions is attractive for its greater stability compared to microbubbles. In this demonstration, we have encapsulated both nanoemulsions and DNA complexes into a single liposomal construct allowing for a more controlled delivery of DNA therapeutics with a single distribution and clearance characteristic. In summary we show the ability to selectively activate transfection events via an ultrasound trigger by packaging DNA complexes with NE into the liposome. Loading efficiency with and without the NE is observed to be 30% which is in line with values cited in the literature. Additionally, we have shown that we are able to use clinically relevant FU as the source of the ultrasound trigger. This opens up the viability of the
liposome-DNA construct to be used in the clinic. Exposure to ultrasound is also not noted to affect the nature of transfection from the ultrasound mediated released DNA complexes.

Chapter 2 in part is currently being prepared for submission for publication of the material. Benchimol, Michael; Barback, Christopher; Esener, Sadik. The dissertation author was the primary investigator and author of this material.
CHAPTER 3. SILICA COATING ADENOVIRUSES PROTECTS FROM NEUTRALIZING ANTIBODIES AND EXPANDS VIRAL TROPISM
Abstract

Although use of viruses has been gaining traction for cancer therapy in the forms of gene delivery or oncolytic viral therapy, immune responses to the viruses that limit repeated administration and therapeutic efficacy has been the main stumbling block to clinical development of therapeutic viral vectors. To address this issue, we have developed a novel silica nanoparticle encapsulation method that solves the problem of the host immune response by concealing the virus while retaining bioactivity of the virus. In this work, silica adenoviral nanoparticles were synthesized and shown to have improved serum stability and are resistant to the effects of neutralizing antibodies in both in vitro and in vivo models. Additionally coated adenoviruses were tested to have a broader spectrum of activity over bare adenoviruses and were able to establish efficient transduction in a number of CAR-negative cell lines that were otherwise resistant to transduction by bare adenoviral vectors.
3.1. Introduction

Non-viral vectors while safe and easy to produce, are often lacking in therapeutic efficacy in their current form. In the pursuit of clinical efficacy, viral vectors with the ability to self-amplify have a distinct advantage over non-viral methods of gene therapy. Viral vectors have been widely studied as promising gene carriers for the subject of gene therapy over comparable non-viral vectors. While non-viral vectors are easy to mass produce and generally have low immunogenicity, viral vectors benefit from a long evolutionary program that grants them efficient access to cells, sustained high levels of transgene expression and the ability to propagate themselves in vivo. While most of the work in the field has been focused on development of conditionally replicative vectors which exploit characteristic deficiencies in disease biology to achieve conditional replication\textsuperscript{16}, recent efforts have also explored the use of non-human pathogens, mainly as oncolytic agents, with limited tropism for healthy tissue but having high affinity for tumor cells\textsuperscript{17}.

Oncolytic viruses can selectively replicate in and kill tumor cells. Over the last two decades significant progress has been made in the preclinical and clinical development of viral based therapy as a platform for the treatment of cancer. Proof of concept studies with Onyx-015 demonstrated the safety of this approach and significant responses were noted among patients treated by intrallesional injections and regional vascular delivery\textsuperscript{3,33}. However, induction of high titers of neutralizing antibodies as well as high levels of antiviral cytokines
was demonstrated among the cancer patients treated with Onyx-015, limiting the efficacy of this approach by systemic delivery. Despite these limitations, intratumoral and regional approaches are being pursued in large, multinational studies with growing evidence of success in melanoma and hepatocellular carcinoma\textsuperscript{20,34}. While much progress has been made in understanding viral lifecycles and biology, their delivery and clearance characteristics remain a major stumbling block for effective therapy\textsuperscript{35–37}. Despite the many advances seen, immune response to viral therapy and issues of host cell tropism remain two major stumbling blocks to the widespread adoption of oncolytic viral therapy.

The issue of immune activation following viral therapy has been widely documented. It has been shown in animal models that prior exposure to a given virus limits therapeutic outcome in mice\textsuperscript{18,19}. Clinical trials of oncolytic viral therapy in humans have noted the induction of high titers of neutralizing antibodies and elevated cytokine levels, which would limit the possibilities for their use in vivo\textsuperscript{38}. Oncolytic viruses currently in clinical trials are derivative of common human pathogens that patients are exposed to on a regular basis\textsuperscript{39}. Prior exposure to these viruses complicates interpretation of the clinical outcome and negates the possibility of repeat therapy to clear residual disease. A secondary concern arising from immune activation is the short persistence time in blood and bioavailability of oncolytic viruses\textsuperscript{21,40,41}. The speed at which oncolytic viruses are cleared from the immune system precludes general systemic delivery of viral oncolytics. Most clinical trials currently underway are
focused on intratumoral, intralesional or intra-arterial delivery of oncolytic viruses for regional therapy\textsuperscript{20,21}. The inability to achieve systemic delivery precludes the ability to properly treat disseminated disease which is the primary stage upon presentation in the clinic. Disseminated disease is implicated in adverse outcomes to conventional therapy and poor response and survival rates. The limitations on repeat dosing and lack of a true systemic therapy option from immune activation is the first and paramount hurdle that needs to be crossed for greater clinical uptake of oncolytic viral therapy.

The issue of host cell tropism is of great concern because it implicates three areas of therapy; the therapeutic targets that can be treated\textsuperscript{42}, variable receptor expression between patients\textsuperscript{43}, and off target toxicities\textsuperscript{44,45}. Just as flu virus that normally infects lung tissues would have difficulty gaining traction in the prostate or pancreas, individuals within a given population have different susceptibilities to different viral systems. Receptors that viruses use to gain entry into a cell have highly variable expression levels in cancer cells, which can lead to off target toxicities in tissues with high expression levels of these receptors (splenic and liver toxicities are often implicated). Re-targeting oncolytic viruses to cell markers that are over expressed in cancer cells would expand the efficacy of the oncolytic viral platform to patients with disseminated disease.

Current approaches to get around the immune response involve viruses being coated with polymers such as polyethylene glycol which shields it from
antibodies. However immune evasion achieved using current encapsulation methods are frequently accompanied by consequent loss of viral activity. In this paper, we will introduce a novel coating material/technique for viruses developed to enable coated viruses to evade immune detection and response. As a test platform, human adenoviruses serotype 5 (Ad5) were coated with silica gel through a template driven process. Silane chemistry has been shown to be compatible with physiological conditions. We have been able to manipulate silane chemistry to achieve coating of viruses into silica nanoparticles without any consequent loss of biological activity of the viruses while retaining the possibility of further downstream functionalization of the virus-silica nanoparticle through silane chemistry. By coating the virus in silica, we are essentially changing the interaction surface the virus presents to the host preventing the host immune system from generating an effective immune response to the virus.

3.2. Materials and methods

Adenoviral stocks, Ad5-RFP, Ad5-CMV-Luc were purchased from Vector Biolabs (USA). Goat Ad5-anti-hexon polyclonal was obtained from Thermo Scientific (USA). Tetremethoxy orthosilicate, poly-L-lysine and HCl were obtained from Sigma Aldrich (USA).
3.2.1 Preparation of viral silica nanoparticles and characterization

Adenoviruses (Ad) were obtained from Vector Biolabs and used without purification. Briefly, $10^8$ pfu of Ad was mixed vigorously with varying concentrations of 0.1% poly-L-lysine solution (PLL, Sigma Aldrich, USA) and left to stand for 5 minutes at room temperature (solution A). Separately, a 13% tetramethoxy orthosilicate v/v (TMOS, Sigma Aldrich, USA) in 1mM HCl (Fischer, USA) solution was prepared (solution B). Solution B is then added drop wise to solution A to a final concentration of 2.5% v/v under vigorous mixing (solution C), then allowed to stand under vigorous mixing at room temperature for a further 1 hour. Solution C containing the silica coated adenoviruses (SiAd) is then spin purified and resuspended in PBS for immediate use or frozen in aliquots at -80°C. Nanoparticle size and measurements were determined using a Zetasizer Nano ZS90 (Melvern).

3.2.2 Serum Stability

Stability of viral particles in serum was assayed according to previous published methods. Viral particles were resuspended in 80% donor human serum preheated to 37°C. Absorbance measurements at 560nm of the viral particles were taken every 10 minutes on a plate reader (TECAN) for 3 hours at 37°C. Particles were agitated briefly prior to absorbance measurements to prevent viral particles from settling.
3.2.3 Cell culture and transfection protocol

In vitro assays were performed on a human pancreatic line (Panc-1), a human ovarian line (SKOV-3) and Chinese hamster ovarian line (CHO-K1). The cell lines were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin/Glutamine (PSG) (MEM-complete). Cells were incubated at 37°C at 5% CO₂ and passaged every 3-4 days when 70% confluent.

Viral transfections were carried out in a 96 well format. 10⁴ cells were split into each well and allowed to attach overnight prior to transfection. Cells were infected with either equivalent tiers of Ad or SiAd for 48 hours then analyzed. Effect on viral titer from encapsulation was not quantified with end titer post encapsulation taken to be the same as the pre-encapsulation viral titer.

3.2.4 FACs analysis

For transfections with RFP reporters, Panc-1 or A549 cells were seeded on a 6 well plate with and left to attach overnight. Varying titers of viruses were then added to the cells and left to incubate for 48 hours, following which cells were detached and fixed. Flow cytometry was acquired using FACSCalibur (BD Biosciences). Analysis was performed using FlowJo 6.3.5.
3.2.5 Antibody neutralization

Goat poly-clonal anti-Ad5-Hexon (PA128357, Thermo Scientific, USA) was obtained from Thermo Scientific. Briefly, serial dilutions of the antibody was incubated with either free or silica coated Ad-RFP at an MOI of 50 for 1hour at 37°C. Viruses were then plated onto PANC-1 or A549 cells and left to incubate for 48 hours before being assayed for RFP transduction.

3.2.6 In vivo neutralization assay

All animal experiments were conducted humanely in accordance with the Institutional Animal Care and Use Committee guidelines. In vivo neutralization of adenoviral vectors was evaluated in a reconstitutive immune model using IV delivery of IgG to nude mice according to published methods. Nude mice (6-8 weeks old) were implanted via sub-cutaneous injection with 1 x 10^6 HT1080 cells. Tumors were left to develop till a volume of 100mm^3 prior to further treatments. At t = 0, mice were injected with PBS or varying concentrations of neutralizing antibodies. Antibodies were left to circulate for 10mins, after which 1 x 10^8 pfu of Ad-Luc or SiAd-Luc were injected IT. Transgene expression was measured by light output via luciferase activity. Mice were imaged 48 hours post viral treatment for luciferase activity by IV delivery of 150mg/kg of D-luciferin (30mg/ml in PBS). All mice were imaged for 30 seconds every minute from 1 – 20 minutes.
3.3. Results

3.3.1 Particle size and zeta measurements

Silica coated viral nanoparticles were prepared (Figure 3.1) by mixing adenoviral (Ad) solutions with polycationic solutions (poly-L-lysine, PLL) and silicic acid solutions, resulting in the absorption of polycationic polymers onto the adenoviral capsid coat with downstream poly-condensation of silica onto the resultant viral-(poly-cationic polymer) template. Coated particles were characterized with scanning electron microscopy (SEM), dynamic light scattering (DLS) and zeta potential measurements. Optimization of reaction conditions gave poly-cationic polymer (PLL) concentrations as the biggest determining factor of particle size and stability. Using varying PLL concentrations of 0.3% to 5% v/v (Figure 3.2), an optimal PLL concentration of 0.83% was established yielding particles with an average size of 199nm.
40nm, PDI 0.20). Decreasing and increasing PLL concentrations resulted in poly-disperse particles with larger average diameters. Varying silicic acid concentrations was not observed to have an effect on particle size distributions. All downstream experiments were carried out using particles made with 0.83% PLL unless otherwise stated. SEM imaging revealed that particles were non-spherical with a bumpy surface morphology. Absence of colloidal silica deposits was indicative of successful templating of the silica poly-condensation reaction. Zeta potential measurements of coated and uncoated adenoviruses gave -11.6mV (± 0.62mV) and -45.4mV (± 0.32mV) respectively (Figure 3.2).
Figure 3.2. Particle size distribution as a function of increasing PLL concentration during silica poly-condensation on adenoviruses. (A) Changes to TMOS concentration has no significant contribution to particle size distribution in the concentrations tested. Zeta potential of Ad and SiAd. (B)
3.3.2 Serum stability of coated viruses

Serum stability of coated and uncoated adenoviruses was analyzed using an absorbance assay. Ad and SiAd were suspended in 100% human serum (pooled) at 37\(^\circ\)C with absorbance at 560nm measured over time. PEG functionalized SiAd was also tested to see if the PEG coating was able to delay onset of opsonization of the viral nanoparticles. Particle concentration was set at 1x 10\(^9\) particles/ml for all samples. Both SiAd and SiAd-PEG showed no noticeable change in absorbance within 3 hours. On the country, bare Ad showed immediate aggregation upon exposure to serum, marked by an increase in 560nm absorbance over time (Figure 3.3). Encapsulation with silica is shown here to enhance serum stability of coated viral particles.

![Figure 3.3. Serum stability of viral particles. Bare viruses, silica coated viruses and PEG functionalized silica coated viruses were added to 80% human serum (pooled) in a 96-well format. Absorbance readings were recorded every 10 minutes at 560nm.](image-url)
3.3.3 Viral viability post coating

Effect of silica coating on viral activity was next evaluated. Bare and silica coated adenoviruses bearing a red fluorescent protein (RFP) transgene were plated upon human PANC-1 cells to evaluate effects of silica coating on viral viability. Treated cells were analyzed using a plate reader, FACS and fluorescent microscopy for RFP expression. Cells treated with bare Ad-RFP showed a dose dependent expression of the red fluorescent signal (Figure 3.4). At a MOI 50 level, silica coated adenoviruses (SiAd) were shown to have a 5.4x increase in RFP signal compared to bare viruses at the same MOI. FACS analysis of Ad and SiAd treated cells both showed a population wide increase in RFP signal (Figure 3.4b) as opposed to the evolution of a super infected sub-population of cells. Cells spiked with empty silica particles showed no change in fluorescence (not shown). Results were confirmed on fluorescent microscopy (Figure 3.5). Silica coated viruses were shown to be still active with potentially greater transduction activity over uncoated viruses.
Figure 3.4. Transduction efficiency of Ad-RFP on PANC-1 cells after coating with silica. (A) RFP fluorescence intensity was measured 2 days post transduction with setups normalized to the MOI 50 level. Silica coated adenoviruses showed significantly higher transduction (p<0.0001) and were protected from proteinase K digest. Secondary coating of PEG on the silica particle was sufficient to ablate transduction (p<0.0001). FACs analysis of PANC-1 cells transduced with free or silica coated Ad-RFP. (B) Cells were infected with Ad-RFP then harvested and analyzed on FACS 2 days post transduction. Both free and silica coated Ad-RFP showed population wide dose response with increasing viral titer. Stability under storage at -80°C. (C) SiAd was frozen down in aliquots in PBS. Cells were transfected using an MOI of 50.
Ad and SiAd were subjected to proteinase K digest for 1 hour at 37\(^\circ\)C to evaluate the integrity of the silica coating as well as protection effect of silica on Ad. SiAd showed a 3.7x decrease in viral titer upon treatment with proteinase K. Bare Ad dropped back to background levels of fluorescence upon exposure to the same conditions, showing that the silica coating has a protective effect on the coated Ad. A secondary test to confirm the proper encapsulation of Ad in a silica matrix involved functionalization of the outer surface of the silica matrix with PEG via silane chemistry. Secondary functionalization of the silica layer in SiAd with PEG-silane (MW 5000 da) ablated transduction, resulting in a 30 fold decrease in measured fluorescence in cells co-cultured with PEGylated SiAd (Figure 3.4a). The silica coating was sufficient to protect the virus from proteinase K digest while allowing for ablation of viral uptake via functionalization with PEG showing the integrity of the encapsulating layer of silica.
Coated viruses also maintained activity in an in vivo model (Figure 3.8). In a test of viral stability when stored at -80°C, SiAd viral particles retained viral viability through 3 months storage at -80°C in PBS. A slight upward trend in mean fluorescence with increasing storage time at -80°C was observed which might be attributed to slight particle aggregation due to non-optimized storage conditions at -80°C.

3.3.4 Neutralization

Availability of viral epitopes for binding to antibodies was tested in a neutralizing assay. Ad and SiAd were incubated with anti-Ad neutralizing antibodies (nAb) prior to being plated upon cells to determine the protective effects of silica on the coated virus. All concentrations of nAb tested gave significant reduction in RFP transduction in bare adenoviruses. IC50 of the neutralizing antibodies on bare adenoviruses was determined to be 8µg/ml.
(Figure 3.6). Silica coated adenoviruses showed no noticeable impact on viral transduction from being incubated with nAb showing that the layer of silica coating is able to prevent neutralization of the encapsulated virus.

![Graph showing fluorescence intensity](image)

*Figure 3.6. Neutralization assay in the presence of antibodies. Free and silica coated Ad-RFP were incubated with varying dilutions of neutralizing antibodies (nAb) prior to being plated onto PANC-1 cells. RFP fluorescence intensity was measured 2 days post transduction. Transduction with free adenoviruses was significantly suppressed in the presence of nAb at the three antibody titers tested: 8µg/ml, 20µg/ml and 40µg/ml (p<0.04, p<0.02 and p<0.007). Silica coated adenoviruses showed little differential impact from incubation with nAb.*

3.3.5 Transduction of CAR negative cell lines

Transduction efficiency of SiAd and Ad was next compared across 2 additional CAR negative cell lines; human ovarian SKOV-3 and Chinese hamster ovarian CHO-K1. A CAR positive cell line, human adenocarcinoma A549, was used as a control. Similar to PANC-1 cells, SiAd transduced cells showed 8 and 28 fold increased fluorescence intensity in SKOV-3 and CHO-
K1 respectively compared to bare Ad at MOI 50. In contrast, A549 cells showed no significant difference in RFP transduction when transduced with SiAd, compared to bare Ad (Figure 3.7). Transduction efficacy of encapsulated viruses were hence uncoupled from CAR expression levels suggesting an alternative uptake pathway to be in effect.

Figure 3.7. Transduction of CAR-negative cell lines compared to A549. Free and silica coated Ad-RFP were plated onto PANC-1, SKOV-3, CHO-K1 and A549 cells for 48 hours. Fluorescence intensity was measured at the end of 48 hours on a plate reader. SiAd showed improved transduction in PANC-1 (3.5x, p<0.0001), SKOV-3 (7.7x, p<0.0002), and CHO-K1 (28.7x, p<0.004). A549 cells showed no significant difference in transduction between Ad and SiAd particles.
3.3.6 In vivo neutralization

Figure 3.8. *In vivo* neutralization of Ad-Luc. Varying concentrations of neutralizing antibodies were delivered intravenously to nude mice bearing HT1080 tumors 10 minutes prior to an IT injection of $10^8$ pfu of Ad or SiAd. Luciferase activity was measured 48 hours later via IV D-luciferin 150mg/kg per mouse. Mice were imaged for light output 5 minutes post IV delivery of D-luciferin.

Ability of Ad and SiAd to establish efficient transgene expression in vivo in the presence of neutralizing antibodies was evaluated in a reconstitutive immune model. Nude mice bearing HT1080 tumors were given intravenously varying concentrations of nAb to establish a pre-existing population of nAb in host serum prior to exposure to Ad or SiAd viral particles bearing a luciferase reporter. Ability of viral particles to facilitate luciferase transgene expression in the presence of neutralizing antibodies in host serum was evaluated via light output 48 hours post viral injections (Figure 3.8). Both Ad and SiAd showed robust luciferase transgene expression in the PBS and low dose nAb (20µg/ml nAb in mouse blood) groups. At the higher dose of 40µg/ml nAb, transgene expression by Ad was severely inhibited with little luciferase activity detected while SiAd maintained robust transgene expression showing the ability to establish robust transduction in the presence of neutralizing antibodies *in vivo*. 
3.4. Discussions

Immune response to virotherapy remains one of the biggest hurdles towards mainstream deployment of therapeutic viruses in the clinic. Previous studies have shown that viral encapsulation is a promising approach that can solve some of the problems related to viral delivery in patients\textsuperscript{25,49–54}. However, current approaches tradeoff between circulation time, viral viability and target specificity\textsuperscript{25}. Silane chemistry has been shown to be compatible with physiological conditions. In this study, we formulated a novel silica coating using silane chemistry to address some of the shortfalls inherent in current approaches. We adopted a template driven silica condensation process whereby silica is being directly deposited upon the viral templates.

In order to achieve true systemic delivery, coated particles have to be below 200nm in diameter\textsuperscript{55,56}. Optimization of reaction parameters for coating viruses in silica revealed PLL concentration as the biggest determining factor of particle size and stability. Mono-dispersed particles with an average size of 199 nm was obtained when the concentration of PLL solution in the reaction was set at 0.83% v/v. Zeta measurements of the viral stock post coating showed a shift towards neutral voltage from -45.4mV pre-coat to -11.6mV post-coat which is in line with expected observations as viral capsid proteins are being conjugated to poly-cationic polymers which should serve to neutralize a portion of the negative charge on the capsid proteins. The shift towards a more neutral charge should also improve stability in blood of the
viruses as highly charged species readily complex to carrier proteins in blood with rapid downstream clearance from the circulation.

To quantify expected stability in blood, both bare and coated adenoviruses were incubated in 80% human serum at 37\(^\circ\)C with absorbance measurements at 560nm taken every 10 minutes as described previously\(^47\). Human serum was used to best mimic physiological conditions. Silica particles functionalized with PEG were also tested to determine effects of the PEG coating on particle stability. The rationale behind taking absorbance readings is that large particles would occlude and scatter more light resulting in an increased absorbance measurement over smaller particles. If particles were to destabilize when exposed to serum and start aggregating, the absorbance measurements would show a concurrent increase allowing us a convenient method for tracking particle stability. Using our setup, bare adenoviruses registered an immediate increase in absorbance that increased over the course of 3 hours post exposure to human serum. Both silica and PEG functionalized silica showed no noticeable change in absorbance over the same time period. The assumption from this methodology results is that absorbance has a direct correlation to particle size, any changes of which over time is indicative of serum instability. From these we draw the conclusions that bare adenoviruses are not expected to be stable in serum, while silica coated adenoviruses and PEG functionalized silica coated adenoviruses both show extended stability in serum. Both PEG functionalized and bare silica coated adenoviruses showed no noticeable difference in stability within 3 hours. The
rapid destabilization of adenoviruses in serum might be attributed to the higher charge density on the bare adenoviruses compared to the silica coated virus. Additionally the viral capsid surface presents a more attractive target for opsonization of serum proteins compared to silica and PEG-silica coated viruses which have an outward presentation of an inert surface that presents a steric barrier to absorption of serum proteins.

One of the inherent drawbacks to current encapsulation methodologies is the loss of viral viability during the encapsulation process\textsuperscript{57,58}. Having established particle size characteristics, we next looked into the effects of silica coating on viral viability. To test viral viability, coated and uncoated Ad encoding a red fluorescent protein (Ad-RFP) were plated onto PANC-1 cells and left to incubate for 2 days. Transduction efficacy was read in terms of the intensity of the RFP signal. Both bare and coated Ad showed a dose response that was independent of silica concentration. Silica coated adenoviruses in particular showed a 5.4x increase in transduction efficacy over bare adenoviruses. To determine the distribution of the RFP signal in transduced cells, transduced cells were harvested and analyzed using FACs. FACs data showed that the fluorescence signal was evenly distributed across the transduced cell population and not concentrated in a subpopulation of super infected cells minimizing the possibility that the increase in RFP signal is due to aggregation of SiAd particles resulting in super infection of a select few cells that had up taken a super virus cluster. As adenoviral transduction efficacy is intimately tied to CAR mediated uptake by host cells\textsuperscript{59–62}, we tested a panel of
CAR-negative cell lines and compared them to a CAR positive control to try understand if the improved transduction is related to cellular uptake of adenoviral particles. Transduction efficacy was markedly improved in CAR-negative cells transduced with SiAd yielding 5 – 30 fold improvements in transduction efficacy over bare Ad. In contrast, SiAd showed no significant difference in transduction efficacy in A549 cells which are CAR-positive. We postulate that since viral epitopes are being hidden under a layer of silica, traditional adenoviral uptake mechanisms do not apply in the case of SiAd, as evidenced by the lack of correlation between CAR expression levels and transduction efficacy.

One of the major concerns in encapsulating viruses is the integrity of the encapsulating layer of silica. The encapsulating layer of silica should fully enclose the virus in order achieve protection and evasion from the host immune response. To test the integrity of the encapsulating layer of silica, silica coated adenoviruses were incubated with proteinase K. Proteinase K digest of the silica coated adenoviruses was severely retarded compared to digest of bare adenoviruses. The results suggest that the adenovirus is properly encapsulated in the silica layer and only offers proteinase K limited access resulting in a significantly retarded proteinase K activity. As a secondary test of the integrity of the silica layer, the encapsulating silica was functionalized with mPEG silane (5kDa). The rationale is if the encapsulated adenovirus was totally hidden and hence invisible to the environment, functionalization of the silica layer would be sufficient to ablate uptake and
hence transduction by the adenovirus. However if viral proteins were being presented to the environment, coating of the silica layer with PEG would have little to no impact upon viral uptake and transduction. Secondary functionalization of the encapsulating silica layer with PEG was sufficient to suppress viral activity by 30 fold which improves our confidence that the adenovirus is indeed being properly encapsulated and protected by the silica layer.

Induction of neutralizing antibodies to therapeutic viruses in patients is a major limiting factor of viral therapy in the clinic\textsuperscript{18,19,63}. Having established the integrity of the silica coat that we applied onto the viruses, we next set forth to test if coating viruses in silica would have any impact on the way coated viruses react to neutralizing antibodies. Antigen recognition is highly dependent on availability and presentation of viral epitopes. Masking viral proteins under a layer of silica should enable the encapsulated viruses to evade detection and neutralization by neutralizing antibodies. Bare and coated adenoviruses were separately incubated with varying concentrations of neutralizing antibodies prior to being plated onto cells. IC\textsubscript{50} of the neutralizing antibodies on bare adenoviruses was determined to be 8\textmu g/ml with almost complete neutralization at 40\textmu g/ml. Significant inhibition of transduction was observed in bare adenoviruses at all concentrations of antibodies tested. In contrast, SiAd showed no noticeable impact from antibody treatment showing that we were able to protect adenoviruses from neutralizing antibodies by encapsulation in silica.
Having established protection from antibodies *in vitro*, we next set out to determine if the same protection phenomena could be replicated in an *in vivo* model. The *in vivo* model presents a more challenging environment for viral therapies due to complex interactions between the adaptive immune response, innate immune elements and the complement system. To simulate the immune response a viral particle would encounter in an individual with pre-existing immunity to the virus, we chose a nude mouse model that had been fortified with neutralizing antibodies against adenoviruses. While nude mice are athymic and hence lack a mature T cell population, they retain an active complement system and macrophage population. Circulating anti-adenoviral IgG would in the presence of an adenovirus, be able to recruit the complement system and macrophages to neutralize and aid in the clearance of the adenovirus. Using our reconstituted immune model, we were able to neutralize transduction by bare Ad-Luc administered IT in the presence of 40µg/ml mouse blood of anti-adenoviral nAb. SiAd-Luc viral particles in contrast were able to establish efficient transduction in a similar concentration of nAb as measured by light output from luciferase activity showing that we were able to achieve protection via encapsulation in silica in an *in vivo* model.
3.5. Conclusions

In conclusion we have developed a silica coating for adenoviruses that is compatible with physiological functions of the virus. Encapsulated viruses have markedly improved serum stability over bare viruses and are size optimized to prolong circulation time in the body. The silica coating is shown to allow coated adenoviruses to efficiently establish an infection in CAR-negative cell lines and more importantly protect the encapsulated virus from immune neutralization. The immune protection extends to *in vivo* models where we were able to efficiently establish viral transduction in the presence of neutralizing antibodies using encapsulated viruses. We believe that this solution is compatible with multiple viral species and can be readily adapted for use in the clinic.

Chapter 3 in part is currently being prepared for submission for publication of the material. Esener, Sadik. The dissertation author was the primary investigator and author of this material.
CHAPTER 4. INORGANIC ADENOVIRUS COMPLEXES FOR ULTRASOUND-DIRECTED VIRAL DELIVERY
Abstract

External stimuli responsive drug nanoparticles show promise in overcoming off target toxicities that are frequently associated with adverse events in the clinic. In this study, we formulated a novel silica based viral nanoparticle containing perfluorononane nanoemulsions that utilizes an external ultrasound stimuli to trigger release of viral particles within regions of ultrasound focus. Viral nanoparticles kept in an inactive ground state were shown to regain viral activity upon exposure to ultrasound. Recovery of activity was shown to be contingent on encapsulation of nanoemulsions within the viral nanoparticle. Additionally silica viral nanoparticles were shown to be resistant to the effects of neutralizing antibodies.
4.1. Introduction

Despite the newer generation of viral vectors with improved safety tolerances towards healthy tissues, off target toxicities remain one of the major drivers of adverse events from virotherapy in the clinic. Efforts to improve disease specificity have largely focused on passive accumulation in diseased tissues or intrinsic active targeting strategies. However these techniques do little to address issues like liver/splenic accumulation, receptor ligand promiscuity and ubiquity of disease markers. For a systemic delivery approach to be effective, it must allow the virus to circulate for ample time to evade the immune system on its indirect path to the target. However, immune evasion should not negatively impact the ability to infect the cell target. Therefore, a triggering mechanism is necessary to convert the virus from a long circulating inactive state to an active infective state, whether it be physical, chemical, or biochemical (receptor-mediated).

Activatable drug complexes that rely on external stimuli for drug release make for attractive alternatives to traditional targeting modalities. The ability to physically direct virus exposure to specific tissues is a key differentiator from biochemical targeting approaches, which depend on the presence of overexpressed surface receptors. Although physically-directed methods require knowledge of the location of the target, they do not require knowledge of the target phenotype. This is especially important in cancer, where there is great heterogeneity between patient tumors, between different tumors in the
same patient, and even within a single tumor.\textsuperscript{29,30} Even if a patient is assessed to present a targetable receptor, it may be only present on a fraction of the patient’s tumor.

Ultrasound is a safe energy which can treat specific volumes precisely and noninvasively. Many approaches are being developed to locally enhance virus delivery with focused ultrasound. Gas-filled microbubbles are often used to sensitize tissues, and porate nearby cells.\textsuperscript{31,32} Unfortunately, few of these approaches have successfully hidden the viruses in a stealth layer. Those that have encapsulated the viruses have often used processes that impair the bioactivity of the virus or do not account for the short circulating half-lives of microbubbles and nanoemulsions. In this study, we formulated a novel silica based viral nanoparticle that incorporates an ultrasound triggering device in the form of perfluorononane (PFN) nanoemulsions (NE). Incorporation of nanoemulsions into the nanoparticle formulation has the advantages of improved stability of and spatial co-localization of nanoemulsions with viral particles. Nanoemulsions have been widely studied as ultrasound therapeutic and/or diagnostic agents, allowing for us to explore the possibility of developing an ultrasound triggered viral release mechanism. Under the action of ultrasound, NE encapsulated within a carrier particle may nucleate a rapid liquid-to-vapor transition known as acoustic cavitation. This highly energetic and localized process results in the disruption of the NE’s encapsulating matrix (silica) and consequent exposure of virus.
4.2. Materials and methods

Adenoviral stocks, Ad5-RFP, Ad5-CMV-Luc were purchased from Vector Biolabs (USA). Goat Ad5-anti-hexon polyclonal was obtained from Thermo Scientific (USA). Tetremethoxy orthosilicate, poly-L-lysine, zonyl FSE, perfluorononane and HCl were obtained from Sigma (USA).

4.2.1 Preparation of nanoemulsions and characterization

Zonyl FSE and perfluorononane (PFN) were purchased from Sigma Aldrich (USA) and used without further purification. 1050µl of PBS was heated to 95°C. 120µl of Zonyl FSE (14% w/v) was added to the heated PBS and briefly vortexed to ensure even mixing. 30µl of 99% PFN was next added to the bottom of the mixture which was then immediately placed into an ice bath, giving a final concentration of 1% Zonyl FSE and 2.475% PFN v/v in PBS. Mixture was then sonicated on ice using a Misonix XL-2000 (Farmingdale, USA) probe type sonicator operated at level 20 for 30 minutes until an evenly distributed PFN nanoemulsion (NE) was obtained. The NE was then dialyzed against DPBS using the Fast SpinDialyzer (Harvard Apparatus, USA) for 12 hours, which allowed the use of large pore membranes for the efficient removal of free surfactants. Nanoparticle size and measurements were determined using a Zetasizer Nano ZS90 (Melvern).
4.2.2 Preparation of ultrasound sensitized viral silica nanoparticles and characterization

Adenoviruses (Ad) were obtained from Vector Biolabs (USA) and used without purification. Briefly, $10^8$ pfu of Ad was mixed vigorously with varying concentrations of 0.1% poly-L-lysine solution (PLL, Sigma Aldrich, USA) and 0.025% v/v of the NE solution from before and left to stand for 5 minutes at room temperature (solution A). Separately, a 13% tetramethoxy orthosilicate v/v (TMOS, Sigma Aldrich, USA) in 1mM HCl (Fischer, USA) solution was prepared (solution B). Solution B is then added drop wise to solution A to a final concentration of 2.5% v/v under vigorous mixing (solution C), then allowed to stand under vigorous mixing at room temperature for a further 1 hour. Solution C containing the silica coated adenoviruses NE complexes (SiAd-NE) is then spin purified and resuspended in PBS for immediate use or frozen in aliquots at -80°c. Nanoparticle size and measurements were determined using a Zetasizer Nano ZS90 (Melvern).

4.2.3 Cell culture and transfection protocol

In vitro assays were performed on a human pancreatic line (Panc-1). The cell line was maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin/Glutamine (PSG) (MEM-complete). Cells were incubated at 37°c at 5% CO$_2$ and passaged every 3-4 days when 70% confluent.
Viral transfections were carried out in a 96 well format. $10^4$ cells were split into each well and allowed to attach overnight prior to transfection. Cells were infected with either equivalent tiers of Ad or SiAd for 48 hours then analyzed. Effect on viral titer from encapsulation was not quantified with end titer post encapsulation taken to be the same as the pre-encapsulation viral titer.

4.2.4 Antibody neutralization

Goat poly-clonal anti-Ad5-Hexon (PA128357, Thermo Scientific, USA) was obtained from Thermo Scientific. Briefly, serial dilutions of the antibody was incubated with either free or silica coated Ad-RFP at an MOI of 50 for 1 hour at $37^0\text{C}$. Viruses were then plated onto PANC-1 or A549 cells and left to incubate for 48 hours before being assayed for RFP transduction.
4.3. Results

4.3.1 Particle size and zeta measurements

![Diagram showing the process of silica coating of Ad.](image)

Figure 4.1. Silica coating of Ad. Negatively charged NE is reacted with a cationic polymer, poly-L-lysine (PLL) to modify the surface charge on the NE. The now positively charged NE attracts negatively charged Ad to form a nucleation complex. As the surface charge on the nucleation complex is still predominantly positive, negatively charged silica precursor and hydroxyl ions are attracted to its surface creating a basic environment suitable for the silica poly-condensation reaction. Viral particles trapped in the silica matrix are rendered inactive via a layer of PEG on the silica nanoparticle.

Ultrasound (US) sensitized viral silica nanoparticles were prepared by sequentially mixing perfluorononane nanoemulsions (NE) with a poly cationic solution then an adenoviral solution (Figure 4.1). The resulting adenoviral-nanoemulsion complex serves as a template for poly-condensation of silica gel following addition of silicic acid to the reaction mixture. A monodispersed population of silica coated viral nanoparticles (SiAd-NE) with an average diameter of 313nm (±70nm) was obtained from the above reaction. Zeta potential measurements of the coated viruses gave -28mV (±8mV), compared to -45.4mV (±0.2mV) of the bare virus and -86.1mV (±7mV) of the NE (Figure 4.2). The shift towards neutrality of the coated viral nanoparticle is indicative of encapsulation of viruses and nanoemulsions within a silica gel matrix.
4.3.2 Ultrasound release mechanics

Perfluorocarbon nanoemulsions are known to reduce the cavitation threshold of the surrounding media. While the phenomenon is postulated to proceed through a droplet-to-bubble transition, much of the actual mechanism remains an unknown. In the current setup, a perfluorononane (PFN) nanoemulsion is co-encapsulated with adenoviruses in an amorphous silica gel matrix through a template driven silica poly-condensation reaction. A secondary layer of PEG was applied to the resultant SiAd-NE nanoparticle to ablate non-specific uptake of the viruses keeping the encapsulated viruses in a default inactive state. Exposure of the SiAd-NE nanoparticle to focused US above the cavitation threshold results in cavitation events which disrupts the encapsulating layer of PEG functionalized silica releasing active adenoviral particles into the local vicinity of the US focal zone (Figure 4.3).
In a test of the US release system, SiAd-NE were subjected to brief pulses of probe sonication with DLS and zeta potential measurements taken of the resultant mixture. DLS measurements of SiAd-NE nanoparticles taken before and after US exposure showed a shift in size from 313nm to 90nm post exposure. Zeta potential measurement of the particles showed no significant changes following US exposure (Figure 4.4). The evolution of the 90nm size peak following US exposure corresponds well to DLS measurements of bare adenoviruses strongly suggesting the disruption of SiAd-NE nanoparticles with consequent release of adenoviral particles. Zeta potential of the released particles however did not reflect the surface charge of bare adenoviruses suggesting that portions of the released virus might still be covered by a silica matrix. Shift in size was not observed in silica coated viral particles without NE suggesting that this is a NE specific phenomenon.
Figure 4.3. Proposed release mechanism for SiAd-NE viral particles. SiAd-NE reaches target site and are exposed to a short pulse of focused US. The NE acts as a focal point for the US pulse resulting in a cavitation event that disrupts the silica coating releasing active viral particles that can mediate viral transduction and infection events.

Figure 4.4. Effects of US exposure on particle size (A) and zeta potential (B) of SiAd-NE. Viral peak is revealed upon exposure of SiAd-NE particles to US. Zeta potential remains unchanged before and after US.
4.3.3 Viral viability post US activation

**Figure 4.5.** Transduction efficiency of SiAd-NE on PANC-1 cells before and after exposure to US. (A) RFP fluorescence intensity was measured 2 days post transduction with setups normalized to the MOI 50 level. SiAd-NE exposed to US showed significantly higher transduction ($p = 0.002$) compared to unexposed SiAd-NE. Exposure of SiAd and PEG functionalized SiAd in the absence of NE or with spiked NE, to US resulted in no significant difference in transduction between exposed and unexposed groups. (B)

We have previously shown that encapsulating viruses in silica has no impact on viral viability. However, subjecting bare viruses to probe sonication is sufficient to ablate viral activity. Hence, we next set out to determine the effect of US exposure on viral activity of SiAd-NE released viral particles. SiAd-NE viral nanoparticles bearing a RFP reporter gene were alternatively left as is or exposed to probe sonication prior to being plated on PANC-1 cells at a MOI 50 level. Treated cells were analyzed using a plate reader and fluorescent microscopy for RFP expression. Results were compared to cells transfected with bare Ad and silica coated adenoviruses without NE. SiAd-NE nanoparticles not exposed to US were shown to have low transduction activity, due to the secondary layer of PEG that ablated nonspecific cell uptake of the
nanoparticles. Exposing SiAd-NE nanoparticles to US in turn resulted in a 23 fold increase in RFP transduction ($p = 0.002$) compared to unexposed nanoparticles. Activity of exposed SiAd-NE nanoparticles was similar to silica coated adenoviruses and much greater than bare Ad (4.8 fold increase over bare Ad for US exposed SiAd-NE compared to 5.4 fold increase for SiAd) (Figure 4.5a). Both SiAd and PEG functionalized SiAd nanoparticles without NE showed no significant changes in transduction ability upon exposure to US. Spike of NE into PEG functionalized SiAd nanoparticles also resulted in no significant changes in transduction upon US exposure (Figure 4.5b). Fluorescent microscopy of Ad and SiAd-NE treated cells both showed a population wide increase in RFP signal (Figure 4.6) as opposed to the evolution of a super infected sub-population of cells. Here we show the ability to conditionally activate transfection events using US as a triggering mechanism.
Figure 4.6. Fluorescent microscopy of PANC-1 cells transduced with Ad-RFP (MOI 50) taken at 2 days post transduction. Ad-RFP was alternatively, complexed with NE and coated with silica, coated with PEG functionalized silica or coated with silica. Setups were then exposed to US or left as-is before being plated onto cells. Cells were imaged on an inverted fluorescent microscope 2 days post transduction for RFP expression. Cells transduced with bare Ad-RFP were used as a control.

4.3.4 Protection from Neutralization

Immune neutralization of therapeutic viruses by the host immune system is one of the biggest hurdles to widespread deployment of viral therapeutics in the clinic. One of the goals of encapsulating viruses in silica, aside from serving as a platform for the targeted release of therapeutic viruses, is to hide viral epitomes from immune detection and neutralization. Availability of viral epitomes for binding to antibodies was tested in a neutralizing assay. Bare Ad and US exposed SiAd-NE were incubated with varying concentrations...
of neutralizing antibodies (nAb) prior to being plated onto PANC-1 cells. IC50 of the nAb on bare adenoviruses was determined to be 8µg/ml with bare viruses showing significantly decreased transduction efficacy across all nAb concentrations. On the contrary, SiAd-NE viral nanoparticles showed no effect of nAb on transduction efficacy show that the silica coating is effective in protecting encapsulated viruses from neutralizing antibodies (Figure 4.7).

![Figure 4.7. Neutralization assay in the presence of antibodies. Bare Ad(RFP) and US exposed SiAd(RFP)-NE were incubated with varying dilutions of neutralizing antibodies (nAb) prior to being plated onto PANC-1 cells. RFP fluorescence intensity was measured 2 days post transduction. Transduction with bare adenoviruses was significantly suppressed in the presence of nAb at the three antibody titers tested; 8µg/ml, 20µg/ml and 40µg/ml. SiAd(RFP)-NE showed little differential impact from incubation with nAb.](image-url)
4.4. Discussion

Off target toxicities is one of the major drivers of adverse events from virotherapy in the clinic. Efforts to improve disease specificity have largely focused on passive accumulation in diseased tissues or intrinsic active targeting strategies. However, these techniques do little to address issues like liver accumulation, receptor ligand promiscuity and ubiquity of disease markers. Externally activatable drug complexes that rely on external stimuli for drug release makes for an attractive alternative to traditional targeting modalities. In this study, we formulated a novel silica based viral nanoparticle that incorporates an ultrasound triggering mechanism in the form of PFN nanoemulsions. Ultrasound has the advantages of using a non-ionizing energy source with deep tissue penetration and narrow focus, allowing for pinpoint activation. Nanoemulsions have been widely studied as an ultrasound sensitizing agent allowing for us to explore the possibility of developing an ultrasound triggered viral release mechanism. Under the action of ultrasound, NE encapsulated within a carrier particle may undergo droplet-to-bubble transition with downstream cavitation resulting in the rupture of the encapsulating matrix of the carrier containing the NE and consequent release of viral particles from an inactive silica encapsulated state to an active burst phase using an ultrasonic trigger.

Silica coated viral nanoparticles containing adenoviruses and NE (SiAd-NE) were formulated by mixing PFN NE with a PLL solution then complexing
them with adenoviral particles. The resultant complex was coated in a silica matrix through a template driven process with a secondary PEG coating applied to ablate nonspecific uptake of viral nanoparticles to keep them in a default inactive state. The resulting viral nanoparticles were mono-dispersed with an average diameter of 313nm. Zeta potential measurements of the bare virus, NE and the final encapsulated product show a marked shift in zeta potential from -45.4mV and -86.1mV pre-coat to -28.3mV post-coat which is in line with expected observations as negatively charged surfactants and viral capsid proteins are being conjugated to poly-cationic polymers which should serve to neutralize a portion of the negative charge. The shift towards a more neutral charge also serves to improve stability in blood of the viral particles as highly charged species readily complex to carrier proteins in blood resulting in rapid downstream clearance from the circulation.

To evaluate the ability of the particle to release activated viral particles in response to an ultrasound stimuli, SiAd-NE was subjected to mild probe sonication. DLS analysis of the resultant mixture showed a shift in average diameter of the particles from 313nm to 90nm. Given the 90nm peak corresponds well to both reported and in-house measured values of adenoviral diameter quantified using DLS, this was strongly suggestive that the application of ultrasound through probe sonication was successful in rupturing SiAd-NE nanoparticles with consequent release of adenoviral particles into solution.
One of the major concerns in exposing viruses to probe sonication and ultrasound induced cavitation events would be the consequent loss of biological activity in the exposed viral particles. To test if SiAd-NE retained bioactivity following US exposure, exposed SiAd-NE nanoparticles were plated onto cells with activity compared to unexposed SiAd-NE, bare adenoviruses and silica coated adenoviruses via a RFP reporter gene. Exposure to US resulted in a 23 fold increase in RFP signal over unexposed SiAd-NE showing that US exposed viral particles retained their bioactivity and transitioned from an inactive state to an active state following US exposure which is in line with DLS size measurements that suggested that SiAd-NE nanoparticles were ruptured with viral particles released into the media. Of interest to note is that released viral particles showed transduction efficacy similar to silica coated adenoviruses as opposed to bare adenoviruses suggesting that released viral particles remain encapsulated in silica. Exposure of silica coated adenoviruses and PEG-silica coated adenoviruses without NE resulted in no change in RFP transduction suggesting that the signal increase when exposing SiAd-NE to US only occurs in the presence of NE suggesting that NE associated cavitation effects might be responsible for the release of viral particles from SiAd-NE. To test the importance of co-localization of the NE and adenoviruses for US based rupturing of SiAd-NE nanoparticles, NE were spiked into the solution of PEGylated SiAd viral particles prior to US exposure. Using this setup, application of US to the particles had no perceived effect on activity of the encapsulated viral particles, indicating that co-encapsulation of NE into
SiAd-NE nanoparticles is required for US based activation. This also implies that the NE from the SiAd-NE formulation is properly encapsulated within the silica coating.

Induction of neutralizing antibodies to therapeutic viruses in patients is a major limiting factor of viral therapy in the clinic\textsuperscript{18,19,63}. Having established the ability to conditionally activate viral release and transduction using an ultrasound trigger, we next set forth to test if silica encapsulated viruses were protected from neutralizing antibodies. Antigen recognition is highly dependent on availability and presentation of viral epitopes. Masking viral proteins under a layer of silica could potentially enable the encapsulated viruses to evade detection and neutralization by neutralizing antibodies. Bare and coated adenoviruses were separately incubated with varying concentrations of neutralizing antibodies prior to being plated onto cells. Significant inhibition of transduction was observed in bare adenoviruses at all concentrations of antibodies tested. In contrast, SiAd-NE showed no noticeable impact from antibody treatment showing that we were able to protect adenoviruses from neutralizing antibodies by encapsulation in silica.
4.5. Conclusion

In conclusion, we have developed a silica viral nanoparticle that releases activated viral particles in response to external ultrasound stimuli. Nanoparticles were shown to release of active viral particles when exposed to ultrasound, leading to downstream activation of transduction events. Ultrasound-responsiveness of the nanoparticle is highly dependent on the co-encapsulation of nanoemulsions into the silica matrix. Coating of viral particles in silica is also shown to protect encapsulated viruses from neutralizing antibodies while preserving infectivity. We believe that this solution is compatible with multiple viral species and can be readily adapted for use in the clinic.

Chapter 4 in part is currently being prepared for submission for publication of the material. Benchimol, Michael; Barback, Christopher; Esener, Sadik. The dissertation author was the primary investigator and author of this material.
CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS
Increasingly, new technologies being developed are pushing the prospect of gene therapy in the clinic closer to mainstream applicability. Much of the focus of the field has been in developing vectors, both viral and non-viral, with improved safety and efficacy profiles. Despite this, safe and efficient delivery and targeting of vectors remains a major hurdle to overcome in order for gene therapy to gain traction in the clinic. As part of the work for this thesis, we have developed two systems to address some of the delivery concerns for gene therapy vectors, one each for non-viral and viral vectors.

In chapter two, we discussed a method for the liposomal encapsulation of DNA complexes with an externally activated ultrasonic trigger. In our method, DNA complexes are co-encapsulated together with an external triggering mechanism (ultrasonic) into a liposome. Using our technology, transfection events can be manually activated via an externally applied stimuli in the form of an ultrasonic pulse. In vitro testing showed that we are able to achieve a 7 fold improvement over unactivated liposomes using a focused ultrasound stimuli. Encapsulation of DNA into liposomes protects the DNA from degradation and clearance issues that plague current lipoplex approaches, while incorporation of an externally activated triggering mechanism allows us to only release DNA complexes in regions of interest while maintaining the bulk of the DNA in an inactive encapsulated state for the rest of the body. The benefits of this technology is that it would allow us a significantly increased therapeutic window in terms of dosing as well circulation time given that transfection events are only being conditionally
activated while the bulk of the DNA complexes remain protected via liposomal encapsulation. However complex manufacturing and material constraints together with inherent limitations in non-viral gene delivery vectors mean that the current methodology espoused would only hold relevance for a subset of gene therapy applications. In order to have an effective arsenal of therapeutic options relevant to gene therapy, there is a need to explore additional treatment modalities that can be used in additional disease indications to complement our work in non-viral vectors, hence our foray into developing nanoparticle applications for viral vectors.

Chapter three covers a new nano-templated process for growing an amorphous silica gel layer over viral vectors with the intention of shielding the viruses from the host immune system while improving upon their performance characteristics. Adenoviruses were used as a test platform to evaluate the functional parameters of the silica coating. Viruses coated with silica suffered no functional impairment to their ability to transduce cells and were made resistant to proteinase K digest and ultrasonication. Additionally, coated viruses were also not subject to the action of neutralizing antibodies showing that we are able to achieve immune protection from at least one aspect of the host immune response. In a reconstituted immune surveillance model, coated viruses retained activity in nude mice that were supplemented with anti-viral antibodies, showing in vivo efficacy of the silica coating in enabling viral vectors to evade the host immune response.
In chapter four, we presented a method for the specific targeting of viral vectors using an external ultrasonic trigger. Using the technology described in the previous chapter, viruses were co-encapsulated with an external triggering mechanism (ultrasonic) in an amorphous silica gel coating. Using this setup, we were able to conditionally activate transduction events for the viral ultrasound particles with exposure to focused ultrasound yielding a 20-fold increase of signal over background. Viral ultrasound particles that were not exposed to ultrasound showed no transduction. Using this modality, we were able to achieve specific activation of viral vectors in regions of interest (like in tumors) while maintaining viral vectors in a silenced state in the rest of the host system. This example is demonstrative of our larger efforts to target viral vectors only to regions of disease in the patient.

We believe that additional work in overcoming the delivery constraints of gene therapy vector systems is crucial to the uptake of gene therapy as a viable option in the clinic. Through the course of our dissertation, we have explored different means of arriving at a suitable construct for delivery of both viral and non-viral gene therapy vectors.

In the field of non-viral gene delivery vectors, we established a novel liposomal construct that encapsulates both our gene delivery vector as well as an ultrasound sensitizing agent. While use of ultrasound to enhance delivery of gene vectors has been studied, ours is the first demonstration wherein the ultrasound sensitizing component is being encapsulated within the confines of
the liposome. This is significant as ultrasound sensitizing agents in the form of microbubbles or perflurocarbon nanoemulsions have relatively short persistence time in blood. By encapsulating them within the liposome, we are able to greatly extend their circulation time allowing for greater flexibility and an extended therapeutic window within which to treat patients. Future direction would focus on improving particle size and loading efficiencies.

For viral vectors, we have developed a novel coating using silica that enables us to retain infectious ability of the viruses while enabling the encapsulated viruses to evade neutralization by nAbs both in vitro and in vivo. As immune clearance of therapeutic viral vectors remains the major stumbling block to widespread clinical utility of virotherapy, we believe that our innovation has potential to disrupt the current state of the art and bring virotherapy closer to reality. The inherent beauty of our system is not only that all reactions are carried out under physiological conditions and are hence compatible with life, but also that the encapsulating matrix forms a flexible support structure that enables easy modifications and secondary functionalizations of the encapsulated viruses that would not be possible otherwise. In a number of preliminary studies, we have shown the ability to ablate CAR dependency of coated adenoviruses enabling them to infect a wider variety of cell types than wild type uncoated adenoviruses. One demonstration of the flexibility of our system is the incorporation of an ultrasound trigger into the matrix of the encapsulated virus. Using this setup, we are then able to conditionally trigger infection of our viral vectors through the application of an external ultrasound
source to the system. This technology in particular hold promise for unresectable diseases as well as minimally invasive surgical procedures where the ability to target multiple pinpoint spots diseased tissue is integral to success of the therapy. Future direction would focus on elucidating the uptake and release mechanisms of the coated viruses and exploring the ability to apply our coating to additional types of viruses.

While additional testing is required to establish the safety and efficacy of our proposed solutions, we believe it is a promising first step that can be built upon to truly revolutionize gene therapy in the clinic.
REFERENCES


