Engineering Translational Multi-Therapeutic Targeted Delivery Vehicles
For Disease Management

by

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SUBMITTED TO THE DEPARTMENT OF CHEMICAL ENGINEERING IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF SCIENCE IN CHEMICAL ENGINEERING
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

MAY 21, 2015

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Engineering Translational Multi-Therapeutic Targeted Delivery Vehicles For Disease Management

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Submitted to the Department of Chemical Engineering on May 21, 2015 in Partial Fulfillment of the Requirements for the Degree of Doctor of Science in Chemical Engineering

ABSTRACT

The complexity of growth, survival, and death signaling pathways in cancer continues to motivate extensive investigations using systems biology approaches to better inform treatments. Many of these drugs and drug combinations, however, are highly toxic, due to lack of targeting and poor pharmacokinetics, and are not easily delivered together due to solubility issues – requiring high levels of patient compliance over the course of treatment whereby lower, less efficacious doses of therapies are administered during multiple intravenous infusions. To address this problem, development of delivery systems that are safe/less toxic, yet capable of housing and delivering higher, more efficacious doses with sophisticated levels of control over the delivery, timing, and/or sequence of release, in order to therapeutically re-wire these signaling pathways, is essential to translate these drug combinations to the clinic. Work detailed herein addresses this challenge by developing controlled approaches for engineering and manufacturing translational nanoscale delivery systems for more efficacious, targeted treatment of cancer - specifically using layer-by-layer nanoscale assembly and liposomal manufacture.

Layer-by-Layer (LbL) assembly of polyelectrolytes on solid substrates, including colloidal systems, is a well characterized, tunable approach for generating functional thin films for a variety of applications, including drug and gene delivery, tissue engineering, and bone regeneration. On the basis of electrostatics, this assembly method allows for incorporation of a broad range of materials; and due to its water-based synthesis, it allows for incorporation of a broad range of therapeutics without significant alteration of biological function. Using this approach, work described in Chapters 2-4 detail the utility of this approach. In Chapter 2, we demonstrate the ability to coat materials atop drug-loaded PLGA nanoparticle substrates that control drug release and nanoparticle pharmacokinetics in a systemic environment. In Chapter 3, we incorporate ligand-functionalized materials atop a drug-loaded liposomal core to promote tissue-level specificity for targeted treatment of cancer. In Chapter 4, we further adapt this approach towards addressing the challenge of scalably and reproducibly manufacturing LbL-functionalized nanoparticle systems by collaborating with Joseph DeSimone at UNC-Chapel Hill. In this work, we combine scalable methods, PRINT® particle fabrication, and spray-LbL deposition, to
generate uniform and functional nanotechnologies that are built-to-order with precise control over composition, size, shape, and surface functionality.

In Chapters 5-7, targeted liposomal technologies were synthesized to deliver drug combinations with different solubilities. Liposomes, representing the first nanomedicine systems approved and employed in the clinic, are well-characterized, simple and versatile platforms for manufacture of functional and tunable drug-carrier systems. Further, functionalization of these systems for tissue-level specificity is readily achieved by ligand conjugation of a lipid that is subsequently inserted into the dual drug-loaded liposomal vesicular membrane. Utilizing the hydrophobic and hydrophilic compartments of liposomes, we achieved high loadings of both hydrophobic and hydrophilic therapies within a singular tissue-targeted (e.g. folate) liposomal system. The resultant nature of compartmentalization inside the vesicular structure promoted the desired sequence of drug release for synergistic cancer therapy, improving tumor cell killing in vitro and significant tumor shrinkage in in vivo murine triple-negative breast cancer and non-small cell lung cancer models. In Chapters 6 and 7, we applied this approach for targeted combination therapeutic delivery for additional drug therapies (e.g. cisplatin-quisinostat (HDAC inhibitor) in a folate-targeted liposome; (+)-JQ1-temozolomide in a transferrin-targeted liposome) and disease targets (non-small cell lung cancer; brain stem gliomas).

Using both LbL and liposomal manufacture, work detailed herein represents a broad skill set for engineering and manufacturing translational targeted multi-therapeutic delivery systems with high levels of control.

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In loving memory of my hero and late father,
Kenneth Reid Morton;
For my cherished grandparents,
Tim Elmo Morton, Shirley Rose Morton, Giles Winford Clayton, Mary Pegram Clayton;
For my loving chocolate (Labrador) inspirations,
Sable and Sadie Mae;
For my supportive and kind older brother and best friend,
Christopher Reid Morton;
And for the best mom anyone could ask for,
Jill Clayton Morton.
Acknowledgements

This section is one of the most difficult to write because it is impossible to include everyone in the exhaustive list of people that made the work done in my Ph.D. possible.

First of all, I want to thank my lord and savior, Jesus Christ. “I can do all things through Him who strengthens me.” Philippians 4:13

Throughout this process, family has been my rock. My parents have always been my biggest supporters. Their sacrifice for my growth and success from a boy to a young man deserves more thanks than I can provide in this lifetime. Believe me... growing up, MIT would have meant “Mission Impossible: Trouble” for my years as a kid. I aspired to be a teenage mutant ninja turtle (not an engineer...), so you can imagine what sort of mischief this inspired. To be where I am at today is a true testament to the loving, caring, brilliant parents they are. From ball fields to the classroom, they were always there to support me in any way possible. My dad called me the “Rock” growing up (maybe because I was hard-headed and didn't listen), but it was my parents who were the true Rock’s in my life. I lost my father physically in December 2013, but I know he is and always will be with me spiritually through my journey in life. To my parents, Jill and the late Reid Morton – I love (and miss you, pops) you dearly.

I also want to thank my brother, Chris Morton, who has also been my best friend throughout my life. He is one of the most kind-hearted and giving individuals I know. He is also, more importantly, as excited to talk and play sports as I am – so special thanks to my big bro for the passionate sports banter on Tiger Woods and Braves/Sox baseball as well as many an hour on the links.

I also would like to thank my grandparents: Giles and Mary Clayton, Tim and Shirley Morton. I was fortunate to have three sets of parents growing up – spoiled by each as much as the other. I always looked forward to sick days at the grandparents where I was healed with homemade caramel cake and cinnamon toast while watching the Jungle Book or Sound of Music. They were so caring and giving – memories I will always cherish.

Of course, I cannot forget to mention the two sweetest, chocolate furry labs, Sable and Sadie Mae, that always energized me with each visit home.

So many teachers have motivated me through the years, from Mrs. Saunders and Mills in 6th grade to Kay Sandberg and Lisa Bullard at N.C. State. All of them have been advisers, friends, colleagues, and mother figures to me – challenging and inspiring me intellectually, developing me professionally, and shaping me as a young man. They set the standard for education – motivating so many each year yet maintaining personal relationships with each, and I am honored to keep them as lifelong friends in my continued journey.

There are too many friends to begin to enumerate them here, but specifically during my Ph.D. I would like to thank Ben Renner and Kevin Shopsowitz – two of my best and most
genuine friends, brothers by all but blood. Lots of Braves baseball talk and good times as roommates for 5 years with the “Sage” Ben Renner, and on the links and hanging out with Kevin usually around a “Morty-Shops” feast consisting of Shops’ homemade pizza dough topped with Morty’s Carolina smoked pork shoulder.

I also want to thank my fitness family – Elena Byrne, Melanie Smithers, Moses Blumenstiel, Stephanie Gayle, Vicky Palay, Izzy Weigner-Lodahl, Stacey Hutchins, Adrian Barnett, Richard Hamilton, Melissa Hyland, and Cindy Carter. Science has no shortage of its frustrations and taking it out in kickboxing, combat, pump, punishment, and jam with these awesome friends has been tremendous support to keep me motivated throughout my Ph.D.

I also want to thank my friends and colleagues in the Hammond lab, specifically Zhiyong Poon (P-dizzle), Nisarg Shah (Nate-dawg), Erik Dreaden (Dr. Dre), Jason Deng (Master Jedi), Mohi Quadir (Dr. Q), Kevin Shopsowitz (Shops), Liz Welch, Brian Aitken, Peter DeMuth, Santi Correa (triple-threat), Noemie Dorval (Noeminem), Nasim (the Dream) Hyder, Chibueze (the Breeze) Amanchukwu, Jouha Min, and Steven Castleberry (Huckleberry). It has been a great lab to work in and a fun ride these past 5 years. I also feel obliged to thank the Langer lab as an honorary lab member, attending last year’s Langer lab outing to the Cape as well as helping their team win the KF summer league. Cheers to Mark Tibbitt, Owen Fenton, Ben Tang, Katy Luly, and so many others for more good times.

Other collaborators include Joseph DeSimone, Kevin Herlihy, Kevin Reuter, Tammy Shen, Kevin Chu, Charles Bowerman – all from UNC-Chapel Hill and who were gracious hosts for 3 months while I worked in this exciting and brilliant lab; also Michael Yaffe, Michael Lee, Eilaf Ahmed, Tim Swager, Katharina Ribbeck, and Jeremiah Johnson from MIT – all of which deserve a heartfelt thanks for their help and support as colleagues and friends in this work. Ed Lawson, Peter Palmer, and their team at Janssen Pharmaceuticals were also instrumental to the success of this work as well as developing me professionally.

Above all collaborators at MIT, I want to extend a heartfelt thanks to my advisor and mentor, Paula Hammond. She is a caring, kind, understanding individual with a great heart and brilliant mind. I am truly indebted to her sacrifice and support as I grew professionally in her lab these past 4 years. I am blessed to have worked with a leading expert in the field of nanotechnology and drug delivery. Paula has been my rock at MIT, supporting me in many endeavors from traveling to the Chapel Hill for exciting work as well attending many national and international conferences to share the great work we have accomplished together. I will always cherish my time in her lab and cannot thank her enough for her leadership and friendship during my time at MIT.

I also want to thank my brilliant thesis committee members: Sangeeta Bhatia and Robert Langer, who were generous with their time to advise and support my work. It was difficult to get my entire committee, including Paula, in the same room with their insanely busy schedules, but when I was able to catch them all together I was fortunate to gain insight from 3 inspirational world experts in the field of nanotechnology and drug delivery – who are equally approachable and honest in their opinions and advice on work and growth as a professional (or where the cool new spots in town are).
Finally, I would like to thank the Koch Institute for Integrative Cancer Research at MIT (and MIT on the whole) for being such an incredible and stimulating place to work and collaborate. I could not ask for a better environment to learn, engage, and take action in the fight against cancer.

Heartfelt thanks,

Stephen Winford Morton
Massachusetts Institute of Technology
May 21, 2015
I, Stephen Winford Morton, would like to thank the generous funding sources that made possible all the work described herein: an NSF graduate research fellowship, Janssen Pharmaceutical TRANSCEND, P30 CA14051 (NCI), 5 U54-CA151884-02 and U54-CA151652 (CCNE), R01-AG029601 (NIA), and R01-EB010246 (NIBIB). U54-CA112967, R01-ES015339, and R21-ES020466, and a Breast Cancer Alliance Exceptional Project Grant. I would also like to thank assistance from the Koch Institute Swanson Biotechnology Center, specifically the Hope Babette Tang (1983) Histology Facility (Kathleen Cormier), the Applied Therapeutics and Whole Animal Imaging Facility (Scott Malstrom), the microscopy core (Eliza Vasile) and the Flow Cytometry Facility (Glenn Paradis, Michael Jennings); also, Bill DiNitale and Institute for Soldier Nanotechnologies, Center for Materials Science and Engineering (CMSE) at MIT, and Chapel Hill Analytical and Nanofabrication Laboratory (CHANL) for assistance and facilities that supported part of this work. I would also like to acknowledge UNC-Chapel Hill and Joseph DeSimone's lab in Chemistry, as well as Liquidia Technologies for graciously allowing me to work in their labs for an extended portion of my Ph.D., as well as generously supporting part of this work.
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Chapter 1. Introduction

Often in clinical settings, drugs are available that effectively treat a particular diseased state; however, the issue remains one of delivery. Free drug administration at the site of action is not always realizable; and in many cases, this form of treatment suffers from poor drug solubility, limited permeability, acute toxicity, and biological multi-drug resistance mechanisms that flush the cell of free drug influx via drug-efflux transmembrane pumps.[1] Further, diseased states are often only accessible via the blood supply, in lieu of invasive surgery, and may exist as metastases in different compartments in the body. Treatment, therefore, requires a systemically administrable formulation that can access these locations; however, introduction of free drug to the systemic environment results in undesirable pharmacokinetics, biodistribution, and lack of selectivity for particular tissues, yielding tissue damage or necrosis at off-target locations.

Drug delivery carriers on the nanoscale, ranging in approximate size from 10-200nm, are promising candidates for systemic delivery of therapeutic agents. These vehicles have been intelligently designed in many different forms, including polymer-drug conjugates, micelles, dendrimers, hydrogels, vesicles (liposomes, polymerosomes), and nanoparticles (polymeric, inorganic, magnetic).[2, 3] Nanocarriers of these types allow for incorporation of a variety of drug classes, including both hydrophobic and hydrophilic small molecules, proteins, and nucleic acids, via non-covalent encapsulation or covalent modification of the carrier material, and can enhance uptake into the tumor, increase specificity of targeting to cancer cells, and protect and maintain drug activity while mitigating off-target toxicity to healthy tissue.[4-8] In this way, they are able to load high concentrations of drug cargo and effectively stabilize them for delivery. This allows for enhanced bioavailability of the drug in circulation, such that the therapeutic window for access to an efficacious dose of drug is maintained for extended periods of time, overcoming the issues with multiple bolus injections of free drug formulations oscillating in and out of this regime, as shown in Figure 1.1.

While nanomaterials offer significant promise in stabilizing drugs for delivery, several of which have been successfully applied in the clinic[9], there remain a number of
challenges to engineering therapeutic nanoparticles for translation. These challenges include\(^{[10, 11]}\):

(1) robust methods for accurate characterization of nanoparticle size, shape, and composition;
(2) scalable approaches for producing nanomedicines with optimized bioavailability and excretion profiles;
(3) particle engineering for maintaining low levels of nonspecific cytotoxicity and sufficient stability during storage;
(4) optimization of surface chemistries for maximum targeted delivery and minimum nonspecific adsorption;
(5) practical methods for quantifying ligand density and distributions on multivalent nanocarriers;
(6) the design of multifunctional nanomedicines for novel combination therapies with supportable levels of bioaccumulation.

In particular for systemic application, a characteristic bolus drug release upon administration to physiological conditions in circulation is problematic for non-covalently encapsulated payloads. Alternatively, polymer-drug conjugates are generally of the size, \(<10\) nm, that are efficiently cleared via renal filtration, and those that do reach the target location are often unable to efficiently release their cargo for therapy because of the design constraints for stability in circulation versus triggered release at the tumor.\(^{[11]}\) Further, carriers must avoid rapid immune-mediated clearance via serum proteins which decorate foreign materials for phagocytosis and clearance by macrophages in the reticuloendothelial system, specifically Kupffer cells in the liver and red pulp cells in the spleen. Filtering on the basis of size is also employed by the fenestrated endothelium of the liver and sinusoidal endothelium of the spleen and is responsible for the clearance of larger particles (generally \(>200\) nm), while renal filtration clears smaller particles (generally \(<20\) nm) from circulation.\(^{[10, 11]}\) Towards this end, particles in the range of 20-200 nm decorated with a hydrophilic, neutral (i.e. poly(ethylene glycol)) or negatively-charged (i.e. hyaluronic acid) corona have been extensively investigated as delivery vehicles.\(^{[2, 8, 11, 12]}\) These have been shown to exhibit enhanced systemic stability with long-circulating half-lives and are also efficient at extravasating into tumor vasculature and accumulating on the basis of the
enhanced permeation and retention (EPR) effect. A passive targeting strategy, EPR-based targeting relies on the leaky, perforated vasculature that results from rapid proliferation of cancerous cells beyond their ability to generate an adequate blood supply, as shown in Figure 1.2. Further, the lymphatic drainage is defective such that particles are able to accumulate in the tumor region without being effectively removed. Active targeting can enhance delivery via cancer cell ligand-receptor interactions, such that delivery is increased to target tissue. Targeted delivery allows for lower doses of drugs to be administered, thereby mitigating adverse side effects while increasing therapeutic potency.\[8,13\] Furthermore, other nanoparticle parameters such as shape and elasticity are critical to the performance of a nanoparticle system for systemic delivery applications.\[14-20\]

After designing a carrier that is both serum-stable and able to accumulate in the tumor region, the challenge of cellular entry with the drug-loaded platform is the next barrier to delivery, particularly for payloads, such as nucleic acids, that require cellular entry but have no means of facilitating internalization independently and are readily degraded without a delivery material host.\[21\] Positively-charged materials are extremely effective at interacting with the negatively-charged glycocalyx on the surface of cell membranes and trafficking into the cell by fluidizing the bilayer, which often also yields a cytotoxic effect. However, positively-charged materials are not stable in circulation and are rapidly cleared and often cytotoxic, therefore not a viable systemic delivery carrier. For serum-stable particles, which are neutral or negatively-charged and with or without the complement of a targeting ligand, these particles rely on the process of pinocytosis ("cellular gulps") or receptor-mediated endocytosis, which can be quite effective at internalizing carriers with size-dependence. The latter introduces the challenge of endosomal escape for the drug to reach its therapeutic target. It is believed that a proton-sponge effect is necessary to mediate endosomal rupture, requiring a positively-charged material to introduce a proton gradient across the endosome such that it bursts and releases the drug; however, the acidic pH environment may degrade or inactivate the drug of interest before it is able to function.\[21,22\]

The paradoxical nature of serum-stability and cellular entry has generated interest in engineering "smart" particles that possess serum-stable properties that are unmasked in the tumor microenvironment. This complementary approach to traditional passively-
accumulating or actively-targeted carriers exploits the nature of tumor progression. For this purpose, designs hone in on the acidity of the localized tumor region, which results from the poorly oxygenated (hypoxic) regions at the tumor due to inadequate vascularization. Stealth particles that have a cue-sensitive (i.e. pH-sheddable) cloak are an emerging technology to enhance delivery for this purpose.\textsuperscript{[2, 21, 23-26]}

The surface chemistry of nanoparticles plays a major role in determining their biological properties. However, many of the current methodologies used to modify the surfaces of nanoparticles are inefficient and labor intensive. Layer-by-layer (LbL) polyelectrolyte deposition is an attractive process for incorporating functionality onto the surfaces of nanocarriers to provide improved stability, enhanced cellular uptake, and targeting capabilities.\textsuperscript{[24, 27-29]} Because small molecule drugs and biologics such as nucleic acids can be included in the nanolayers, it also affords the ability to incorporate additional therapeutics that act as synergistic drug combinations\textsuperscript{[30, 31]} on a single nanoscopic platform and in a fashion such that the release of each can be programmed.\textsuperscript{[32, 33]} Layer-by-Layer (LbL) assembly\textsuperscript{[34]} of polyelectrolytes on solid substrates is a well-characterized, tunable approach\textsuperscript{[35]} for generating functional thin films on the basis of complementary interactions, such as electrostatics or hydrogen bonding, for a variety of applications, including drug and gene delivery\textsuperscript{[28, 36, 37]} and tissue engineering.\textsuperscript{[38, 39]} For these applications, this technique is advantageous in that the incorporation of a broad range of therapeutics (e.g., proteins, nucleic acids, and small-molecule drugs) and is carried out under physiological conditions, non-covalently, thereby preserving the native properties of the cargo while stabilizing it for delivery. Furthermore, surface-limited, sequential adsorption affords nano-scale precision over the composition of each layer; these advantages, when combined with the ability to incorporate a broad range of therapeutics and materials with diverse functionalities, greatly facilitate the development of drug delivery platforms with sophisticated control over the spatial and temporal release of film constituents, allowing for the construction of a customizable therapeutic delivery platform\textsuperscript{[40-43].}

While LbL has been successfully implemented on macroscopic surfaces with great promise for many biomedical applications for many years\textsuperscript{[28, 43-53]}, it has only been more recently established as a viable tool to coat nanoscale materials systems\textsuperscript{[24, 27, 28, 43, 54-75]} to
achieve particles that can be sustained for systemic delivery. The ability to generate conformal LbL coatings on nanoscale templates opened new and exciting opportunities for nanoparticle technologies with applications in drug and nucleic acid delivery\cite{76-87}; however, the translation of LbL-based nanocarriers for systemic applications required further examination of how LbL film architectures can be manipulated to overcome systemic delivery barriers and to sustain drug delivery in such complex biological settings. Previously, we investigated the impact of different nanoparticle-bound film architectures on physiological stability, elucidating key control variables necessary to generate a serum-stable particle, as well as the effect of terminal polymer layers on subsequent biodistribution of the nanocarriers\cite{27-1}. LbL architectures that act as highly effective stabilizing layers were demonstrated to impart long circulation times and EPR-based passive targeting to the resulting systems\cite{88}. We have also developed dynamic LbL layers that ‘shed’ at the lower pH of hypoxic tumor microenvironments and subsequently reveal a positively charged surface that rapidly mediates uptake by tumor cells\cite{24}. Similar demonstrations of dynamic LbL particle design for tumor targeting\cite{25,72,74} along with the ability of LbL nanofilms to deliver multiple constituents for combination therapies\cite{71,89} and to modulate drug and carrier biodistribution, lay a framework for continued development of LbL-based nanoparticles towards scalable, built-to-order systems\cite{75} that are key for unlocking new biomedical opportunities for these platforms – detailed in the subsequent Chapters 2 – 4.

Further work detailed herein (Chapters 5 – 7) focuses on the use of liposomal technologies to deliver combinations of small molecule therapeutics with vastly different solubilities (hydrophobic-hydrophilic small molecules) that are readily compartmentalized within the liposomal structure\cite{90,91} – a limitation discovered experimentally to the rapid and scalable deployment of LbL nanoparticles for this purpose (issues with achieving therapeutic loadings of both drugs). Combination treatments have been explored for more intransigent tumor types such as triple negative breast cancer (TNBC), but with little significant gain thus far reported\cite{92-94}. Similarly, non-small cell lung cancer (NSCLC) is often non-responsive to single chemotherapy treatments, and is prone to recurrence. Although numerous drug combinations have been examined in oncology in the past, in almost all cases, these drugs were administered simultaneously in simple combinations,
without manipulation of drug presentation, scheduling and duration of dose; recent work\cite{95-97} further supports that many combinations of previously screened existing drugs may be highly potent if combined in the correct order and relative dose. To capitalize on the importance of order and timing for multi-drug therapies (Chapter 5) in complex cellular systems, it is key to devise a means of a) enabling bioavailability of the drugs, including hydrophobic small molecule inhibitors; b) targeting each of the drugs with equal or similar efficacy to the tumor; and c) releasing the drug in a manner that enables temporally-controlled release of each agent.

To achieve this within a single nanoparticle platform, liposomes, which have been investigated extensively and approved for several cancer treatments,$^{98-101}$ were employed. Previous research has illustrated the versatility of these systems, ranging from demonstrated pH responsive behavior to the development of theranostic systems$^{102,103}$. Furthermore, these systems were particularly attractive due to their inherent ability to compartmentalize vastly disparate drugs to enable encapsulation and delivery of multiple drug types without the need for two or more independent drug formulations that may undergo differences in pharmacokinetics and uptake. Recent studies have shown that only 25% of cells contain plasmids delivered from two co-administered nanoparticles$^{104}$, indicating that uptake of both drugs by the same cell cannot be presumed. Furthermore, the injection of each nanoparticle drug formulation at different times to achieve staging can lead to increased cost and more complex dosing schedules that are inconvenient for the patient. A more critical concern in the use of two separate nanoparticle formulations is the potential change in vascularity and tumor tissue morphology due to the first drug, which may impede delivery of the second nanoparticle system to the same tumor cells. Finally, increased treatments mean higher opportunity for systemic toxicity from the nanocarrier. For this reason, multi-therapeutic liposomal formulations were investigated as the nanoparticle basis for work described in Chapters 5 – 7.
Figure 1.1. Therapeutic window following drug administration. Blue = ideal drug nanocarrier formulation. Red = repeated bolus injection (arrow) of free drug formulations.
Figure 1.2. Passive (EPR-based) and active (receptor-ligand specificity) targeting strategies.\textsuperscript{[9]}
References

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This chapter is in part adapted from:

Introduction

As LbL nanoparticle technology moves towards clinical translation, it is essential to establish these systems as drug-stabilizing carriers; the focus of this work is on small molecule therapeutics. The challenges and approaches to small molecule delivery are well documented; characteristic bolus release upon injection is one of the most significant limitations to efficacious treatment for this class of therapeutics. Once it is freed from the carrier, drug is often rapidly cleared, reducing its plasma concentration, and producing significant off-target cytotoxicity. The ability to control small molecule release and drug distribution at a cellular and tissue level is, therefore, an active area of investigation.

Little information regarding the real-time fate of small molecule release from delivery platforms in vivo is understood following systemic administration. The current work seeks to demonstrate LbL nanoparticle material systems as viable candidates for small molecule drug delivery, as well as to develop a robust, systematic approach for screening a library of materials for incorporation in these engineered systems. LbL films have been previously demonstrated as effective small molecule delivery agents with an enhanced level of control over release. This work examines LbL architectures as dually functional films on nanoparticle surfaces – acting as membranes that control rate of drug release from the nanoparticle core and thus impact pharmacokinetics of the drug, as well as the hydrated, protein-resistive coatings that modulate blood circulation half-life and biodistribution for both the drug and carrier. The current study probes a series of nanoparticle architectures assembled on a biodegradable poly(lactic-co-glycolic acid) (PLGA) drug-loaded nanoparticle core. Using in vivo imaging for simultaneous drug and particle fluorescence tracking in vivo, this work provides a framework for assessment of LbL nanoparticles as small molecule delivery agents. This technique for live animal imaging is a convenient and robust means of probing delivery pharmacokinetics and
biodistribution. It affords the capability of drug and particle monitoring following administration to a single animal, allowing for high throughput in vivo screening of delivery systems. The current study demonstrates this capacity, evaluating LbL nanoparticles using multiple indicators of stability and performance, in an effort to advance the technology towards therapeutic settings.

**Materials and Methods**

All chemicals were purchased from Sigma-Aldrich, except for hyaluronic acid (Lifecore Biomedical) and doxorubicin-HCl (LC Laboratories). Release dialysis float-a-lyzers were purchased from Spectrum Laboratories. NCR nude and BALB/C female mice were furnished by Taconic.

**Nanoparticle Synthesis.** PLGA particles were prepared under aseptic conditions via an emulsification-diffusion method with slight modifications to a previously established protocol.[106] Briefly, 50 mg PLGA was dissolved in 3 mL acetone with 0.5 mL of 10mg/mL drug (cardiogreen/doxorubicin) in methanol. This solution was subsequently added to 10mL 10% BSA in PBS and sonicated at RT. The emulsion was stirred overnight at 1000 rpm. The resulting particle suspension was purified via centrifugation (15,000 g, 30 min) prior to LbL assembly. LbL assembly was conducted by introducing ~10% of the recovered drug-loaded nanoparticles in excess polyelectrolyte solution (5 mg/mL for PLL, DXS; 1mg/mL for HA, Alg) at pH 7.4 under agitation for 30 min. The particle suspension was purified from the polyelectrolyte solution via centrifugation (15,000 g, 30 min), and this process was repeated iteratively for deposition of each layer. Final particle suspensions were characterized by dynamic light scattering (DLS) and zeta potential analysis (Malvern Instruments, ZS90) and stored at 4°C prior to testing.

**in vitro Drug Release.** Particle formulations were suspended in 1X PBS and incubated in 2 mL dialysis float-a-lyzers (3.5KDa MWCO) while agitated at 37°C in 1X PBS under sink conditions. Small aliquots were collected at various time points, replaced with fresh
solution, and analyzed by high-performance liquid chromatography (HPLC, Agilent Technologies).

**in vivo experimentation.** For biodistribution experiments, NCR nude female mice (Taconic) were used. To attenuate gut fluorescence, an alfalfa-free special diet (AIN-93M Maintenance Purified Diet from TestDiet) was administered to the mice 1 week prior to and during experimentation. Nanoparticle formulations suspended in 1X PBS were administered via the tail vein. *In vivo* imaging (IVIS, Caliper Instruments) was performed at regular time points. Full scale biodistribution harvesting of relevant organs was conducted at a relevant time point and imaged using the IVIS to quantify recovered fluorescence. Circulation persistence experiments were performed in BALB/c female mice from Taconic. Nanoparticle formulations were administered via the tail vein. Blood collection was obtained from the retro-orbital sinus (~0.2-0.3mL, diluted in ~0.1mL 0.5 M EDTA) and imaged using the IVIS to quantify recovered fluorescence. For cardiogreen recovery experiments, feces were recovered from three BALB/c mice per experimental group and fractional recovered fluorescence (IVIS) was quantified across a 48 h window. For co-injection experiments, free polymer corresponding to the terminal layer polymer used for the NP architecture being investigated were diluted in PBS and co-injected at normalized concentrations of particles against 10 mg/kg and 20 mg/kg free polymer in NCR mice. Experiments were performed under the guidance and supervision of the MIT Department of Comparative Medicine and Committee on Animal Care.

**in vitro opsonization experimentation.** Texas Red-labeled polymer nanoparticles (company) were incubated with fluorescently labeled protein (Human IgG-FITC, Sigma) for 1h. Bound protein was quantified using a nanodrop spectrophotometer (GE Nanovue Plus). These particles were subsequently incubated with the murine macrophage cell line (J774A.1, ATCC) for 1h and cell-associated fluorescence was quantified via fluorescence-activated cell sorting (BD LSR II). Geometric mean cell-associated fluorescence was used for display of particle association with the cells.
Results and Discussion
The schematic for generating films using electrostatic LbL assembly is presented in Figure 2.1.A. The PLGA core particles used in this work are generated from carboxylic acid terminated PLGA, and present a net negative charge on their surfaces, which provide a means for initiating LbL assembly. Iterative adsorption of alternately charged polyelectrolytes is accomplished by incubation of particles in aqueous polycation and polyanion solutions, with intermediate rinse steps, to generate LbL nanoparticles. Although this study focuses on the materials tabulated in Appendix A.T, a much larger library of film components, along with a diverse range of therapeutics, can be generated for incorporation on this platform. Towards this end, materials with relevant biological functionalities are of particular interest, such as negatively-charged linear polysaccharides including hyaluronic acid (HA), dextran sulfate (DXS), and alginate (Alg). Each of these polysaccharides have demonstrated anti-fouling properties as hydrophilic brush-like hydrated coatings to minimize protein adsorption and opsonization\cite{107-110}; as such, they present promising biodegradable, biomimetic alternatives to the traditional and widely-reported use of poly(ethylene glycol)\cite{111}. Further, HA is a known ligand for CD44 receptors\cite{112}, which are characteristically overexpressed in many aggressive cancer cell types, including triple-negative breast cancer.\cite{113} Alg is known to be an essential protective coating for pathogenic bacteria to evade host immune responses.\cite{114} Each of these materials has been incorporated in the current study.

Characterization of the resulting LbL particles is shown with regard to particle hydrodynamic size and charge in Figures 2.1.B and 2.1.C. Following deposition of six layers on a 125 nm diameter polymer core, particles range in hydrodynamic size from 180nm for DXS-terminated NPs to 250nm for Alg-terminated NPs, based on a number distribution. The dynamic light scattering data taken as a function of the number of deposited bilayers indicates that the nanoparticles grow at a rate of approximately 18 nm in diameter with each bilayer pair, corresponding to nanolayer thickness increases of 9 nm per adsorbed polymer pair, over the first five adsorbed layers. This relatively linear growth is consistent with traditional LbL processes\cite{115}. The three different LbL film architectures are similar in size until the sixth and final adsorbed polyanion layer.
DXS-terminated LbL nanoparticles continue to grow linearly; whereas the particles terminated with HA and Alg both indicate a significant increase in thickness with the final adsorbed layer, which is 55 and 77 nm, respectively. In LbL assembly, it has been observed that the thickness and density of the layer deposited is highly dependent on pH for weak polyelectrolytes due to changes in the degree of ionization along the backbone.[116] Furthermore, certain weakly charged polyelectrolytes, including hyaluronic acid, can undergo exponential growth behavior due to interdiffusion of the absorbing species above a critical thickness.[117,118] This increased increment may be further impacted by the high degree of hydration of the high molecular weight HA (500K MW) and Alg (200K MW) backbones, and to the partially charged nature of the carboxylic acid groups along the glycopolymer backbone. The surface charge of the particles is reversed with each deposition step, confirming that the film is building in a stepwise fashion driven by alternating electrostatic adsorption. The overall surface potential was (+/-) 50-60mV (in ultrapure 18 MΩ water, 25°C), independent of terminal layer.

Previous work established a minimum number of four layers for generation of serum-stable particles, with diminishing returns beyond eight layers.[27] Independent optimization conducted for the current PLGA-based particle template was found to be consistent with these findings; therefore, this study reports data from six-layered (6L) particles with the following nanoparticle architecture: a biodegradable PLGA nanoparticle core (acid-terminal, hydrodynamic diameter = 125nm; ζ-potential = -50mV) containing a drug, which is layered with polycationic/polyanionic pairs deposited iteratively on top of this substrate, terminated with a final polyanion. The systems were assembled with poly-L-lysine (PLL) as the polycation and DXS as the intermediate polyanion, and are indicated as PLGA_{drug}/(PLL/DXS)_{2}/PLL/X_{term}, where X_{term} is either HA, Alg, or DXS.

To facilitate our investigation of the in vivo fate of drug cargo after systemic circulation, we sought a good candidate for a conventional chemotherapeutic, doxorubicin (logP (pH 7.4) = 1.27), which inhibits topoisomerase II, resulting in DNA damage thus disabling cellular replication. The model drug chosen for systematic stability assessments of different nanoparticle architectures is cardiogreen (CG, λ_{ex} = 740nm, λ_{em} = 820nm, logP (pH 7.4) = -0.29), a hydrophilic near-IR dye that absorbs light at 820nm, where there is
minimal whole-animal auto-fluorescence. The fundamental chemical characteristics of both small molecules are also similar; they are hydrophilic with low molecular weights (543.5 g/mol for doxorubicin and 775g/mol for CG) and are significantly charged ($pI_{CC} = 3.3; pI_{Dox} = 11$) under physiological conditions with water solubilities in the range of 1mg/mL and similar lipophilicities ($logP_{CC} = -0.29; logP_{Dox} = 1.27; pH 7.4$). Additionally, CG is cleared from circulation readily, with a serum half-life of 2-3 minutes (compared to 10 minutes for doxorubicin), and in a manner that traffics exclusively via the liver, such that its clearance from the body can be monitored in the liver and gall bladder via in vivo imaging, and corroborated with recovered fluorescence from the feces in addition to the relevant biodistribution and blood sampling. These advantageous properties make CG a particularly good choice to model the in vivo fate of small molecule therapeutics in systemic delivery carriers, as multiple readouts, including biodistribution, persistence data via blood and feces collection, are generated from a single animal to guide particle engineering.

To demonstrate the similarity of CG to conventional small molecule chemotherapeutics, release profiles for both doxorubicin and CG were determined for the uncoated NP core as well as the layered LbL nanoparticle architectures under physiological conditions ($37^\circ C$, stirred in large excess of 1X phosphate buffered saline (PBS)) using high performance liquid chromatography (HPLC) to determine quantitative and fractional release. The resulting release curves for these two molecules are compared in Figures 2.2.A and 2.2.B. The in vitro release characteristics are similar, with both exhibiting the same general release profiles and similar fractional release in the coated (60% released after 48h, following from initial drug loadings of 10ug/mL for CG-loaded NP formulations and 50ug/mL for Dox-loaded NP formulations) and uncoated (100% released after 48h) versions, proving CG to be an effective model of doxorubicin for the demonstration of LbL NP technologies as small molecule delivery agents. The observed release kinetics is typical of biodegradable particle cores and consistent with mathematical models accounting for small molecule diffusion-based release (initial logarithmic-phase release) and bulk erosion (inflection after initial diffusion-based release plateaus) of the PLGA matrix. The characteristic diffusion-erosion based release is observed for both coated and uncoated systems; however, the LbL coated systems show significant benefit in small molecule sequestration, minimizing the characteristic bolus release observed initially (from $\sim 35\%$
uncoated core to ~20% coated after 1h), and sustaining release of the therapeutic over longer time scales of up to 4-5 days in vitro. Greater control and modulation of bolus release is important to diminish systemic toxicity while the nanoparticle is en route to the tumor site, particularly for cytotoxic chemotherapeutics. Extended release of drug from the nanoparticle once it has reached its target location may also have therapeutic value for certain types of molecular drugs. The variable terminal layer had little to no effect on overall release profiles, despite the range of functionalities and molecular weights used for the end-capping layer. Drug release from the NP is characteristic of the bulk film properties of the LbL multilayer, and relatively independent of the end-capping layer. It thus appears that the terminal layer primarily serves as a functional interface directing the biodistribution and blood circulation behavior of the delivery system, while the bulk film impacts the pharmacokinetics of the encapsulated therapeutic. These findings suggest the LbL nanoparticle technology can provide a modular platform for systemic drug delivery.

To understand the impact of these particle architectures on the pharmacokinetics of the model drug and nanocarrier used for delivery, simultaneous tracking of both the near-IR labeled polycationic layer 1, PLL700 (modified with Cy5.5 dye, λex,max = 673 nm, λem,max = 707 nm), and encapsulated model drug, CG, was conducted using in vivo imaging (IVIS) following systemic administration via the tail vein. This approach to systematically screen libraries of LbL NP architectures as therapeutic vehicles is an exciting, novel means of generating multiple readouts, including biodistribution and persistence in the animal (blood, feces sampling), from longitudinal studies of animals. In this way, nanoparticle stability and its pharmacokinetic behavior is assessed based on persistence in circulation and biodistribution over time at λex = 640 nm, λem = 700 nm (PLL700 channel), while drug stability and pharmacokinetics is also evaluated by biodistribution and circulation data, as well as recovered drug fluorescence in feces as a function of time at λex = 745 nm, λem = 820 nm (CG820 channel). Due to the hepatic-specific clearance of CG, the feces serve as a quantitative measure of drug clearance from the animal to further assess drug bioavailability as a function of time with each NP system investigated.

The IVIS images achieved for biodistribution studies are shown in Figure 2.3 for both the drug (3A) and NP (3B) channel. Significant differences in the accumulated signal intensities were observed for different formulations of the layered architectures in the
liver, relative to the controls, within the first 30 minutes post-administration on both the PLL700 and CG820 channels. Normalization of the liver-associated CG fluorescence (circled and identified with an arrow) with total fluorescence from the amount injected, shows significant reduction of CG accumulation in the liver when administered as cargo in LbL-NPs versus free or uncoated PLGA NPs. The quantified signal for liver accumulation at 5 minutes post-injection for the free drug (70%) and uncoated nanoparticle (55%) formulations are much higher when compared to the layered architectures, Alg-term (15%), HA-term (17%), and DXS-term (28%). At the carrier channel for PLL700, 50% injected dose is associated with the liver after 5 minutes for free PLL700, as compared to 12% for Alg-terminated 6L NPs, 15% for HA500K-terminated, 25% for DXS-terminated. The full 48h stability panels at both channels are shown in Appendix A.2-3, with quantification of the total radiant efficiency from the whole animal as a function of time displayed for the panel of images at each channel to gauge persistence of the individual NP components (drug, particle). These data reflect the improved biodistribution and overall enhanced persistence of the coated CG-loaded NP formulations, relative to the uncoated polymer-encapsulated and free drug formulations.

The results from in vivo whole-animal imaging were further substantiated by harvesting of the relevant clearance organs. This biodistribution data was collected 30 minutes post-administration (Figures 2.4.A and 2.4.B, Appendix A.4.). Significantly reduced drug clearance is evident for the coated systems within the first 30 minutes post-injection, with 55% ID/g associated with the liver after 30 minutes for free cardiogreen, as compared to 43% for the uncoated PLGA CG-loaded core, 33% for DXS-terminated, 18% for HA-terminated, and 20% Alg-terminated systems. Layered architectures also exhibit minimal clearance during this time frame as delivery carriers, with 70% ID/g associated with the liver for control free PLL700, 32% for DXS-terminated, 20% for Alg-terminated systems, and 22% for HA-terminated systems. The similarity in percent recovery of fluorescence between the particle and drug channel from this biodistribution data, along with the in vivo images in Figure 2.3, illustrate co-localization of the drug with the delivery platform for the initial period post-administration (up to 4h); however, as shown in Appendix A.2 and A.3, this does not hold at long time points. At 8h and beyond, due to
small molecule out-diffusion ("leakage") from the LbL NP and subsequent bulk-erosion based release observed \textit{in vitro}, the particle and drug localize in the liver at different times.

Further, recovered fluorescence from the feces can also serve as readouts for drug clearance in the animal. As shown in Figure 2.4.C, CG is recovered in the bile following longer time intervals for the coated systems. The DXS-terminated system peaks in fractional CG recovery at only the 4-8h time span post-injection as compared to Alg- and HA-terminated systems, which peak in the 8-12h time period. This further corroborates the enhanced persistence and drug bioavailability that the coated delivery systems can promote.

To try to rationalize the observed differences in feces drug recovery and biodistribution of DXS-terminated systems as compared to the other two coated systems, we hypothesized that, as previously implicated,\cite{1,24,125} the sulfonated polysaccharide dextran sulfate preferentially binds liver receptors (e.g. liver endothelial cells, Kupffer cells) that scavenge blood for soluble macromolecules. From the biodistribution and feces recovery results, it is clear that HA- and Alg-terminated systems delay particle and drug clearance as well as exhibit improved biodistribution. These results implicate that this potential receptor-mediated mechanism for clearance impacts drug biodistribution as well as that for the carrier agent. This is further illustrated by co-injection of free terminal layer polymer with the corresponding polymer-terminated 6-layered NP. Significant reduction in CG signal was observed only when free DXS was co-injected with the DXS/sulfonate-terminated NPs, as evident in Figure 2.4D. Free polymer competition for receptor-binding suggests that the sulfonated terminal layer preferentially interacts with the liver endothelial cells,\cite{125,126} promoting receptor-mediated endocytosis that significantly affects the clearance of DXS-terminated LbL particles. The differential impact of a co-injection of free terminal layer polymer was quantified by normalizing the liver-associated fluorescence obtained from co-injection studies with signal intensities for particle-only studies (30 minute stability panel shown in Appendix A.5.). No effect was observed for corresponding co-injections with Alg- and HA-terminated systems, in contrast to DXS-terminated nanoparticles, with liver-associated uptake, implying receptor-independent liver uptake.
Additionally, in vitro opsonization of the different NP architectures (Appendix A.8.) showed little dependence on protein adsorption and no dependence on subsequent macrophage-associated fluorescence as a function of terminal layer choice, further suggesting that all coatings have similar protein resistive properties and that the mononuclear phagocyte system clearance is likely not the primary reason for the different liver signal intensities (respective to uncoated and PLL controls). Poorer biodistribution of the DXS-terminated particles results relative to other coatings guided particle and drug persistence experiments for only the more optimal systems, HA- and Alg-terminated particles. Results on drug and carrier stability in circulation for these systems are presented in Figures 2.4.E and 2.4.F (raw data shown in Appendix A.6.). The particles exhibit improved pharmacokinetic properties in circulation, with Alg-terminated NPs characterized by half-lives, based on a two-compartment model, of 0.59h and 7.18h and HA-terminated NPs characterized by half-lives of 0.50h and 7.861h. The lifetime of drug from these platforms is also, compared to the reported free drug half-life of 2 min and uncoated PLGA core half-lives of 0.15h and 1.87h, extended to 0.52h and 4.17h for Alg-terminated NPs and, for HA-terminated NPs, to 0.48h and 4.54h (based on a 2-compartment model).

These findings are consistent with previously reported PEGylated versions of PLGA nanoparticles that show 30-65% particles remaining (for various MW PEG shells) in serum 3h post-i.v. administration with liver-associated clearance of the particles remaining at or below 20% up to 5h post-administration.[127] This is important because, in addition to imparting similar hydration properties to the particle surface like PEG, LbL can also provide a surface that is molecularly targeted for cellular engagement and/or cue-sensitive (e.g. pH-responsive[241]) for exploiting the cellular environment in hypoxic tumor compartments. Alternatively, PEG can be used exclusively as a hydration layer for minimizing protein adsorption, opsonization, and subsequent clearance by the major players in the mononuclear phagocyte system (MPS) system – a property that in fact can also negatively impact interactions with target cells.[128,129] These findings clearly highlight the benefit of LbL-functionalized nanoparticle systems as extended release platforms, particularly for small molecule therapeutics, allowing for enhanced drug and particle persistence in circulation and that promote hydrated, protein-resistive properties for more
efficacious systemic drug delivery. This provides much promise towards development of these systems as molecularly targeted entities, in addition to improved biological performance observed in this investigation.

Small molecule sequestration in delivery platforms for extended periods in circulation remains a significant challenge to selective delivery of therapeutics to the target tissue; however, the coatings investigated here demonstrate an enhanced level of control over diffusional bolus release following administration, as well as an ability to enhance the circulation half-life for longer periods of time in vivo. The findings in this investigation, namely the improved pharmacokinetic behavior observed with Alg- and HA-terminated NPs via biodistribution, feces drug recovery, and persistence in circulation, are consistent with documented anti-fouling characteristics of HA[130-132] and Alg.[110, 125, 133] These systems present exciting new opportunities for enhancing delivery of small molecule therapeutics; however, due to the accelerated rate at which the uncoated PLGA drug-loaded core is hydrolytically degraded and unloaded in circulation (with short half-lives and poor biodistribution, see Appendix A.7.), the LbL system can be further adapted to different core materials and encapsulation approaches (e.g. covalent attachment of therapeutics to core materials or film components), which is currently under active investigation in our laboratory to probe whether the benefits of enhanced persistence, improved biodistribution, and controlled drug release provided by LbL-coated systems will promote more efficacious EPR-based and molecular targeting strategies.

Conclusion

LbL is a facile approach for generating functional thin films for enhanced systemic delivery of nanoscopic systems. This self-assembly method allows for incorporation of a broad range of materials; and due to its water-based synthesis, it allows for incorporation of a diverse set of therapeutics without significant alteration of biological function. To facilitate continued development of these systems, the current study establishes a two color imaging methodology that enables efficient, high throughput in vivo screening of a library of material architectures and establishes each as systemic drug carriers. Using live animal in vivo imaging, multiple readouts, including biodistribution, persistence, and feces drug
recovery can be performed, longitudinally assessing *in vivo* fate of both particle and drug in a single animal model. Further, this work demonstrates LbL-drug delivery particles with enhanced persistence and improved drug/carrier biodistribution using biomimetic alternatives to poly(ethylene glycol), specifically Alg and HA, as terminal layers for this NP delivery vehicle. In total, LbL provides a powerful tool to engineer NP systems that control drug release while incorporating materials that are dynamic, protein-resistive, and molecularly-targeted for more efficacious systemic treatment of diseased states.
Figure 2.1. Synthesis and characterization of a Layer-by-Layer PLGA nanoparticle (LbL NP) system with cardiogreen encapsulated for multi-color imaging. (A) LbL deposition on NP template. (B) Materials library used in current study. (C) Representative mean hydrodynamic diameter (based on a number distribution) and (D) zeta potential analysis, conducted in ultrapure water at 25°C. All particles in current study are 6 layers (6L) with the following NP architecture: PLGA_{50:50}\text{drug}/(PLL/DXS)_2/PLL/X_{term}, with drug = doxorubicin (Dox) or cardiogreen (CG) and X_{term} = terminal layer (DXS, Alg, HA_{500K}). (PLGA) poly(lactic-co-glycolic acid), (PLL) poly(L-lysine), (DXS) dextran sulfate, (Alg) alginate, (HA) hyaluronic acid; n = 3, mean +/- SEM. PDI of uncoated PLGA NPs is 0.1; functionalized NPs ranged from 0.15 for DXS-terminated NPs to 0.2-0.25 for HA-coated and Alg-coated NPs. Representative scanning electron micrograph of LbL NPs (HA-coated) shown in Appendix A.1.
Figure 2.2. In vitro release of uncoated and coated cargo-loaded (cardiogreen, doxorubicin) PLGA LbL NPs. (A) Cardiogreen (CG)-loaded NP formulations and (B) Doxorubicin (Dox)-loaded NP formulations in PBS, pH 7.4 at 37°C under agitation (3.5kDa MWCO) as measured by high-performance liquid chromatography (HPLC). (PLGA) poly(lactic-co-glycolic acid), (PLL) poly(L-lysine), (DXS) dextran sulfate, (Alg) alginate, (HA) hyaluronic acid, (CG) cardiogreen, (Dox) doxorubicin; n = 3, mean +/- SEM.
Figure 2.3. Stability assessments of different LbL PLGA nanoparticle architectures assessed by drug (CG<sub>820</sub>) and particle (PLL<sub>700</sub>) tracking via in vivo whole animal fluorescent imaging.

(top of each image panel) Schematic detailing the fluorescent trackers used for stability assessment of NP architectures. CG<sub>820</sub> is the cargo encapsulated in the LbL NP architecture with the first polycationic layer of the 3 bilayer structure labeled with Cy5.5. (A) CG<sub>820</sub> stability panel, surveying drug bioavailability up to 30 minutes post-injection. (i) free CG<sub>820</sub>; (ii) PLGA<sub>50:50</sub>CG; (iii) HA-terminated 6L NP; (iv) Alg-terminated 6L NP; (v) DXS-terminated 6L NP. Representative 48 h study shown in Appendix A.2. IVIS images at CG channel (λ<sub>ex</sub> = 745 nm, λ<sub>em</sub> = 820 nm), surveyed up to 30 min and subsequently opened. Region of interest analysis shows ~70% injected dose associated with the liver (white dashed circle identified with an arrow) after 5 minutes for free CG, ~55% for PLGA<sub>50:50</sub>CG, ~28% for DXS-terminated and ~15% for Alg- and HA<sub>500K</sub>-terminated 6L NPs; n = 3. (B) Carrier stability panel, surveying nanoparticle biodistribution up to 30 minutes post-injection. (i) free PLL<sub>700</sub>; (ii) DXS-terminated 6L NP; (iii) HA-terminated 6L NP; (iv) Alg-terminated 6L NP. Nanoparticle tracking facilitated by labeling of first polycationic layer (PLL) in 6L NP architecture with Cy5.5. Representative 48 h study shown in Appendix A.3. IVIS images at PLL<sub>700</sub> carrier channel (λ<sub>ex</sub> = 640 nm, λ<sub>em</sub> = 700 nm) were surveyed up to 30 minutes. Region of interest analysis yields ~50% injected dose associated with the liver (white dashed circle identified with an arrow) after 5 minutes for free PLL<sub>700</sub> as compared to ~25% for DXS-terminated, ~15% for HA<sub>500K</sub>-terminated, ~12% for Alg-terminated 6L NPs. (PLGA) poly(lactic-co-glycolic acid), (PLL) poly(L-lysine), (DXS) dextran sulfate, (Alg) alginate, (HA) hyaluronic acid, (CG) cardiogreen; n = 3.
**Figure 2.4.** Pharmacokinetic data for the PLGA LbL nanocarriers (PLL700, CG820), as a function of terminal layer and the encapsulated drug, CG820.

(A,B) Biodistribution profile of carrier via PLL700 tracking (λex = 640 nm, λem = 700 nm and CG820 (λex = 745 nm, λem = 820 nm) 30 minutes post-injection (n=3). Raw data shown in Appendix A.4. (C) Profile of CG recovery in murine feces (3 total mice feces/experimental group). λex = 745 nm, λem = 820 nm. Data presented as fraction initial dose over a 48h period. (D) Dose-dependent co-injection of free polymer with corresponding polymer-terminated 6L NP. Liver-associated CG820 fluorescence (λex = 745 nm, λem = 820 nm) collected 15 min following iv administration. Data normalized to average value associated with 6L NP without free polymer co-injection. Raw data shown in Appendix A.5. (E,F) Circulation profile of CG820-loaded NP formulations for both the carrier (λex = 640 nm, λem = 700 nm and encapsulated drug (CG820). Raw data shown in Appendix A.6. (PLGA) poly(lactic-co-glycolic acid), (PLL) poly(L-lysine), (DXS) dextran sulfate, (Alg) alginate, (HA) hyaluronic acid, (CG) cardiogreen; n = 3, mean ± SEM.
References

Chapter 3. Osteotropic therapy via targeted LbL nanoparticles.

This chapter is in part adapted from:

Introduction

The development, maintenance, and repair of bone require cell-mediated remodeling to sustain the structural integrity of the tissue. Disturbances in the physiological processes of osteoblast-mediated bone deposition and osteoclast-mediated bone resorption are observed in many bone-related disease states, such as osteosarcoma, cancer metastasis to bone, osteoporosis, and Paget’s disease of the bone.[134] Several therapies have been developed to combat these pathologies,[134-136] however, the clinical outcomes for patients with these diseases continue to be very poor. The arsenal of available agents to treat patients has not made any substantial impact in improving their survival, and new methods for therapy are critical. Engineering a robust delivery platform with bone-tissue level specificity to treat these diseases can improve therapeutic efficacy, lower systemic toxicity, and improve disease management.

Significant fundamental work in the area of bone biology has uncovered the potential of bone-specific agents, such as bisphosphonates.[136,137] These compounds act as pharmacophores, whereby the pyrophosphate-like structure coordinates calcium ions within the hydroxyapatite mineral in bone with high affinity and specificity. Accumulation of bisphosphonates further promotes inhibition of bone resorption by inducing apoptosis in osteoclasts that are responsible for this action. As such, this class of pharmaceutics is a potent drug for promoting homeostasis between osteoclast and osteoblast activity in, for example, osteoporosis, while also promoting tissue-specific binding and localization.

Bone targeting systems[138-142] have been synthesized to utilize the specificity of a variety of ligands, including bisphosphonates, tetracycline and derivatives, sialic acid, and bio-inspired materials containing similar functionalities[136]; however, systems capable of therapeutic delivery for treatment of bone-related diseases remain limited. Towards this end, the modularity of Layer-by-Layer (LbL)-functionalized nanomedicine systems[41,77,143-.
presents an attractive opportunity to incorporate these bone-specific, highly watersoluble ligands on the surface of nanotechnology for targeted drug delivery.

In this chapter, the synthesis of tissue-targeted LbL nanoparticles, specifically manufactured to hone in on bone tissue, is reported. To achieve this, a polyelectrolyte, poly(acrylic acid) (PAA), was functionalized with a bisphosphonate, alendronate, and subsequently electrostatically-assembled in a nanoparticle coating. The functionalized particles accumulated in subcutaneous 143B osteosarcoma xenografts, where they delivered their payload, doxorubicin, in a mouse model. The targeted particles significantly attenuated tumor burden and extended animal survival, in some cases even completely eliminating tumors. The results described herein establish LbL as a modular approach to develop targeted drug carriers via adsorption of ligand-functionalized, aqueous polyelectrolytes for tissue-specific targeting, further developing these systems towards clinical application.

**Materials and Methods**

**PAA-Alendronate conjugation.** Alendronate (Alfa-Aesar) was conjugated to poly(acrylic acid) (Sigma) through the primary amine functional group of alendronate. Half of the free carboxyl end group of the poly(acrylic acid) was targeted for coupling. As both of the reacting species were only soluble in water, we have used water soluble 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as the coupling agent[150]. To perform the coupling chemistry, alendronate (1.0 eq.) was added to a solution of poly(acrylic acid) (0.5 equivalents considering all carboxylic groups) in 100 mL water. The solution was stirred for 10 minutes after which DMTMM (2.0 equiv.) was added to the resulting solution. The reaction was allowed to run for 12 hours at room temperature. After the reaction period, the solution was dialyzed against water for 36 hours for complete removal of coupling agent and unreacted alendronate. The dialyzed solution of alendronate-conjugated poly(acrylic acid) was lyophilized to yield a white foam-like product in 65% yield. 1H NMR (in D2O): 1.33 – 1.9 ppm [broad signal, poly(acrylic acid)], 2.10-2.40 ppm [broad signal, poly(acrylic acid)], 2.88 ppm (2H, alendronate). The degree of conjugation was estimated according to the assay protocol reported previously after the
hydrolysis of the conjugate at pH 10 with 0.1M NaOH\(^{[151]}\). The estimated degree of functionalization was found to be 43.2%.

**LbL on QDs.** Carboxyl-modified quantum dots (QD800, Life Technologies) were suspended in dilute solution (~10μL stock in 1mL DI water) and injected in excess 10mL polyelectrolyte (5mg/mL for PLL-Hydrochloride (4-15K, Sigma); 1mg/mL PAA\(_{450K}\)-Alendronate) while under agitation at 4°C. Each layer was incubated for ~30 minutes, prior to purification via ultracentrifugation (12,500 RPM, 30m minutes – 1st layer required longer spins, subsequent purifications are much more expedient) and washing with DI water, repeated twice prior to introduction to the subsequent polyelectrolyte. The final functionalized particle was re-suspended in 1X PBS after the 2nd wash and filtered in a 0.45μm filter for further experimentation.

**Liposome synthesis.** Liposomes were formulated at a mass ratio of 56:39:5 (DSPC:Cholesterol:POPG – all purchased from Avanti Polar Lipids). These three components were dissolved in a 2:1 mixture of chloroform:methanol. A thin film of these materials was generated by rotary evaporation at 40°C, 150 mbar for approximately 10 minutes. This film was allowed to desiccate overnight for complete drying. Hydration of the lipid film was conducted at 65°C under sonication in 300 mM citric acid buffer (pH 4) for 1 hour, after which they were filtered through a 0.2 μm PES syringe filter and allowed to cool to room temperature. The pH of the liposomal suspension was then adjusted to 6.5 by addition of 300 mM sodium carbonate buffer to create a gradient between the exterior and interior compartments. Doxorubicin (LC Laboratories) at a feed ratio of 3 mg drug to 50 mg lipids was then added in a 0.9% sodium chloride solution to load via a pH gradient method. The final drug-loaded system was subsequently purified out of the high salt buffers and any excess, unloaded drug via centrifugal filtration (100K MWCO Millipore) and transferred to DI water for subsequent functionalization via LbL. Blank liposomes were prepared in the same fashion; however, no drug was added.

**LbL on liposomes.** Liposomes were diluted in 1 mL DI water (from a 50 mg batch prepared in a final suspension of 5 mL DI water – use ~200 μL stock to 800 μL DI water) and injected
in excess polyelectrolyte under agitation at 4°C as previously described for QDs. Incubate for ~30 minutes, purify via ultracentrifugation at 10,000 RPM for ~10 minutes – repeat twice prior to introduction to subsequent polyelectrolyte. The final, functionalized liposomal system was filtered through a 0.45 µm filter and suspended in 1X PBS for further experimentation.

**Physicochemical characterization.** Dynamic light scattering and zeta potential analysis was conducted in 10 mM sodium chloride at 25°C using a Malvern ZS90 zeta-sizer. High-performance liquid chromatography (Agilent technologies, 1:1 acetonitrile to pH 5 water mobile phase) and nanodrop absorbance measurements (480nm) were performed to quantify doxorubicin concentrations in the prepared samples. Cryo-TEM was conducted by imaging a frozen dilute sample of the liposomal suspension at 120 kV.

**In vitro Binding/Cytotoxicity.** 143B osteosarcoma cells and human mesenchymal stem cells were seeded in 96-well tissue culture plates at a density of 1x10^3 cells/well and allowed to proliferate for 48 hours. PLL-Cy5.5 labeled particles were quantified based on fluorescence, serially diluted in cell culture media and added to the cells. After 24-48 hours, cells were washed 3X with PBS. Binding was measured by quantifying the amount of residual fluorescence in the well. Cytotoxicity was measured using a standard MTT assay.

**Fluorescence-activated cell sorting analysis.** Measurements were performed using a BD LSRFortessa (BD biosciences, San Jose, CA). PLL-Cy5.5 fluorescence was collected following excitation at 640 nm and detected at 710/50 nm (AF700 channel). Cell-association data presented as representative histogram overlays of data collected in triplicate following 143B cell incubation with blank, PLL-Cy5.5 coated, PAA-Alendronate functionalized LbL liposomes for 2 hours at 37°C in 96-well plates. Final samples ran for analysis were washed 3X with optiMEM and trypsinized immediately prior to introduction to the flow cytometer. Blue is the representative treatment; red is the control sample without treatment.

**Confocal Microscopy.** Images were taken using a Nikon A1R Ultra-Fast Spectral Scanning Confocal Microscope (Nikon instruments Inc., Melville, NY). 143B cells were seeded in a
CELLview glass bottom dish (Greiner Bio-One GmbH, Germany) at 1 x 10^5 cells per well and grown overnight. Cells were then incubated with blank, PLL-Cy5.5 coated, PAA-Alendronate functionalized LbL liposomes (or QDs) for 2 hours at 37°C. At the end of this period, cells were washed, fixed via paraformaldehyde, permeabilized via Triton-1X, and stained with phalloidin-568 for 30 minutes, followed by addition of DAPI for an additional 10 minutes, after which they were washed 3X and imaged.

**Xenograft development/targeting/treatment/monitoring – NCR nude.** Cells were mixed in a 1:1 ratio with BD Matrigel™ Basement Membrane Matrix to a final density of 5 x 10^6 cells/0.1 mL injection. 0.1mL injections of the matrix-cell suspension were performed in each rear hind flank of NCR nude mice (Taconic). Tumors were allowed to grow until a visible tumor was established, approximately 25 mm^2 in tumor area prior to treatment (determined via caliper measurements via the longest length and width dimensions); for tumor targeting, xenografts were allowed to grow near terminal size (1 cm in diameter) to reduce effects of EPR. Treatments were injected in 0.1mL 1X PBS at the concentration and treatment regimen indicated in the figure legend. Imaging was performed using whole-animal fluorescence imaging (IVIS, Xenogen, Caliper) at the time points indicated pre- and post-administration at the given fluorescence filters. For targeting experimentation, functionalized, fluorescent systems were administered at a radiant efficiency of ~3x10^9 (700 channel)/~1x10^9 (800 channel) in a 0.1mL injection. This was determined by imaging the vial prior to injection and diluting as necessary in 1X PBS. Tissue harvest (liver, spleen, kidneys, heart, lungs, tumors, ‘gutted’ skeleton) was performed at the terminal point of the experiment and imaged for fluorescence recovery against an untreated control for auto-fluorescence determination. Tumor sizes for the remediation study were monitored via caliper measurements, whereby areas based on the largest visible length and width dimensions were measured. Depth proved challenging to reliably measure at early time points due to skin thickness, therefore it was not included for a final volume determination for real-time tumor size measurements.

**Pharmacokinetics (circulation) – immune-proficient BALB/c.** Fluorescent systems (PLL-Cy5.5 coated LbL-targeted empty liposomes) were administered in 0.1mL 1X PBS injections
in BALB/c mice (Taconic) at a relative concentration of \( \sim 3 \times 10^9 \) (700 channel)/\( \sim 1 \times 10^9 \) (800 channel) in a 0.1mL injection, based on fluorescence in the IVIS prior to injection. Mice were imaged pre- and post-nanoparticle administration using whole-animal fluorescence imaging (IVIS, Xenogen, Caliper) at the time points indicated and at the given fluorescent filters. Retro-orbital bleeding was performed in a cohort of injected mice to determine the serum half-life of the particles, presented on the basis of fluorescence recovery in these isolated blood samples (\( \sim 0.1 \text{mL blood/time point done in 3 different mice for each time point plotted} \)). Fluorescence recovery was determined by imaging in the IVIS immediately following isolation and dilution 1:1 in EDTA to prevent coagulation. Final data is normalized to an untreated control blood sample to remove artifacts of auto-fluorescence. The data was fit to a two-compartment model (two-phase decay in PRISM®), from which the fast and slow half-lives characterizing the data were extracted.

**Histology.** Tumors were resected at the terminal point of experimentation and fixed in 4% paraformaldehyde (PFA) for 48 hours and transferred to 70% ethanol until processed. Tissue was embedded in paraffin wax and sections were stained with routine hematoxylin and eosin (H&E) and Masson’s trichrome stain.

**Statistical Analysis.** Experiments were performed in triplicates, or otherwise indicated. Data were analyzed using descriptive statistics, single-factor analysis of variance (ANOVA), and presented as mean values ± standard deviation (SD) from three to eight independent measurements. Statistical comparisons between different treatments were assessed by two-tailed t tests or one-way ANOVA assuming significance at \( P < 0.05 \).

**Results and Discussion**

LbL-targeted nanoparticle systems were generated by covalent modification of PAA (\( M_w \sim 450K \), Figure 2.1.A) with alendronate (Figure 2.1.B) at 40% functionalization of the total carboxylic groups on the PAA molecule (Figure 2.1.C). Alendronate was coupled via its amino functional handle to PAA through an amide bond. As both of the components were water soluble, we used methyl morpholine-based water-soluble coupling agents to
perform amide coupling (Appendix B.1). The immobilization of the molecule onto the PAA backbone was confirmed by $^{31}$P and $^1$H NMR. The functionalized PAA-Alendronate polymer was subsequently used as the polyanionic layer in LbL assembly, iteratively adsorbed on a solid nanoparticle substrate in alternation with polycationic poly-L-lysine (PLL), as schematically illustrated in Figure 3.1.D. PAA is a well-characterized weak polyanion with a high charge density and a non-erodible backbone that has been listed as an approved excipient in the FDA’s Inactive Ingredient Guide and is used in clinically-approved drug formulations as a stabilizer and thickener to tune the rheological properties of the injectable or topically-applied therapeutic. As such, PAA presented a suitable material for introducing tissue specificity and targeting via alendronate functionalization.

To establish these systems as osteotropic delivery systems, initial investigations employed a 25 nm quantum dot (QD) (CdSeTe core) for imaging purposes to understand how the coating directed particle association with osteosarcoma cells (143B) in vitro and in vivo, as well as other hard tissue in vivo. These particles were fabricated as shown in Figure 3.1.D, with linear film growth observed for three bilayers of (PLL/PAA-Alendronate) to a final z-average hydrodynamic diameter of 115 nm, with a PDI of 0.19, and zeta-potential of $-39 \text{ mV}$ (10 mM NaCl in DI water, 25°C; Appendix B.2).

Incubation of LbL-targeted QD$_{705}$ nanoparticles with 143B cells showed significant binding and cell uptake after 2 hours at 37°C (see Figure 3.2.A), where red is representative of QD$_{705}$ nanoparticle fluorescence. Nanoparticle binding, on the basis of QD$_{705}$ fluorescence, was further characterized and observed to be dose-dependent for a range of concentrations, over which little cytotoxicity (48 hour incubation) was observed, as shown in Figure 3.2.B. The number of bilayers (1, 2, and 3, whereby 1, 2, and 3 layers of the targeted PAA-Alendronate polymer are incorporated on the particle surface) was also investigated. These results are shown Appendix B.3. It was observed that the in vitro binding affinities and cytotoxicity profile were similar across different bilayer numbers. Previous work[^73] established the improved biological performance of QD nanoparticles with 3 or more bilayers; therefore, we focused on the 3-bilayer LbL-targeted QD particles for in vivo assessment of targeting.

To evaluate this system in vivo, LbL-targeted QD$_{800}$ nanoparticles were administered via the tail vein in NCR nude mice with ectopically induced 143B osteosarcoma xenografts.
Particle distribution in live animals was tracked using whole-animal in vivo fluorescence imaging (Figure 3.3.A). Immediately following administration, the particles rapidly accumulated in the bone tissue regions, particularly in the parietal region of the cranium, spinal column, and hind limb regions. At later time points, particles trafficked to the 143B xenografts, consistent with targeted interactions with the tumor matrix. Controls for uncoated, untargeted QDs and coated QDs with unconjugated PAA showed no specific affinity for hard tissue and had little to no accumulation in the xenografts, suggesting the enhanced permeation and retention effect does not account for much of this tumor localization (Appendix B.4). Tumor-specific accumulation was significant and observed over the course of 8 days (Figure 3.3.B). Such a long period of accumulation may be due to the strong binding affinity between alendronate and the osteosarcoma tissue, leading to much longer residences times of the nanoparticle in the tumor. The tumors were resected after 8 days, along with necropsy of other relevant tissue, and analyzed via recovered fluorescence to investigate post-mortem particle distribution. Significant localization of particles was observed in xenografts (accounting for ~30% fluorescence recovered), relative to ~40% in the liver and smaller fractions in each of the other organs (spleen, kidneys, heart, lungs) harvested (Figure 3.3.C, Appendix B.5). Accumulation in the liver is a common challenge for all nanoparticle delivery platforms, though we have shown in other work that the use of hyaluronic acid can lower accumulation considerably compared to other systems. Future work will investigate its use for introducing tissue specificity via ligand functionalization.

To visualize the biodistribution of both the coating and nanoparticle in real time, the bisphosphonate-targeted polymer, PAA-Alendronate, was labeled with a near-IR, Cy5.5 dye and adsorbed onto the surface of QD800 to allow for two-color in vivo imaging of the targeted, 3 bilayer (PLL/PAAcys.s-Alendronate)_3 nanoparticle. We observed co-localization of the two components – the PAA-Alendronate outer layer and the quantum dot - in the xenografts (Figure 3.3.D, Appendix B.5) for up to 9 days, further substantiating these systems as serum-stable targeted platforms for delivery.

Next, we applied the coating, (PLL/PAA-Alendronate), to drug-loaded particle systems for targeted delivery and treatment of diseased tissue. For this purpose, the coatings were adapted to a liposomal carrier for use in drug delivery (Figure 3.4.A). Initial
work focused on empty, negatively-charged liposomal carriers, containing DSPC (1,2-
distearoyl-sn-glycero-3-phosphocholine), cholesterol, and POPG (1-palmitoyl-2-oleoyl-sn-
glycero-3-phospho-(1’-rac-glycerol)), functionalized with one bilayer of PLL/PAA-Al, which
yielded 170 nm nanoparticles with a zeta-potential of -20 mV (Appendix B.6), to validate
these systems for LbL-targeted delivery. Due to size considerations for systemic delivery,
fabrication was truncated after a single bilayer due to the significant increase in size
associated with adsorption of a single bilayer (80-90 nm per bilayer). Differences in bilayer
thickness relative to films on QDs could be due to the relative fluidity of the liposomal
substrate. Consistent with results for the LbL-targeted QDs, binding of the LbL-
functionalized liposomes to 143B cells was observed to be dose-dependent and not
apparently cytotoxic (Figure 3.4.B). Rapid internalization after a 2 hour incubation with
the 143B cells, visualized by confocal microscopy (Figure 3.4.C) and confirmed via flow
cytometry (Figure 3.4.D), was also observed. The high levels of red, diffuse nanoparticle
fluorescence (visualized by incorporation of PLL-Cy5.5 in the LbL coating), along with a
marked shift in cell-associated fluorescence observed by flow cytometry, suggested this
system enabled high levels of nanoparticle binding and uptake, providing a promising
platform for LbL-targeted drug delivery.

Osteosarcomas are known to be responsive to conventional chemotherapeutics,
such as doxorubicin, so we next explored the incorporation of doxorubicin in the liposomal
carrier for subsequent LbL functionalization. We were able to incorporate doxorubicin into
stable DSPC:Cholesterol:POPG liposomes with high drug-loading efficiency (97%) at 5.5
w/w% (drug/lipid). During subsequent coating with a single bilayer of (PLL/PAA-
Alendronate), no drug loss was observed, and liposomal morphology remained unchanged,
as illustrated in Figure 3.4.A. The LbL-functionalized doxorubicin-loaded liposome was
characterized by a hydrodynamic diameter of approximately 210 nm in size with a zeta-
potential of -20 mV, measured in 10 mM NaCl (Appendix B.6). The targeted liposomal
carriers were found to be particularly potent against 143B cells, with high levels of toxicity
over a wide-range of doxorubicin-loaded concentrations following incubation for 24 hours
and 48 hours (Figure 3.4.E), whereas the uncoated control showed about 8-fold lower
levels of toxicity (Figure 3.4.F).
Translating these systems in vivo, we first investigated the pharmacokinetics of the coated liposomal system in the absence of drug. Shown in Figure 3.5.A is the circulation profile of this system on the basis of fluorescence recovery following systemic administration of the PLL-Cy5.5 labeled carriers to immune-proficient BALB/c mice. Based on a two-compartment model, the targeted carriers exhibited half-lives of 0.23 hours (fast) and 18.7 hours (slow), indicative of a stable, long-circulating system that could promote enhanced delivery of loaded therapeutics.

In tumor-bearing NCR nude mice, the same PLL-Cy5.5 labeled, targeted system was observed to accumulate in the target diseased tissue rapidly, with the ability to persist in the tumor up to the terminal point of the study - 100 hours (Figure 3.5.B, Appendix B.7). Biodistribution results (Figure 3.5.C, Appendix B.7) at the terminal point of this investigation further corroborated these systems as stable delivery platforms that preferentially locate in osteosarcoma xenografts at high levels (~35% on the basis of fluorescence recovery, relative to ~47% in the liver, ~7% in the kidneys). Elevated amounts of recovered fluorescence in the liver suggested that hepatic clearance is the primary means of excretion; however, the large number associated with the liver, in addition to the values reported for other tissue, is not meant to be wholly quantitative, as the data is reported as percent of the total fluorescence recovered from only the tissue collected. The relative ratios of fluorescence between tissue confirmed that we were able to achieve a high level of tumor specificity relative to the other tissue, which validated this system for further investigation towards therapeutic delivery.

After observing high levels of tumor localization for the targeted empty liposomal formulation, the efficacy of the Lbl-targeted doxorubicin-loaded liposomes was evaluated against 143B osteosarcoma xenograft-bearing NCR nude mice. Initial attempts at determining the optimal dosing regimen (drug concentration and number/timing of injections) employed a dose escalation study of serial 1 mg/kg, 2 mg/kg, and 3 mg/kg (based on doxorubicin loading) injections starting at day 10 post-tumor inoculation with each treatment separated by one week. As observed in Figure 3.6.A, untreated osteosarcoma tumors grew beyond terminal size (diameter > 1 cm) after 19 days post-inoculation. Treated mice for both the uncoated and coated versions survived repeated dosing out to 30 days, after which comparisons in terminal tumor size were drawn. As
observed visually in Figure 3.6.A and quantified in Figure 3.6.B, LbL-targeted doxorubicin-loaded liposome treated mice showed significantly reduced terminal tumor sizes relative to the uncoated doxorubicin-loaded liposome control (characterized by measurements on the longest x-y dimensions). This observation is consistent with enhancement of the therapeutic potency of the loaded drug due to preferential accumulation of the targeted system in the 143B xenografts.

Results from the dose escalation study indicated that a higher dose was necessary to effectively remediate the tumor. For this purpose, LbL-targeted doxorubicin-loaded liposomes were administered at a doxorubicin concentration of 5 mg/kg, along with an uncoated, drug-loaded control at the same concentration. Xenograft-bearing mice were allowed to develop tumors to an area of ~25 mm² [measured in the longest length and width dimensions] after which they were treated, with repeated injections one week apart for 3 total treatments until a terminal point at day 40. We observed enhanced efficacy of the LbL-targeted doxorubicin-loaded liposome, relative to the uncoated control, as determined by caliper measurements and visual inspection of the terminal point harvested xenografts (Figure 3.6.C). Varying levels of therapeutic benefit for the targeted group were observed from complete remediation (NT - no tumor observed) to tumor maintenance (tumor reduction of 30% from day 0 on average), whereas the uncoated control group mice showed significant tumor growth (several beyond the maximum allowable tumor burden of 1 cm in diameter; growth of 550% from day 0 on average). These results highlight the capabilities of the LbL-targeted nanoparticle approach for highly effective chemotherapeutic treatment in tumor-bearing mice.

Histological analysis via a Masson’s trichrome stain of recovered osteosarcoma (143B) tumor tissue following treatment with each the targeted and untargeted doxorubicin-loaded liposomal formulations at 5 mg/kg is displayed in Figure 3.7. Initially, the tumor demonstrates an abundance of 143B cells (stained red) and connective tissue (stained blue) in the tumor mass (left column). Particles coated with alendronate for targeting the tumor tissue resulted in significant cell death (right column, top row). A lower level of cell death resulted in tumors with uncoated nanoparticles (right column, middle row). Virtually no change in the tumor infrastructure was observed in untreated animals (right column, bottom row). Micro-CT analysis of these tumors immediately prior to
resection (Appendix B.8) complements this data, clearly demonstrating the enhanced efficacy of the targeted system relative to the uncoated control. These observations suggest the potency of this approach to deliver the payload in an efficacious manner to the 143B osteosarcoma solid tumor.

**Conclusion**

LbL is a versatile platform to functionalize nanoparticles in ways that promote improved biological performance. Prior art has established LbL as a means to impart protein-resistive, long-circulating properties to nanoparticle systems, with a means to control biodistribution of both the carrier and drug in a complex systemic environment. This investigation further demonstrates the modularity of this approach to impart targeting capabilities to the nanoparticle to diseased tissue to achieve enhanced treatment outcomes. We capture this potential with the synthesis of osteotropic nanoparticles via incorporation of alendronate-functionalized PAA as a means of surface modification of drug-loaded liposomes. Different nanoparticle core substrates may be used for imaging and treatment for a variety of bone diseases. While surgical resection of a primary tumor will continue to be the first-line of treatment, subsequent treatment with the osteotropic nanoparticles has the potential to decrease recurrance rates and increase successful outcomes. These functional nanoparticles are also highly promising for future investigations towards treatment of bone-localized metastases of invasive cancer cell types such as breast and lung cancer. The potential to further generalize this approach towards the built-to-order manufacture of different targeted delivery systems continues to provide much promise for LbL nanoparticles.
Figure 3.1. Achieving bone tissue level specificity of LbL coated nanoparticles.

(A) Aqueous anionic polyelectrolyte, poly(acrylic acid) [PAA, MW 450K], functionalized with the (B) bisphosphonate targeting moiety, alendronate [for high specificity to hydroxyapatite in bone], to yield (C) the aqueous, ligand-functionalized polymer at 40% side-chain functionalization (Appendix B1), which is used for complementary, iterative adsorption to the polycationic component (poly-L-lysine, PLL) in the film on the NP substrate, schematically illustrated in (D).
Figure 3.2. In vitro assessment of PAA-Alendronate LbL-targeted QD NPs incubated with 143B osteosarcoma cells. 
(A) Confocal microscopy of 143B cells incubated with the LbL-targeted QD_{705} core NPs for 2 hours. Blue staining representative of a Hoechst nuclear stain, green representative of a phalloidin stain of the actin filamentous cell structure, and red representative of the nanoparticle fluorescence (QD_{705} fluorescence). Scale bar representative of 20 \mu m. (B) Binding and relative cytotoxicity of 3 bilayer [PLL/PAA-Alendronate] LbL-targeted QD_{800} core NPs following incubation for 2 hours in 143B cells. Fluorescence emission data corresponds to that of the nanoparticle core (QD_{800}).
Figure 3.3. In vivo evaluation of PAA-Alendronate LbL-targeted QD800 core NPs.

(A) Representative live-animal imaging of LbL-targeted QD800 core NPs following systemic administration to 143B osteosarcoma xenograft-bearing NCR nude mice. Imaging conducted at $\lambda_{ex} = 640$ nm, $\lambda_{em} = 800$ nm for up to 8 days. Hind-flank xenografts identified in the pre-injection image; arrows at 5 minutes and 9 hours indicate binding to native bone tissue. (B) Quantification of fold tumor-specific accumulation normalized to tissue auto-fluorescence pre-injection for the systemically administered LbL-targeted QD800 core NPs, as visualized in (A). n = 3 mice (6 tumors); data presented as mean +/- SEM. (C) Biodistribution data corresponding to endpoint of (A) (quantified as percent recovered fluorescence following harvest of relevant tissue after 8 days post-administration). n = 3 mice (6 tumors); data presented as mean +/- SEM. (D) Co-localization of QD800 NP core with LbL-film containing labeled PAA-Alendronate700; top row [PAA-Alendronate700 channel] - imaging conducted at $\lambda_{ex} = 640$ nm, $\lambda_{em} = 700$ nm, bottom row [QD800 channel] - imaging conducted at $\lambda_{ex} = 640$ nm, $\lambda_{em} = 800$ nm.
Figure 3.4. In vitro evaluation of PAA-Alendronate LbL-targeted liposomal NPs in 143B cells. 
(A) Cryo-TEM of LbL-targeted doxorubicin-loaded liposomal NPs. Scale bar representative of 200 nm. (B) Binding (2 hour incubation) and relative cytotoxicity (48 hour incubation) of LbL-targeted empty liposomal NPs over a range of concentrations, based on fluorescence, in 143B cells (λex = 675 nm, λem = 710 nm; tracked using PLL700 as cationic component in LbL film). (C) Confocal microscopy of 143B cells incubated with the LbL-targeted empty liposomal NPs (tracked via PLL700 polycationic component in LbL film) for 2 hours. Blue staining representative of a Hoescht nuclear stain, green representative of a phalloidin stain of the actin filamentous cell structure, and red representative of the nanoparticle fluorescence (PLLcy5.5 polymer shell fluorescence). Scale bar representative of 10 μm. (D) Representative cell-associated NP fluorescence following a 2 hour incubation of 143B cells with the
**LbL-targeted empty liposomal NPs.** The in vitro cytotoxicity of LbL-targeted doxorubicin-loaded liposomal NPs and the corresponding uncoated doxorubicin-loaded liposomal control following 48 hour (blue) and 72 hour (red) incubation periods over a range of doxorubicin concentrations.
Figure 3.5. Biological evaluation of PAA-Alendronate LbL-targeted empty liposomal NPs. LbL-targeted empty liposomal NPs tracked via PLL70 polycationic component in surface coating. Circulation data in (A) normalized to % remaining particles recovered, on the basis of fluorescence [λ_ex = 640 nm, λ_em = 700 nm], immediately following systemic administration to immune proficient BALB/c mice; data presented as mean +/- SEM (n = 3). Two-compartment model fit to determine half-lives displayed. Data includes background subtraction of blood auto-fluorescence. (B) Quantification of fold tumor-specific accumulation normalized to tissue auto-fluorescence pre-injection for the systemically administered LbL-targeted blank liposomal core NPs to 143B xenograft-bearing NCR nude mice; data presented as mean +/- SEM (n = 3). (C) Biodistribution data corresponding to endpoint of (B) (quantified as percent recovered fluorescence [λ_ex = 640 nm, λ_em = 700 nm] following harvest of relevant tissue after 100 hours post-systemic administration); data presented as mean +/- SEM (n = 3).
Untreated - 19d post inoculation

Treatment groups - 30d post inoculation
Treatment Regimen: 1mg/kg 10d, 2mg/kg 17d, 3mg/kg 24d

Untargeted:

Targeted:

A

B

Tumor Area (mm²)

 Untreated  Untargeted  Targeted

19d  30d

0 50 100

***

Tumor Area (mm²)

Untargeted  Targeted

19d  30d

0 50 100

*** p = 0.003

C

Treatment groups - 40d post inoculation
Treatment Regimen: 5mg/kg 22d, 28d, 35d

Untargeted  Targeted

NT  NT

0 20

5mg/kg [Dox]  5mg/kg [Dox]  5mg/kg [Dox]
Figure 3.6. 143B xenograft tumor remediation following treatment with doxorubicin-loaded liposomal formulations.  
(A) in vivo tumor remediation of 143B xenografts in NCR nude mice for Lbl.-targeted doxorubicin-loaded liposomal NPs, against untreated and uncoated doxorubicin-liposomal NPs. Untreated control xenografts were sacrificed 19 days post-inoculation of xenograft due to tumor burden exceeding 1 cm. Untargeted and targeted Dox-liposomal formulations were sacked at 30 days post-inoculation following a dose-escalation study with the following treatment regimen: day 10 at 1 mg/kg, day 17 at 2 mg/kg, day 24 at 3 mg/kg. Terminal point shown with final tumor area displayed in (B). Statistics are from an unpaired t-test, two-tailed to compare the untargeted and targeted formulations at each time point. Data presented as mean +/- SEM; n = 4. (C) Caliper measurements for in vivo tumor remediation with repeated dosing of 5 mg/kg for both targeted and untargeted formulations at 22 days, 28 days, and 35 days post-inoculation (displayed as day 0, day 6, and day 13 in (C)). Statistics are from an unpaired t-test, two-tailed to compare the untargeted and targeted formulations; *p < 0.05; **p < 0.01; ***p < 0.001. Data presented as mean +/- SEM; n = 4. Resection of tumors from terminal point of (C), n = 4 for each group, displayed from the final caliper measurement.
Figure 3.7. Representative histological sections of 143B tumors following treatment with doxorubicin-loaded liposomal formulations. Tumors were harvested at 18 days post-treatment corresponding to the terminal point in Figure 3.6.C. (left column) tumors prior to treatment; (right column) tumors at the 18 day terminal point post treatment of 3 repeated injections at 5 mg/kg doxorubicin. (top row) tumors treated with PAA-Alendronate coated doxorubicin-loaded liposomes, (middle row) uncoated dox-loaded liposomes, and (bottom row) untreated animals. Masson's trichrome stain – red = 143B osteosarcoma cells, blue = connective tissue; scale bars representative of 100 μm.
References

Scalable methods, PRINT particle fabrication and spray-assisted layer-by-layer deposition, are combined by Paula Hammond, Joseph DeSimone and co-workers on page 4707 to generate uniform and functional nanotechnologies with precise control over composition, size, shape, and surface functionality. A modular and tunable approach toward the design of built-to-order nanoparticle systems, spray coatings on PRINT particles are demonstrated to achieve technologies capable of targeted interactions with cancer cells for applications in drug delivery.
Chapter 4. Scalable manufacture of built-to-order nanomedicine: spray-LbL on PRINT® nanoparticles

This chapter is in part adapted from:

Introduction

A significant limitation in the design of new nanotechnologies for drug delivery is the balance between efficacy, safety, and scalability – the three major hurdles to streamlined approval towards the clinic and, ultimately, adaptation in the pharmaceutical industry. Oftentimes, these aspects of nanomedicine are competing, and impose challenging design requirements on systems synthesized in the laboratory. Resource-intensive syntheses or purification procedures, limited material yields, and the difficulty of precisely controlling particle size, morphology, and composition for increasingly complex systems are some of the major challenges that continue to prevent the translation of promising technologies away from the bench-top. For this reason, it is highly desirable to conceive of simple, scalable, and highly controlled methodologies for the manufacture of multi-functional nanoparticles that possess the necessary physicochemical characteristics to be clinically relevant.\[7, 10, 155\]

Particle fabrication using the Particle Replication in Non-wetting Templates (PRINT®) process is a well-established, scalable approach\[19\] for rapidly manufacturing particles with exquisite control over particle geometry (size, shape) and composition (cargo, carrier system), important parameters for optimizing cellular engagement and in vivo pharmacokinetics.\[19, 156-159\] Through a roll-to-roll process, an elastomeric mold is implemented in a high-throughput fashion to generate monodisperse particles with well-defined geometries. By varying the properties of the elastomeric mold, particles can be produced with a dynamic range of shapes and sizes, varying from 10 nm - 200 μm. Further, particles prior to recovery are presented in an ideal, ordered spatial arrangement that presents an attractive opportunity for high-throughput surface functionalization methodologies, such as LbL. The ability to fabricate precisely controlled nanotechnologies
on a large scale is highly attractive towards expedient approval through regulatory pathways.\cite{12,160}

Purification and surface functionalization of PRINT® particles, however, endures all of the challenges faced by other synthesis techniques. Following particle fabrication, purification from the solubilized transfer adhesive film is limited by traditional means (e.g. ultracentrifugation, tangential-flow filtration). Post-purification modification is generally required to achieve effective surface modification using the more traditional techniques available to the biomaterials community at-large for yielding functional particle systems. Oftentimes, these bioconjugation or functionalization chemistries are difficult and expensive to scale and result in significant material loss. The ability to control surface characteristics is essential to improve performance of nanotechnologies regarding enhanced specific molecular interactions with target cells, as well as avoid non-specific clearance \textit{in vivo} via protein-resistive properties.\cite{128} Surfaces largely mediate interactions at the interface of biology; therefore, the desire to incorporate a technology with the ability to include a library of materials on the surface of PRINT® particles in an analogous high throughput, scalable fashion is highly desirable, and one that holds much promise for the future of multi-functional nanotechnology manufacture.

Towards the development of a complementary, scalable approach for the surface functionalization of roll-to-roll assembled functional nanoparticles, we demonstrate that spray-assisted Layer-by-Layer (Spray-LbL) deposition may be used to generate highly-controlled functional coatings in a rapid, reproducible, and facile manner on a generalizable platform of particle systems, as illustrated in Scheme 4.1. Spray-LbL has been shown\cite{161,162} to conformally coat materials on the nanoscale in a controllable fashion, with extremely thin layers deposited on the surface of various charged substrate materials, including 3D structures such as electrospun mats. The combination of PRINT® and LbL technologies offers an expansive toolbox for producing functional particles for a variety of applications, including catalysis, microelectronics, photovoltaics, and cosmetics, in addition to nanomedicine.\cite{13,163} Bringing PRINT® technology and Spray-LbL functionalization together, this work demonstrates a scalable, reproducible approach for fabrication of functional carriers with exquisite control over particle composition, geometry, and surface
properties, providing an exciting platform for large-scale manufacture of highly-controlled multi-functional nanocarriers.

**Materials and Methods**

All materials and equipment necessary for PRINT® particle fabrication were maintained in a clean room under controlled conditions at UNC-Chapel Hill. Bulk material, including rolls of poly(ethylene terephthalate) [PET], poly(vinyl alcohol) [PVA]-coated PET, and pre-made molds cast from a perfluoropolyether (PFPE) material, were provided by Liquidia Technologies. All chemicals used were provided by Sigma Aldrich, except for hyaluronic acid, provided by Lifecore Biomedical, and Cy5.5-NHS from Lumiprobe.

**PRINT® Nanoparticle Fabrication.** PRINT® particle fabrication follows a well-established protocol. Briefly, the polymeric material, poly(lactide co-glycolic acid) [PLGA], used for particle molding was dissolved in dichloromethane. A film of PLGA was cast using a mayer rod (#3) on a high energy PET backing prior to lamination of this film to the pre-cast mold at 320°F. The filled mold from this step was laminated at 320°F to a transfer adhesive film (PVA) cast on a PET backing. The particles were preserved in this state via vacuum seal under N₂ atmosphere until ready for functionalization.

**Vapor-phase Glutaraldehyde Crosslinking.** Molded nanoparticles were delaminated from the mold onto the transfer adhesive film. Sections of these harvested particles were subjected to PVA-crosslinking conditions in an enclosed chamber via vapor-phase glutaraldehyde crosslinking to further immobilize the particles for spray-LbL deposition. This was done via incubation of the particle arrays with vials containing 50% glutaraldehyde (in water) and 10% aqueous acid (HCl) for 15h. Arrays following crosslinking were removed for subsequent functionalization.

**Spray Layer-by-Layer Deposition.** Materials were deposited onto the surface of crosslinked nanoparticle arrays by aerosolization of polyelectrolytes for spray times of ~3 seconds, with ~3 second wash steps between each layer. Polyelectrolytes were sprayed at
a concentration of 1mg/mL. A final water rinse was used prior to drying and subsequent analysis and/or recovery of functionalized particles. Particles recovered following spray-LbL were harvested by sonication of the functionalized particle arrays in water for ~15 minutes. The collected particles were subsequently purified via centrifugation and filtration through a 0.45μm syringe filter.

**Nanoparticle Characterization.** Images to assess iridescence of the nanoparticle arrays following each processing step were obtained by photography with a 5-megapixel camera (iPhone 4). Contact angle measurements and images (Rame-Hart model 500 goniometer with Nikon camera) were obtained immediately following drop-casting a bubble of water on the NP array. Dynamic light scattering (Malvern ZS90) was used to determine the hydrodynamic diameter of the nanoparticles. Zeta potential measurements were also performed using the Malvern ZS90. Measurements were conducted in 10mM NaCl in millipore water at 25°C.

Scanning electron micrographs were collected using a JEOL 6700 high resolution microscope. Sample preparation included drop-casting on a silicon wafer followed by sputter coating with gold-palladium (~3nm). Transmission electron micrographs were collected using a JEOL 2010 Advanced High Performance TEM. Sample preparation included drop-casting on a carbon/formvar-coated copper grid. Atomic force microscopy data was collected using a MultiMode™ atomic force microscope with a NSC15/AIBS, 325kHz, 46N/m tip from μmasch in tapping mode.

Confocal microscopy (Nikon A1R scanning confocal microscope) was conducted following incubation of the functionalized nanoparticles (tracked via labeled PLL, PLLcy5.5) at 37°C with BT-20 cells. Fixed samples included a DAPI and Phalloidin-488 stain, in addition to particle tracking Cy5.5 label. Flow cytometry (BD LSRFortessa) was performed in parallel with confocal microscopy to quantitatively assess particle association (tracking via PLLcy5.5) with cells following incubation at 37°C. Particles administered were normalized against the fluorescent intensity (λex=675nm, λem = 710nm), as measured by a Tecan Microplate Reader, of the labeled polycationic layer deposited initially on the NP surface.
**Results and Discussion**

Using the widely-reported top-down approach of PRINT® particle fabrication, 200x200x200nm PLGA nanoparticles (PLGA\(_{200x200nm}\)) were fabricated and collected on a low molecular weight polyvinyl alcohol (PVA) base coating atop a polyester support film. While immobilized on the support film, the particles are arranged in a highly ordered 2-D hexagonal array. The PVA supported particles were visualized by AFM and SEM. The regular spatial arrangement of PRINT® particles coupled with the negative surface charge of the acid-terminated PLGA NPs in water make them ideal candidates for rapid LbL polyelectrolyte functionalization through spray-assisted LbL deposition (Spray-LbL). Initial attempts to deposit aqueous solutions of polyelectrolytes onto the supported PRINT particles resulted in a loss of the particles due to detachment from the substrate. This process could be visualized by the loss of diffraction-based iridescence characteristic of the ordered nanoparticle arrays (**Figure 4.1.B, 4.1.C**); this characteristic iridescence is indicative of highly ordered particles on the harvesting layer, allowing for a convenient qualitative assessment of particle immobilization or detachment. The loss of particles was confirmed with AFM and SEM, which essentially show a complete absence of particles after the Spray-LbL process due to the water solubility of the PVA backing. The reason for choosing PVA as the support material for PRINT particles is in fact to enable facile particle harvesting (see **Appendix C.1**).

To avoid PVA dissolution, vapor-phase crosslinking using concentrated glutaraldehyde and acid was employed to selectively crosslink the PVA adhesion layer to reduce its water solubility, while avoiding any chemical changes to the relatively impermeable and inert PLGA PRINT® nanoparticles. Contact angle measurements were carried out to study the effects of the vapor-phase cross-linking reaction on the wettability of the PRINT® films. As shown in the **Figure 4.1.A**, pre-crosslinked NP arrays show considerable wettability, owing to the presence of highly water-soluble and hydrophilic PVA polymer. After cross-linking, the contact angle increased from 26° to 78° (**Figure 4.1.D**), demonstrating that the films become considerably more hydrophobic due to the formation of a glutaraldehyde cross-linked PVA adhesive. Crosslinking conditions were also varied with results shown in **Appendix C.2**. As a result, the cross-linked films can be placed...
in water without particle detachment. Following crosslinking, it was observed that particles could be readily functionalized, as evidenced by maintenance of iridescence from the NP array following each processing step (Figure 4.1.E, 4.1.F). This step therefore allowed for the deposition of water-based polyelectrolytes using Spray-LbL.

As shown in Scheme 4.1, Spray-LbL on PRINT® nanoparticles, following vapor-phase cross-linking of the PVA support films, is achieved by spraying an aqueous solution of cationic polyelectrolyte is sprayed onto the NP containing films for 3 seconds and, after briefly rinsing with water (3 seconds), an anionic polyelectrolyte is subsequently sprayed onto the films (3 seconds). This cycle can then be repeated indefinitely to control the thickness of the polyelectrolyte coating. In the final step, the particles are harvested by sonicating the films in water, which causes the particles to detach from the PVA substrate.

Using AFM, the shape, persistence and uniformity of the particles were monitored with each processing step, beginning with the initial NP array, followed by crosslinking, and subsequent Spray-LbL deposition, as shown in Figure 4.2 (corresponding amplitude images shown in Appendix C.3). The versatility of this platform was further demonstrated by coating a second PRINT® PLGA particle type, 80x80x320nm [PLGA80x320nm]. AFM confirms that the particles are coated in a manner that maintains the uniformity of the PRINT® particle template while building a film, observed by slight increase in height (~5nm/bilayer), which avoids bridging of neighboring particles and without causing significant loss of particles during functionalization.

Particles were subsequently recovered via sonication, purified via filtration, and concentrated using ultracentrifugation following Spray-LbL. A variety of techniques were then used to illustrate the uniformity of the particles obtained from this fabrication method. Dynamic light scattering analysis is shown in Table 4.1, with the corresponding histograms shown in Appendix C.4. For both particle types, uniformity of the particle population is maintained as evidenced by the PDI of functionalized particles (see Table 4.1: PRINT® PLGA200x200nm particles - original 0.01, 0.06 following deposition of three bilayers of PLL/HA500K; PRINT® PLGA80x320nm particles - original 0.05). A slight decrease in size following crosslinking is observed for both particle types, consistent with the amount of material exposure to the crosslinking contraction forces on the PVA adhesive layer. The change in size for functionalized particles is indicative of a very thin LbL film,
approximately 15-20nm for 3 bilayers of PLL/HA_{500K}, based on dynamic light scattering (Table 4.1) and confirmed with electron microscopy (Figures 4.3, 4.4). Histogram overlays also illustrate the thin coatings applied to the particle surface (Appendix C.4). Characteristic surface charge reversal of the LbL deposition is also observed, as evidenced by samples taken at different steps in the functionalization process. $\zeta$-potential, measured at 25°C in 10mM NaCl, of pre- and post-XL NPs are approximately the same, while deposition of materials post-XL results in surface charge characteristics consistent with the material deposited (see Appendix C.4). A range of polyelectrolyte multilayers were explored, including poly-L-lysine (PLL)/hyaluronic acid (HA) [PLL/HA_{500K}], PLL/dextran sulfate (DXS) [PLL/DXS], PLL/poly(acrylic acid) [PLL/PAA], chitosan/HA [Chit/HA_{500K}], and chitosan/PAA [Chit/PAA] (see Appendix C.4), further illustrating a primary advantage of LbL as a means of tailoring the surface properties of these NP systems. These films, while diverse in the range of materials incorporated, behaved similarly in coating the NP systems without compromising the particle shape or significantly increasing the size beyond a few tens of nanometers.

Recovered and purified particles visualized by electron microscopy, including SEM and TEM, are displayed in Figures 4.3 and 4.4. Figures 4.3.A and 4.3.B are representative of the uncoated, crosslinked PRINT® PLGA_{200x200nm} particle array, along with the corresponding recovered particles observed by both SEM (Figure 4.3.C) and TEM (Figure 4.4.B). The coated array is shown in Figure 4.3.D and 4.3.E for direct comparison. A thin film coating is observed on the particles in such a fashion that individual particle integrity is maintained prior to recovery. Subsequent recovery yields the results in Figure 4.3.F, where a thin coating of approximately 20nm is observed to surround the entire particle surface by TEM (Figure 4.4.B). Observation of a conformal coating surrounding the entire nanoparticle is a surprising result due to the inaccessibility of the spray deposition to the immobilized surface; however, it can be explained by the unique feature of the electrostatic self-assembly LbL process to “self-heal” defects in the adsorbed film.$^{[164]}$ The excess film that corrects for this defect in spray LbL on PRINT® is observed in Figure 4.3.E, whereby the film surrounding the individual nanoparticle collapses in a manner that completely and smoothly seals the nanoparticle with deposited film around the harvested particle following suspension. This phenomenon is also observed for the PLGA_{80nmx320nm} NPs.
Displayed in Figure 4.3.G and 4.3.H are the uncoated, crosslinked PLGA_{80x320nm} NP arrays, followed by the recovered particles visualized by both SEM (Figure 4.3.I) and TEM (Figure 4.4.E). Compared to the coated NP arrays in Figure 4.3.J and 4.3.K, the particles clearly grow in size in both dimensions following film deposition. This thin film is also observed following recovery in using both SEM (Figure 4.3.L) and TEM (Figure 4.4.E), where a coating of 20nm is observed around the entire particle. From these data, it is clear that we can conformally coat PRINT® PLGA NPs with films around the entire particle surface and in a manner that preserves the monodispersity of the platform.

After demonstrating the feasibility of spray functionalization and observing a conformal coating surrounding two different PRINT® NP templates with a variety of coating materials, it was important to examine the ability to control film growth using Spray-LbL. Control over film thickness will allow precise tuning of drug release for therapeutic-containing NP systems as well as functionalization of these material systems in such a way that does not significantly impact the NP size or shape, which is a primary advantage of PRINT® manufacture. From Figures 4.3, 4.4.B, 4.4.E, it was observed that three bilayers of PLL/HA (3 seconds/spray) generate a film of approximately 15nm. To observe whether thicker films could be generated, high molecular weight polymers (Chitosan, 200K; HA, 500K) were sprayed (3 seconds/spray) alternately to yield a 5 bilayer film. As expected, this film was found to be significantly thicker, approximately 40nm by TEM (Figure 4.4.C, 4.4.F), while conformally coating the NP surface so as to not compromise the NP shape. SEMs of the coated backings and subsequent harvested and purified coated NPs for each PRINT® template are displayed in Appendix C.5. TEMs of each corresponding uncoated NP systems are displayed in Figure 4.4.A and 4.4.D for reference. This effectively demonstrates the capability of Spray-LbL as a means of tuning film thickness by incorporating higher molecular weight polymers with more bilayer coatings. Additional parameters to tune film thickness are longer spray times and higher concentrations of material sprayed.

As an additional test of particle functionalization, the polycationic layer, poly-L-lysine (PLL), was labeled with a Cy5.5 dye (Lumiprobe, Cy5.5-NHS ester). Deposition was tracked using fluorescence imaging and shown to significantly increase following layers 1 and 3, consistent with the subsequent addition of PLL_{Cy5.5}. This is also demonstrated by
directly comparing PLL/DXS films cast both with and without PLLCy5.5 using confocal microscopy (Appendix C.6).

While particle functionalization is clearly demonstrated and in a reproducible manner, questions regarding biological functionality remained as this component of the carrier system is critical to the translational relevance of the technology. Using the labeled PLLCy5.5 Spray-LbL functionalized NP systems, particles were incubated with a triple negative breast cancer cell line, BT-20. These cells, like many aggressive cancer cell types, characteristically overexpress CD44 receptors[22], which are a convenient target for the natural ligand, hyaluronic acid.[165-168] Previous work has also shown much promise for HA-coated systems to provide a serum-stable, stealth-like platform for delivery.[27] Investigations of cell-associated fluorescence in combination with confocal microscopy, shown in Figure 4.5.A-4.5.D, confirmed that these coatings are functional, as shown with enhanced levels of cell uptake for the HA-coated systems. Further, increased uptake for HA-coated systems is clear relative to the non-specific uptake of the DXS-terminated system, suggesting that film construction can be finely tuned towards targeting specific cell populations. In this way, Spray-LbL on PRINT® presents exciting opportunities to not only functionalize particles scalably, reproducibly, and with extremely thin coatings but also in a manner that molecular engagement of target cells can be achieved.

Particle shape also plays a significant role in cellular internalization, as shown in Figure 4.5.E-4.5.G. Coated PLGA80x320nm NPs ([PLL/HA]₃) exhibited nearly 10-fold higher levels of cell association relative to identically-coated PLGA200x200nm NPs within 2h of treatment, as determined by FACS. This enhanced level of uptake is further complemented by confocal microscopy, from which significantly more punctate NP fluorescence is observed inside the cells for the PLGA80x320nm NPs. The impact of particle size and shape on cellular internalization is of particular interest towards designing effective carrier systems that further mediate endocytosis, in addition to surface chemical functionality. This proves highly promising towards combination of optimal PRINT® technologies with target-specific Spray-LbL architectures to maximize therapeutic impact of these NP systems.
Conclusion

Spray-LbL on PRINT® is a significant advance in the design of nanotechnology, as it provides an exciting platform for large-scale production of built-to-order functional nanoparticle systems, whereby we have precise control of the physicochemical characteristics of the NP systems developed. This includes the ability to finely tune the NP shape, size, chemical composition, and surface characteristics towards manufacture of systems that maximize cellular entry and optimize drug and NP pharmacokinetics in vivo. Further, the ability to rapidly and scalably manufacture these systems is realizable, as PRINT® technology is capable of roll-to-roll NP fabrication while spray can be applied in a continuous fashion. This compatibility in continuous manufacture and subsequent functionalization of NP systems presents a highly attractive opportunity for highly reproducible manufacture of customizable NP systems for a wide variety of applications.
Scheme 4.1. Spray-LbL on PRINT® nanoparticles.
PRINT® particles were fabricated and used while immobilized in a post-harvesting array for Spray-LbL. Arrays were subsequently crosslinked under vapor-phase glutaraldehyde/concentrated acid conditions, followed by LbL application (sequential deposition of polycation/wash/polyanion/wash comprising one bilayer). Functionalized particles were harvested by sonication of the arrays in water and purified by filtration and ultracentrifugation.
Figure 4.1. Contact angle and particle iridescence characterization pre- and post-functionalization.
(a) Contact angle measurement and image of pre-crosslinked NP array. (b) Particle iridescence observed in the ordered particle array post-PRINT® fabrication. (c) Loss of iridescence following spray of water-based polyelectrolytes, indicative of complete loss of particles. (d) Contact angle measurement and image of post-crosslinked NP array (glutaraldehyde/HCl vapor phase crosslinking). Particle iridescence is maintained following (d) 15h crosslinking and (f) Spray-Lbl deposition of [PLL/HA]₃.
Figure 4.2. AFM characterization (height) of pre- and post-functionalized NP arrays. (a) Pre-crosslinked PLGA$_{200\times200\text{nm}}$ NP array; (b) PLGA$_{200\times200\text{nm}}$ NP array following crosslinking; (c) PLGA$_{200\times200\text{nm}}$/(PLL/HA$_{500\text{k}}$)$_3$. (d) Pre-crosslinked PLGA$_{80\times320\text{nm}}$ NP array; (e) PLGA$_{80\times320\text{nm}}$ NP array following crosslinking; (f) PLGA$_{80\times320\text{nm}}$/(PLL/HA$_{500\text{k}}$)$_3$. Scale bars representative of 2um. Color scale representative of linear gradient from 0 [bottom] to 200 nm [top].
Figure 4.3. SEM characterization of particles pre- and post-Spray-LbL functionalization.
SEM of (a,b) uncoated, crosslinked 200x200nm PLGA NP array; (c) purified uncoated, crosslinked 200x200nm PLGA NPs; (d,e) coated 200x200nm PLGA NP array [(PLL/HA)$_3$]; (f) purified coated 200x200nm PLGA NPs [(PLL/HA)$_3$]; (g,h) uncoated, crosslinked 80x320nm PLGA NP array; (i) purified uncoated, crosslinked 80x320nm PLGA NPs; (j,k) coated 80x320nm PLGA NP array [(PLL/HA)$_3$]; (l) purified coated 80x320nm PLGA NPs [(PLL/HA)$_3$]. Scale bars representative of 200nm.
Figure 4.4. TEM characterization of particles pre- and post-LbL functionalization - control film thickness.
TEM of (a) uncoated, crosslinked 200x200nm PLGA NPs; (b) purified coated 200x200nm PLGA NPs [(PLL/HA)$_3$]; (c) purified coated 200x200nm PLGA NPs [(Chit/HA)$_3$]; (d) uncoated, crosslinked 80x320nm PLGA NPs; (e) purified coated 80x320nm PLGA NPs [(PLL/HA)$_3$]; (f) purified coated 80x320nm PLGA NPs [(Chit/HA)$_3$]. Scale bars representative of 100nm. TEMs of uncoated, uncrosslinked NPs are displayed in Appendix C.10.
Figure 4.5. In vitro assessment of variable spray LbL coatings and PRINT® NP shape on cell uptake - tracking with PLLCy5.

Confocal microscopy of (a) PLGA200x200nm/PLL/HA500K NPs [L2 HA], (b) PLGA200x200nm/(PLL/HA500K)3 NPs [L6 HA], (c) PLGA200x200nm/PLL/DXS NPs [L6 DXS] incubated with BT-20 cells for 6h at 37°C. NPs labeled with PLLCy5 as first polycationic component in film and normalized in dose based on the absorbance of this layer on the functionalized NPs. (d) Mean cell-associated fluorescence [Cy5.5 channel (λex = 640nm, λem = 700nm)] for each NP formulation displayed in (a)-(c). Data presented as the average +/- SEM of triplicate measurements. Confocal microscopy of (e) PLGA80x320nm/(PLL/HA500K)3 NPs and (f)
PLGA_{200x200nm}/(PLL/HA_{500k})_x NPs incubated with BT-20 cells for 2h at 37°C. NPs labeled with PLL_{Cy5.5} as first polycationic component in film and normalized in dose based on the absorbance of this layer on the functionalized NPs. (g) Mean cell-associated fluorescence [Cy5.5 channel (\lambda_{ex} = 640nm, \lambda_{em} = 700nm)] for each NP formulation displayed in (e)-(f). Data presented as average +/- SEM of triplicate measurements. Confocal microscopy images representative of overlays of individual fluorescence channels displayed below [from left to right under each overlay: DAPI nuclear stain, phalloidin-488 stain, NP fluorescence – Cy5.5].
Table 4.1. Nanoparticle physicochemical characterization.
Dynamic light scattering data, based on z-average hydrodynamic diameter, and zeta-potential measurements were conducted at 25°C in 10mM NaCl/deionized water. Data is presented for purified, uncoated PLGA<sub>200x200nm</sub> and PLGA<sub>80x320nm</sub> NPs, purified PLGA<sub>200x200nm</sub> and PLGA<sub>80x320nm</sub> following crosslinking, and coated NPs: PLGA<sub>200x200nm/(PLL/HA<sub>500K</sub>)<sub>3</sub></sub> and PLGA<sub>80x320nm/(PLL/HA<sub>500K</sub>)<sub>3</sub></sub>. Data for mean hydrodynamic diameter and zeta-potential are presented as average +/- standard error of the mean for triplicate measurements.
References

Chapter 5. Liposomal compartmentalization of a hydrophobic-hydrophilic combination chemotherapy regimen (erlotinib, doxorubicin) for staged, targeted delivery against triple-negative breast cancer and non-small cell lung cancer.


Introduction

Cancers represent the end states of accumulated genetic transformations that disrupt normal cell signaling events, including those involved in DNA repair, cell cycle regulation, and cell death by apoptosis \cite{169, 170}, enabling these mutant cells to proliferate and metastasize. Paradoxically, although defects in DNA-damage signaling and response underlie tumor development, they also provide a mechanism for therapeutic tumor cell killing by conventional anticancer cytotoxic therapies, such as chemotherapy or radiation therapy. Undesired effects of these DNA-damaging treatments include the development of highly resistant residual tumors and toxicity to proliferative non-tumor tissues, such as the bone marrow and epithelial lining of the gastrointestinal tract. Therefore, there is an important clinical need to identify potent therapeutic strategies that target multiple tumor cell-specific survival pathways to enhance the extent of tumor cell killing and potentially reduce total drug exposure during treatment. Most drug-screening efforts have focused on careful selection of drug cocktails on the basis of the underlying biology of the tumor or the response to individual agents. Unfortunately, much less is known about the positive and negative drug-drug interactions for many combination therapies, and the influence of relative dose, dose duration, or timing of delivery has been much less frequently explored\cite{169, 170}.

Our recent work validated the impact of timing of drug delivery on the efficacy of multiagent chemotherapy: A pair of drugs that are not particularly beneficial as singular therapies, are effective when used in combination if a specific time lag between the administration of each drug is employed \cite{30, 95-97}. Thus, systematic study of the adaptive
responses of signaling networks in tumor cells following an initial drug exposure could be used to design highly effective drug combinations\[^{30}\].

We recently demonstrated that triple negative breast cancer (TNBC) cells and non-small cell lung cancer (NSCLC) cells are dramatically sensitized to the effects of DNA-damaging chemotherapy by prolonged, but not acute, suppression of epidermal growth factor receptor (EGFR) signaling \[^{30}\]. This sensitization effect resulted from the rewiring of signaling networks upon persistent EGFR inhibition, which unmasked a caspase-8-dependent cell death pathway critical to the ability of doxorubicin and other genotoxins to more effectively kill tumor cells in culture \[^{30}\]. This work demonstrated the importance of drug order and timing for maximizing synergistic effects of combination chemotherapy for cancer. However, translating these new findings into the clinic with existing delivery methods is likely to prove challenging due to patient-specific differences in pharmacokinetics, the differing pharmacodynamic parameters for each drug, and the difficulties in targeting both drugs to the same tumor cells in the proper temporal sequence.

To overcome these challenges, we created a robust nanoparticle-based delivery platform capable of producing a precise time-staggered drug release in vivo. Nanoparticles are colloidal material systems commonly comprised of organic (such as lipids or polymers) or inorganic (such as silica, iron, or gold) materials and are generally 200 nm or less in size. These structures are commonly employed as vectors for controlled drug delivery by containing and protecting the therapeutics from metabolism or destruction. Using this approach, we designed a single nanoparticle construct to serve as a dual hydrophobic-hydrophilic depot for timed sequence of both a EGFR inhibitor and a DNA-damaging agent. This technology facilitated delivery into mice, achieving intracellular co-localization of both drugs and time-sequenced delivery of the synergistic drug combinations. Such an approach, if successful in patients, should mitigate off-target side effects and increase the bioavailability of the drugs, thereby expanding the therapeutic efficacy of selected chemotherapy combinations identified by ongoing systems pharmacology and signaling-based studies.
**Materials and Methods**

All lipid components were purchased from Avanti Polar Lipids, except for cholesterol from Sigma. All therapeutics were purchased from LC laboratories, except for cisplatin (cis-diamineplatinum(II) chloride) from Sigma. All other chemicals (citric acid, sodium citrate, sodium carbonate) and solvents (chloroform, methanol, phosphate buffered saline) were purchased from Sigma.

**Study Design.** Erlotinib and doxorubicin as a combination therapeutic treatment have been previously shown to enable synergistic cell death in certain cell types when administration of the therapeutics is manually time-staggered[31]. We sought to achieve this temporal control over release in a systemically-administrable nanoparticle formulation. For efficacy evaluation of these systems, power analysis was performed with G*Power Analysis, using repeated-measures ANOVA, between-factors test. We assumed an effect size of 0.5, error probability of 0.05, power of 0.95, and a correlation of 0.2. To achieve statistical significance, 15 animals bearing dual rear xenografts were used – 5 in each experimental group. Endpoints were pre-determined for evaluation of the biological performance of these systems, as well as for those experiments investigating tumor burden of the animals following treatment. All experiments were randomized and non-blinded.

**Liposome preparation.** Liposomes were formulated at a mass ratio of 56:39:5 (DSPC:Cholesterol:POPG). These three components were dissolved, along with the small-molecule inhibitor (weight ratio to total lipid weight = 3:50), in a 2:1 mixture of chloroform:methanol. A thin film of these materials was generated by rotary evaporation at 40°C at 150 mbar. This film was dessicated overnight until completely dry. Hydration of the lipid film was conducted at 65°C under sonication in 300 mM citric acid buffer (pH 4) for 1h, Functionalization with DSPE-PEG<sub>5K</sub>-Folate, DSPE-PEG<sub>2K</sub>, with or without DSPE-PEG<sub>2K</sub>-Cy5.5 was conducted post-fabrication using a post-insertion technique in which micelles (in 0.9% sodium chloride solution) of the components desired to incorporate on the liposomal surface were incubated with the prepared, drug-loaded liposomes under sonication at 65°C for 30 minutes, after which they were filtered through a 0.2 μm polyethersulfone (PES) syringe filter and cooled to room temperature. The pH of the
liposomal suspension was then adjusted to ~6.5 by addition of 300 mM sodium carbonate buffer to create a gradient between the exterior and interior compartments. The cytotoxic (doxorubicin - 3 mg, cisplatin - 10 mg) was added in a 0.9% sodium chloride solution (1 mL) to load through a pH gradient method. To facilitate solubilization of the cytotoxic agents, the dispersed solution was sonicated at 65°C for 5 minutes. The final combination drug-loaded system was subsequently exchanged into PBS (pH 7.4) following centrifugal filtration (100K MWCO Millipore) to remove the high salt buffers (citric acid, sodium carbonate) and any unloaded drug. Empty (lacking drug) liposomes (NFP) were formulated using the same procedure.

**Liposome characterization.** Dynamic light scattering and z-potential analysis was conducted in 10 mM sodium chloride at 25°C using a Malvern ZS90 zeta-sizer. High-performance liquid chromatography (Agilent technologies) and nanodrop absorbance measurements (345 nm for inhibitor; 480 nm for doxorubicin) were used to validate drug loading of the inhibitor ($\lambda_{abs} = 345$ nm) and doxorubicin ($\lambda_{abs} = 480$ nm). Cisplatin concentration was quantified by a colorimetric assay using o-phenylenediamine against a standard curve. Cryo-transmission electron microscopy (cryo-TEM) was conducted by imaging a vitrified dilute sample of the liposomal suspension at 120 kV and 77 K.

**Nanoparticle Drug Release In Vitro.** Liposomes were incubated under sink conditions [1 L sink for 1 mL liposome suspension] in 1X PBS under agitation in 1 mL 3.5 K MWCO float-a-lyzers (Spectrum) at 37°C. PBS was replenished each day of the experiment. Samples were taken of the liposomes to quantify remaining drug concentrations by HPLC (following dissolution in a 50:50 mixture of acetonitrile:pH 5 water) and absorbance measurements (nanodrop) [345 nm for erlotinib, lapatinib, /gefitinib, or /afatinib; 480 nm for doxorubicin].

**Confocal Microscopy.** Images were taken using a Nikon A1R Ultra-Fast Spectral Scanning Confocal Microscope (Nikon instruments Inc., Melville, NY). BT-20 and A549 cells were seeded in CELLview glass-bottom dishes (Greiner Bio-One GmbH, Germany) at $1 \times 10^5$ cells per well and grown overnight in Opti-MEM (Gibco, Life Technologies). For data displayed
in Fig. 2, cells were then incubated with empty (no drug, NP-Cy5.5, NFP-Cy5.5) Cy5.5-labeled liposomes for 1 h at 37°C. At the end of this period, cells were washed, fixed with paraformaldehyde, permeabilized with Triton-1X, and stained with phalloidin-568 for 30 minutes, followed by the addition of DAPI for an additional 10 minutes, after which they were washed (with Opti-MEM) and imaged (in Opti-MEM) at the DAPI (excitation 360 nm, emission 460 nm) and Cy5.5 channel (excitation 640 nm, emission 700 nm). For data displayed in fig. S8, live-cell imaging was performed by exposing A549 cells to DFP-Cy5.5 or free doxorubicin in Opti-MEM and serially imaging the cells at the DAPI (excitation 360 nm, emission 460 nm), doxorubicin (excitation 480 nm, emission 560 nm), and Cy5.5 (excitation 640 nm, emission 700 nm) channels.

**Flow Cytometry.** Measurements were performed using a BD LSR Fortessa-HTS coupled with a high-throughput system for the 96-well plate format (BD biosciences, San Jose, CA). Doxorubicin fluorescence was measured following excitation at 488 nm and detected at 530 nm; Cy5.5 fluorescence was measured following excitation at 640 nm and detected at 710 nm. Cell-association data presented as geometric mean fluorescence collected in triplicate following cell incubation with empty and doxorubicin-loaded Cy5.5-labeled liposomes for 1h at 37°C.

**Apoptosis.** Measurements were conducted as previously described[^30]. Briefly, following the treatment time course, cells were washed, trypsinized, fixed in 4% paraformaldehyde for 15 min at room temperature, resuspended in ice-cold methanol, and incubated overnight at −20°C. Cells were then washed twice in PBS-Tween and stained with antibodies against cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP). Secondary Alexa-conjugated antibodies were used for visualization in a BD FacsCaliber flow cytometer.

**Western Blotting.** Experiments were performed as previously described[^30]. Briefly, cell lysates were prepared in a manner that enabled samples to be used for both Western blot analysis and reverse phase protein microarray. Cells were washed twice in PBS and lysed directly on the plate in a buffer containing 50 mM Tris-HCl, 2% SDS, 5% glycerol, 5 mM
EDTA, 1 mM NaF, 10 mM, β-glycerophosphate, 1 mM PMSF, 1 mM Na₃VO₄, and phosphatase and protease inhibitors (Roche complete protease inhibitor tablets and PhosSTOP tablets). Crude lysates were filtered using an AcroPrep 96-well 3.0 μm glass fiber/0.2 μm BioInert filter plate (Pall), and normalized for protein content using the BCA protein assay (Pierce). For Western blots, lysates were run on 48-well pre-cast gels and transferred using a semi-dry fast transfer apparatus onto nitrocellulose membranes (i-PAGE, E-BLOT, Invitrogen). Blots were blocked in Odyssey Blocking Buffer (LiCOR Biosciences), incubated overnight with primary antibody, stained with secondary antibodies conjugated to an infrared dye, and visualized using an Odyssey flat bed scanner (LiCOR Biosciences).

Antibodies for gH2AX, pERK, and cleaved caspase-8 were purchased from Cell Signaling Technologies. Antibodies against b-actin were purchased from Sigma.

Raw signals for each protein of interest were quantified and background subtracted using the Li-COR Odyssey software and divided by b-actin signals to normalize for loading differences, and then each normalized signal was divided by a reference sample contained on each gel for gel-to-gel normalization.

**Pharmacokinetics.** All animal experimentation adhered to the NIH Guide for the Care and Use of Laboratory Animals and was in accordance with institutional guidelines.

BALB/c female mice (Taconic) were systemically-administered (tail vein) empty Cy5.5-labeled liposomes (NFP-Cy5.5) at a concentration of 3 mg/mL (corresponding radiant efficiency, measured by the whole-animal imaging system – IVIS [Xenogen, Caliper Instruments] of 0.1 mL sample injected in each mouse ~1 x 10¹⁰). Whole-animal fluorescence imaging (IVIS – Xenogen, Caliper Instruments) was performed at the indicated time points for one cohort of mice (n = 3), and a separate cohort was used for retro-orbital bleeds to determine the circulation half-life of the system. Imaging and circulation data presented is normalized to auto-fluorescence determination (imaged animals, isolated blood) obtained prior to injection. Analysis of circulation data based on recovered
fluorescence normalized to pre-injection blood auto-fluorescence is displayed with a two-compartment model fit (both slow and fast half-lives presented).

**Tumor Targeting and Regression.** Tumor targeting data was obtained by imaging a Cy5.5-labeled DSPE-PEG-Cy5.5 lipid that was inserted into the liposomal membrane. This dye-labeled lipid was functionalized using a DSPE-PEG-NH2 lipid (Nanocs) and Cy5.5-NHS (Lumiprobe) at a 1:1 ratio in DMSO at 4°C for complete labeling of the lipid. Final dye-labeled lipid product was lyophilized and stored as a powder at -20°C. Tumor-targeting data was collected using a near-IR imager (IVIS, Xenogen, Caliper Instruments) at λ<sub>ex</sub> = 675 nm, λ<sub>em</sub> = 720 nm. % injected dose (ID) calculations were performed from fluorescence intensity of the tumors, following background subtraction of pre-injection tissue autofluorescence, and normalized to the amount of fluorescent material injected. Luminescent xenograft tumors were seeded following stable transfection of BT-20 and A549 cells with the firefly luciferase plasmid. This enabled assessment of tumor size by a visual and quantifiable luminescent readout generated by whole-animal imaging (Xenogen, Caliper Instruments). Cells were mixed in a 1:1 ratio with BD Matrigel<sup>TM</sup> Basement Membrane Matrix to a final density of 5 x 10<sup>7</sup> cells/0.1 mL injection. 0.1 mL injections of the matrix-cell suspension were performed in each rear hind flank of NCR nude mice (Taconic). Tumors were allowed to grow until a visible tumor was established and luminescence monitoring was indicative of solid tumor growth (see fig. S3, ~7 x 10<sup>8</sup> radiance final luminescent readout from each xenograft). Xenograft-bearing NCR nude mice were then systemically-administered therapy (DFP, DEFP) in a 0.1 mL injection at a concentration of 2 mg/kg (based on doxorubicin loading) and monitored for 32 days. Luminescence images were obtained by 0.1 mL intraperitoneal injections of 30 mg/kg D-luciferin (Caliper) and imaging (IVIS, Xenogen, Caliper) with an open luminescence filter 15 minutes post-injection. These data are presented, along with region of interest quantification of radiance corresponding to the xenograft-specific luminescence for each mouse treated. Data is normalized for each mouse against the tumor luminescence prior to injection and presented as fold luminescence above this measurement. N = 5 for each treatment (untreated, DFP, DEFP) and data corresponds to mean +/- SEM for each treatment group. An unpaired, two-tailed t-test comparing the single drug (DFP) and combination drug
(DEFP) systems was performed to determine statistical significance. A control simulating the simultaneous release of both doxorubicin and erlotinib was performed in vivo by the systemic co-administration of DFP liposomes (1 mg/kg) and an erlotinib-cyclodextrin complex (CD-Erl) in PBS (1 mg/kg, hydroxypropyl-beta-cyclodextrin) 22 days after xenografts were established. Subsequent booster doses of erlotinib were systemically administered at 1 mg/kg on days 2 and 4. Live-animal bioluminescence images of hindflank A549 xenografts shown prior to treatment (pre) and at days 3, 10, and 30 were collected. Quantification of luminescence (radiance [photons]), normalized to pre-injection tumor luminescence, is displayed as average +/- standard deviation fold luminescent change (normalized to pre-injection tumor luminescence).

**Statistical Analyses.** Prism 5 (GraphPad) was used for all analyses. Results are presented as mean ± SEM, unless otherwise noted. Efficacy data was analyzed by an unpaired, two-tailed t-test and repeated measures one-way ANOVA comparing all groups to assess significance in treatment. P < 0.05 was considered significant.

**Results**

As is typical of most EGFR inhibitors (log P ~ 2 to 6), the EGFR inhibitor erlotinib is highly hydrophobic (log P 2.7); whereas, as is typical of most DNA-damaging agents (log P ~ 1.5 to -1.5), doxorubicin is relatively hydrophilic (log P 0.9). Therefore, this particular drug combination presents a challenge in achieving high concentrations of both drugs in a single nanoparticle. Liposomes, however, provide a unique opportunity to achieve this capability by virtue of their vesicular structure [172,173]. By utilizing the lipid envelope for storage of the hydrophobic drug and the aqueous interior to house the hydrophilic drug, liposomes enable incorporation of high concentrations of both therapeutics and the potential to present them in a shell first, core second fashion to produce the desired sequential staggered release [30] – release of the hydrophobic small molecule EGFR inhibitor, erlotinib, from the shell of the nanoparticle prior to unloading of the cytotoxic, doxorubicin, from the core.

We fabricated liposomes containing doxorubicin and erlotinib using a lipid film-hydration method [174] (Figure 5.1A). Lipid vesicles were formed following hydration in an
acidic citric acid buffer under high heat and sonication in the presence of the hydrophobic inhibitor, erlotinib. Subsequent pH-driven loading of doxorubicin in the interior of the vesicles resulted in the final dual-drug liposomal system (see Materials and Methods) (Figure 5.1.B). Dynamic light scattering showed that the liposomes prepared in this two-step drug-loading process were of uniform size on the basis of their polydispersity index (PDI) (Table 5.1). We prepared both single-drug (D, doxorubicin) and dual-drug (DE, doxorubicin + erlotinib) liposomes, containing a mixture of lipids in a 56:39:5 mass ratio of DSPC (distearylphosphatidylcholine):cholesterol:POPG [1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol)] with z-average hydrodynamic sizes (physical size of the nanoparticle in suspension) relevant for systemic administration and with a ζ-potential, measure of surface charge, of -29 mV, indicative of a negatively charged phospholipid exterior. The drugs effectively accumulated in these, yielding a 2.5:1 mass ratio of doxorubicin:erlotinib within the liposomes, from an equal mass supply (3 mg of each drug per 50 mg of lipid used) during fabrication, and the encapsulation efficiency was higher for doxorubicin than for erlotinib (Table 5.1).

From the compartmentalization of drug in the liposome, we predicted that erlotinib, which is sequestered in the exterior lipid bilayer membrane compartment, would be released before doxorubicin, which is concentrated in the hydrophilic core of the liposomes. To determine the relative release rates of these two drugs, we measured the amount of drug remaining in the liposomes at serial time points following incubation in phosphate-buffered saline (PBS) at physiological pH (7.4) and temperature (37°C). After 24h, 60% of the erlotinib had been released, compared to only 20% of the doxorubicin (Figure 5.2.A). This differential fractional release suggested that spatial control of therapeutic loading into liposomes could enable presentation of this drug combination in the desired fashion for synergistic killing, particularly because the effective local concentration of erlotinib necessary for EGFR inhibition (~100 nM) is much lower than that required for doxorubicin-induced cytotoxicity (2-10 μM) [175, 176].

To determine if the differential release rates from the liposome recapitulated the synergistic cell killing observed previously [30], we applied the dual-drug and single-drug liposomes to BT-20 TNBC and A549 NSCLC cell lines. We observed an increase in apoptosis, monitored by the number of cells positive for cleaved caspase-3 and cleaved...
poly(ADP-ribose) polymerase (PARP), in response to either liposome, but DE liposomes were more effective at both 24h and 48h compared to the single-drug D liposomes (Figure 2B). Importantly, the enhanced cleavage of caspase-8, which we have shown previously occurs only in cells treated with time-staggered EGFR-doxorubicin combinations and not in cells co-treated simultaneously with EGFR inhibitors and doxorubicin [30], was detected in both cell lines upon DE liposome treatment (Figure 5.2.C).

Reduced abundance of phosphorylated extracellular signal-regulated kinase (pERK) occurs in response to EGFR inhibition. We observed reduced pERK abundance in the DE liposome-treated cells, confirming that EGFR activity had been effectively suppressed by release of erlotinib from the dual-loaded nanoparticles (Figure 5.2.D).

Molecular characterization of liposome-treated cells revealed equivalent amounts of DNA damage, as assessed by γH2AX formation, produced by exposure to either D liposomes or DE liposomes (Figure 5.2.E). Although both liposomal formulations induced substantial DNA damage (Figure 5.2.E), the DE formulation produced sustained inhibition of EGFR as indicated by reduction in pERK for up to 72h (Figure 5.2.D) and were more cytotoxic, as indicated by the larger apoptotic response (Figure 5.2.B-C).

To further improve the utility of this time-staggered drug delivery for in vivo use, we prepared liposomes with folate and polyethylene glycol (PEG) as a means of minimizing protein adsorption and subsequent nonspecific clearance, while promoting tumor targeting [177-182]. Folate is a commonly used ligand for targeted cancer delivery; because it enhances delivery of therapeutics to tumor cells on which folate receptor alpha is abundant at the cell surface. Of particular interest for this investigation, as many as two out of three patients with NSCLC and TNBC, as well as many other cancer cell types including tumors of the ovary and prostate, have tumors with abundant folate receptors [183]. Thus, to enhance tumor targeting, we included a folate-functionalized lipid, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol-5000 (DSPE-PEG5K), with a 5K PEG linker (DSPE-PEG5K-Folate) in the lipid formulation at a 0.5 mole% ratio to the total lipid composition. We also included shorter 2K PEG linker lipid (DSPE-PEG2K) at an equivalent 0.5 mole% ratio to minimize protein adsorption and opsonization of the liposomes while still enabling access to the folate group on the longer PEG linker (Figure 5.3.A). This type
of targeted liposomal design has been used to deliver single-drug therapies\cite{177, 178, 180-182, 184}.

The resulting single drug-loaded liposomes with doxorubicin, folate, and PEG (DFP) and the dual drug-loaded liposomes with doxorubicin, erlotinib, folate, and PEG (DEFP) were uniform, with a slight reduction in negative z potential compared to the uncoated liposomes, due to the addition of PEG to the surface, and a corresponding \(~10\%\) increase in size from the uncoated liposomes (Table 5.1). Thus, the addition of folate and PEG on the drug-loaded liposomal exterior only resulted in a moderate increase in particle size, which is important for systemic administration to minimize physical filtration of the nanoparticles by the liver and spleen and enhance tumor accumulation.

To demonstrate the cell-targeting capabilities of these functionalized liposomes, we labeled doxorubicin-loaded, folate-functionalized liposomes with a near-infrared (IR) dye Cy5.5 (DFP-Cy5.5) by including the Cy5.5-labeled 2K PEG lipid conjugate DSPE-PEG2K-Cy5.5 at 0.1 mole\%, which enabled fluorescent tracking\cite{180, 181} in vitro and in vivo. We also created nonfunctionalized PEG liposomes labeled with Cy5.5 (DP-Cy5.5), but lacking the folate (Appendix Table D.1), so that we could determine the effect of folate targeting on the cellular uptake of the liposomes. We analyzed nanoparticle uptake by confocal microscopy (Fig. 3B) and flow cytometry (Figure 5.3.C) of both BT-20 and A549 cells following 1-hour incubation with either Cy5.5-labeled liposomes containing the folate for cell targeting (DFP-Cy5.5) or lacking the folate (DP-Cy5.5). Following incubation with the targeted liposomes (DFP-Cy5.5), both cell lines exhibited particle fluorescence (red) throughout the cell cytosol, suggesting uptake and endosomal escape of the liposomal contents (Figure 5.3.B). Analysis by flow cytometry confirmed that both cells lines exhibited enhanced uptake of folate-containing liposomes compared with that of the liposomes without folate (Figure 5.3.C) and showed that saturation occurred at high concentrations of folate-containing liposomes. Not only did the folate-containing liposomes result in substantially greater uptake of the nanoparticles by the cells, but also increased the concentration of doxorubicin associated with each cell line (Figure 5.3.C). For the untargeted systems, it is likely that most of the doxorubicin that accumulates in the cells results from nonspecific liposomal uptake or occurs by pinocytosis of the free drug that is released into the media by the liposomes. We compared the uptake and nuclear
accumulation of DFP-Cy5.5 liposomes with that of free doxorubicin by confocal microscopy, which confirmed that uptake and nuclear accumulation were delayed when delivered by the nanoparticles (Appendix D.1).

To evaluate these systems for tumor remediation in vivo, we first examined the pharmacokinetics of the folate-PEG-functionalized empty nanoparticles (lacking drugs) injected intravenously into healthy BALB/c mice, which are immune proficient. The folate-PEG-functionalized, Cy5.5-labeled empty liposomes (NFP-Cy5.5) exhibited relatively low liver accumulation as a function of time (14%, 23%, 17%, 14%, 12% injected dose respectively, based on fluorescence recovery, at 30min, 9h, 24h, 48h, 72h, respectively) (Figure 5.4.A) In the circulation, NFP-Cy5.5 exhibited half-lives of 2.2h and 11.1h, representing rapid and slow elimination phases calculated on the basis of a two-compartment model (Figure 5.4.B). Furthermore, visualization of firefly luciferase-expressing tumors revealed NFP-Cy5.5 liposome accumulation in the tumors of xenograft-bearing nude mice up to 30 days following a single injection the folate-targeted liposomes; we observed 2% injected dose at 9h, 5% injected dose at 24h, and 7% injected dose after 30 days based on fluorescence recovery (Figure 5.4.C). We also observed lower intensity liposome fluorescence in other tissues, such as the liver, kidneys, and brain (Appendix D.2). Thus, the formulated folate-targeted liposomal systems accumulated appreciably in the target tissue (xenograft tumors), as well as the liver, kidneys, and brain, for a prolonged period of time.

Because tumor size in BT-20 and A549 xenograft models scaled with bioluminescence of the firefly luciferase-expressing tumors measured with the live animal imaging system (Appendix D.3), we could quantify tumor size in situ during treatment. We compared the effectiveness of the administration of DEFP liposomes or DFP liposomes, in limiting or reducing tumor growth in the BT-20 TNBC and A549 NSCLC xenograft models (Figure 5.5.A, B; Appendix A.4, 5). A single administration of 2 mg/kg of the dual drug-loaded (DEFP) liposome 22 days after the tumors had been injected (Appendix D.3) produced tumor regression, whereas the single drug-loaded DFP liposomes and the untreated control mice exhibited continued tumor growth (Figure 5.5.A, B) In some of the untreated control mice injected with A549 cells, tumors grew rapidly, reaching the
maximum allowed tumor burden (Appendix D.4, 5). Significant tumor shrinkage was only observed for combination therapy-treated mice bearing xenografts for each cell line.

We also tested the effectiveness of codelivery of doxorubicin and erlotinib. Because it was difficult to achieve simultaneous delivery with a single liposomal nanoparticle for this drug combination, we designed single drug delivery systems for each drug in the combination therapy and administered them together to mimic a co-release behavior. We co-injected 2 mg/kg DFP with cyclodextrin-erlotinib (hydroxylpropyl-β-cyclodextrin) in phosphate-buffered saline at 0.5 mg/kg in A549 xenograft-bearing mice, followed by repeated dosing of cyclodextrin-erlotinib at days 2 and 4 to supply additional erlotinib to match the slow, sustained release of doxorubicin (Appendix D.6). Although some inhibition of tumor growth was observed at day 3, later the tumors exhibited increased growth. Thus, simultaneous, sustained coadministration was a less effective treatment, resulting in tumor growth. In contrast, tumor shrinkage and regression was achieved with staged combination therapeutics packaged in the same nanoparticle delivery vehicle.

So far, we extensively characterized a time-staggered doxorubicin:erlotinib combination liposome that effectively and significantly reduced xenografted tumor growth. We examined if this platform could be adapted for delivery of other therapeutic combinations. We interchanged the cytotoxic drug encapsulated in the aqueous compartment [doxorubicin (D) or cisplatin (C)], and incorporated one of the following (RTK) inhibitors of signaling by EGFR family members into the lipid envelope, erlotinib (E), gefitinib (G), afatinib (A), or lapatinib (L). The physicochemical properties of the nanoparticles, including size distribution and drug loading efficiency, with the different drug combinations were similar (Tables 5.2, 3). In vitro drug release kinetics were similar for each of the combinations, with rapid release of the RTK inhibitor followed by a sustained release of the cytotoxic agent (Figure 5.6.A, Appendix D.7). We examined the ability of each drug combination-folate-targeted liposome (DAFP, DEFP, DGFP, DLFP, CAFP, CEFP, CGFP, CLFP) to mediate cell death in culture by analyzing caspase-3 and PARP cleavage in BT-20 and A549 cells (Figure 5.6.B, C). Some combinations were more effective at promoting tumor cell death, whereas others had little effect on the absolute magnitude of cell death but accelerated the rate at which the cells died. Compared to the A549 cells, BT-20 cells were typically more sensitive to the cytotoxicity effects of any of the
single cytotoxic agents or the dual drug-loaded liposomes, exhibiting >80% cell death in most conditions. Within each cell line, there were some differences in cytotoxicity. For example, the afatinib-cisplatin combination was the most effective of the cisplatin-containing combinations in promoting A549 cell death (~20%); whereas ~40-50% of these cells were apoptotic when exposed to any of the doxorubicin-containing combinations. For the BT-20 cells the cisplatin-erlotinib and cisplatin-afatinib combinations produced the fastest apoptotic response, which might be an important determinant of efficacy in vivo; however, the maximum percent of apoptotic BT-20 cells was similar for all dual drug-loaded liposome combinations. The cisplatin combinations were generally as effective at producing cell death as cisplatin alone, with the exception of the cisplatin-afatinib (CA) combination, which produced a higher maximal cell death than any other combination or cisplatin alone in A459 cells.

We also examined the different doxorubicin- and RTK-loaded liposomes for caspase-8 cleavage, as well as their effect on the extent of DNA damage, as indicated by the abundance of γH2AX, and on downstream signaling, as indicated by phosphorylation ERK abundance (Appendix D.8).

**Discussion**

Here, we utilized knowledge of synergistic combination therapies that re-wire signaling pathways and networks [30]. However, the synergistic therapeutic response exhibits a pronounced dependence on timing and sequence of drug release. Therefore, we packaged the drugs together in a systemically administrable tumor-targeted drug carrier, a liposome-based nanoparticle. Some success towards implementing synergistic cancer therapies using engineered delivery systems had been achieved previously [172, 173, 185-191]. These prior strategies have largely focused on the simultaneous release of drugs from a single delivery platform using various techniques, including covalent linkage of drug to the material comprising the nanoparticle or encapsulation within the nanomaterial. Although these approaches have demonstrated the enhanced therapeutic efficacies of drug combinations, there has been no development to date of time-staggered release platforms that are specifically designed to engage and rewire cancer survival pathways.
We found that differential release rates of the drugs occurred in the correct sequence from a single nanoparticle platform and that the dual drug-loaded liposomes were cytotoxic to cultures A549 cells and BT-20. Molecular readouts (caspase-8 cleavage, pERK, γH2AX) further confirmed that the multidrug liposomes disrupted cell function in the expected sequence with early and sustained suppression of EGFR signaling, thereby rendering the cells susceptible to apoptosis in response to later exposure to toxic amounts of DNA-damaging agents. Furthermore, modification of the combination drug-loaded liposomes to include folate and PEG promoted tumor targeting in vivo for enhanced efficacy against A549 and BT-20 xenograft tumor models compared to single drug treatment alone.

The efficacy of these staggered release nanoparticle systems containing a small-molecule inhibitor with a cytotoxic agent presents a new therapeutic option for cancer therapy. By exploiting the intrinsic two-compartment property of a liposomal system, combination therapeutics with different physiochemical properties (hydrophilic and hydrophobic) were compartmentalized with reasonable efficiencies in a core-shell fashion that facilitated the staggered release observed. Because liposomal systems provide high levels of control over fabrication and modularity, these type of nanoparticle systems should enable customizable targeting to specific tumors, as well as loading of therapeutic agents tailored to specific treatment regimens.

The drugs that we tested were naturally hydrophobic (erlotinib, afatinib, gefitinib, lapatinib) or hydrophilic (doxorubicin and cisplatin), and this nanoparticle system should be adaptable to combinations that have chemically similar properties as these two combinations (RTK inhibitor and cytotoxic agent). However, staggered release that relies on the core versus outer layer loading of the two drugs may not always be attainable. Therefore, other technologies, such as layer-by-layer assembly of polyelectrolytes, can facilitate coatings on the exterior of drug-loaded nanoparticles to incorporate both hydrophilic and hydrophobic molecules, to introduce compartmentalization of two or more drugs and enable control over timing and sequence of drug release. Further, conjugation of one therapeutic to the nanoparticle core, followed by encapsulation or coating on this core could further promote the staggered release necessary to elicit synergism between drug combinations. Finally, combinations that require delivery to the
same cell for optimal synergistic efficacy further demonstrate the importance of encapsulating multi-drug combinations within a single nanoparticle formulation.

The complexity of growth, survival, and death signaling pathways in cancers continues to motivate investigation using systems biology approaches to inform treatments against cancer\(^\text{95, 192}\). Furthermore, with the growing appreciation that the pathways change in response to treatment or drug exposure, development of delivery systems that are safe, yet capable of sophisticated levels of control over the delivery, timing, or sequence of release, to therapeutically rewire these signaling pathways are essential to translate findings to the clinic. Here, we addressed this challenge by developing a simple delivery platform to target specific cancer cell types that are responsive to sets of synergistic drugs with predetermined staged treatment regimens. Future tuning and adaptation of these systems using nanomaterials design approaches should greatly enrich the range of signaling pathway-based drug combinations and synergistic time-dependent release profiles that can be achieved for targeted tumor therapy through dynamic network rewiring.
Figure 5.1. Characterization of combination therapeutic-loaded liposomal system.

(A) Cryogenic transmission electron micrograph (cryo-TEM) of dual drug-loaded liposomes. Scale bar representative of 100 nm. (B) Schematic of dual loading of a small molecule inhibitor (erlotinib, blue) into the hydrophobic, vesicular wall compartment and a cytotoxic agent (doxorubicin, green) loading into the aqueous, hydrophilic interior.
Figure 5.2. Evaluation of doxorubicin-erlotinib dual drug-loaded liposomal system in vitro.  
(A) Drug release from dual-drug-loaded liposomes in an excess volume of pH 7.4 PBS at 37°C under agitation.  
(B) Comparative cytotoxicity of dual drug-loaded liposome relative to the single drug-loaded liposome in BT20 (TNBC) and A549 (NSCLC) cell lines. This was measured by staining against cleaved caspase-3 and poly(ADP-ribose) polymerase [PARP]. (C) Cleaved caspase-8 in BT-20 cells (upper) and A549 cells (lower) following addition of the indicated liposomes. In the gel images, red is actin and green is cleaved caspase-8. Quantification shown below stained gel images corresponds to relative signal of cleaved caspase-8 to actin. Data are presented as mean +/- SEM or 3 experiments. (D) The dynamics of pERK in BT-20 cells (upper) and A549 cells (lower) following addition of the indicated liposomes. The data are representative of 3 experiments and, for quantification, pERK abundance was normalized to actin. (E) γH2AX formation in BT-20 cells (upper) and A549 cells (lower) following addition of the indicated liposomes. The data are representative of 3 experiments and, for quantification, γH2AX abundance was normalized to actin. D liposomes, doxorubicin only (single drug); DE liposomes, doxorubicin and erlotinib (dual drug).
Figure 5.3. Folate decoration of combination doxorubicin-erlotinib therapeutic-loaded liposomes for targeted delivery to TNBC (BT-20) and NSCLC (A549) cells.

(A) Schematic of addition of DSPE-PEG2K [mole% ratio (0.5%)] to minimize nonspecific protein binding, DSPE-PEG2KCy5.5 [mole% ratio (0.1%)] for fluorescent tracking, and DSPE-PEG5K-Folate [mole% ratio (0.5%)] for cell-targeted delivery. (B) Cell uptake of the folate-targeted liposomes in BT-20 and A549 cells, visualized by confocal microscopy. Blue = nuclei, labeled with DAPI; green = actin, labeled with phalloidin Ph586; red = DSPE-PEG2KCy5.5-labeled DFP liposomes. Lower panels represent the fluorescence in each channel, upper panel is a merged image. (C) Cell-associated fluorescence measured by flow cytometry as a function of nanoparticle (NP) concentration in both cell lines after incubation with folate-containing or absent liposomes at 37°C for 1h. Data on left corresponds to Cy5.5 (λex = 675nm, λem = 710nm) corresponding to liposomal association for both targeted (DFP-Cy5.5, squares) and untargeted control (DP-Cy5.5, circles). Data on right corresponds to the amount of doxorubicin associated with the cells [doxorubicin fluorescence, (λex = 480nm, λem = 560nm)] for both targeted (DFP-Cy5.5, squares) and untargeted control (DP-Cy5.5, circles). Data presented as mean +/- SEM of 3 experiments.
Figure 5.4. Biological performance of folate-targeted liposomal system in vivo.

(A) Biodistribution panel of folate-targeted liposomes containing no drug (tracked through Cy5.5 fluorescence) that were intravenously administered to BALB/c mice. In situ quantification (region identified with circle at 30 min post-injection) of liver-associated nanoparticle fluorescence (normalized to injected dose) presented above each time point. (B) Circulation data displayed as percent injected dose (ID), on the basis of nanoparticle Cy5.5 fluorescence recovered in blood samples. Half-life calculated based on a two-compartment model and presented as mean +/- SEM. (C) Tumor visualization (left, visualized as firefly luciferase expressed in the xenografted cells) and nanoparticle visualization (right, visualized by Cy5.5 fluorescence) 30 days after injection of single 0.1 mL administration of folate-targeted empty liposomes (NFP-Cy5.5) to either BT-20- and A549-xenograft bearing NCR nude mice bearing BT-20 xenografts or A549 xenografts on the hind flanks. The same animals are shown in the left and right images. See fig. S2 for tissue necropsy.
Figure 5.5. Effect of dual drug-loaded or single drug-loaded, folate-targeted liposomes on A549 and BT-20 tumor size.

(A) A549-luciferase expressing, xenograft-bearing NCR nude mice; n = 5, quantification representative of mean +/- SEM. (see fig. S5 for all 5 mice) (B) BT-20-luciferase expressing, xenograft-bearing NCR nude mice; n = 5, quantification representative of mean +/- SEM. (see fig. S4 for all 5 mice). Tumor-imaging data for dual-drug (DEFP, top), single-drug (DFP, middle), and untreated control, along with luminescence quantification (reported as fold initial tumor luminescence, presented on a semilog plot) corresponding to tumor size as a function of time, following a single administration of 1 mg/kg drug-loaded liposomal formulations. Animals with tumor reaching 1 cm were sacrificed. An unpaired, two-tailed t-test comparing the DFP and DEFP at the terminal 32d time point shows statistical significance with p-values of 0.0057 and 0.001 for treated A549 and BT-20 xenograft bearing mice, respectively. A one-way ANOVA comparing all treatments for the duration of the experiment (all time points) was also performed for each xenograft cell line and p-values of 0.0024 and 0.0010 were obtained for each A549 and BT-20 cells, respectively.
Figure 5.6. Developing the RTK inhibitor-cytotoxic agent combination liposomal systems as a platform for dual-drug delivery.

(A) In vitro drug release from dual-drug loaded folate-targeted liposomal formulations. Top represents the release from folate and PEG-containing liposomes (FP) with doxorubicin and the indicated RTK inhibitor. Bottom represents the release from folate and PEG-containing liposomes (FP) with cisplatin and the indicated RTK inhibitor. Liposomes were incubated with pH 7.4 PBS at 37°C under agitation in sink conditions. Data presented as mean of triplicate experiments. (B) Cytotoxicity of dual drug-loaded (RTK and doxorubicin) liposomes compared with that of single drug-loaded (doxorubicin) liposomes against BT-20 and A549 cells. Data presented as mean of triplicate experiments. (C) Cytotoxicity of dual drug-loaded (RTK and cisplatin) liposomes compared with that of single drug-loaded (cisplatin) liposomes against BT-20 and A549 cells. Data presented as mean for triplicate experiments. D = doxorubicin; A = afatinib; E = erlotinib; G = gefitinib; L = lapatinib; C = cisplatin; F = folate; P = PEG.
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<th>Liposomal formulation</th>
<th>Mean $z_{\text{avg}}$ d$_h$ (nm) +/- SEM</th>
<th>PDI</th>
<th>$\zeta$ potentials (mV)</th>
<th>Encapsulation Efficiency</th>
<th>w/w % (drug/lipid)</th>
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<td>DE</td>
<td>136 +/- 10</td>
<td>0.13</td>
<td>-29</td>
<td>D: 97%</td>
<td>D: 5.4%</td>
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<tr>
<td>D</td>
<td>126 +/- 16</td>
<td>0.15</td>
<td>-27</td>
<td>D: 97%</td>
<td>D: 5.5%</td>
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<td>DFP</td>
<td>156 +/- 7</td>
<td>0.1</td>
<td>-17</td>
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<td>D: 5.5%</td>
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<tr>
<td>DEFP</td>
<td>151 +/- 14</td>
<td>0.16</td>
<td>-15</td>
<td>D: 97%</td>
<td>D: 5.5%</td>
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Table 5.1. Dynamic light scattering, polydispersity index, and potential measurements for the doxorubicin-erlotinib dual-drug and single-drug uncoated and coated (FP) liposomal systems. Measured in 10 mM NaCl at 25°C. Encapsulation efficiency (% loaded from amount supplied) and mass loading ratio (g/g drug/lipid) as determined by high-performance liquid chromatography. DE, doxorubicin and erlotinib; D, doxorubicin; DFP, doxorubicin, folate, PEG; DEFP, doxorubicin and erlotinib, folate, PEG.
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<th>Liposomal Formulation</th>
<th>Mean $z_{avg} d_h$ (nm) +/- SEM</th>
<th>PDI</th>
<th>Encapsulation Efficiency</th>
<th>w/w % (drug/lipid)</th>
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<tr>
<td>DFP</td>
<td>156 +/- 7</td>
<td>0.1</td>
<td>D: 97%</td>
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<td>DAFP</td>
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<td>DLFP</td>
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<td>0.13</td>
<td>D: 97% L: 70%</td>
<td>D: 5.5% L: 3.8%</td>
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Table 5.2. Dynamic light scattering and polydispersity index measurements for the various dual-drug and single-drug coated (FP) liposomal formulations - doxorubicin + afatinib/erlotinib/gefitinib/lapatinib. Measured in 10mM NaCl at 25°C. Encapsulation efficiency (% loaded from amount supplied) and mass loading ratio (g/g drug/lipid) as determined by high-performance liquid chromatography. D = doxorubicin; A = afatinib; E = erlotinib; G = gefitinib; L = lapatinib; C = cisplatin; F = folate; P = PEG. Corresponding formulations without folate-PEG (FP) are shown in table S1.
Table 5.3. Dynamic light scattering, polydispersity index, and zeta-potential measurements for the various multi-drug coated (FP) liposomal formulations – cisplatin + afatinib/erlotinib/gefitinib/lapatinib. Measured in 10mM NaCl at 25°C. Encapsulation efficiency (% loaded from amount supplied) and mass loading ratio (g/g drug/lipid) as determined by high-performance liquid chromatography. D = doxorubicin; A = afatinib; E = erlotinib; G = gefitinib; L = lapatinib; C = cisplatin; F = folate; P = PEG. Corresponding formulations without folate-PEG (FP) are shown in Table S1.

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<th>Liposomal Formulation</th>
<th>Mean $z_{avg}$ $d_n$ (nm) +/- SEM</th>
<th>PDI</th>
<th>Encapsulation Efficiency</th>
<th>w/w % (drug/lipid)</th>
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<td>CFP</td>
<td>183 +/- 12</td>
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<td>177 +/- 6</td>
<td>0.06</td>
<td>C: 58%</td>
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<tr>
<td>CEFP</td>
<td>183 +/- 13</td>
<td>0.16</td>
<td>C: 60%</td>
<td>C: 10.5%</td>
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<tr>
<td>CGFP</td>
<td>162 +/- 8</td>
<td>0.09</td>
<td>C: 55%</td>
<td>C: 9.4%</td>
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<tr>
<td>CLFP</td>
<td>204 +/- 11</td>
<td>0.11</td>
<td>C: 60%</td>
<td>C: 10.2%</td>
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References


Introduction

Histone deacetylase inhibitors (HDACi) are promising anti-cancer therapeutics that have demonstrated activity in altering gene expression, inducing apoptosis and cell cycle arrest in various cancer cells, as well as promoting degradation of oncogenes. One such HDACi developed by Janssen Pharmaceuticals, Inc. is JNJ-26481585, also known as quisinostat. It has been shown to have potent anti-tumor activity and favorable oral bioavailability, biodistribution, and tissue penetration than the current clinically approved HDACi, vorinostat. Despite this, free drug administration of HDACi, including quisinostat, is dose-limiting and shows a potent cardiotoxicity that prevents its immediate clinical application.

Lung cancer is the deadliest form of cancer for both men and women in the United States and worldwide. Over 80% of tumors are non-small cell lung carcinoma (NSCLC), with most cases diagnosed at a late stage, beyond the point where they can be cured by surgery alone. Despite chemotherapy and radiation, the mainstays of treatment for inoperable tumors, the overall prognosis is grim, with a 5-year survival of ~15%. Targeted therapies have shown substantial benefit in select patients; EGFR inhibitors significantly improve survival of patients with adenocarcinomas with EGFR mutations. Similarly effective therapies are, however, presently lacking for the majority of patients. Conventional cytotoxic therapies for advanced NSCLC involving platinsates with a second agent chemotherapeutic agent have shown modest benefits.

Towards this end, we sought to address this clinical need by packaging and delivering quisinostat combination cisplatin in a singular nanoparticle formulation for more effective treatment of NSCLC.

Materials and Methods

All lipid components were purchased from Avanti Polar Lipids, except for cholesterol from Sigma and DSPE-PEG2k/DSPE-PEG2k-NH2 from Nanocs, Inc. Quisinostat was provided by Janssen Pharmaceuticals, Inc. Cisplatin (cis-diamineplatinum(II) chloride) was purchased
from Sigma. All other chemicals (citric acid, sodium citrate, sodium carbonate, folate, transferrin) and solvents (chloroform, methanol, phosphate buffered saline) were purchased from Sigma.

**Liposome preparation.** Liposomes were formulated at a mass ratio of 56:39:5 (DSPC:Cholesterol:POPG). These three components were dissolved, along with the small-molecule inhibitor (weight ratio to total lipid weight = 3:50), in a 2:1 mixture of chloroform:methanol. A thin film of these materials was generated by rotary evaporation at 40°C at 150 mbar. This film was dessicated overnight until completely dry. Hydration of the lipid film was conducted at 65°C under sonication in 300 mM citric acid buffer (pH 4) for 1h. DSPE-PEG2k-Folate was prepared by reacting DSPE-PEG2k-NH$_2$ with folate in the presence of DCC/pyridine as previously described.$^{[179,207,208]}$ Functionalization with DSPE-PEG5k-Folate, DSPE-PEG2k, with or without DSPE-PEG2k-Cy5.5 was conducted post-fabrication using a post-insertion technique in which micelles (in 0.9% sodium chloride solution) of the components desired to incorporate on the liposomal surface were incubated with the prepared, drug-loaded liposomes under sonication at 65°C for 30 minutes, after which they were filtered through a 0.2 μm polyethersulfone (PES) syringe filter and cooled to room temperature. The pH of the liposomal suspension was then adjusted to ~6.5 by addition of 300 mM sodium carbonate buffer to create a gradient between the exterior and interior compartments. The cytotoxic (doxorubicin – 3 mg, cisplatin – 10 mg) was added in a 0.9% sodium chloride solution (1 mL) to load through a pH gradient method. To facilitate solubilization of the cytotoxic agents, the dispersed solution was sonicated at 65°C for 5 minutes. The final combination drug-loaded system was subsequently exchanged into PBS (pH 7.4) following centrifugal filtration (100K MWCO Millipore) to remove the high salt buffers (citric acid, sodium carbonate) and any unloaded drug. Empty (lacking drug) liposomes (NFP) were formulated using the same procedure. Cryo-preservation was done following a previous published protocol.$^{[209]}$

**Liposome characterization.** Dynamic light scattering and z-potential analysis was conducted in 10 mM sodium chloride at 25°C using a Malvern ZS90 zeta-sizer. High-performance liquid chromatography (Agilent technologies) and nanodrop absorbance
measurements (345 nm for inhibitor; 480 nm for doxorubicin) were used to validate drug loading of the inhibitor ($\lambda_{\text{abs}} = 345\text{nm}$) and doxorubicin ($\lambda_{\text{abs}} = 480\text{nm}$). Cisplatin concentration was quantified by a colorimetric assay using o-phenylenediamine against a standard curve [171]. Cryo-transmission electron microscopy (cryo-TEM) was conducted by imaging a vitrified dilute sample of the liposomal suspension at 120 kV and 77 K.

**In vitro assessment.** Liposomes were incubated under sink conditions [1 L sink for 1 mL liposome suspension] in 1X PBS under agitation in 1 mL 3.5 K MWCO float-a-lyzers (Spectrum) at 37°C. PBS was replenished each day of the experiment. Samples were taken of the liposomes to quantify remaining drug concentrations by HPLC (following dissolution in a 50:50 mixture of acetonitrile:pH 5 water) and absorbance measurements (nanodrop) [345 nm for erlotinib, lapatinib, /gefitinib, or /afatinib; 480 nm for doxorubicin]. IC50 determined by a dose-escalated MTT cytotoxicity assay with the value empirically determined by the fit to the data – find value at which 50% cell death occurs corresponding to fit on a death vs. log(concentration) plot.

**Pharmacokinetics.** BALB/c female mice (Taconic) were systemically-administered (tail vein) empty Cy5.5-labeled liposomes (NFP-Cy5.5) at a concentration of 3 mg/mL (corresponding radiant efficiency, measured by the whole-animal imaging system – IVIS [Xenogen, Caliper Instruments] of 0.1 mL sample injected in each mouse ~1 x 10^10). Whole-animal fluorescence imaging (IVIS – Xenogen, Caliper Instruments) was performed at the indicated time points for one cohort of mice (n = 3), and a separate cohort was used for retro-orbital bleeds to determine the circulation half-life of the system. Imaging and circulation data presented is normalized to auto-fluorescence determination (imaged animals, isolated blood) obtained prior to injection. Analysis of circulation data based on recovered fluorescence normalized to pre-injection blood auto-fluorescence is displayed with a two-compartment model fit (both slow and fast half-lives presented).

**Gross toxicity.** BALB/c mice were dosed intraperitoneally with free and liposomal encapsulated therapeutics at the given concentrations (dose-escalated). Body weight of
cohorts of 5 mce/treatment was recorded during and following repeated doses on the indicated days. Data is presented as weight versus day from first administration.

Tumor Targeting and Regression. Tumor targeting data was obtained by imaging a Cy5.5-labeled DSPE-PEG-Cy5.5 lipid that was inserted into the liposomal membrane. This dye-labeled lipid was functionalized using a DSPE-PEG-NH2 lipid (Nanocs) and Cy5.5-NHS ester (Lumiprobe) at a 1:1 ratio in DMSO at 4°C for complete labeling of the lipid. Final dye-labeled lipid product was lyophilized and stored as a powder at -20°C. Tumor-targeting data was collected using a near-IR imager (IVIS, Xenogen, Caliper Instruments) at λ_ex = 675 nm, λ_em = 720 nm. % injected dose (ID) calculations were performed from fluorescence intensity of the tumors, following background subtraction of pre-injection tissue autofluorescence, and normalized to the amount of fluorescent material injected. Necropsy of relevant tissue (liver, kidneys, spleen, heart, lungs, brain, and tumors) was preformed, with images displaying recovered fluorescence in each tissue via IVIS whole animal fluorescence imaging shown. Luminescent xenograft tumors were seeded following stable transfection of BT-20 and A549 cells with the firefly luciferase plasmid. This enabled assessment of tumor size by a visual and quantifiable luminescent readout generated by whole-animal imaging (Xenogen, Caliper Instruments). Cells were mixed in a 1:1 ratio with BD Matrigel™ Basement Membrane Matrix to a final density of 5 x 10^7 cells/0.1 mL injection. 0.1 mL injections of the matrix-cell suspension were performed in each rear hind flank of NCR nude mice (Taconic). Tumors were allowed to grow until a visible tumor was established and luminescence monitoring was indicative of solid tumor growth (see fig. S3, ∼7 x 10^8 radiance final luminescent readout from each xenograft). Xenograft-bearing NCR nude mice were then systemically-administered therapy (DFP, DEFP) in a 0.1 mL injection at a concentration of 2 mg/kg (based on cisplatin loading) and monitored for 50 days. Luminescence images were obtained by 0.1 mL intraperitoneal injections of 30 mg/kg D-luciferin (Caliper) and imaging (IVIS, Xenogen, Caliper) with an open luminescence filter 15 minutes post-injection. These data are presented, along with region of interest quantification of radiance corresponding to the xenograft-specific luminescence for each mouse treated. Data is normalized for each mouse against the tumor luminescence prior to injection and presented as fold luminescence above this measurement. N = 5 for each
treatment and data corresponds to mean +/- SEM for each treatment group. An unpaired, two-tailed t-test comparing the single drug (cisplatin/quisinostat) and combination drug (cisplatin + quisinostat) systems was performed to determine statistical significance. Quantification of luminescence (radiance [photons]), normalized to pre-injection tumor luminescence, is displayed as average +/- standard deviation fold luminescent change (normalized to pre-injection tumor luminescence).

**Statistical Analyses.** Prism 5 (GraphPad) was used for all analyses. Results are presented as mean ± SEM, unless otherwise noted. Efficacy data was analyzed by an unpaired, two-tailed t-test and repeated measures one-way ANOVA comparing all groups to assess significance in treatment. P < 0.05 was considered significant.

**Results and Discussion**

We fabricated liposomes containing cisplatin (C) and quisinostat (Q) using a lipid film-hydration method[174] (Figure 6.1.A) with the same lipid composition and fabrication protocol as that described in Chapter 5. The final functionalized liposomal system was targeted using 0.5mol% surface folate functionalization, which was shown to significantly enhance NP uptake in NSCLC A549 cells in Chapter 5. Physicochemical characterization of the fabricated liposomes is shown in Figure 6.1.B. Fabricated cisplatin-containing folate-PEG functionalized liposomes (CFP) were observed to be ~150nm with a narrow PDI of 0.13 by dynamic light scattering. Dual drug (cisplatin + quisinostat)-containing folate-PEG functionalized liposomes (CQFP) were observed to be ~180nm with a narrow PDI of 0.04. Loading of quisinostat was conducted analogous to erlotinib in Chapter 5, whereby it was loaded in the lipid bilayer by incorporating the small molecule in the organic phase during thin-film formation, while cisplatin was driven in the aqueous center by a pH-gradient method analogous to that of doxorubicin described in Chapter 5. To further solubilize cisplatin, sonication and heat was applied to the saline-drug containing solution prior to loading into the pre-formed liposomes. The final folate-PEG functionalized dual drug liposomes were visualized by cryogenic-TEM shown in Figure 6.1.C.
Drug release properties of this dual drug system was characterized in PBS under agitation at 37°C and is shown in Figure 6.2.A. Quisinostat was released on a faster time scale than that of cisplatin, likely due to the nature of the core-shell compartmentalization of the drugs. Approximately 50% of quisinostat was released after ~2 days, while that of cisplatin was achieved after 4 days. The given drug loading and release properties of the dual drug folate-targeted liposomal system was sufficient to elicit a strong anti-cancer response in A549 cells in vitro, significantly reducing the IC50 of cisplatin used in the treatment after 48h and 72h relative to a single drug cisplatin-only folate-targeted liposomal construct. This data presented in Figure 6.2.B suggests an added or synergistic benefit of delivering quisinostat and cisplatin together.

To evaluate this system for tumor remediation in vivo, we first examined the pharmacokinetics of the folate-PEG-functionalized empty nanoparticles (lacking drugs) injected intravenously into healthy BALB/c mice, which are immune proficient. The folate-PEG-functionalized, Cy5.5-labeled empty liposomes exhibited relatively low liver accumulation as a function of time (14%, 23%, 17%, 14%, 12% injected dose respectively, based on fluorescence recovery, at 30min, 9h, 24h, 48h, 72h, respectively) (Figure 6.3.A). From blood isolation, the NPs were characterized by serum half-lives of 2.2h and 11.1h, representing rapid and slow elimination phases calculated on the basis of a two-compartment model (Figure 6.3.B). Furthermore, visualization of firefly luciferase-expressing A549 tumors revealed the Cy5.5-tracked empty liposomes accumulate in the tumors of xenograft-bearing nude mice significantly (~8% ID) as early as 48h following a single injection of the folate-targeted liposomes – shown in Figure 6.4.A. We also observed little to no fluorescence in the kidneys and heart for these formulations, which was essential given the potent renal toxicity of cisplatin and cardiotoxicity of quisinostat – shown Figure 6.4.B. Gross toxicity of liposomal and free quisinostat as well as liposomal dual drug cisplatin + quisinostat formulations was conducted and is displayed in Figures 6.5, 6. Primary initial toxicity concern centered around the cardiotoxicity of quisinostat, with previous work suggesting an MTD of free drug quisinostat at ~10mg/kg; however, we observed little gross toxicity as measured by weight change in the mice following 3 repeat injections at days 0, 2, and 4 out to 24 days. As shown, toxicity requiring euthanasia (body weight change > 15%) for the given dose range and treatment regimen was not observed,
therefore no MTD was observed in this particular experiment. This data suggests the liposomal carrier does not introduce additional toxicity versus the free drug; we anticipate that it may also be able to prevent heart toxicity by controlling the release of the drug \textit{in vivo}, but more detailed histological studies would be needed to study this effect. Gross toxicity evaluation of the dual drug (quisinostat + cisplatin) construct showed toxicity requiring euthanasia was only at 20mg/kg (Figure 6.7), suggesting the MTD for this formulation lies in the range of 10-20mg/kg for this given treatment regimen. Free cisplatin was not tested as its MTD is known to be \textasciitilde{}4mg/kg,\textsuperscript{[210]} below the tested range.

With promising \textit{in vitro} results and \textit{in vivo} biological performance (pharmacokinetics, tumor targeting, and toxicity) in immune-proficient BALB/c mice and tumor-bearing ncr nude mice, we evaluated the therapeutic efficacy of these systems against a hind flank A549 mouse model. Using luminescent signal as a means of tracking tumor progression (described and validated in Chapter 5), significant differences in the treatment outcomes were observed, as shown in Figure 6.8, with the combination drug liposome promoting tumor regression (\textasciitilde{}30\% reduction in tumor luminescence) over the course of the first three weeks, with overall tumor maintenance observed by the end of 50 days (+/- 5\% of initial tumor luminescence) following initial treatment. In comparison, each of the single drug controls showed overall tumor growth (\textasciitilde{}30\% for Pt only, \textasciitilde{}80\% for HDAC only) at this dose range, with singular benefit in slowing tumor growth relative to the untreated control (\textasciitilde{}130\% growth by the end of day 50).

For clinical application, a final set of experiments evaluating the ability to preserve and store these systems was performed. As presented by the light scattering data in Figure 6.8, one measure of nanoparticle stability is a measure of the size of the nanoparticle over time; typically we anticipate seeing little or no significant change in size, as may occur with aggregation, during storage. We have shown that these systems are capable of lyophilization and storage out to 3 months with marginal impact of resuspended size (\textasciitilde{}20\% increase) and effectively no change on drug loadings, presenting this system as an attractive approach towards treatment of NSCLC.
**Figure 6.1.** Characterization of cisplatin-quisinostat dual-drug coated (FP) liposomal system. 

(A) Schematic of liposomal system containing cisplatin in the core and quisinostat in the lipid bilayer. (B) Physicochemical characterization: hydrodynamic diameter, polydispersity index (PDI), and loading efficiency of each drug. (C) Cryogenic-TEM of cisplatin-quisinostat loaded liposomal system.
Figure 6.2. In vitro release and efficacy of cisplatin-quisinostat dual-drug coated (FP) liposomal system. (A) Drug release study of cisplatin+quisinostat loaded liposomal system in 1X PBS at 37°C under agitation. (B) IC50 of cisplatin (Pt) and cisplatin + quisinostat (Pt + HDAC) loaded liposomal systems against A549 cells.
Figure 6.3. Pharmacokinetics of FP coated empty liposomal carrier.

(A) Biodistribution of blank folate-functionalized liposomal system following systemic administration in BALB/c mice with the corresponding liver-associated accumulation data, calculated as % injected dose, at each time point as quantified from IVIS whole animal fluorescence imaging. (B) Circulation persistence data of systemically administered folate-functionalized liposomal system. Quantified as % injected dose with two-phase decay model fit to data to determine two half-lives characteristic of the system.
**Figure 6.4.** Biodistribution of FP coated liposomal carrier in A549 xenograft bearing ncr nude mice.  
**(A)** Tumor targeting capabilities of folate-functionalized liposomal system. **(left)** IVIS luminescence of A549 hind flank xenografts; **(right)** corresponding nanoparticle fluorescence overlay showing particle distribution in tumor bearing mice in the left image.  
**(B)** Biodistribution data of folate-functionalized liposomal nanoaprticles 48h following systemic administration. Necropsied tissue corresponds to mice displayed in right image of panel A.
Figure 6.5. Gross toxicity of free and encapsulated quisinostat formulations following intraperitoneal administration in immune-proficient BALB/c mice.

Three repeat injections (at doses: 5, 10, 20 mg/kg) were performed, each 2d apart and monitored out for 3 weeks beyond the final treatment. Weight reduction for “free” drug treatment averaging 6.7%, 9.1%, 9.2% for 5, 10, and 20mg/kg, respectively. Weight reduction for liposomal drug treatment averaging (n=3) 5.4%, 4.7%, 6.7% for 5, 10, and 20mg/kg, respectively.
Figure 6.6. Gross toxicity evaluated as body weight change in BALB/c mice treated intraperitoneally with liposomal formulations and corresponding free drug controls. Repeat administration on days 0, 7, and 14. Mice monitored for one week following final administration. Cisplatin MTD 7 mg/kg.
Figure 6.7. In vivo efficacy of single drug and multi-drug cisplatin-quisinostat loaded liposomal systems in A549 hind flank xenograft bearing ncr nude mice. Data presented as fold luminescence signal normalized to initial tumor size as quantified by luminescent signal. Dosage of 5mg/kg for cisplatin only and dual drug-loaded NPs; 4mg/kg for HDACi only liposome – corresponds to concentration of HDACi in dual drug-loaded liposomal system with 5mg/kg cisplatin (5:4 drug loading ratio). Repeat administrations performed on days 0, 5, and 10.
Figure 6.8. Cryo-preservation of dual-drug cisplatin-quisinostat FP coated liposomal construct. Data shown for resuspension of lyophilized particles both with and without lyopres (the preservation media).
References


Chapter 7. Liposomal delivery of JQ1 and temozolamide systemically across the blood-brain barrier for treatment of brain-stem gliomas

Introduction

Approximately 18,000 new diagnoses of primary malignant central nervous system (CNS) tumors occur annually in the United States resulting in 13,000 deaths. Gliomas comprise approximately 80% of primary malignant CNS tumors, and carry one of the highest mortality rates of all tumors. There is currently no cure for gliomas. The most common and most aggressive form of glioma is glioblastoma multiforme (GBM). Despite an improved molecular understanding of GBM, there remain no effective treatments to cure this brain cancer. The current clinical standard includes surgery followed by radiation therapy plus temozolomide resulting in a median survival of only 15 months. The prognosis has not changed substantially over 20 years.

Gliomas can arise anywhere in the CNS, however brainstem gliomas (BSG) occur in the midbrain, pons, medulla oblongata, and upper cervical spinal cord, critical areas of the brain that control crucial life functions. BSGs commonly occur in children, comprising approximately 20% of primary pediatric CNS tumors and are the leading cause of death for children with brain tumors. Given the delicate anatomy in which BSGs are found, surgery is often not an option for these patients, and radiation therapy is the current standard of care for these tumors.

Many cancer cells are deficient in their ability to effectively repair DNA damage. This is apparent in glioma cells, where deficient DNA repair results in genomic instability and a high rate of DNA mutations that can lead to more aggressive tumors and resistance to therapy. Radiotherapy is partially effective against glioma by exploiting this deficient tumor DNA repair and causing extensive DNA damage that can be repaired in normal cells, but not in tumor cells. Using a novel discovery platform for genes and small molecules that alter the response to DNA damage, a bromodomain-containing epigenetic reader Brd4 was identified as a modulator of ionizing-radiation-induced intracellular signaling and cell survival. A range of small molecule inhibitors of Brd4, including JQ1, was found to further alter DNA damage signaling and demonstrate activity against glioma cells.
One factor that limits the utility of many potent chemotherapy agents against gliomas is the blood-brain barrier (BBB), which restricts entry of many small molecules into the brain. The BBB is comprised of capillary endothelial cells that act as a highly selective permeability barrier between the blood in circulation and the extracellular fluid in the central nervous system. This barrier mediates entry of molecules via a number of different mechanisms, including a) transcellular lipophilic pathways for lipid soluble agents, b) paracellular pathways through the endothelial tight junctions for water soluble agents, c) transport proteins for amino acids and glucose, d) receptor-mediated transcytosis (e.g. transferrin, insulin, folate), and e) adsorptive transcytosis for plasma proteins such as albumin.[220] The BBB also contains efflux pumps such as P-glycoprotein and multidrug resistance pump-1 (MRP-1) that can recycle therapeutics back into circulation.

A number of drug delivery strategies have been employed for treatment of CNS-related disease, including direct injection via a catheter into the brain or cerebrospinal fluid driven either by diffusion (natural gradient) or convection (pump), implants of devices purposed as drug depots, delivery via trans-nasal pathways, and systemic solutions in combination with barrier disruption mechanisms, such as ultrasound, microwaves, radiation, infection, or injection of various substances such as hyperosmolar mannitol.[221] Limitations of localized delivery include limited diffusion of the drug away from the site of injection and risk of infection following direct disruption of the CNS tissue.[223] Systemic delivery promises to overcome these limitations as a less invasive option for more efficient delivery throughout the brain where the delivery system would preferentially bind and deliver cargo to target cells via receptor-mediated interactions; however, issues of tissue selectivity and biodistribution continue to hinder these systems towards clinical application.

Regarding systemic delivery, a number of molecular receptor targets, including transferrin[224-226], insulin, and low-density lipoprotein, have been investigated towards the deployment of nanomedicine designed to traverse the BBB.[227-229] Further selectivity following BBB penetration towards gliomas can be achieved with molecular targets such as those promoting angiogenesis (e.g. integrins, VEGF, ILs) or invasiveness (e.g. MMP-2). One such promising strategy employed a chlorotoxin molecular ligand, which is a 36 amino acid
peptide isolated from deathstalker scorpion venom that selectively binds isoforms of MMP-2 and chloride conductance channels unique to gliomas.\textsuperscript{[230-232]} BBB-penetrating decoration has been investigated on a number of different nanoparticle cores, including liposomes, solid-lipid nanoparticles, dendrimers, gold nanoparticles, carbon nanotubes, quantum dots, and magnetic nanoparticles.\textsuperscript{[233]} Nanoparticles employing this targeted approach show significant promise towards development of a systemic delivery agent to CNS tissue, several of which are currently being evaluated in the clinic.\textsuperscript{[233-235]}

\textbf{Materials and Methods} \\
(+)-JQ1 was purchased from Cayman Chemical. All lipid components were purchased from Avanti Polar Lipids, except for cholesterol from Sigma and DSPE-PEG\textsubscript{2K}/end-functionalized DSPE-PEG\textsubscript{2K}-\textsubscript{(NH\textsubscript{2}/maleimide) from Nanocs, Inc. Chlorotoxin was purchased from Enzo Life Sciences. Cholesterol, temozolamide, citric acid, sodium citrate, sodium carbonate, and solvents (chloroform, methanol, phosphate buffered saline) were purchased from Sigma.

\textbf{Liposome preparation.} Liposomes were formulated at a mass ratio of 56:39:5 (DSPC:Cholesterol:POPG). These three components were dissolved, along with the small-molecule inhibitor (+)-JQ1 (weight ratio to total lipid weight = 3:50), in a 2:1 mixture of chloroform:methanol. A thin film of these materials was generated by rotary evaporation at 40\textdegree C at 150 mbar. This film was dessicated overnight until completely dry. Hydration of the lipid film was conducted at 65\textdegree C under sonication in 300 mM citric acid buffer (pH 4) for 1h. DSPE-PEG\textsubscript{2K}-folate was synthesized as reported in Chapter 5. DSPE-PEG\textsubscript{2K}-HA\textsubscript{10k} was functionalized by reacting HA with DSPE-PEG2k-NH2 under the same conditions as those described for folate in Chapter 5. DSPE-PEG\textsubscript{2K}-Chlorotoxin/Transferrin was prepared by reacting a maleimide-functionalized DSPE-PEG\textsubscript{2K} lipid with thiolated-transferrin/chlorotoxin (obtained by reacting each with Traut’s reagent at a 1:40 ratio of protein:Traut’s in pH 8.5 borate-EDTA buffer (0.15M sodium borate, 0.1mM EDTA) and finally purified using amicon ultracentrifugation using previously established protocols.\textsuperscript{[230, 236]} DSPE-PEG\textsubscript{2K}, with or without DSPE-PEG\textsubscript{2K}-Cy5.5, and with or without a ligand-functionalized lipid (0.5 mol\% folate, 2 mol\% transferrin, 2 mol\% chlorotoxin, 5 mol\% HA\textsubscript{10k}) was conducted post-fabrication using a post-insertion technique in which micelles
(in 0.9% sodium chloride solution) of the components desired to incorporate on the liposomal surface were incubated with the prepared, drug-loaded liposomes under sonication at 65°C for 30 minutes, after which they were filtered through a 0.2 µm polyethersulfone (PES) syringe filter and cooled to room temperature. The pH of the liposomal suspension was then adjusted to ~6.5 by addition of 300 mM sodium carbonate buffer to create a gradient between the exterior and interior compartments. The cytotoxic, temozolomide (10mg), was added in a 0.9% sodium chloride solution (1 mL) to load through a pH gradient method. To facilitate solubilization of the cytotoxic agents, the dispersed solution was sonicated at 65°C for 5 minutes. The final combination drug-loaded system was subsequently exchanged into PBS (pH 7.4) following centrifugal filtration (100K MWCO Millipore) to remove the high salt buffers (citric acid, sodium carbonate) and any unloaded drug. Empty (lacking drug) liposomes (NFP) were formulated using the same procedure.

**Liposome characterization.** Dynamic light scattering and zeta-potential analysis was conducted in 10 mM sodium chloride at 25°C using a Malvern ZS90 zeta-sizer. High-performance liquid chromatography (Agilent technologies) was used to validate drug loading of (+)-JQ1 ($\lambda_{\text{abs}} = 260$nm, elution ~10min) and temozolomide ($\lambda_{\text{abs}} = 260$nm, elution ~2min).

**Cranial window technique for intravital multiphoton imaging of intracranial tumors.** Mice were anesthetized using ketamine and xylazine in accordance with institution-approved animal protocols. A cranial window was fashioned using a handheld micro-drill (Ideal Micro-Drill™) and the skull carefully removed exposing the underlying brain parenchyma (Fig.). Mice were immediately positioned on the stage of an inverted Olympus FluoView 1000 multiphoton laser scanning confocal microscope for imaging through the cranial window. Mice were anesthetized using 2% isoflurane during the imaging sessions. For visualization of NP transport across the BBB, NPs were labelled with Cy3 and injected intravenously 24 hours prior to intravital imaging to allow for uptake. For visualization of intracranial tumors, GFP-expressing U87MG tumors were induced for 10 to 14 days prior
to creation of the cranial window and imaging through the multiphoton microscope. Composite images were compiled using ImageJ software.

**Biodistribution.** For biodistribution experiments, NCR nude female mice (Taconic) were used. To attenuate gut fluorescence, an alfalfa-free special diet (AIN-93M Maintenance Purified Diet from TestDiet) was administered to the mice 1 week prior to and during experimentation. Nanoparticle formulations suspended in 1X PBS were administered via the tail vein. *In vivo* imaging (IVIS, Caliper Instruments) was performed at regular time points. Necropsy of relevant organs was conducted at a relevant time point and imaged using the IVIS to quantify recovered fluorescence.

**Histology.** Brains were resected at the terminal point of experimentation and embedded and frozen in OCT medium until processed. Tissue was sectioned coronally at 20um thick with 10 sections performed 100um apart and mounted in DAPI medium on a glass slide with a coverslip for confocal imaging.

**Stereotactic intracranial implantation of U87MG human glioma xenograft tumors.** Human U87MG glioblastoma cells were obtained through ATCC and maintained in 10% FBS (Gibco) and DMEM. For generation of stable cell lines, cells were transduced with a mir30-based pMLS vector encoding both GFP and luciferase (gift from M. Hemann). For xenograft implantation, 6 week old male NCR nude mice (Taconic) were anesthetized using 2% isofluorane, ketamine, and xylazine. The skull was exposed through a midline scalp incision and a hole was made through the right hemicranium using a 16 gauge needle (2 mm lateral to the midline, 2 mm anterior to the lambdoid, 3 mm below the dura). Mice were then place in a stereotactic frame (Stoelting Co., IL) and $1 \times 10^5$ cells suspended in PBS were injected slowly into the right cerebrum using a 32 gauge Neuros syringe (Hamilton). Bone wax was applied to occlude the hole in the skull and the scalp was closed using interrupted sutures. All procedures were performed under sterile conditions. Tumor growth was monitored using bioluminescence imaging using an IVIS Spectrum-bioluminescent and fluorescent imaging system (Xenogen Corporation). Brain tumors were also imaged using a Varian 7-Tesla whole-mouse MRI system (Varian/Agilent). Tumors
were visualized by bioluminescence and magnetic resonance imaging modalities. Efficacy of transferrin-targeted liposomal drug carriers was evaluated by systemically administering a single dose at 2mg/kg then observing extension of survival for the animal cohorts in each treatment, generating the Kaplan-Meier curve shown in Figure 7.7.

**Results and Discussion**

There are currently no chemotherapeutic or biological agents that have proven additional benefit over radiation therapy for high-grade pediatric BSGs. Even with radiation therapy, the median survival for children with malignant BSGs is <1 year.\(^{[237]}\) New leads toward effective therapy are a significant unmet need in this devastating disease. Using a unique automated microscopy and image analysis platform to interrogate the DNA damage response (DDR), Floyd et al probed RNAi libraries for modulators of DDR signaling,\(^{[219]}\) which identified Brd4 as a novel and potent regulator of early signaling events in the DDR. Brd4 contains tandem bromodomains that bind to acetylated lysine. A small molecule inhibitor of bromodomain binding, JQ1, had potent effects on DDR signaling in glioma cell lines. Additionally, Floyd et al found that among these glioma cell lines, those with known defects in DDR repair genes become exquisitely sensitive to ionizing radiation after pretreatment with JQ1. Delivery of JQ1, however, remains a challenge for delivery due to stability and solubility issues for clinical application – usually requiring direct intrathecal or intracranial delivery of the drug.

To overcome these limitations, we have developed nanoparticle technology to stabilize and solubilize the drug for systemic application. The nanoparticle platform is a liposomal construct described in Figure 7.1.A. Similar to the construct described in Chapter 5, we have fabricated dually loaded liposomal carriers by a traditional thin film-hydration method\(^{[238]}\) with the same lipid composition (56:39:5 DSPC:Cholesterol:POPG), loading and compartmentalizing the inhibitor (+)-JQ1 and cytotoxic cisplatin as shown. For targeted transport of these therapeutics, a number of targeting ligands including transferrin and chlorotoxin, were chosen for transport across the BBB as well as targeting glioma cells, while other systems decorated with PEG, hyaluronic acid, and folate, which is overexpressed on glioma cell surfaces,\(^{[239]}\) were also studied in parallel to determine the optimal system for delivery and efficacy. Physicochemical characterization of the fabricated
Targeted liposomes in Figure 7.1.B shows sizes between 130 – 170 nm with PDIs around 0.15. Targeted liposomes (e.g. transferrin, chlorotoxin) have been shown to traverse the BBB at appreciable levels, preferentially localizing in the brain to deliver their cargo, and more specifically in areas with direct access to the brainstem. Liposomal technology is readily adaptable to carry a wide range of small molecules, siRNAs, biological agents, as well as combinations of cargos analogous to the system developed in Chapter 5, and subsequently functionalized with a hybrid surface for both targeting and stealth (e.g. resistant to opsonization and clearance) capabilities.

To investigate the capability of these fabricated systems to cross the BBB, we developed an intravital imaging technique that allowed us to visualize the BBB and the engagement of the NPs with this barrier following tail vein systemic administration. Surgically removing the skull of the mouse, exposing the brain and blood vessels, and fluorescently tracking the transit of our Cy3-labeled NPs using a multiphoton confocal imager achieved visualization. Using this technique, we tested each of the different NP formulations – PEG, HA, folate, transferrin, and chlorotoxin. Intravital imaging results show rapid uptake of the NPs into the blood vessel walls in the brain (Figures 7.2.A-D, white arrowheads) but only the chlorotoxin- and transferrin-conjugated particles demonstrate passage across the BBB into the surrounding brain matter (Figures 7.2.B, C). This is consistent with published reports of success using chlorotoxin\textsuperscript{[230]} and transferrin\textsuperscript{[230,236]} as ligands to facilitate delivery of NPs across the BBB. Although the particles tagged with folate also showed rapid uptake into the blood vessel walls (Figure 7.2.D), they did not demonstrate diffusion across the BBB, serving as a good negative control that the penetration was ligand specific.

To further assess the ability of these NPs to penetrate and reside in the brain, we administered the targeted formulations in both immunodeficient ncr nude and immune-proficient BALB/c mice and monitored the distribution of the NPs throughout their bodies and organs using live animal fluorescence imaging. Shown here in Figure 7.3, we observed significant accumulation in the brain, \(~1.3-1.7\%\) injected dose based on recovered fluorescence, for both the chlorotoxin (CTX)- and transferrin (Tf)-functionalized NPs respectively at 24 hours post-injection, which we expected due to their known capacities for traversing the BBB and targeting glioma cells. Control PEG and HA-targeted NPs
showed no appreciable accumulation, whereas folate showed some, ~0.9% injected dose at 24 hours (data not shown), likely owing to the expression of folate receptors on the blood side in the choroid plexus3 and possible other avenues of entry yet unknown.

**Figure 7.3** informed our selection and further screening of the chlorotoxin- and transferrin-targeted liposomal systems for efficacy evaluation. In both ncr nude and BALB/c mice, significant BBB penetration and distribution of NPs to the brain was observed for both targeted systems, as shown in **Figure 7.4**, with slightly higher amounts observed for the transferrin-targeted system (~1.7% ID) relative to the chlorotoxin-targeted system (~1.3% ID). Enhanced levels of NP accumulation (shown in red, nuclei stained with DAPI mount shown in blue) in the brain is further shown in **Figure 7.5** from fluorescent imaging of coronal brain sections of treated non-tumor bearing ncr nude mice. Similar results were obtained from coronal brain sections of treated non-tumor bearing BALB/c mice. No apparent accumulation was observed for PEG- and HA-functionalized liposomal systems, while some accumulation was visualized for folate-targeted liposomal systems – consistent with data presented in **Figures 7.3, 4**.

We are currently testing the adult human glioma cell line, U87MG, and have successfully implanted these cells into the brains (**Figure 7.6.A**) of ncr nude mice that can be visualized by MRI (**Figures 7.6.B, C**). Using the U87MG animal model for initial efficacy evaluation, we tested our transferrin-targeted liposomal nanoparticles, observing significant colocalization of the NPs with the glioma brain xenografts as early as 24h post-administration and out to 5d (**Figure 7.7**). From necropsy data shown in **Figure 7.4**, the amount could be as high as ~1.7% injected dose, based on the entry of transferrin-targeted liposomal manufactured systems in healthy ncr nude mice. Further experimentation investigating whether the glioma xenograft affects the BBB and entry of our NPs into the brain is ongoing.

Efficacy evaluation is currently in progress. Preliminary results are shown in **Figure 7.8**, where transferrin-targeted single drug and dual drug ((+)-JQ1, cisplatin) liposomal nanoparticles were tested at 2 mg/kg single systemic tail vein administration (each drug, drug loading ratio ~1:1 in dual drug nanoparticle) against the U87MG brain xenograft. Shown in the Kaplan-Meier data, extension of life was observed for the single drug controls for 3-4 days longer than that of the control non-treated mice. For the dual drug system,
extension of life 12 days beyond the non-treated control and 8-9 days beyond the corresponding single drug liposomal controls. Testing of free drug controls at the same drug concentration is currently underway to characterize the impact of drug encapsulation as well as benefit of multi-drug therapy for this glioma animal model.

Final therapeutic evaluation of this transferrin-targeted dual drug liposomal system will include combination radiation to investigate any synergism between treatments, as suggested by previous reports. To accomplish this, we have designed a stereotactic rodent radiation device, which is compatible with the Cesium source irradiator in the Koch Institute (Figure 7.9.A). The device has interchangeable collimators, which allow us to administer focused doses of irradiation to the brain or other regions of the body (Figure 7.9.B).

**Conclusion**

We have successfully adapted the liposomal technology developed in Chapter 5 towards targeting (e.g. transferrin- and chlorotoxin-targeted lipids) and delivering small molecule chemotherapeutics (e.g. cisplatin) and inhibitors (e.g. JQ1) for treatment of brainstem gliomas, which are plagued by poor prognoses. Future work will incorporate radiation therapy with the liposomal drug carriers to screen for synergism to create effective treatment algorithms for this devastating disease.
Figure 7.1. Liposomal construct for dual-drug (+)-JQ1-temozolomide targeted carriers. 

(top) Nanoparticle design. (bottom) A list of the different ligands attached to our nanoparticles and the diameters ($z_{avg}$ $d_h$) of these particles. PEG: poly(ethylene glycol); Tf: transferrin; Fol: folate; HA: hyaluronic acid; CTX: chlorotoxin.
Figure 7.2. Intravital multiphoton imaging of nanoparticles crossing the blood-brain barrier in mice. 
(A) Imaging through a cross section of blood vessels running through the skull (shown in white) shows uptake of red fluorescent chlorotoxin nanoparticles into the walls of blood vessels (white arrows). (B) Imaging deeper into the brain shows the nanoparticles coursing through a blood vessel (outlined in white) with diffusion of particles into the surrounding brain matter. (C) Red fluorescent transferrin nanoparticles line a blood vessel in the brain (white arrows) and demonstrate diffusion into the surrounding brain. The skull is shown in blue and outlined in white. (D) Red fluorescent folate nanoparticles show uptake into the blood vessel wall (white arrows) but do not show diffusion across the BBB.
Figure 7.3. Representative distribution data of each fabricated nanoparticle system in non-tumor bearing mice. (left) ncr nude and (middle) BALB/c mice at 8h post-systemic administration. Poly(ethylene glycol) (PEG); transferrin (Tf); folate (FOL); chlorotoxin (CTX); hyaluronic acid (HA); Li: liver; S: spleen; K: kidneys; H: heart; Lu: lungs; B: brain. (right) Representative distribution data of each fabricated nanoparticle system in nude mice after 24h.
Figure 7.4. Biodistribution of targeted liposomal carriers in non-tumor bearing mice. 
(Left) Representative distribution data of chlorotoxin (C) and transferrin (Tf)-targeted nanoparticle formulations 8h post-systemic administration in ncr nude (top) and BALB/c (bottom) mice relative to an untreated, autofluorescence control. (Right) Representative distribution data of chlorotoxin (CTX)- and transferrin (Tf)-functionalized nanoparticles in mice at 24h post-injection (Li: liver; S: spleen; K: kidneys; H: heart; Lu: lungs; B: brain).
Figure 7.5. Histological sections of ncr nude brains 24h post-systemic administration of functionalized nanoparticle systems. Polyethylene glycol (PEG); folate (FOL); chlorotoxin (CTX); Tf (transferrin); hyaluronic acid (HA)-targeted systems. Blue = DAPI stain of nuclei; Red = Cy5.5 tracker lipid in liposomal formulation.
Fig 7.6. Mouse models of human glioma.

Human glioma cells, U87MG, are implanted into the brains of mice and visualized using bioluminescent imaging (A) as well as magnetic resonance imaging (MRI) (B, C).
Figure 7.7. Biodistribution data of transferrin-functionalized liposomal carriers in U87MG brain-implanted tumors.
Whole-animal imaging demonstrating colocalization of fluorescently-tracked Cy5.5-labeled transferrin-targeted liposomal nanoparticles with luminescently-labeled M059K brain xenografts. Imaging shown 24h and 5d post-systemic administration of NPs.
**Figure 7.8.** Kaplan-Meier curve following treatment of U87MG brain tumor implants with single drug and dual-drug temozolamide-(+)-JQ1 transferrin-targeted liposomal carriers. (black) vehicle control – transferrin-targeted blank liposome; (blue) temozolamide-loaded transferrin-targeted liposome; (red) – (+) JQ1-loaded transferrin-targeted liposome; (grey) [temozolomide + (+)-JQ1]-loaded transferrin-targeted liposome. Vehicle dosed at equivalent lipid concentration; drug-loaded systems dosed at 2mg/kg of each drug.
Figure 7.9. Small animal irradiation platform for combination radiation-systemic chemotherapy. 
(top) The lead irradiator box has interchangeable collimators which will deliver focused irradiation to rodents. 
(bottom) Shown are radiation beam characteristics for a 1 cm circular field created with custom lead shielding apparatus designed for use with the Cesium137 irradiator housed in the Koch Institute mouse facility.
References

Chapter 8. Framing the impact of this work and future directions.

Chapters 2 – 4 focus on systems utilizing the capabilities of layer-by-layer (LbL) customization for design of nanoparticle systems for a variety of disease targets with the added dimensionality of scalably manufacturing these systems, making them attractive for clinical adaptation.

Future impact of this work –

Chapter 2: This work is a fundamental study on LbL films atop nanoparticle platforms and how that influences drug release and the distribution of the nanoparticle in a systemic setting. It establishes a framework with which to screen LbL nanoparticle designs and understand how new biomaterials and/or particle designs compare relative to existing architectures and NP constructs.

Chapter 3: This work establishes a method for customizing and incorporating biomaterials atop NP platforms via LbL for tissue targeting. The approach of functionalizing and incorporating a material via LbL atop a NP platform presents an array of opportunities for disease management beyond cancer – conceivably any disease target of interest. Regarding moving this work forward to the clinic, more advanced osteosarcoma models (directly in bone tissue) are necessary to test, as well as a metastatic model, if possible to establish, that localizes in bone – that would be the highest impact result.

Chapter 4: This work establishes a scalable method for designing built-to-order medicinal systems on the nanoscale and that could be easily adapted to the microscale for a variety of applications. The tunability of this approach for NP engineering opens up a number of exciting opportunities in the field of biotechnology – treatment, diagnostics. Regarding the specific technique, adjusting the hydrophobicity of the sacrificial adhesive layer to facilitate spray-assisted LbL would be the next technical step towards streamlining the approach for future scalable manufacturing purposes.
Overall, the systemic use of LbL NPs has been shown to exhibit much promise, and opportunities for clinical development exist in this area with existing technologies developed in our lab. Next steps would be to consider work on systems for issues where local application (creams/rinses/i.d. injections/incorporation in microneedles) is necessary, such as vaccines or immunotherapy. Due to the nature of the films to directly incorporate a wide variety of therapeutics while also serving as surfaces for targeting and controlled drug release, these systems would be ideal for applications local to the diseased tissue/site of interest to serve as drug depots for managing the issue.

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Chapters 5 – 7 focus on the use of liposomal systems without LbL functionalization towards disease management. Liposomal formulations became of interest due to their current clinical use and ease in manufacturing and manipulation for the desired compartmentalization and drug release, as well as tissue targeting, necessary for the applications explored in this work.

**Chapter 5:** This work takes advantage of the nature of the liposomal structure to load and compartmentalize hydrophobic and hydrophobic drug combinations together in high quantities to achieve the desired time-staggered release of the two drugs for synergistic cancer cell killing – herein lies the novelty, not necessarily in the technology itself. We also demonstrate this technology to successfully deliver a range of therapeutic combinations for treatment of TNBC and NSCLC. Future work with this system lies in screening the nanoparticle formulation against more advanced tumor models, such as the autochthonous NSCLC model in the Jacks’ lab, as well as testing in large animal models such as monkeys. A current collaborative effort with Merrimack pharmaceuticals is being conducted to further validate and develops this system towards clinical application.

**Chapter 6:** This work adapts the technology in Chapter 5 with two different drugs for treatment of NSCLC, further demonstrating the versatility of this system. Future work with this system lies in screening the nanoparticle formulation against more advanced tumor
models, such as the autochthonous NSCLC model in the Jacks’ lab, as well as testing in large animal models such as monkeys. A current collaborative effort with Janssen pharmaceutical employees is being exploited to investigate the possibilities of VC funding and development of this technology external to the company towards clinical application. Again the novelty lies in the delivery of the agents together, not in the technology, so we are also interested in developing a proprietary LbL formulation designed to achieve the same efficacy.

**Chapter 7:** This work adapts the technology in Chapter 5 with two different drugs as well as incorporating a range of new targeting approaches for traversing the BBB and treating brain stem gliomas, further demonstrating the versatility of this system. The technology is not novel in the field, but the capabilities in delivering the combinations of drugs investigated is exciting, along with the potential to rapidly screen particle designs and combinations with other treatment modalities, such as radiation therapy. Future development here lies in developing a more highly controlled and patentable approach in particle engineering by incorporating LbL and functional materials with the ligands of interest for BBB penetration and glioma cell targeting ala work in Chapter 3. This would not only open a number of exciting opportunities for developing a proprietary blend that could be clinically developed (and IP protected) but also allow for more fundamental studies on blends using combinations of ligands with high levels of control over the surface of the nanoparticle for BBB engagement and cancer cell targeting/treatment.

Overall, the liposomal technology is exciting and readily adaptable to the clinic – likely much moreso than the LbL formulations described herein due to the current use of liposomal products in the clinic. The issue lies in IP protection. The systems developed are based on previous systems, with little novelty lying in the technology itself but rather the action of the technology (staggered release leading to enhanced cancer cell killing, delivering unique combinations of drugs for various disease targets), hence the interest in future work focusing on utilizing the knowledge gained with LbL to develop proprietary blends that can achieve the same level of success with added IP protection and entrepreneurial prospects.
Appendix A. Architecture, Biological Performance of LbL Nanoparticles

Although this study focuses on a number of biodegradable materials and conventional therapeutics, a much larger library of film components, along with a diverse range of therapeutics, can be generated for incorporation on this platform.

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<th>Cores</th>
<th>Polycation</th>
<th>Polyanions</th>
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<td>PLGA (50:50&lt;sup&gt;a&lt;/sup&gt;; 65:35&lt;sup&gt;b&lt;/sup&gt;; 75:25&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>Poly-L-Lysine&lt;sup&gt;d&lt;/sup&gt; (PLL)</td>
<td>Dextran Sulfate&lt;sup&gt;e&lt;/sup&gt; (DXS)</td>
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<sup>a</sup>38-54K  <sup>b</sup>24-38K  <sup>c</sup>4-15K  <sup>d</sup>4-15K  <sup>e</sup>9-20K  <sup>f</sup>500K  80-120K

**A.1.** Representative scanning electron micrograph (SEM) of LbL PLGA NPs (HA-terminal). 1μm scale bar.
A.2. CG drug stability - biodistribution of LbL PLGA NPs in ncr nude mice. 
(left) 48h IVIS imaging panel ($\lambda_{ex} = 745$ nm, $\lambda_{em} = 820$ nm) surveying drug bioavailability as a function of nanoparticle architecture. (i) free CG$_{820}$; (ii) PLGA$_{50:50}$CG; (iii) DXS-terminated 6L NP; (iii) HA$_{5000}$-terminated 6L NP; (iv) Alg-terminated 6L NP. (right) Whole-animal region of interest analysis yielded from the series of images demonstrates enhanced persistence of drug in the animal.
A.3. NP stability – biodistribution of LbL PLGA NPs.

(Left) 48h IVIS imaging panel ($\lambda_{ex} = 640$ nm, $\lambda_{em} = 700$ nm) surveying carrier bioavailability as a function of nanoparticle architecture. (i) free PLL$_{700}$ (ii) DXS-terminated 6L NP; (iii) HA$_{500}$K-terminated 6L NP; (iv) Alg-terminated 6L NP. (Right) Whole animal region of interest analysis yielded from the series of images. Whole-animal region of interest analysis yielded from the series of images demonstrates enhanced persistence of the layered nanoparticles in the animal.
A.4. Biodistribution data 30 minutes after systemic administration in ncr nude mice.  
*(left) PLL700 tracking* - $\lambda_{ex} = 640$ nm, $\lambda_{em} = 700$ nm; (i) free PLL700; (ii) Alg-terminated 6L NP; (iii) HA500K-terminated 6L NP; (iv) DXS-terminated 6L NP; *(right) CG820 tracking* - $\lambda_{ex} = 745$ nm, $\lambda_{em} = 820$ nm; (i) free CG820; (ii) PLGA50:50CG; (iii) HA500K-terminated 6L NP; (iii) Alg-terminated 6L NP; (iv) DXS-terminated 6L NP.
A.5. Dose-dependent co-injection of free polymer with corresponding terminated 6L LbL PLGA NP formulations. (left: 10 mg/kg, right: 20 mg/kg) co-injection of 6L nanoparticle architectures with corresponding terminal layer free polymer. IVIS imaging survey circulation of drug ($\lambda_{ex} = 745 \text{ nm}, \lambda_{em} = 820 \text{ nm}$). (i) Free HA$_{500K}$ + HA$_{500K}$ 6L NP; (ii) HA$_{500K}$-terminated 6L NP; (iii) Free Alg + Alg-terminated 6L NP; (iv) Alg-terminated 6L NP; (v) Free DXS + DXS-terminated 6L NP; (vi) DXS-terminated 6L NP. Region of interest analysis was conducted 15 min post-injection to capture liver-associated drug fluorescence ($\lambda_{ex} = 745 \text{ nm}, \lambda_{em} = 820 \text{ nm}$) shown in bottom graph. Data is normalized to maximal radiant efficiency in data set for each NP architecture.

(top) IVIS quantification of recovered fluorescence at carrier channel, $\lambda_{ex} = 640$ nm, $\lambda_{em} = 700$ nm; (bottom) IVIS quantification of recovered fluorescence at CG820 channel, $\lambda_{ex} = 745$ nm, $\lambda_{em} = 820$ nm.
A.7. Variation of core template by modulating L:G ratio in PLGA and effect on biodistribution in ncr nude mice.

(top and bottom, left) PLGA_{65:35CG} core; (top and bottom, right) PLGA_{75:25CG} core - (top, left and right) $\lambda_{ex} = 640$ nm, $\lambda_{em} = 700$ nm; (i) free PLL200, (ii) DXS-terminated 6L NP, (iii) HA500K-terminated 6L NP, (iv) Alg-terminated 6L NP; (bottom, right and left) $\lambda_{ex} = 745$ nm, $\lambda_{em} = 820$ nm; (i) free CG820, (ii) PLGA_{65:35CG} core, (iii) HA500K-terminated 6L NP, (iv) Alg-terminated 6L NP, (v) DXS-terminated 6L NP. Similar in vivo stability profiles to that of PLGA^{50:50CG} (Figure 2.3); however, significantly higher CG loadings could be achieved by tuning the L:G ratio. Typical loadings (based on UV-Vis absorption via HPLC) are: PLGA^{50:50CG} - 10 ug/mL; PLGA^{65:35CG} - 20 ug/mL; PLGA^{75:25CG} - 50 ug/mL. 30 min stability assessment representative of 48 h panel of images (data not shown). Data deemed consistent with findings in main text and not shown to impact the stability of the drug in the core.
A.8. Opsonin (IgG) and subsequent uptake by macrophages in vitro as a function of terminal layer on LbL PS beads.

(Left) Opsonin binding of fluorescently labeled IgG (Human IgG-FITC) (blue = absorption pre-opsonin incubation; red = absorption post-opsonin incubation), and subsequent (right) opsonization by a murine macrophage cell line (J774A.1) of LbL nanoparticles as a function of terminal layer. Particles used for this investigation were polystyrene Texas red-labeled latex beads (PSTXR). Experimental groups include: blank PSTXR core, PSTXR/PLL, and PSTXR/PLL/Xterm, where Xterm was varied for DXS-, Alg-, and HA500K-terminated systems; n = 3, mean +/- SEM.
Appendix B. Osteotropic therapy via LbL Nanoparticles.

Approximately 40% functionalization achieved.
B.2. Osteotropic PAA-Alendronate LbL QD characterization.

(A) Dynamic light scattering histogram overlay of pre- and post-LbL functionalized QD800 NP core. (B) Atomic Force Micrograph of QD800/(PLL/PAA-Al)3 NPs. (C) ζ-potential data following each LbL deposition step.
B.3. Variation of number of layers and impact on biological performance of PAA-Alendronate targeted LbL NPs. (A) in vitro binding of 1- and 2-bilayer targeted LbL NPs [QD$_{800}$/PLL/PAA-Alendronate]. (B) in vivo fluorescence imaging (640nm, 800nm) following systemic administration of 4L targeted LbL NPs. (C) quantification (n = 3 mice, 2 hind flank tumors/mice) of tumor-specific accumulation above pre-injection autofluorescence. (D) representative biodistribution data at terminal point from imaging (7d) following systemic administration of 2-bilayer LbL-targeted QD$_{800}$ NPs. Li = liver; S = Spleen; K = Kidneys; H = Heart; Lu = lungs; T = Tumors.
B.4. Biodistribution of uncoated and PAA untargeted QD NPs. 
(top) uncoated carboxylate-modified QD_{800} NPs. (bottom) untargeted, 3-bilayer (PLL/PAA) Lbl-functionalized NPs.
B.5. Biodistribution and tumor targeting of PAA-Alendronate targeted QD NP tracking the core and LbL shell simultaneously.

(A) Fold tumor specific accumulation as a function of time of the Cy5.5-labeled PAA-Al terminal layer polymer on the QD 800 surface. (B) Corresponding representative biodistribution (n = 3) of the mice at the terminal point (7d). Tissue harvest (liver, spleen, kidneys, heart, lungs, tumors, and gutted skeleton [ventral and dorsal]) displayed for same animal at terminal point for both channels [PAA-AlCy55; QD 800]. (C) Representative biodistribution data corresponding to quantification in Figure 3.4.D at terminal point. Li = liver; S = Spleen; K = Kidneys; H = Heart; Lu = lungs; T = Tumors.
B.6. Physicochemical characterization following functionalization of empty and doxorubicin-loaded liposomal carriers. Dynamic light scattering overlays of formulated liposomes and corresponding functionalization via LbL for both (A) empty liposome core (for PK data in BALB/c) and (B) doxorubicin-loaded liposomes in efficacy studies.

(A) In vivo imaging of NCR nude mice at 100h following systemic administration of Lbl-targeted empty liposomal NPs. (B) Corresponding biodistribution data of harvested tissue and skeleton (dorsal), along with quantification presented as percent recovered fluorescence for tissue (sans skeleton). (C) Representative biodistribution (ventral in vivo fluorescence imaging) following systemic administration of Lbl targeted NPs (empty lipo/PLL\textsuperscript{55}/PAA-Al) in BALB/c mice for PK studies (presented in Figure 3.5). Li = liver; S = Spleen; K = Kidneys; H = Heart; Lu = lungs; T = Tumors.
B.8. microCT scans following treatment of 132B xenografts with PAA-Alendronate targeted liposomal carriers. Initial and final representative μCT images of tumor burden (red arrows) in live animals treated with Lbl-targeted doxorubicin-loaded liposomes.
Appendix C. Scalable Manufacture of Built-to-order Nanomedicine: Spray-LbL on PRINT® Nanoparticles

C.1. AFM of PRINT® NPs pre- and post-spray LbL. Particles washed off following deposition (right) on non-crosslinked harvested particle array (left).
C.2. Varying crosslinking conditions, incubation period – characterizing crosslinking of PVA via contact angle measurements of PRINT® NP arrays. Crosslinking at 37% HCl results in significant loss of particles beyond 0.5h (which was also found not to be long enough to maintain particles during functionalization), presumably due to acid-catalyzed hydrolysis of PLGA, therefore no data shown for this sub-optimal crosslinking condition.
C.3. AFM amplitude data corresponding to Figure 4.3.
C.4. Dynamic Light Scattering (DLS) Intensity distribution histograms displayed throughout Spray-LbL deposition on PRINT® PLGA200x200nm nanoparticles. ζ-potential analysis ran concurrently with DLS characterization.
(top, left) Overlay of coated PRINT® PLGA 200x200nm particles with "thin" [(PLL/HA)$_3$] and "thick" [(Chit/HA)$_3$] coatings. (bottom, left) Overlay of coated PRINT® PLGA 80x320nm particles with "thin" [(PLL/HA)$_3$] and "thick" [(Chit/HA)$_3$] coatings. (right) Coated (Chit/HA)$_3$ 200x200nm and 80x320nm PLGA particle arrays and subsequent harvested and purified coated NPs.
C.6. Tracking deposition with Spray-LbL on PRINT® using AFM, fluorescence imaging. *(top)* AFM of each layer on PRINT® from crosslinked particles (2h) to L4 (PLL/DXS)$_2$. *(bottom)* fluorescence imaging using LiCor *(left)* and confocal microscopy *(right)* to track PLL$_{Cys-5}$ deposition.
Tracking deposition of each layer via AFM in a 3 bilayer, Chitosan/HA Spray-LbL film. 200x200nm particle arrays were crosslinked for 15h prior to functionalization.
C.8. Spray-LbL on PRINT® PLGA_{80x320 nm} NPs.
(top) uncoated, pre-crosslinked 80x320 PLGA particle arrays. (bottom) coated (PLL/HA), 80x320 PLGA particle arrays.
C.9. Spray-LbL on PRINT® PLGA200x200nm NPs.

(left) Uncoated, pre-crosslinked PRINT® PLGA 200x200nm particle arrays. (right) Coated (PLL/HA)$_3$ 200x200 PLGA particle arrays.
C.10. Uncoated, pre-crosslinked TEMs of PRINT® NPs. 
(A) PRINT® PLGA$_{200 \times 200}$nm NPs and (B) PLGA$_{80 \times 320}$nm systems as visualized under TEM.
Appendix D. Liposomal compartmentalization of a hydrophobic-hydrophilic combination chemotherapy regimen (erlotinib, doxorubicin) for staged, targeted delivery against triple-negative breast cancer and non-small cell lung cancer.

D.1. Tumor growth following BT-20 and A549 xenograft seeding at day 0 (n = 5). Treatment demonstrated in Figure 5.5 began on Day 22 (terminal point for data above).
D.2. In vitro release experimentation – release of inhibitors (afatinib, erlotinib, gefitinib, lapatinib) and cytotoxic (doxorubicin) from dual-drug coated (FP) liposomal formulations.

Release study conducted under sink conditions and agitation at 37°C with variation on pH of PBS buffer (adjusted by titration of citric acid buffer, pH = 4) to 7 and 5.5, respectively, as denoted on the graph.
D.3. Cleaved Caspase-8, phosphorylated ERK (pERK), and phosphorylated H2AX (pH2AX) western blot quantification as function of time and treatment in both NSCLC and TNBC cancer cells. 

(A) A549 and (B) BT-20 cells. Ordinate representative as relative signal intensity with time in hours plotted on the abscissa. 

D = doxorubicin liposome; DA = doxorubicin + afatinib liposome; DE = doxorubicin + erlotinib liposome; DG = doxorubicin + gefitinib liposome; DL = doxorubicin + lapatinib liposome.
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D.4. Characterization of non-functionalized multi-drug and single-drug loaded liposomes (without folate-PEG). Mean hydrodynamic diameter, polydispersity index (PDI), and the drug loading characteristics are presented.
D.5. Biodistribution of Cy5.5 labeled blank FP coated liposomes administered to tumor-bearing ncr nude mice. Time point from end of experiment (BT20 - 48d post-injection; A549/MDA-MB-468 - 55d post-injection). Li = liver; S = spleen; K = kidneys; H/Lu = heart/lungs; T = tumors; B = brain. Single systemic injection of liposomal formulation.
D.6. Tumor luminescence data temporally resolved following treatment of A549 xenograft bearing ncr nude mice with single and dual drug doxorubicin-erlotinib FP coated liposomal carriers. Remediation panels for (right to left) untreated, dox-only targeted liposome (DFP), and combination therapy liposome (doxorubicin:erlotinib targeted liposome; DEFP) in A549 (NSCLC) xenografts. Panels display tumor luminescence detected via bioluminescence imaging post-treatment. A single administration was conducted immediately after the "pre" image. Mice were imaged up to 35d as displayed.
D.7. Tumor luminescence data temporally resolved following treatment of BT-20 xenograft bearing ncr nude mice with single and dual drug doxorubicin-erlotinib FP coated liposomal carriers. Remediation panels for (right to left) untreated, dox-only targeted liposome (DFP), and combination therapy liposome (doxorubicin:erlotinib targeted liposome; DEFP) in BT20 (TNBC) xenografts. Panels display tumor luminescence detected via bioluminescence imaging post-treatment. A single administration was conducted immediately after the “pre” image. Mice were imaged up to 35d as displayed.
D.B. Release of doxorubicin visualized by confocal microscopy from FP coated liposomal carriers. Time course comparing uptake and release of doxorubicin for the liposomal doxorubicin (DFP) (A) and free doxorubicin (B). Nuclear stain via Hoescht (blue), doxorubicin fluorescence observed in green, nanoparticle (NP) fluorescence observed in red (Cy5.5).

Treatment displayed at top: co-injection of doxorubicin-loaded liposome (DFP, folate-PEGylated) + erlotinib-cyclodextrin complex in PBS (0.5mg/kg, hydroxypropyl-beta-cyclodextrin) co-injection at beginning of treatment, followed by subsequent boost doses of erlotinib at 0.5mg/kg on day 2 and 4. Live-animal bioluminescence images of hind-flank A549 xenografts shown prior to treatment and at days 3, 10, and 30. Quantification of luminescence (radiance [photons]), normalized to pre-injection tumor luminescence, displayed at bottom. Average +/- standard deviation fold luminescent change (normalized to pre-injection tumor luminescence) at day 3 (0.83 +/- 0.61), day 10 (1.74 +/- 0.47), and day 30 (2.37 +/- 1.2) is plotted.