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UNIVERSITY OF CALIFORNIA,
IRVINE

Chemically-induced Extracellular Blebs for Versatile Cancer Therapy

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Chemical and Biochemical Engineering

by

Dominique Ingato

Dissertation Committee:
Professor Young Jik Kwon, Chair
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Associate Professor Melissa B. Lodoen
Assistant Professor Han Li

2018

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DEDICATION

To

my parents, Steven and Cheryl,
my sister, Isabelle,
my family
and my friends

for their constant love and support.

In remembrance of

my grandparents,
Vincent Ingato (1929-2018)
Mary Ingato (1928-2014)

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PUBLICATIONS

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Kim CS, Ingato D, Petra W-S, Chen Z, Kwon YJ. Stimuli-disassembling gold nanoclusters for diagnosis of early-stage oral cancer by optical coherence tomography. Nano Convergence, 2018, 5(3), pp. 1-11

Ingato D, Lee JU, Sim SJ, Kwon YJ. Good things come in small packages: Overcoming challenges to harness extracellular vesicles for therapeutic delivery. Journal of Controlled Release, 2016, 241, pp. 174–185

Hong CA, Cho SK, Edson JA, Kim J, Ingato D, Pham B, Chuang A, Fruman DA, and Kwon YJ. Viral/Nonviral Chimeric Nanoparticles To Synergistically Suppress Leukemia Proliferation via Simultaneous Gene Transduction and Silencing. ACS Nano, 2016, 10 (9), pp. 8705–8714

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ABSTRACT OF THE DISSERTATION

Chemically-induced Extracellular Blebs for Versatile Cancer Therapy

By

Dominique Antoinette Ingato

Doctor of Philosophy in Chemical and Biochemical Engineering

University of California, Irvine, 2018

Professor Young Jik Kwon, Chair

Extracellular vesicles (EVs) have recently gained momentum in the field of therapeutic delivery due to their promise as therapeutic carriers. Biomolecules are naturally transported by EVs for cell-to-cell communication, and EV interactions with target cells are thought to be specific to cell type. Naturally produced extracellular blebs (EBs), a category of EVs that are derived specifically from blebbing of the cell membrane, transport cargo to target cells while maintaining the surface properties of the cells from which they were derived.

Although EBs have been used for preclinical drug delivery and gene therapy, obtaining sufficient samples of well-characterized blebs for clinical studies is still a major challenge. A major focus of my work has been on developing and characterizing a method for large-scale production of EBs. More specifically, this dissertation explores a method for controlling EB production by sulfhydryl blocking reagents. The optimized formulation of sulfhydryl-blocking reagents described in this dissertation led to an order of magnitude increase in EB production as characterized by protein quantification assay. Furthermore,

this increase in EB production was achieved in a fraction of the time required for natural production of EBs.

With the aim of utilizing EBs for cancer therapy, the efficacy of the production method was assessed for two applications: delivery of a chemotherapeutic drug, and presentation of a model cancer antigen for immunotherapy. Studies confirmed that in tumor-challenged mice treated with chemotherapeutic-loaded, nano-sized EBs, there was significantly slowed tumor growth and improved survival compared to treatment with free drug or liposomal drug. The chemotherapeutic-loaded, nano-sized EBs demonstrated improved cellular uptake, facilitated intracellular drug release and targeted accumulation in the tumor, and they avoided accumulation in vital organs in comparison to a commercial liposomal formulation. In a separate study, antigen-presenting micro-sized EBs produced by sulfhydryl-blocking expressed H-2Kb bound to SIINFEKL and effectively activated T cells *in vitro* and cytotoxic T lymphocytes *in vivo*. Micro-sized EBs performed as well as whole cell therapy in terms of slowing tumor growth and improving survival outcomes while providing a cell-free alternative therapy.

EBs have a broad range of potential health applications beyond drug delivery and immunotherapy; these include gene therapy, immune modulation, tissue regeneration, and pathogen suppression. EB production by sulfhydryl blocking is highly promising for overcoming a current technological gap and allowing EB-based therapeutics to progress more rapidly to clinical trials.

In summary, this dissertation provides a study of an innovative method of EB production including evaluation of two therapeutic applications.

CHAPTER 1: Introduction

Parts of this chapter have been adapted from:

Ingato, D., Lee, J.U., Sim, S.J., Kwon, Y.J. (2016), Good things come in small packages:

Overcoming challenges to harness extracellular vesicles for therapeutic delivery. J Control Release, 241: 174-185.

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1.1 A brief introduction

The fields of drug delivery and gene therapy rely on nano-sized carriers for effective delivery of precious cargo to the designated target site.¹⁻³ Therapeutic delivery agents have two key objectives: protect cargo from the harsh environment of the body and release cargo at the appropriate site without inducing immunogenic response.⁴⁻⁶ In order to achieve these goals, a variety of viral⁷ and non-viral⁸ nanocarriers with specific properties dependent upon the type of cargo and desired site of delivery have been used. Nevertheless, issues with non-specific cytotoxicity, poor biocompatibility, and low efficacy of delivery still remain major challenges in the field.^{9,10} This dissertation investigates a method for overcoming multiple challenges in the field of therapeutic delivery with extracellular vesicles as therapeutics.

1.2 Extracellular vesicles (EVs) as therapeutics

Extracellular vesicles (EVs) have recently become an exciting option for nano-scale delivery.¹¹ They invite a unique chance to harness naturally produced biological carriers for

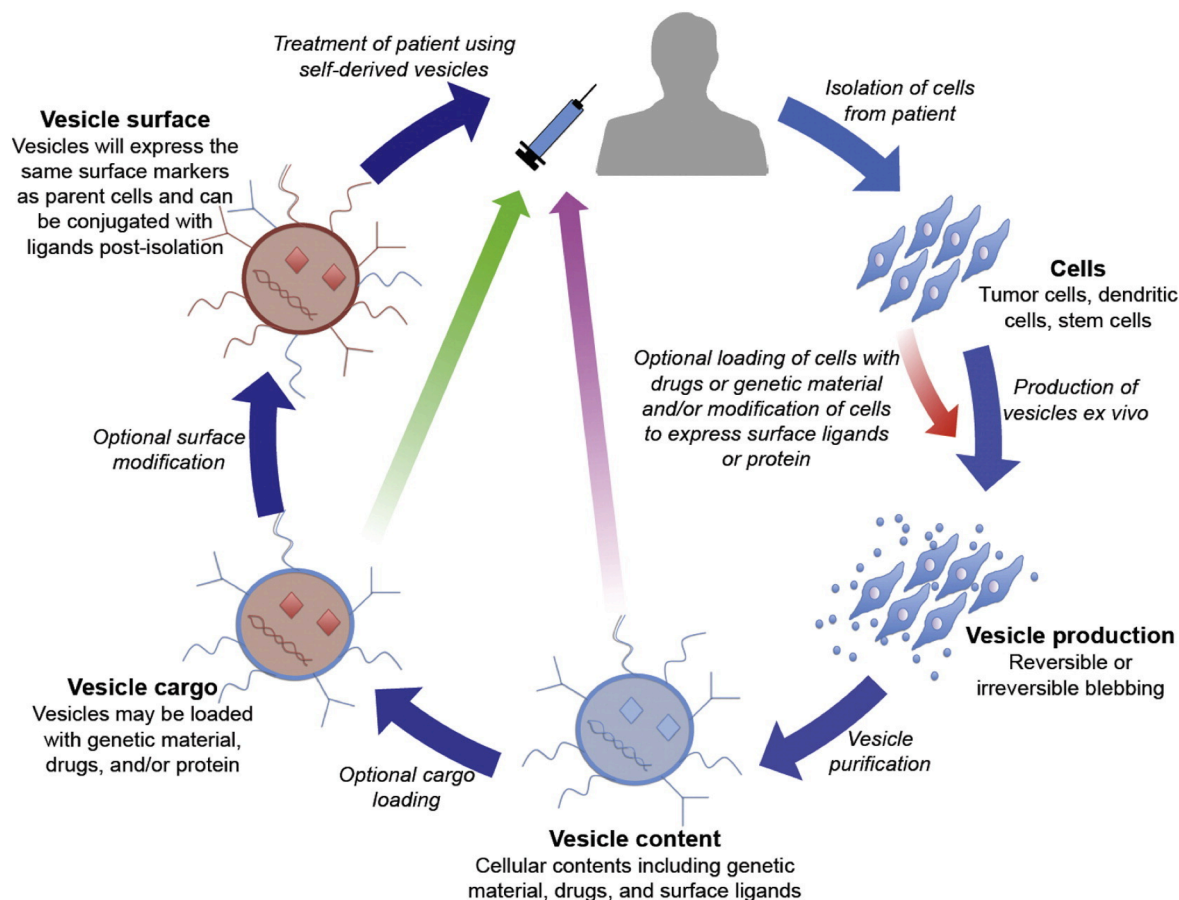


Figure 1.1. Schematic demonstrating the process of personalized vesicular delivery. A patient provides a specific primary cell line for generation of EVs. The EVs are loaded or modified as required. Finally, the EVs are used as therapeutic delivery agents for personalized therapy.

treatment of disease.¹² As diagrammed in **Figure 1.1**, extracellular vesicles offer a compelling opportunity to develop personalized therapeutic delivery carriers.¹³ The concept is relatively simple. Cells are harvested from a patient and used to produce vesicles *in vitro*. These vesicles are then loaded with cargo for delivery to the patient's diseased tissue; in some cases, they may undergo surface modification to achieve improved

targeting. Such personalized treatment leaves low chance of inducing immunogenic response¹⁴ and could improve targeting¹⁵ based on specific surface interactions of vesicles and cells within diseased tissue.

Despite their high potential in therapeutic delivery, vesicle-based therapeutics have been slow to progress to clinical studies due to problems associated with characterization and mass production.^{16–18} The low yield associated with *ex vivo* production of vesicles is a major challenge that leads to a bottleneck in the production process.¹⁹ This review summarizes current research in therapeutic delivery *via* extracellular vesicles and thoroughly examines methods for overcoming challenges associated with EVs in therapeutic delivery.

1.3 Formation and composition of EVs

Vesiculation is a key factor in numerous biological processes, and naturally occurring EVs range dramatically in size and function. In some cases, EVs were named based on the cells from which they were derived; this has led to confusion in the nomenclature and the development of terminology including dexosomes, epididimosomes, argosomes, prostasomes, etc.²⁰ However, for clarity and the purpose of this review, EVs will be loosely grouped into three core categories shown in **Figure 1.2**: exosomes, microvesicles, and apoptotic bodies.

Exosomes, ranging from 30 to 100 nm in diameter, are produced from inward budding of endosomal compartments called multivesicular bodies (MVBs).²¹ In some cases, MVBs fuse with lysosomes and are degraded; however, MVBs may alternatively fuse with the plasma membrane and release their contents into the extracellular space.²² Originally,

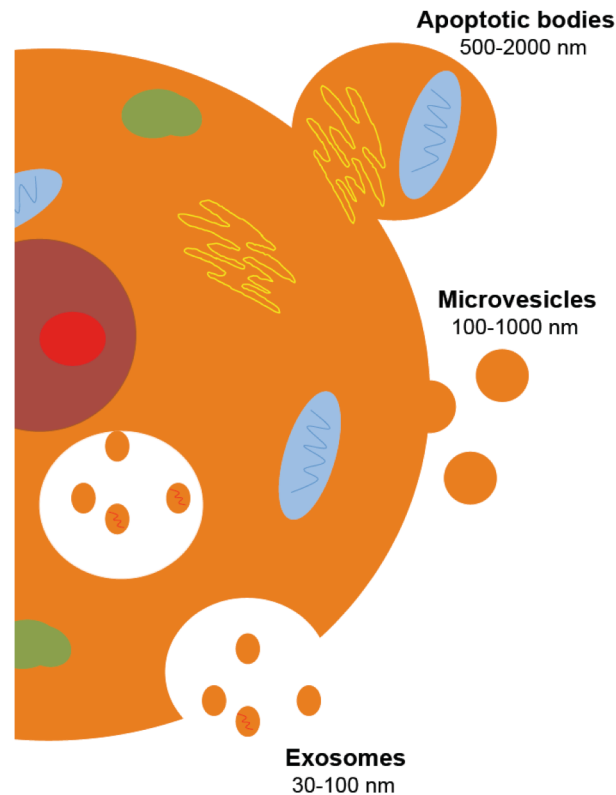


Figure 1.2. The categories of extracellular vesicles.^{28,148,149} Apoptotic bodies are only produced during cell death, while microvesicles and exosomes are released throughout all stages of the cell cycle. Exosomes and microvesicles are within the appropriate size-range for effective therapeutic delivery.

exosomes were believed to be alternatives to lysosomes as they were known to excrete obsolete proteins from the cell. It is now apparent that exosomes have a variety of functions and are capable of transporting mRNA and miRNA²³ between cells to aid in angiogenesis²⁴, proliferation and survival.²⁵

Slightly larger than exosomes, microvesicles (~100-1000 nm) bud directly from the plasma membrane, transporting cargo away from the cell. Although it has been shown that specific molecules are enriched in microvesicles²⁶, the mechanism by which this occurs is not fully understood. In general, cells release microvesicles at a relatively slow rate;

conversely, cancer cells produce these EVs at a rapid rate²⁷ which is hypothesized to aid in their proliferation and survival.

The largest of the EVs, apoptotic bodies are micron-sized carriers of organelles and form as a cell begins to degrade during apoptosis.²⁸ The relatively large size range of apoptotic bodies excludes them from being considered as potential carriers. For the purpose of this review, the focus will be kept on exosomes and microvesicles as potential therapeutic carriers.

1.4 Biological function of EVs

EVs have a wide-array of intrinsic functions. They participate in tissue repair and immune surveillance^{29–34}, transport transcription factors and mRNAs^{32,35–38}, and activate cell surface receptors with protein and lipid ligands.^{30,39} All in all, they possess the ability to control cellular and biological function through multiple dynamic mechanisms.

More importantly, studies have shown that EVs have the potential to be immunostimulatory or immunosuppressive.⁴⁰ Therefore, EV populations can be selected as delivery vehicles with the goal of avoiding immunogenic response. Since exosomes have been shown to express MHC class I and class II molecules from their cell of origin²¹, utilizing exosomes derived from a patient's own cells should be sufficient to avoid immunogenic response.

Clearly the intrinsic functions of EVs must be accounted for when designing therapeutic carriers. A refined selection of EVs will result in an effective therapeutic carrier²³ that has the ability to target a specific population of cells⁴¹ for delivery of desired cargo without inducing immunogenic response.⁴²

1.5 Methods of mass production of EVs

1.5.1 Intracellular calcium levels

There have been several studies demonstrating that an increase in intracellular calcium levels leads to extracellular vesicle formation.^{43–47} Interestingly, this phenomenon has been reported for both exosomes and microvesicles despite the differences in their biogenesis. For example, in 2003, Savina *et al.*⁴³ reported an increase in exosome release when intracellular calcium levels were elevated *via* monensin-stimulation. This was due to a heightened propensity of multivesicular bodies to fuse with the plasma membrane at high Ca^{2+} concentrations. Calcium-dependence also comes into play during microvesicle formation, and Miyoshi *et al.*⁴⁷ demonstrated that increases in Ca^{2+} concentration lead to degradation of cytoskeletal proteins and increased membrane blebbing.

1.5.2 External stress

External stress such as thermal stress⁴¹, hypoxia⁴⁸, radiation⁴⁹ and microenvironmental pH⁵⁰ has been shown to increase EV production. The majority of current studies have focused on EV release from malignant cells as a means of tumor-promotion. In 2009, Wysoczynski and Ratajczak⁴⁹ showed that increased EV production from lung cancer cells exposed to hypoxia and radiation led to induction of pro-angiopoietic factors in stromal cells. That same year, Parolini *et al.*⁵⁰ demonstrated that a key protein involved in melanoma progression is delivered *via* cancer-derived exosomes produced under acidic conditions; as shown in **Figure 1.3**, exosome production is stimulated at pH 6 in comparison to the control at pH 7.

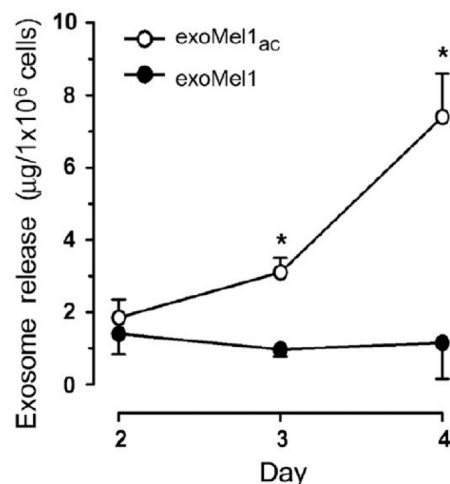


Figure 1.3. Stimulated exosome secretion in acidic conditions. Mel1 cells cultured at pH 6 (light marker) release more exosomes than those cultured at pH 7.4 (dark marker).⁵⁰ Figure is reprinted with permission from the American Society for Biochemistry and Molecular Biology.

EVs produced *via* stress also have the ability to promote immunosuppression; when exposed to thermal and oxidative stress, leukemia and lymphoma cells produce specific ligand-bearing exosomes contributing to immune evasion.⁴¹ Although stress certainly causes increased EV production, the composition of EVs produced under such conditions is markedly different.⁵¹ Future studies should aim to characterize changes in biological functions of EVs resulting from stress as a means to evaluate the potential of this method of mass production.

1.5.3 Sulfhydryl blocking leading to cytoskeletal fixation

In 2012, Sezgin *et al.*⁹² published a protocol for producing and isolating microvesicles using sulfhydryl-blocking reagents⁵²⁻⁵⁴ to stimulate vesicle production. **Figure 1.4** shows these microvesicles prior to and following isolation and purification.⁵⁵ Blebbing, the protrusion and retraction of portions of the plasma membrane, is a result of changes in hydrostatic pressure, which are counteracted by cytoskeletal mechanisms.⁵⁶ As

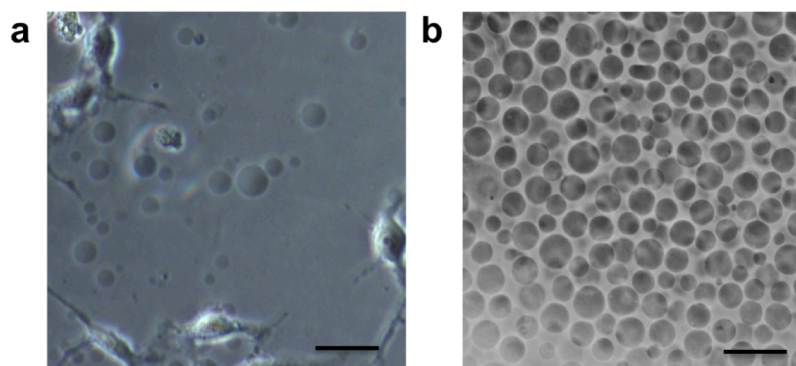


Figure 1.4. Giant plasma membrane vesicles produced from adherent cells (a) and isolated (b) *via* centrifugation. Vesicles were produced by incubating HeLa cells with paraformaldehyde and dithiothreitol to stimulate blebbing as described by Sezgin *et al.*⁵⁵ Scale bar = 20 μm .

diagrammed in **Figure 1.5**, the opposing forces between the hydrostatic pressure of the cytoplasm and the retraction of the actin filaments determines whether a vesicle is released or the bleb retracts⁵⁷ and so blebbing relies heavily on actin and myosin function.⁵⁸ Therefore, impeding cytoskeletal function *via* incubation with sulfhydryl-blocking reagents is a method of inducing rapid EV formation. Although the focus of their work was to model membrane rafts, their method of producing vesicles is relevant and important to the field of vesicular delivery.

In 2013, Zeng *et al.*⁵⁹ published an extensive article correlating blebbing rate and average bleb size with percentage of reagent in solution. Concentration not only affects actin-myosin function but also correlates with media osmolality; this confirms the importance of hydrostatic pressure and actin-myosin function on blebbing. In addition to chemical reagents, biological actin/myosin inhibitors and chemical crosslinking agents have been used to induce vesiculation by hindering actin functionality and halting bleb retraction.^{55,56}

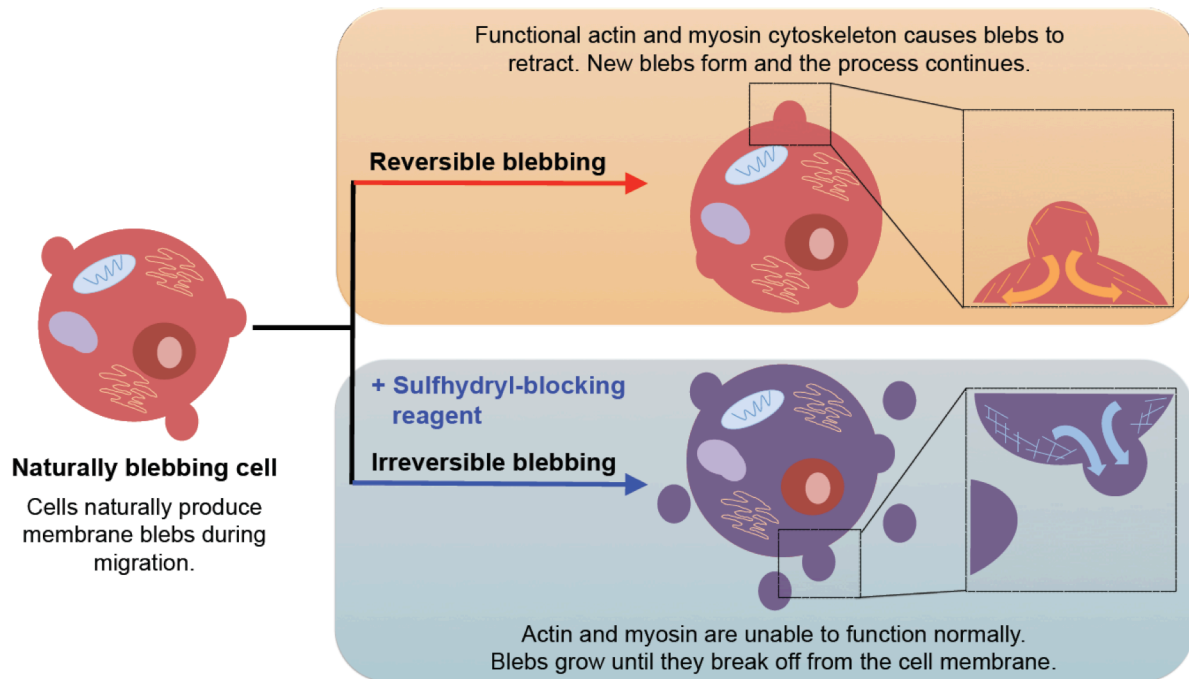


Figure 1.5. Mechanism of EV production *via* exposure to sulfhydryl blocking reagents. A functional cytoskeleton retracts blebs that form due to hydrostatic pressure differences. When actin/myosin function is inhibited, blebbing leads to the release of vesicles.

1.6 The scope of the study

The goal of this study was to evaluate a theoretically feasible method for mass production of EVs to be used in multiple therapeutic applications. Considering all methods of inducing EV formation, cytoskeletal inhibition *via* sulfhydryl-blocking reagents is highly advantageous because of its extremely rapid rate of EV production. While calcium- and stress-induced EV production requires between 12 hours and several days to achieve significantly improved yield^{43,50}, this can be done in just one hour with sulfhydryl-blocking reagents⁵⁵. Additionally, EVs generated in this way should, in theory, be relatively simple to characterize and modify since they should have the same membrane composition and intravesicular cytosol components as their parent cells. In this dissertation, EV production

by sulfhydryl-blocking was characterized and assessed for drug delivery and immunotherapy applications.

1.7 Summary of the dissertation

Chapter 1 introduces extracellular vesicles as promising potential therapeutic carriers. EVs are biologically active, intrinsically transporting cargo between cells. Moreover, they can be loaded with specific cargo for distribution and/or engineered to achieve enhanced uptake. Although studies have already demonstrated therapeutic delivery using EVs, various challenges must be overcome before EV technology is ready for the clinic. Since the properties of EVs are dependent upon their cell of origin and the conditions of their formation, establishing clear characterization practices is essential to ensuring reproducibility and safety.

Identifying methods for mass production of EVs is crucial for achieving high EV yields necessary for clinical trials. In **Chapter 2**, a method to overcome these setbacks by preparing cell-derived vesicles induced by sulfhydryl-blocking is investigated. Applicable to most cell types, this chemical blebbing approach enables efficient, quick, and simple harvest and purification as well as easily scalable production. Applications with Nanovesicles Induced by Sulfhydryl-blocking (NIbS) for drug delivery and micro-Extraellular Blebs (μ EBs) for immunotherapy are described in the following chapters.

In **Chapter 3**, Nanovesicles Induced by Sulfhydryl-blocking (NIbS), in a desirable size range for therapeutic delivery, are loaded with the chemotherapeutic drug, doxorubicin (DOX), resulting in NIbS/DOX. Cellular uptake and intracellular release of DOX was improved using NIbS/DOX compared to a liposomal formulation. It was also confirmed

that in tumor-challenged C57BL/6 mice NibS/DOX significantly slowed tumor growth and led to improved survival compared to treatment with free drug or liposomal drug. NibS are a promising therapeutic carrier for improving cancer treatment outcomes since they are easy to prepare at a large scale, good candidates for drug loading, and capable of efficient administration of therapeutic agents with avoided non-specific major distribution in vital organs.

Antigen-presenting microvesicles produced as a result of sulfhydryl-blocking are good candidates for immunotherapy. In **Chapter 4**, a pilot study involving cancer vaccination with antigen presenting micro-extracellular blebs (μ EBs) is described. Cancer vaccination is achieved using bone marrow dendritic cell derived micro-extracellular blebs (μ EBs) in an optimal size range from a controlled cell stage. In tumor-challenged C57BL/6 mice, μ EBs performed as well as whole cell therapy in terms of slowing tumor growth and improving survival outcomes while providing a cell-free alternative therapy. Note that this study is only a pilot study, and further work elucidating the efficacy of μ EBs for cancer vaccination will be continued in Young Jik Kwon's laboratory.

Chapter 5 summarizes this dissertation. It also provides insights regarding future directions of the research including additional applications for extracellular vesicles produced by sulfhydryl-blocking.

CHAPTER 2: Scalable production of extracellular vesicles

Parts of this chapter have been adapted from:

Ingato, D., Edson, J.E., Zakharian, M., Kwon, Y.J. (2018), Cancer Cell-Derived, Drug-Loaded Nanovesicles Induced by Sulfhydryl-Blocking for Effective and Safe Cancer Therapy. ACS Nano, In press.

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2.1 Introduction

While EVs are a hypothetically superior class of therapeutic carrier with intrinsic biocompatibility, their limited scalability of production and no set standards of quality control, such as size range, biochemical markers, and purity, have hindered their progression to clinical translation.^{16,60} Cells in culture naturally produce EVs but at a rate significantly below the requirements for therapeutic administration, which has been attempted to be mitigated by exposing cells to endosomal trafficking regulators⁶¹, modified proteins⁶² and external stressors.^{41,48-50} However, these time- and labor-intensive processes directly affect cell activities and make it difficult to preserve the composition⁴⁸ and biological functions⁴¹ of EVs at a desired cell stage.

Chemically-induced EV production could be a promising method for rapid and large-scale production. The cell membrane blebs when exposed to a sulfhydryl-blocking reagent.⁵⁵ This cell-blebbing phenomena has been adopted to study membrane raft phases using giant plasma membrane vesicles.^{52,55} Despite the current utility of giant plasma membrane vesicles, their large size range and accompanying unacceptable polydispersity

are not suitable for therapeutic and diagnostic delivery, demanding fine-tuned preparation of EVs *via* sulfhydryl-blocking. For the first time, chemically-induced cell-blebbing *via* sulfhydryl-blocking for producing nano-sized EVs in a desirable quantity is shown, resulting in Nanovesicles Induced by Sulfhydryl-blocking (NIbS). NIbS are easily generated from presumably all types of cells with a high yield. After loading with a desired therapeutic modality for chemotherapy, gene therapy, and/or immunotherapy, NIbS could be used to treat the patient from whom they originated.

2.2 Experimental

2.1.1 Cell culture

A mouse lymphoma cell line (EL4) was obtained from the American Type Culture Collection (ATCC) and grown in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). Cell were incubated at 37°C with 5% CO₂ and 100% humidity.

2.2.2 NIbS production and isolation

10⁷ EL4 cells/mL in the culture media were centrifuged at 200 *g* for 5.5 min and the cell pellet was resuspended and incubated with 25 mM paraformaldehyde (Thermo Fisher Scientific, Waltham, MA) and 2 mM dithiothreitol (Thermo Fisher Scientific, Waltham, MA) in DPBS for 2 h at 37°C with 5% CO₂. To isolate NIbS, cells in the vesiculation buffer were removed by centrifugation at 200 *g* for 5 min at room temperature followed by removal of cell debris and microvesicles at 9,300 *g* for 10 min at room temperature. NIbS were

concentrated with a 30 kDa centrifugal filter (EMD Millipore, Temecula, CA) at 3,200 *g* for 15 min at room temperature and rinsed with DPBS, repeated twice with an equivalent volume of DPBS. The concentration of NlbS was determined by measuring protein content using a BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA). Briefly, 25 μ L serial dilutions of NlbS were plated with 200 μ L of Working Reagent. After 30 min incubation at 37°C, the absorbance was measured at 562 nm. Protein concentration was determined by the absorbance, comparing NlbS serial dilutions to BCA standards.

2.2.3 NlbS characterization

Size distributions of NlbS were measured by dynamic light scattering (DLS) particle analysis using a Zetasizer Nano (Malvern Instruments, Malvern, United Kingdom). Morphology of NlbS was observed by transmission electron microscopy (TEM) using a JEM-2100F field emission electron microscope (JEOL Ltd, Tokyo, Japan). Briefly, 10 μ L of NlbS in water were dropped onto an ultrathin carbon film coated 400 mesh copper grid that had been cleaned *via* glow discharge. After 15 seconds, excess liquid was wicked away from the surface with a Kimwipe followed by dropping 1% uranyl acetate in deionized water (Ted Pella, Inc. Redding, CA) on the grid for 10 seconds. The NlbS-coated grid was imaged with a JEOL 2100F Multipurpose Field Emission Transmission Electron Microscope (JEOL Ltd, Tokyo, Japan) at 200 kV.

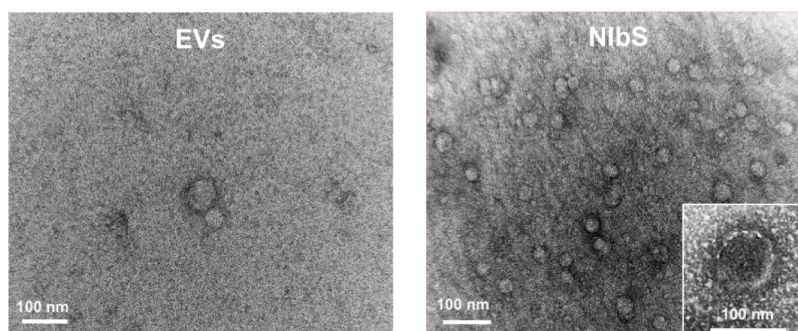


Figure 2.1. Characterization of cancer cell-derived EVs and NibS (An image of a larger NibS in the insert for detailed morphology and vesicular structure). Representative TEM images of naturally-occurring EVs and NibS. Murine T-lymphoma EL4 cells (10^7 /mL) were suspended in serum-free DMEM for 48 h (production of naturally-occurring EVs) or DPBS supplemented with 25 mM PFA and 2 mM DTT for 2 h (vesiculation buffer for NibS production), followed by isolation, purification, and characterization by transmission electron microscopy.

2.3 Results and discussions

2.3.1 Production and morphology of NibS

Biocompatible, cell-derived nano-vesicles were prepared in an easily scalable process that is used to produce cell membrane vesicles: blocking sulfhydryl groups with paraformaldehyde (PFA) along with a disulfide-reducing agent, dithiothreitol (DTT). The size range of EVs produced by sulfhydryl-blocking extends to the nanoscale under the optimized conditions of treating murine T cell lymphoma EL4 cells with 25 mM paraformaldehyde (PFA) and 2 mM dithiothreitol (DTT) for 2 h and more monodispersed NibS than conventionally collected EVs were obtained (**Figure 2.1**). In addition, more than an order of magnitude more NibS were obtained after 2 h-vesiculation than naturally-occurring EVs collected over 48 h, indirectly measured by protein amount (**Figure 2.2**). Naturally-produced EVs are released in cell culture media with significant levels of impurities such as proteins, cell debris, and smaller molecules (e.g., peptides, lipid, amino

acids, etc.), common and pre-dominant impurities generated by cells.^{63,64} However, NIBS are produced for a significantly shorter period of time in DPBS supplemented by PFA and DTT, resulting in a supernatant that is richer in EVs. NIBS are on average approximately 30 nm in diameter (polydispersity index [PDI] of 0.240) as analyzed by dynamic light scattering (DLS) (data shown in Chapter 3), which matched the size observed in TEM images (**Figure 2.1**). Naturally-occurring EVs were highly polydispersed in a range of 25 to 700 nm with a PDI of 0.884. Based on these results, chemically-induced vesiculation *via* sulfhydryl-blocking is a promising strategy for generating large quantities of monodispersed, nano-sized EVs for therapeutic purposes.

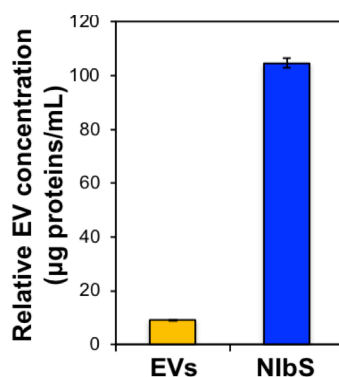


Figure 2.2. Relative production of naturally-occurring EVs (48 h) and NIBS (2 h) from EL4 cells by protein amount ($n = 3$). Murine T-lymphoma EL4 cells (10^7 /mL) were suspended in serum-free DMEM for 48 h (production of naturally-occurring EVs) or DPBS supplemented with 25 mM PFA and 2 mM DTT for 2 h (vesiculation buffer for NIBS production), followed by isolation, purification, and quantification by BCA assay.

2.3.2 Controlling NIBS size distribution

Beyond scalable production of EVs, the method developed in this study also allows for size-control of NIBS by varying the osmotic pressure of the vesiculation buffer (**Figure 2.3**). Nanovesicle size is crucial for taking advantage of size-dependent passive targeting

via the hypothetical enhanced permeability and retention (EPR) effect⁶⁵ for cancer-targeted delivery. Precise control of NibS' size will also be important for quality control purposes.

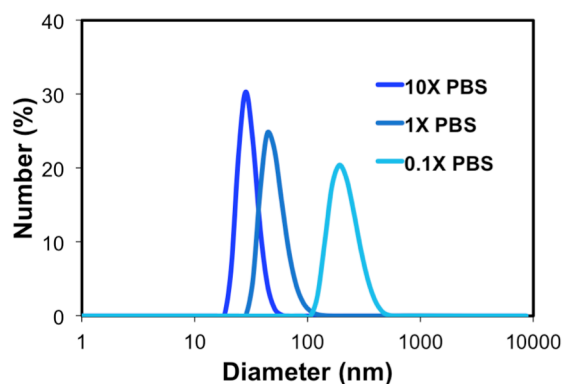


Figure 2.3. Effect of osmotic pressure on NibS production. EL4 cells ($10^7/\text{mL}$) ($n=2$) were suspended in 0.1X, 1X or 10X PBS (representing three orders of magnitude of increasing osmolarity) with 25 mM paraformaldehyde and 2 mM DTT for 2 h at 37°C. NibS were isolated as described in the experimental section and their size distribution was analyzed *via* dynamic light scattering.

2.4 Summary

In contrast to naturally occurring EVs produced in culture media over several days, NibS production in vesiculation buffer containing sulfhydryl-blocking reagents for only few hours results in an EV-rich supernatant and allows for more achievable purification and characterization. Research aiming to develop a consistent set of parameters for obtaining and characterizing pure batches of EVs would greatly benefit from this technology. The following chapters will explore therapeutic applications of EVs produced by sulfhydryl-blocking.

CHAPTER 3: Therapeutic-loaded nano-vesicles for effective and safe cancer therapy

Parts of this chapter have been adapted from:

Ingato, D., Edson, J.E., Zakharian, M., Kwon, Y.J. (2018), Cancer Cell-Derived, Drug-Loaded Nanovesicles Induced by Sulfhydryl-Blocking for Effective and Safe Cancer Therapy. ACS Nano, In press.

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3.1 Introduction

Toxicity and immunogenicity remain common, fundamental challenges for clinical translation of nano-scale therapeutic carriers.^{9,10} Recently, extracellular vesicles (EVs) have gained significant momentum in the field of therapeutic delivery due to their unique advantages resulting from their intrinsic biocompatibility.¹¹ Specifically, EVs in a size range of 30-100 nm are the most widely studied for therapeutic delivery.²¹ Cells naturally utilize EVs for transporting vital biomacromolecules such as mRNA and microRNA between cells,²³ and exogenous RNA-loaded EVs have been used to achieve targeted, tissue-specific delivery.¹⁴ Additionally, protein- and drug-loaded EVs demonstrated similar therapeutic successes.⁶⁶⁻⁷¹

As discussed in Chapter 2, chemically-induced cell-blebbing *via* sulfhydryl-blocking can be harnessed to produce nano-sized EVs in a desirable quantity, resulting in Nanovesicles Induced by Sulfhydryl-blocking (NIbS). After loading with a desired therapeutic modality for chemotherapy, gene therapy, and/or immunotherapy, NIbS could

be used to treat the patient from whom they originated. This approach ensures the delivery of therapeutics with nearly complete biocompatibility, allowing for personalized therapy as well as allogenic applications. Our proof-of-principle study demonstrates that NibS are capable of efficient and safe administration of a chemotherapeutic agent to the tumor site in a murine tumor model.

3.2 Experimental

3.2.1 Cell culture

A mouse lymphoma cell line (EL4) was obtained from the American Type Culture Collection (ATCC) and grown in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). Cells were incubated at 37°C with 5% CO₂ and 100% humidity.

3.2.2 Drug loading and characterization

NibS (25 µg/mL by protein content) (1 mL in DPBS) were incubated with DOX (1 mg/mL) in DPBS mixing on a rotator for 12 h at 37°C. Un-encapsulated DOX was removed and NibS were concentrated with a 30 kDa centrifugal filter (3,200 g for 15 min at room temperature) and the collected NibS were rinsed with an equivalent volume of DPBS three times by centrifugal filtration. 100 µL of vesicles were lysed *via* sonication for 15 min at room temperature and 40 kHz and the DOX fluorescence determined by fluorescence spectroscopy (BioTek, Winooski, VT) (ex. 485 nm, em. 595 nm) was compared with a

calibration curve to calculate its concentration. The DOX loading was determined to be 3.2 mg DOX/mg protein.

3.2.3 Drug release

For drug release studies, a 1000 kD Float-A-Lyzer was used. NibS/DOX and Doxil were diluted to 10 µg DOX/mL in DPBS in the inner compartment and kept incubating at 37°C on a shaking plate. At each time point, a sample 100 µL was removed from the outer compartment and centrifugal filtered at 30 kDa MWCO, 3,200 g, at room temperature for 15 min. The remaining NibS, concentrated on the filter, were re-suspended in an equivalent volume of DPBS and analyzed by fluorescence spectroscopy to determine the concentration of DOX remaining in the liposomes and NibS. The amount of release DOX from NibS/DOX and Doxil was decided by subtracting the measured concentration from the initial DOX concentration of 10 µg/mL.

3.2.4 Eradication of cancer cells *in vitro* and intracellular localization

The capability of NibS/DOX in killing EL4 cells was evaluated using a conventional MTT assay. Briefly, EL4 cells were plated at 20,000 cells/well on a 96 well plate in 100 µL of culture media. Cells were incubated for 24 or 48 h with DOX, Doxil or NibS/DOX at varying concentrations. The cells were then incubated in culture media supplemented by 1 mg/mL thiazolyl blue tetrazolium bromide (Thermo Fisher Scientific, Waltham, MA) for 1 h at 37°C. After the plate was centrifuged at 560 g at room temperature for 10 min and the media was aspirated, 100 µL of DMSO was added per well in order to dissolve the formazan crystals. The absorbance of each well was measured at 560 nm and the relative viability of

the cells was determined by comparing the absorbance of the well containing the cells without drugs.

Confocal imaging was performed using an Olympus Laser Confocal Microscope (Olympus Corporation, Tokyo, Japan). Briefly, EL4 cells were plated at 20,000 cells/well on a 96 well plate in 100 μ L of culture media. Cells were incubated for 3, 6, or 12 h with DOX, Doxil, or NlbS/DOX at 100 μ g DOX/mL. Cells were stained with either CellLight Early Endosomes-GFP (Thermo Fisher Scientific, Waltham, MA) or LysoTracker Green DND-26 (Thermo Fisher Scientific, Waltham, MA). At each time point, 20 μ L of cells were transferred to a 6 well culture slide and 10 μ L of NucBlue (Thermo Fisher Scientific, Waltham, MA) to stain the nucleus. Finally, the samples were fixed by addition of 4% paraformaldehyde prior to imaging. The amount of endosome/lysosome containing DOX was measured by analyzing red (DOX)/green (endosome/lysosome) pixel overlap using MATLAB.

3.2.5 Anti-tumor effect on tumor-challenged mice

All animal studies were carried out using IACUC-approved procedures. EL4 tumors were established in the right flank of 12-week old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) by subcutaneous injection of 10^6 EL4 cells in 100 μ L DPBS. After eight days, tumors were clearly visible, and the mice were intravenously injected with a single dose of DOX, Doxil, or NlbS/DOX (8 mg DOX/kg) in a total volume of 100 μ L DPBS. As a control, a group of mice received DPBS alone. Because of sub-consistent tumor growth, the mice carrying a tumor in a comparable size were used. Tumor size was measured over 40 days post treatment using a digital caliper and its volume was calculated by a equation

$W^2L/2$ where W and L represent the width and the length of a tumor. The statistical significance was analyzed by one-way analysis of variance (ANOVA) and Tukey honest significant difference (HSD) *post-hoc* tests. Survival was analyzed by long-rank test.

For pharmacokinetics and biodistribution experiments, tumors were established and mice were treated with DOX, Doxil, or NlbS/DOX as described above. Blood samples in 20 μ L were collected at varying time points and suspended in 100 μ L of acidified alcohol, 0.075 N HCl (Thermo Fisher Scientific, Waltham, MA) in 90% isopropanol (Thermo Fisher Scientific, Waltham, MA). The blood was immediately analyzed for DOX concentration by fluorescence spectroscopy as described above. The mice were sacrificed at 24 h of post treatment and various organs (e.g., lung, liver, heart, spleen, and tumor) were extracted. The organs were cryo-pulverized by freezing in liquid nitrogen and then manual pulverization with a mortar and pestle. Finally, the organs were re-suspended in 1 mL of acidified alcohol and homogenized by sonication. The DOX concentration was measured by fluorescence spectroscopy.

3.2.6 Data analysis

Statically analysis was completed by one-way ANOVA and the Tukey HSD *post-hoc* test.

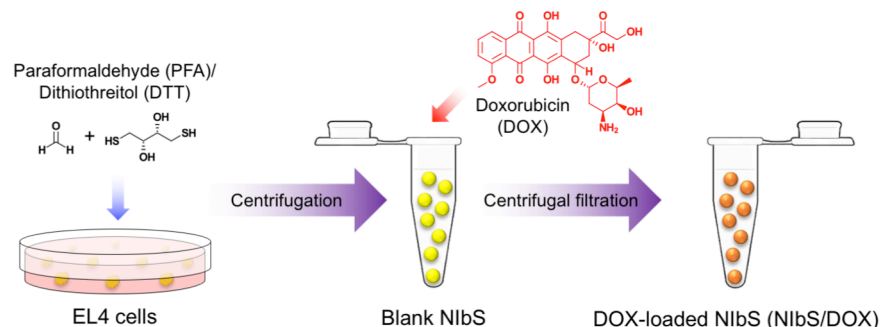


Figure 3.1. Production and drug loading of NibS derived from cancer cells. The scheme shows the highly facile method for cancer cell-derived, doxorubicin (DOX)-loaded NibS (NibS/DOX) production and isolation. To produce NibS, EL4 (murine lymphoma) cells were treated with 25 mM PFA and 2 mM DTT in PBS to chemically induce EV production. After isolation by a series of centrifugation and centrifugal filtration steps, NibS were loaded with DOX by incubation, followed by further purification by centrifugal filtration.

3.3 Results and discussions

3.3.1 Drug loading and characterization

DOX is clinically used to treat many cancer indications but is also known for high instances of systemic toxicity. With the aim of improving drug delivery to the tumor site, EL4-derived NibS were loaded with DOX (**Figure 3.1**). When NibS were incubated at body and storage temperatures of 37 and 4°C, respectively, they demonstrated significantly improved stability than naturally-occurring EVs (**Figure 3.2**). It was hypothesized that DOX-encapsulating-NibS (NibS/DOX) would improve DOX circulation in blood, while shielding the drug from rapid absorption, and releasing it once it was internalized by a cell. NibS were loaded with DOX by incubation for 12 h at 37°C prior to purification by three subsequent washes using centrifugal filtration (MWCO 30 kDa). The filter was selected for high NibS recovery⁷² and efficient removal of vesiculation reagents (**Figures 3.3**), free proteins, and free DOX. A single centrifugal filtration alone removed about 99.9, 85, and 84% of paraformaldehyde assessed NMR, free proteins, and DOX (data not shown),

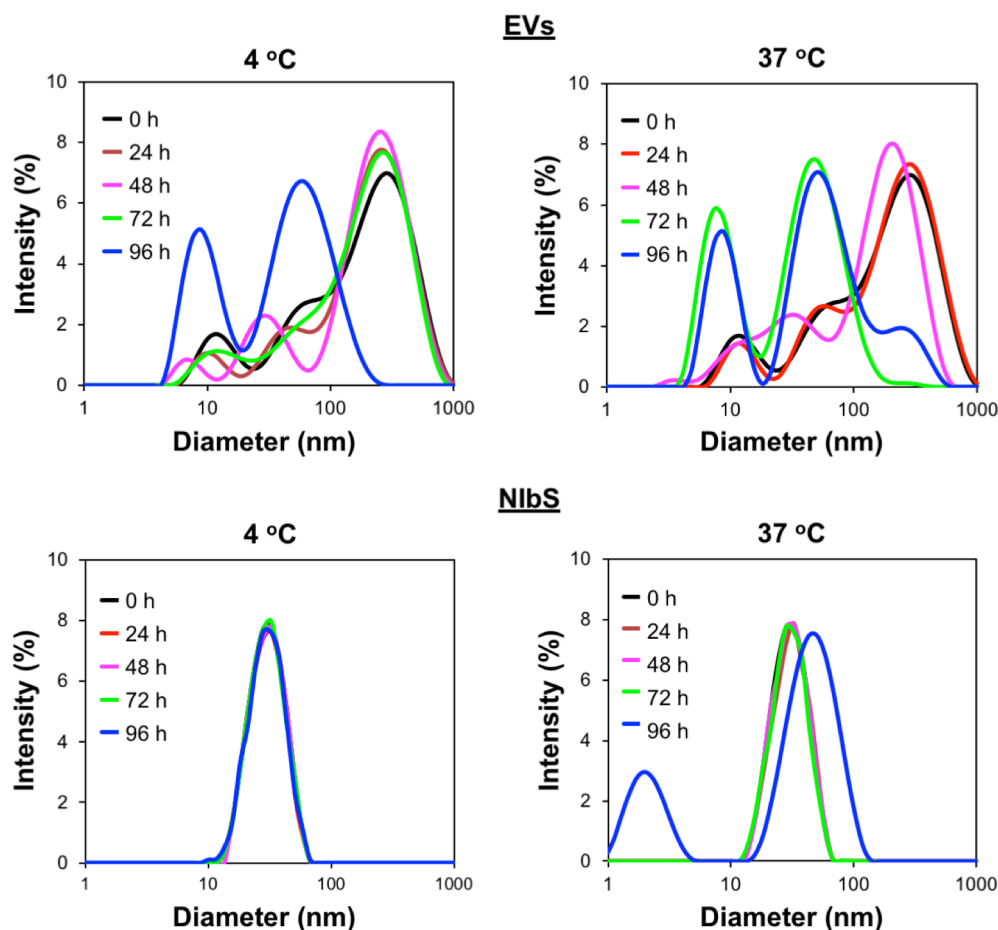


Figure 3.2. Stability comparison of EVs and NibS. Naturally-occurring EVs produced in serum-free media and NibS produced in media with blebbing reagents were isolated and purified. Both EVs and NibS were resuspended in DPBS and incubated at 4°C and 37°C, and size distribution of particles in the suspension was assessed every 24 h by DLS.

respectively, from the supernatant containing NibS/DOX. The resulting NibS/DOX were found to be similar in size to DOX-free, Blank NibS with slight increase in average size to 37 nm in diameter by DLS (**Figure 3.4**) but without noticeable morphological or size changes observed by TEM (data not shown). Optimized methods for production, isolation, and drug-loading generated large quantities of NibS/DOX, and all procedures employed are facilely scalable for mass production.

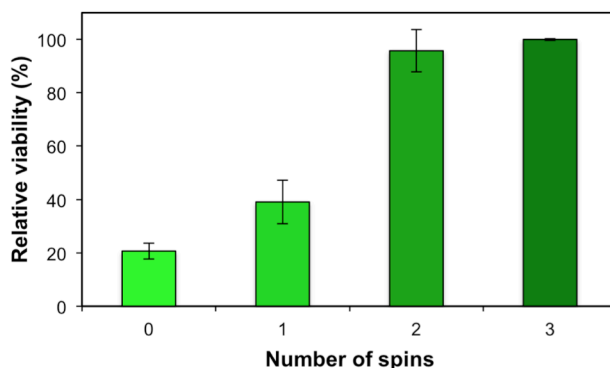


Figure 3.3. Cytotoxicity by residual paraformaldehyde after centrifugal filtration. Vesiculation buffer underwent three centrifugal filtration steps with a 30 kDa centrifugal filter at 3,200 *g* for 15 min. After 24 h incubation with the retentate, cell viability was quantified using conventional MTT assay (n = 2).

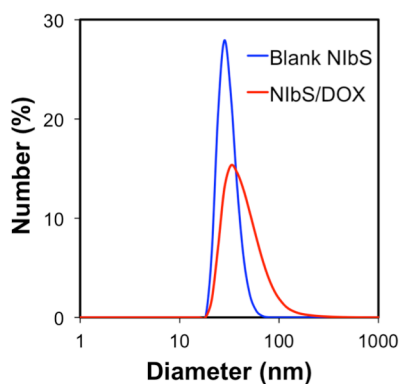


Figure 3.4. The size distributions of DOX-free (blank) NibS and NibS/DOX analyzed *via* dynamic light scattering (DLS) particle analysis.

3.3.2 Drug release, uptake, and cancer cell eradication *in vitro*

Liposomes are commercially used to encapsulate DOX for prolonged circulation and lowered cardiovascular toxicity (e.g., Doxil®, Myocet®, and Caelyx®).⁷³ However, the polyethylene glycol (PEG)-tethered surface limits the uptake of liposomal DOX by cancer cells⁷⁴ while promoting clearance by the reticuloendothelial system (RES), altogether leading to poor therapeutic efficacy.⁷⁵ Most notably, the use of highly stable lipids with a

transition temperature (T_m) higher than the body temperature such as hydrogenated soy phosphatidylcholine (HSPC, $T_m=53^\circ\text{C}$) hinders intracellular DOX release.⁷⁶ NibS overcome the limitation of the liposomal formulation by releasing DOX at faster rate than Doxil both in the presence and absence of serum (**Figure 3.5**). While 84.3% DOX was released from Doxil in the absence of serum, NibS/DOX achieved almost complete DOX release (99.9%),

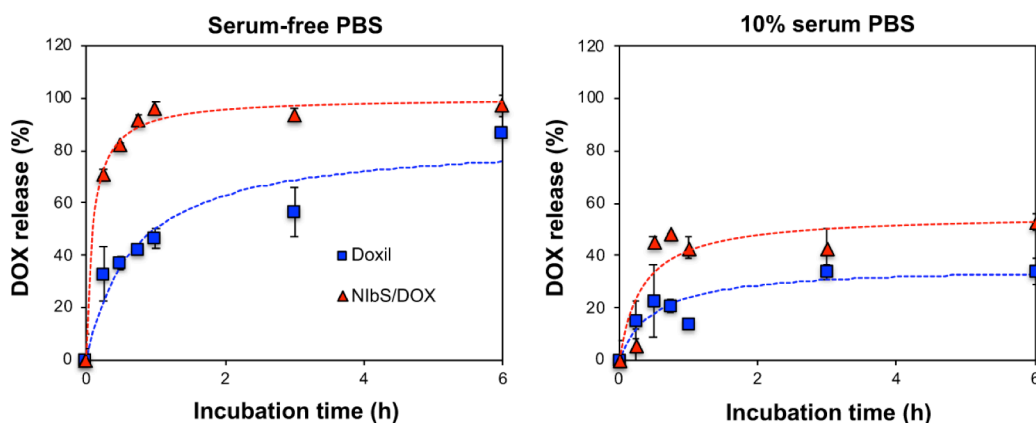


Figure 3.5. DOX release from NibS/DOX and Doxil with or without serum. NibS/DOX and Doxil (10 μg DOX/mL in DPBS) in a dialysis device were incubated at 37°C on a shaking plate. At each time point, a sample of the external compartment DPBS was taken for DOX quantification by fluorescence measurements. Drug release reached 50% of the maximum at 0.68 and 0.10 h for Doxil and NibS/DOX, respectively, in serum-free media, and 0.50 and 0.33 h for Doxil and NibS/DOX, respectively, in serum-containing media ($n = 3$).

with the required time for 50% DOX release (t_{50}) of 0.68 and 0.10 h, respectively. In the presence of 10% serum, 35.8% DOX was released from Doxil (58% reduction) and 55.7% DOX was released from NibS/DOX (43% reduction) with t_{50} of 0.50 h (28% decrease) and 0.33 h (230% increase), respectively. Clearly, serum reduced overall DOX release from both Doxil and NibS/DOX in a comparable way (58 vs. 43% reduction), implying similar non-specific absorption on their surfaces.⁷⁷ However, the proteins absorbed on the liposomal surface of Doxil may have disrupted its structure and facilitated DOX release,^{78,79} while the

proteins absorbed on the surface of NibS/DOX may have contributed to stabilization as reported regarding the cell surface.⁸⁰

EL4 cells treated with NibS/DOX for up to 12 h exhibited strong DOX fluorescence indicating efficient intracellular uptake while Doxil-treated cells did not (**Figure 3.6a**). After 6 h-treatment with NibS/DOX, cells showed significantly increased DOX levels in both the endosome and the lysosome (**Figure 3.6b**), in contrast to those incubated with Doxil. The mechanism behind the inferior uptake of Doxil to that of NibS/DOX is most likely attributed to its PEGylated surface which has been shown to sterically prevent uptake by

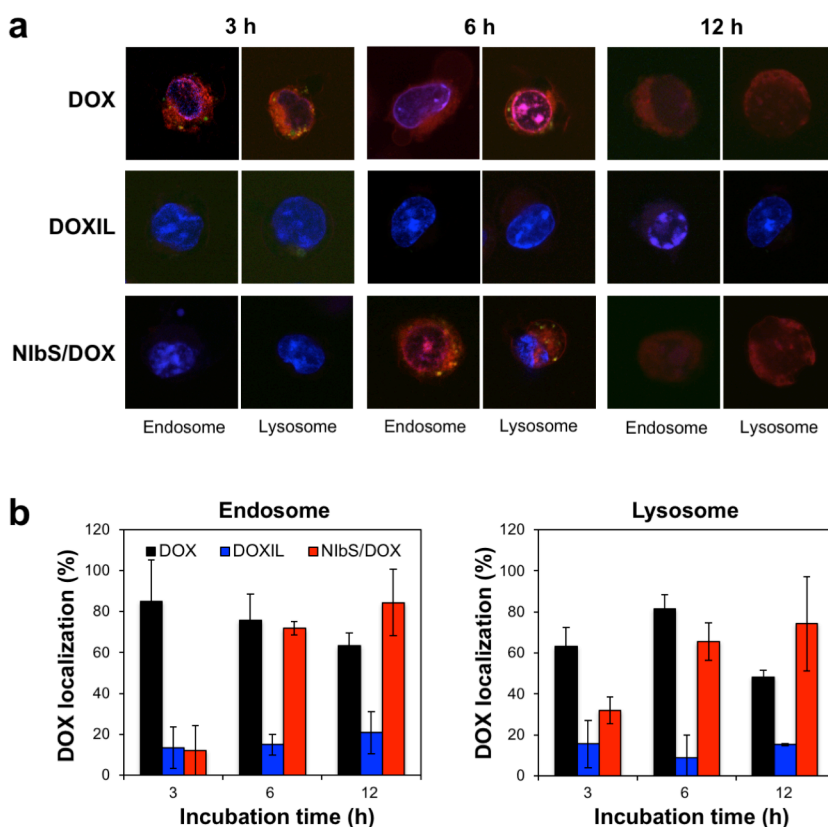


Figure 3.6. Cell uptake and intracellular distributions of free DOX, Doxil, and NibS/DOX. (a) Confocal micrographs of EL4 cells incubated with free DOX, Doxil, or NibS/DOX at a concentration of 100 μg DOX/mL for 3, 6, or 12 h prior to imaging (Green: endosomes or lysosomes, Red: DOX). (b) Quantified intracellular localization of DOX ($n = 2$) over 3, 6, and 12 h in the endosome and the lysosome. While Doxil showed minimal uptake over the timeframe shown, NibS/DOX were taken up and accumulated in the endosome within 6 h.

cells and drug release.⁸¹ Free DOX is known to rapidly penetrate the cell membrane and

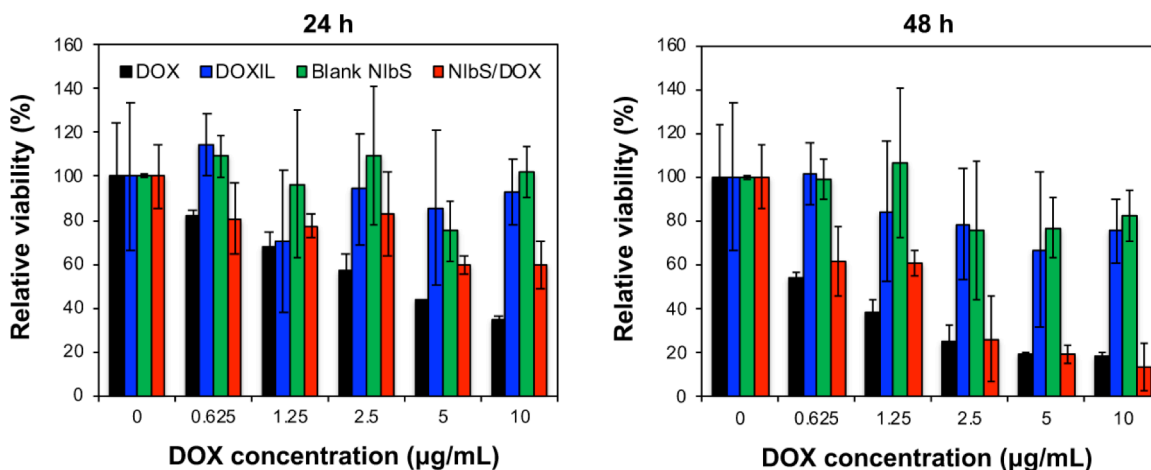


Figure 3.7. Eradication of cancer cells *in vitro* by NibS/DOX. EL4 cells were treated over 24 or 48 h with DOX, Doxil, NibS/DOX, or Blank NibS. Relative viability was determined by MTT assay (n = 3). At the concentrations of 10 µg/mL for 24 h, and 5 and 10 µg/mL for 48 h, DOX and NibS/DOX treatments were statistically significant ($p < 0.01$) from DOX and Doxil. Differences in relative viability after treatment with DOX and NibS/DOX were statistically insignificant after 48 h of incubation at the concentration range tested.

bind to intracellular nucleic acids⁸², resulting in instantaneous, high intracellular accumulation including within the endosome and the lysosome (**Figure 3.6a and b**), attributing to systemic toxicity *in vivo*⁸³.

When incubated with EL4 cells *in vitro*, NibS/DOX eradicated the cells at a comparable efficiency to that of free DOX in one or two days (**Figure 3.7**). NibS/DOX were not as efficient as free DOX at 24 h-treatment but caught up at 48 h of treatment, demonstrating gradual but eventually efficient DOX release inside cells over time. Doxil was found to be as inefficient as blank NibS (**Figure 3.7**) most likely due to poor uptake by the cells and retarded DOX release inside a cell (**Figure 3.6**).⁸⁴ The gradually elevated anti-cancer activity of NibS/DOX approaching that of DOX at 48 h-treatment implicates the

combined advantages of initial long circulation and high biocompatibility like that of Doxil, followed by ready DOX release in cells like that of free DOX, overcoming the current limitations of each modality for efficient cancer therapy.

3.3.3 Effective and targeted tumor eradication *in vivo*

As a proof-of-concept study to assess the potential of NibS/DOX for clinical translation, EL4 tumor-bearing C57BL/6 mice were treated with NibS/DOX (**Figure 3.8 and 3.9**). Intravenously injected NibS/DOX were more effective at interfering with tumor growth than free DOX ($p = 0.019$) or Doxil ($p = 0.016$) (**Figure 3.8a**), and significantly prolonged the survival of the animals compared to free DOX and Doxil over the entire experiment (60 days) ($p < 0.020$) (**Figure 3.8b**). It was noted that half of the animals treated with NibS/DOX survived to the end of the study (**Figure 3.8b**). In contrast to NibS/DOX-treated animals, the tumor growth in the animals treated with blank NibS was slightly faster than that in non-treated animals (*i.e.*, DPBS-injected) but was not statistically significant ($p = 0.980$). Animals treated with blank NibS exhibited fast tumor growth and

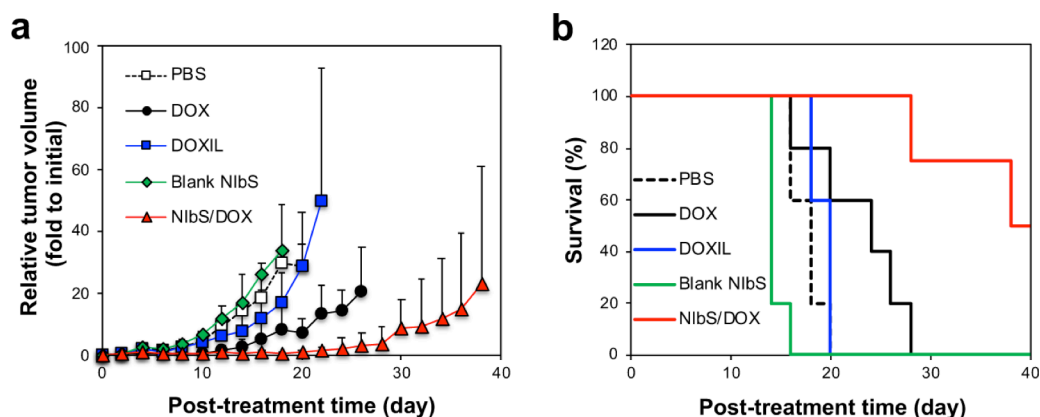


Figure 3.8. Efficient therapeutic efficacy against murine EL4 tumors by EL4-derived NibS/DOX. (a) EL4 tumor growth in C57BL/6 mice ($n = 5$) and (b) survival of the animals treated with DOX, Doxil, and NibS/DOX (8 mg DOX/kg). The animals were intravenously injected with drug in the tail vein and tumor size was measured every two days.

the lowest survival rates among all tested animal groups (**Figure 3.8b**). This could be attributed to recently reported observations that growth of tumor cells is aided by tumor-derived EVs that transport RNAs.^{85,86} Histology also confirmed the most significant necrosis and the highest Caspase-3 activation in the tumor of NlbS/DOX-injected mice 5 days post-treatment (**Figure 3.9**).

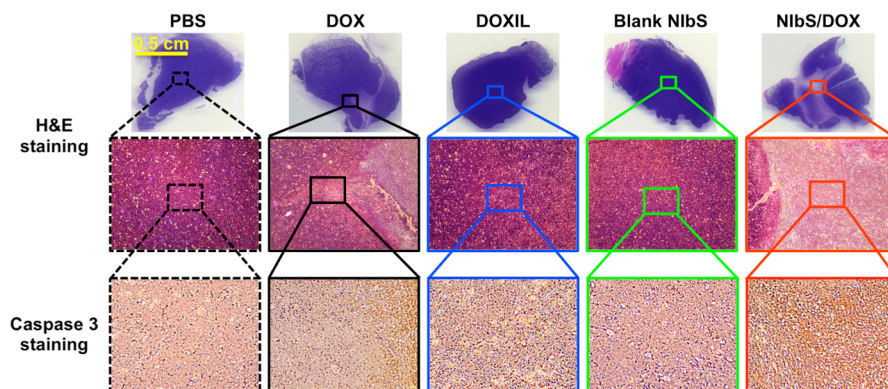


Figure 3.9. Histopathological confirmation of NlbS/DOX's anti-tumor effect. Tumor histology in C57BL/6 mice treated with intravenous injection of PBS, DOX, Doxil, Blank NlbS, and NlbS/DOX (8 mg DOX/kg) in the tail vein (n = 3). After 5 days, mice were sacrificed, and tumors were harvested, sliced, and stained with H&E or for Caspase-3.

In order to obtain clues as to how NlbS/DOX efficiently eradicated the tumor *in vivo*, blood samples were collected from C57BL/6 mice treated with DOX, Doxil, or NlbS/DOX at various post-injection time points and analyzed for DOX concentration. Doxil and NlbS/DOX were significantly more effective in retaining drug in blood circulation than free DOX ($t_{0.5}=9.5$ min) (**Figure 3.10a**). Due to the PEG corona protecting against protein absorption and the highly stable HSPC lipid component, Doxil showed the longest circulation time ($t_{0.5}=99$ min), while its PEGylated surface led to uptake in the liver.⁸⁷ The intermediate circulation time of NlbS/DOX ($t_{0.5}=41$ min) could leverage sufficient

circulation (over 150 times in mice) and accumulation in the tumor. The DOX accumulation over 24 h in various organs demonstrated that NibS were able to deliver DOX to the tumor, as efficiently as Doxil, without consequence of accumulation in the lungs and the liver,

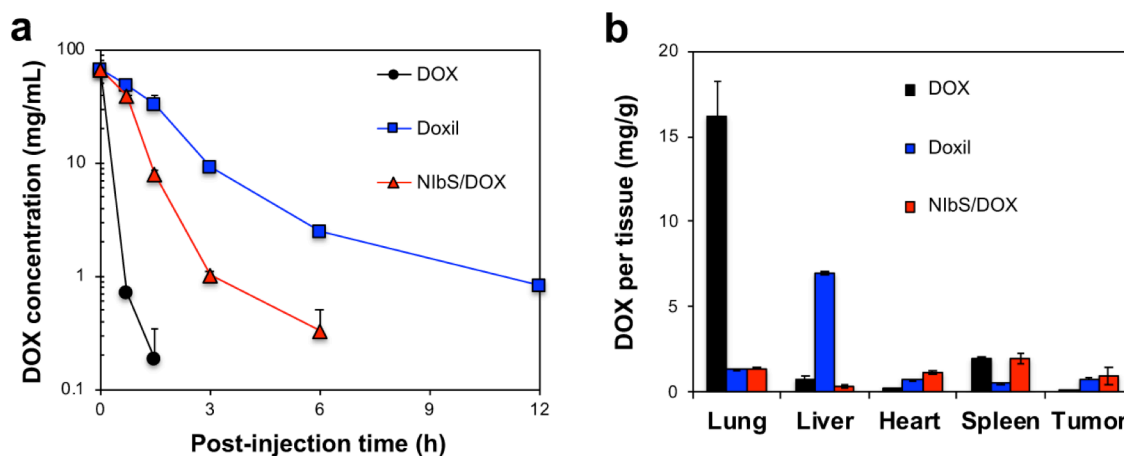


Figure 3.10. Pharmacokinetics and biodistribution of EL4-derived NibS/DOX *in vivo*. (a) DOX concentration in the serum ($n = 3$) over 12 h and (b) DOX accumulation in organs ($n = 3$) for 24 h. For pharmacokinetics study, C57BL/6 mice were intravenously injected with DOX, Doxil, and NibS/DOX (8 mg DOX/kg), blood was drawn at different post-treatment times and diluted in series, and the DOX concentration in blood was measured by fluorescence quantification. For the DOX accumulation study, animals were sacrificed 24 h after treatment and their organs were cryo-pulverized, homogenized, and analyzed for DOX content.

unlike DOX and Doxil, respectively (**Figure 3.10b**). The tumor-targeted accumulation of NibS/DOX along with avoided accumulation in the lungs and liver is of major importance, as non-specific accumulation of chemotherapeutic agents (e.g., free DOX) in vital organs such as the lungs and liver is a major clinical concern leading to adverse side effects.^{88,89} Small molecule drugs (e.g., DOX) are known to heavily accumulate in a highly vascular tissue (e.g., lungs), and the porous paths and interactions with Kupffer cells retain

particulate drugs (e.g., Doxil) in the liver.⁹⁰ The results shown in **Figure 3.8 and 3.10**

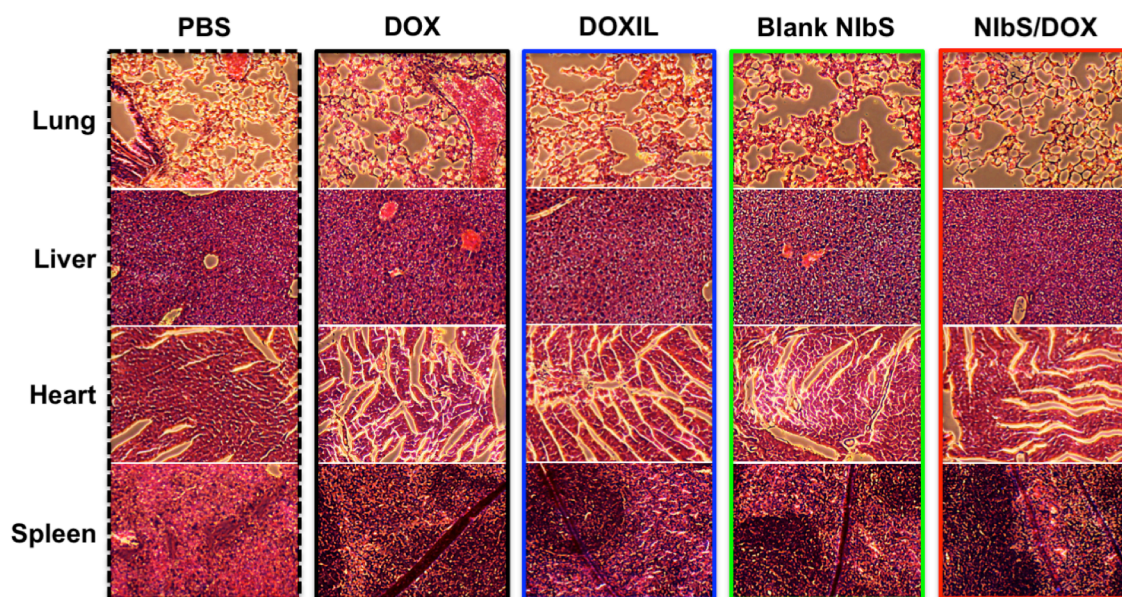


Figure 3.11. H&E staining of major organs. C57BL/6 mice were intravenously injected with PBS, DOX, Doxil, Blank NibS, and NibS/DOX (8 mg DOX/kg) in the tail vein. After 5 days, mice were sacrificed and organs were harvested and sliced, followed by H&E staining.

demonstrate the high potential of drug-loaded NibS for efficient cancer therapy (**Figures 3.8a and b**) with avoided adverse side effects (**Figures 3.10b**). The body weight of the animals was not a good biocompatibility indicator for this study because EL4 tumors are minimally invasive to animals without significant adverse effects and the animal weights kept increasing mainly due to rapid growth of the EL4 tumors (data not shown). The H&E staining of lung, liver, heart, and spleen showed no clear difference between treatment group (**Figure 3.11**), most likely due to the low DOX dose (8 mg/kg) and single administration.^{91,92} In order to assess the potential toxicity *in vivo* by any residual vesiculating agents, animals were injected with a single dose of Blank NibS as in the tumor treatment experiment. Mouse body weight, spleen and liver weight, complete blood count

(CBC), and liver enzyme levels demonstrated no obvious *in vivo* toxicity in the animals by NibS themselves over a month (**Figure 3.12**).

3.4 Summary

Doxorubicin-loaded NibS demonstrated efficient anti-cancer efficacy *in vitro* and *in vivo*, via improved cellular uptake, facilitated intracellular drug release and targeted accumulation in the tumor, and they avoided accumulation in vital organs in comparison to a commercial liposomal formulation. Treatment with drug-loaded NibS significantly slowed tumor growth and led to improved survival in tumor-bearing mice bearing. Although this chapter focuses on their use as chemotherapeutic-delivery vehicles for cancer therapy, NibS have a broad range of potential health applications. NibS could improve delivery of other drugs for chemotherapy and/or nucleic acids for gene therapy. They could also be used to augment tumor antigen presentation for immunotherapy.

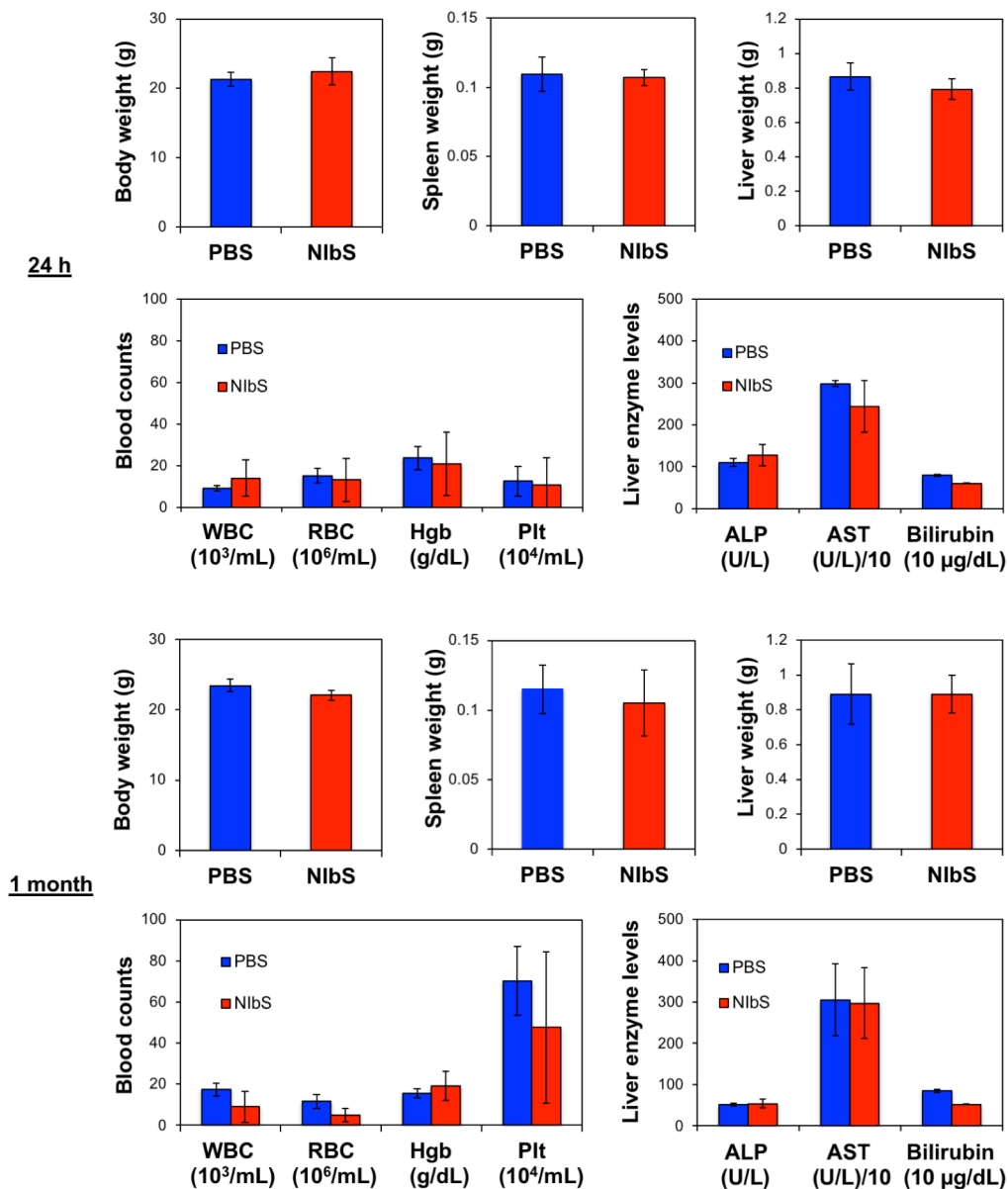


Figure 3.12. *In vivo* toxicity of Blank NibS. C57BL/6 mice were intravenously injected with DPBS or Blank NibS as given in the tumor treatment study. After 24 h or 1 month, mouse body weight, spleen and liver weight, complete blood count (CBC), and liver enzyme levels were compared.

CHAPTER 4: Dendritic cell-derived micro-extracellular blebs (μEBs) with controlled size and maturation markers for cancer vaccination

4.1 Introduction

Immunotherapy aims to overcome tumor immune evasion by activating tumor-reactive T cells to mediate antitumor response.⁹³ Studies have reported that bone marrow dendritic cells (BMDCs) pulsed with tumor antigen *ex vivo* are able to act as anti-cancer vaccines, presenting antigen *in vivo* and stimulating T cell response to eradicate tumor cells.^{94,95} However, variability associated with whole-cell vaccine formulations has made them non-ideal candidates for immunotherapy.⁹⁶ Additionally, live cells are challenging to store and have risk for potential reprogramming once administered.^{97,98}

Extracellular vesicles (EVs) have recently been recognized as a promising therapeutic platform.⁶⁰ EVs maintain the intrinsic biocompatible benefits of cell therapy while gaining advantage with better storability, minimized risk, and ability to tailor size based on desired application. Several groups have demonstrated EVs as effective carriers of therapeutic cargo *in vitro* and *in vivo*.^{66,67} However, few EV therapies have progressed to clinical trials in part due to high-levels of variability in EV formulations. Reducing variability associated with EVs is necessary to ensuring safety and therapeutic efficacy.⁹⁹ Antigen-pulsed dendritic cell derived EVs are a promising cell-free option for cancer immunotherapy,^{100,101} but intrinsic issues with heterogeneity of EV populations has slowed their progression to the clinic.⁹⁹ Improving homogeneity, in particular maturation state of the parent cell¹⁰² and EV size¹⁰³, will be critical to achieving effective immunotherapy.

Due to the naturally slow production of extracellular vesicles by untreated cells, recent studies have aimed to develop methodologies for increasing vesiculation rate^{43,48,50}. Sulphydryl-blocking is one method which results in rapid blebbing⁵⁵, allowing for potential control over the parent cell maturation state and improvement of homogeneity of extracellular blebs. In our previous work, we demonstrate that sulphydryl-blocking leads to production of blebs across a vast size range from the nano- to microscale. Here, we demonstrate that size-selected micro-extracellular blebs (μ EBs) produced by sulphydryl-blocking maintain maturation characteristics of their parent cells (**Figure 4.1**) and serve as

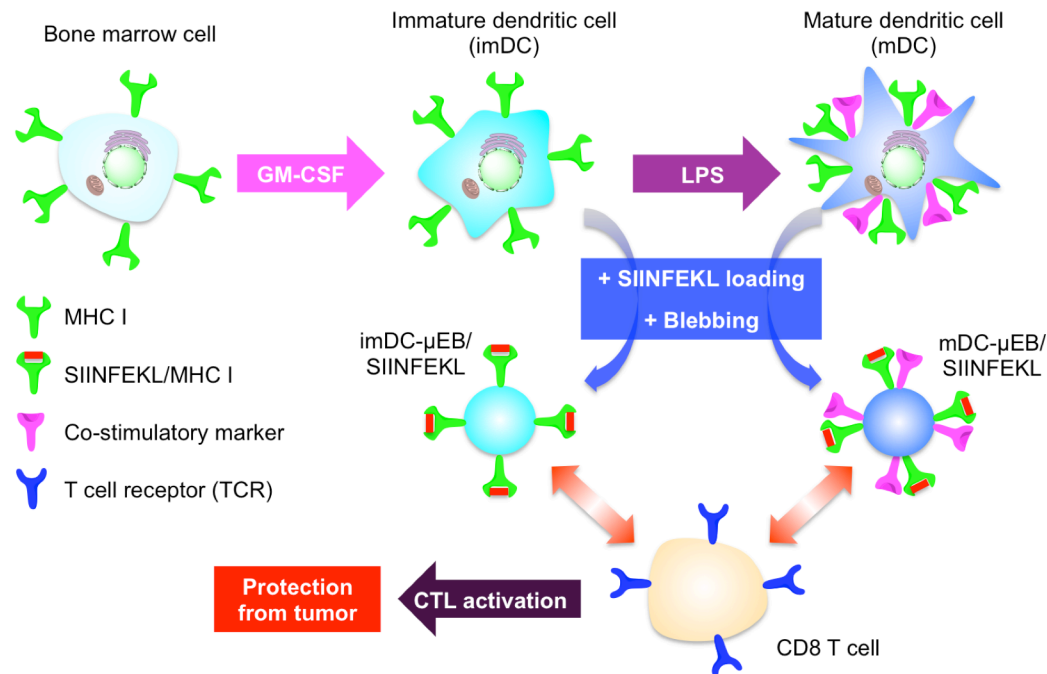


Figure 4.1. Production of cell stage-specific μ EBs. μ EB production occurs rapidly and blebs are visible within 1 hour of sulphydryl-blocking reagent addition. This process is significantly faster than naturally occurring extracellular bleb production, and enables μ EBs to be produced from cells of a defined maturation stage.

efficient cell-free cancer vaccines.

4.2 Experimental

4.2.1 Cell culture

A mouse lymphoma cell line (EL4) was obtained from the American Type Culture Collection (ATCC) and grown in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). An OVA expressing derivative of EL4 (E.G7-OVA) was obtained from the American Type Culture Collection (ATCC) and grown in RPMI (Thermo Fisher Scientific, Waltham, MA) supplemented with 0.40 mg/mL Geneticin (Thermo Fisher Scientific, Waltham, MA), 10% FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). B3Z CD8⁺ T cell hybridomas were grown in RPMI (Thermo Fisher Scientific, Waltham, MA) supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA), 0.055 mM 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA), 10% FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). Cells were incubated at 37°C with 5% CO₂ and 100% humidity.

4.2.2 Bone-marrow isolation and bone marrow dendritic cell (BMDC) culture

Bone marrow was isolated from femurs of 12-week of C57BL/6 mice (Charles River Laboratories, Wilmington, MA). After mice were euthanized, the femurs were isolated and the bone marrow flushed out with a 25-gauge needle using RPMI (Thermo Fisher Scientific,

Waltham, MA) supplemented 10% FBS (Gemini Bio Products, West Sacramento, CA). Red blood cells were lysed using Red Blood Cell Lysis Buffer (Thermo Fisher Scientific, Waltham, MA). Remaining cells were cultured in RPMI (Thermo Fisher Scientific, Waltham, MA) supplemented 10% FBS (Gemini Bio Products, West Sacramento, CA) and 20 ng/mL rmGM-CSF (R&D Systems, Minneapolis, MN). On day 7 of culture, the percentage of BMDCs in the population was assessed with anti-mouse CD11c antibody (BioLegend, San Diego, CA). Cells were then incubated with 20 ng/mL lipopolysaccharide (Sigma Aldrich, St. Louis, MO) for 24 h to induce maturation. The percentage of mature BMDCs (mBMDCs) in the population was assessed with anti-mouse CD40 antibody (BioLegend, San Diego, CA).

4.2.3 Preparation and isolation of SIINFEKL-presenting μ EBs

Immature BMDCs (imBMDCs) were incubated with 50 μ M SIINFEKL for 1 h prior to maturation. 10^6 BMDCs/mL in the culture media were centrifuged at 200 *g* and for 5.5 min and the cell pellet was resuspended and incubated with 25 mM paraformaldehyde (Thermo Fisher Scientific, Waltham, MA) in DPBS for 12 h at 37°C with 5% CO₂. To isolate μ EBs, cells in the vesiculation buffer were removed by centrifugation at 200 *g* for 5 min at room temperature followed by concentration of μ EBs at 9,300 *g* for 10 min at room temperature. μ EBs were washed three times with 10 mL of DPBS. SIINFEKL-presentation was assessed with anti-mouse H-2Kb bound to SIINFEKL antibody (BioLegend, San Diego, CA).

4.2.4 T cell hybridoma activation assay

10 μ L of extracellular vesicles in DPBS were incubated with 30,000 B3Z cells in 100 μ L/well of RPMI supplemented with 10% FBS for 24 h at 37°C with 5% CO₂. After 24 h, the

plate was spun down and the supernatant removed. Cells were resuspended in CPRG buffer consisting of 90% DPBS, 10% NP-40 (Sigma Aldrich, St. Louis, MO), and 0.6 mg/mL chlorophenol red- β -D-galactopyranoside (Sigma Aldrich, St. Louis, MO) and incubated at room temperature for 12 h. The assay was assessed by measuring absorbance at 595 nm compared to set standards of B3Z cells incubated with BMDCs and known SIINFEKL concentration.

4.2.5 Cytotoxic T lymphocyte assay

12 wk-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were vaccinated by s.c. injection with DPBS, OVA protein, SIINFEKL peptide, SIINFEKL-presenting BDMCs, or SIINFEKL-presenting BMDC μ EBs. With the exception of the control (DPBS) group, each group received vaccinations that were equivalent to a dosage of 100 μ L of 50 μ M SIINFEKL as quantified by CPRG assay. Vaccinations were given twice 14 days apart. Seven days after the second injection, mice were sacrificed, spleen cells were isolated, and spleen cells were incubated in various E:T with PKH26 (Sigma Aldrich, St. Louis, MO)-stained E.G7-OVA cells. After 4 h, cells were stained with 1 mM YoPro1 (Thermo Fisher Scientific, Waltham, MA) and analyzed for viability by flow cytometry.

4.2.6 Tumor challenge study

12 wk-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were vaccinated by s.c. injection with DPBS, OVA protein, SIINFEKL peptide, SIINFEKL-presenting BDMCs, or SIINFEKL-presenting BMDC μ EBs. With the exception of the control (DPBS) group, each group received vaccinations that were equivalent to a dosage of 100 μ L

of 50 μ M SIINFEKL as quantified by CPRG assay. Vaccinations were given twice 14 days apart. 7 days after the second injection, mice were injected s.c. with 300,000 E.G7-OVA cells in the right flank. Tumor growth was monitored by measuring the tumor size with calipers every 48 h.

4.3 Results and discussions

4.3.1 Production and characterization of μ EBs derived from BMDCs

μ EBs were efficiently produced from BMDCs in culture by blocking sulfhydryl groups with paraformaldehyde (PFA). Sulfhydryl-blocking led to vast vesicle production (**Figure 4.2a**) in a short time frame, 2 hours. This is favorable to natural microvesicle production, which requires several days of cell culture and results in significant levels of

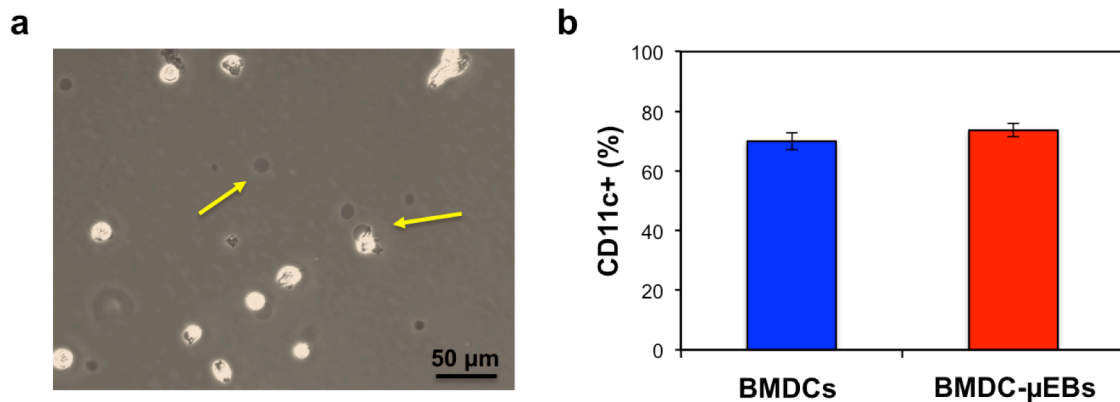


Figure 4.2. Characterization of microvesicles derived from BMDCs by sulfhydryl-blocking. (a) Representative image BMDCs producing μ EBs (yellow arrows) after incubation with sulfhydryl-blocking reagent. (b) Assessment of CD11c expression on the surface of BMDC-derived μ EBs. BMDCs (10^7 /mL) were suspended in DPBS supplemented with 25 mM PFA for 2 h, followed by isolation of microvesicles by centrifugation. Protein expression was assessed by antibody labeling and flow cytometry.

impurities including proteins and cell debris.^{63,64} The resulting μ EBs were isolated by centrifugation and range in size from 0.05 μ m to 5 μ m. Although previous studies have primarily focused on using nano-scale exosomes as vaccines^{100,104}, our studies assess EVs of various discrete size ranges.

μ EBs were assessed for CD11c, a specific marker of bone marrow derived dendritic cells (BMDCs). Culture of cells in granulocyte-macrophage colony-stimulating factor (GM-CSF) led to relatively high levels of CD11c expression on μ EBs (**Figure 4.2b**). The percentage of CD11c positive μ EBs is in agreement with the percentage of CD11c positive cells from which they were derived. This is an indication that μ EBs maintain surface protein expression of their parent cells. Although other studies have shown that extracellular vesicles express proteins found on their parent cells¹⁰⁵, to our knowledge this is the first study to show that blebs produced *via* sulphydryl-blocking maintain a surface protein expressed on their parent cells. mEBs were further assessed to determine whether they would mimic the characteristics expression of proteins associated with maturation of their parent BMDCs.

4.3.2 Control of maturation properties of μ EBs

While immature BMDCs (imBMDCs) are known to induce tolerance, mature BMDCs (mBMDCs) prime immune cells,¹⁰² and studies by Segura et. al have shown EVs derived from mBMDCs to be more effective at stimulating T cells.¹⁰⁶ With the aim of producing μ EBs from a range of immature to mature BMDCs, the cells were assessed for CD40, a costimulatory molecule that is upregulated in mBMDCs, after incubation with 20 ng/mL lipopolysaccharide (LPS). BMDCs reached maximal maturation after approximately 12

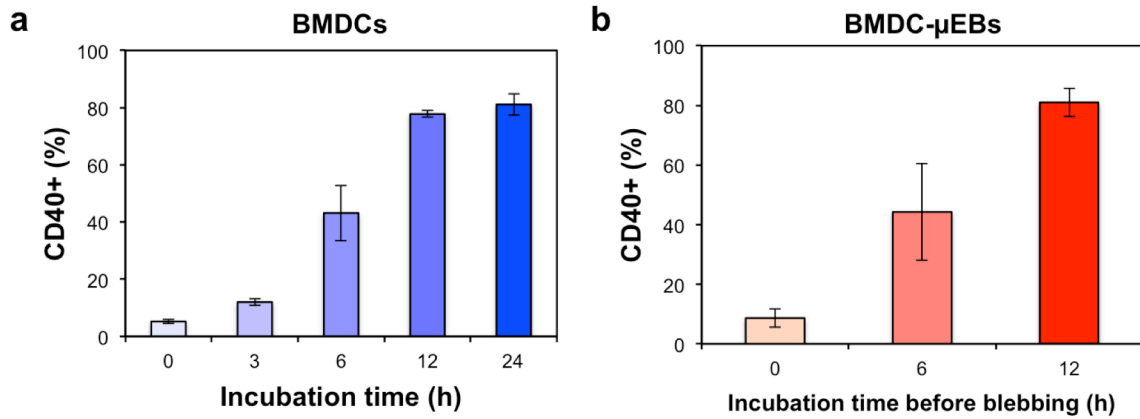


Figure 4.3. CD40 expression in BMDCs and μ EBs post incubation with lipopolysaccharide. (a) imBMDCs were incubated with LPS, and CD40 expression was assessed over time by antibody labeling and flow cytometry. (b) imBMDCs were incubated with LPS and treated with sulfhydryl-blocking reagent at the time point shown. μ EBs were collected and assessed by CD40 expression by antibody labeling and flow cytometry.

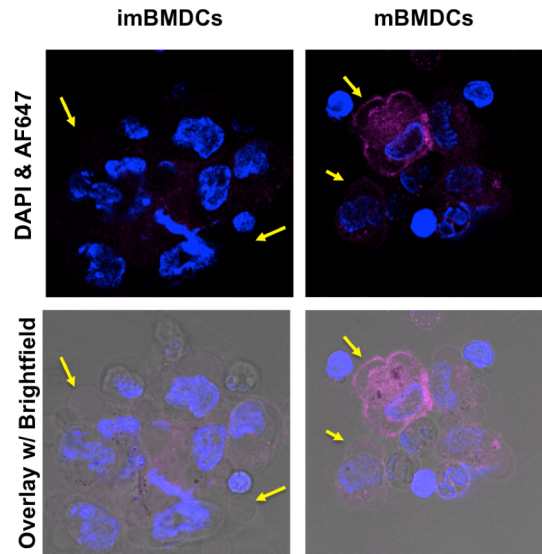


Figure 4.4. Visualization of CD40 presentation by μ EBs. Blebbing imBMDCs and mBMDCs were labeled with Alexa Fluor 647 anti-CD40 (pink) and imaged by confocal microscopy. Yellow arrows point out several blebs.

hours (**Figure 4.3a**). In further studies, it was shown that μ EBs derived from BMDCs after

set lengths of exposure to LPS maintained similar expression of CD40 to their parent cells (**Figure 4.3b**). These results were confirmed by confocal microscopy (**Figure 4.4**). Since it has been shown that mBMDCs are more favorable for applications in immunotherapy¹⁰⁶, this result is of particular interest, demonstrating that sulfhydryl-blocking offers a means

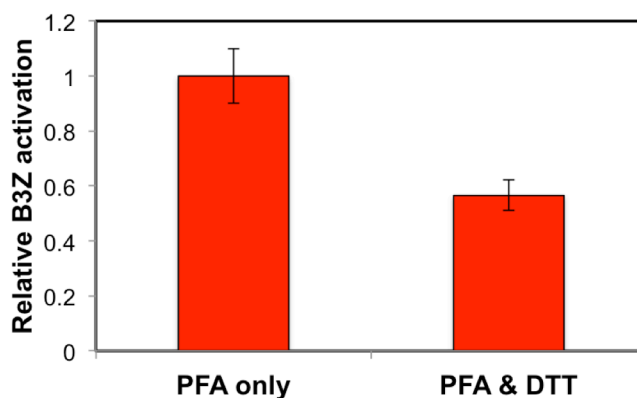


Figure 4.5. Antigen presentation reduction in the presence of DTT. Equivalent T cell hybridoma activation by μ EBs produced in 25 mM paraformaldehyde or 25 mM paraformaldehyde with 2 mM dithiothreitol was determined by β -Galactosidase Assay (CPRG) assay.

to achieve “mature” μ EBs. Additionally, to our knowledge, this is the first report demonstrating precise control over extracellular vesicle properties based on cell maturation. Further studies investigated whether μ EBs derived from mBMDCs present antigen.

4.3.3 Antigen presentation by μ EBs produced by sulfhydryl-blocking BMDCs

A concern with using chemical reagents to induce vesiculation of antigen-presenting cells is that antigens and other proteins expressed on the surface of the blebs may be

impaired. For initial studies with antigen-presentation, mBMDC-derived μ EBs were produced using PFA along with a disulfide-reducing agent, dithiothreitol (DTT), as is used in the production of giant plasma membrane vesicles.⁵⁵ Due to its ability to reduce disulfide bonds, it was found that DTT reduced antigen presentation (**Figure 4.5**). Additionally, PFA alone was capable of inducing high levels of vesiculation and was therefore used without DTT. Further studies showed that antigen presentation was found to be most effective in

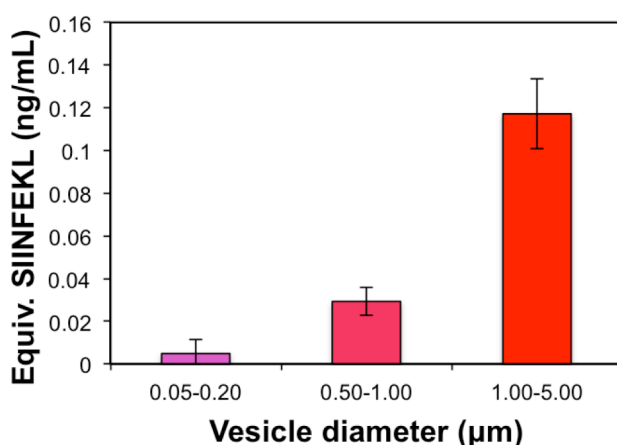


Figure 4.6. Size dependent antigen presentation by μ EBs. Equivalent T cell hybridoma activation by μ EBs isolated to fall in various size ranges was determined by β -Galactosidase Assay (CPRG) assay.

μ EBs isolated to be in the size range of ~ 1 -5 μ m in diameter (**Figure 4.6**). The greater efficacy of larger sized EBs was expected, as numerous other studies have found a correlation between increasing particle size and antigen delivery.¹⁰⁷ Further studies to assess the cause of the correlation between EB size and T cell hybridoma activation were not completed, but it is hypothesized that the greater surface area associated with larger particles, in addition to the curvature particles, plays a role in their efficacy. For future studies, the μ EBs refer to those isolated to fall within the size range: 1-5 μ m. In this study, a class I (Kb)-restricted peptide epitope of ovalbumin (OVA), SIINFEKL, was used; class I

major histocompatibility complex (MHC) molecule, H-2Kb, has been shown to present SIINFEKL.¹⁰⁸ BMDCs were incubated with the peptide SIINFEKL prior to maturation, and MHC Class I presentation of the peptide was assessed for both imBMDCs and mBMDCs. In

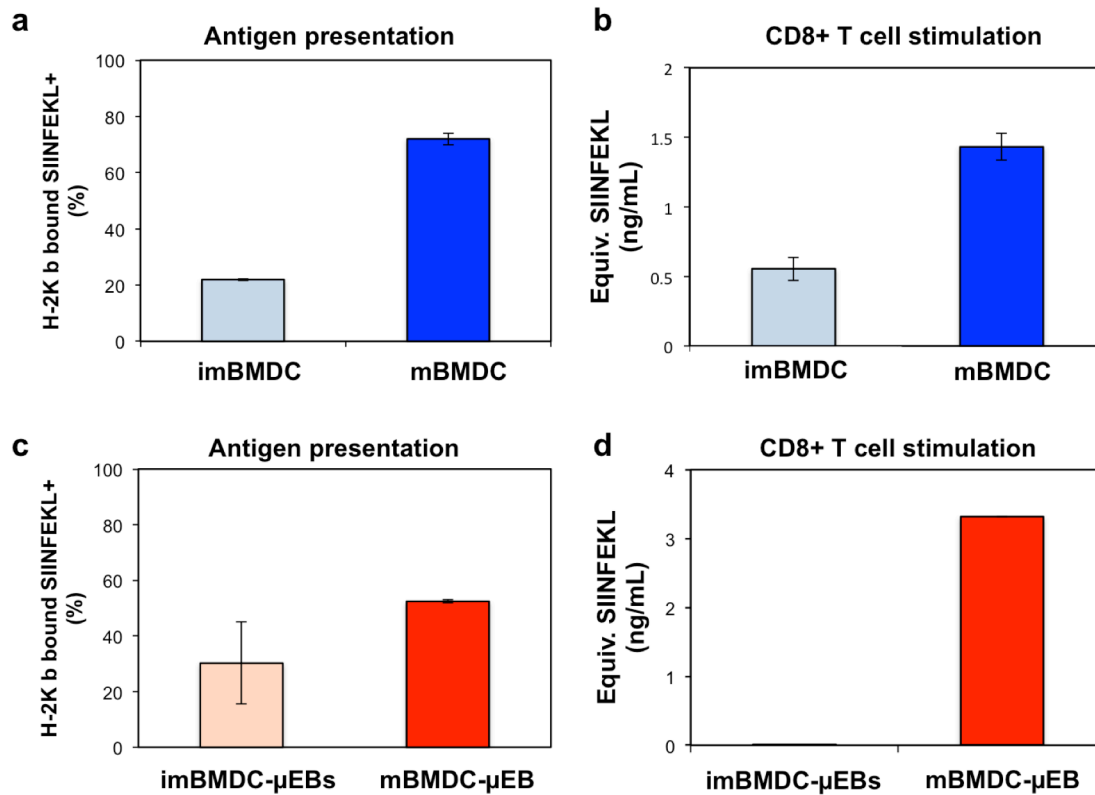


Figure 4.7. Antigen expression and *in vitro* presentation by BMDCs and μ EBs. (a and c) Assessment of H-2K b bound SIINFEKL on the surface of BMDCs and μ EBs derived from imBMDC or mBMDCs was determined by antibody labeling and flow cytometry. (b and d) Equivalent T cell hybridoma activation by BMDCs and μ EBs derived from imBMDCs or mBMDCs was determined by β -Galactosidase Assay (CPRG) assay.

agreement with a study by Kukutsch et al.¹⁰⁹, we found greater antigen presentation by μ EBs derived from mBMDCs than from imBMDCs (**Figure 4.7a,c**). Since BMDC maturation stage is critical to prime immune cells rather than induce tolerance¹⁰², it is essential to rapidly produce blebs from cells while they are in a fixed maturation stage. The benefits of sulfhydryl-blocking for bleb production are therefore twofold: faster production, and

fixation of parent cells. With this promising result, the next studies explored efficacy of antigen presentation *in vitro*.

SIINFEKL presentation was similarly higher on μ EBs derived from mBMDCs. To assess whether μ EBs derived from SIINFEKL-presenting BMDCs were capable activating B3Z T cell hybridomas, a β -Galactosidase Assay (CPRG) assay was used to measure B3Z cell activation, which was then correlated to an equivalent amount of activation induced by BMDCs cultured in a given SIINFEKL peptide concentration. The successful activation of B3Z by μ EBs indicates that μ EBs produced from sulphydryl-blocking by PFA maintain functionality. μ EBs derived from mBMDCs activated B3Z *in vitro* to a greater extent than those from imBMDCs (**Figure 4.7b,d**). Previous studies have shown that cross presentation of antigen is enhanced when LPS exposure occurs shortly after antigen capture¹¹⁰, aligning with the result that mBMDC derived μ EBs show increased antigen presentation ability.

4.3.4 *In vivo* activation of cytotoxic T lymphocytes by μ EBs

Cytotoxic T lymphocytes (CTLs) are activated when they interact with MHC I molecules with antigenic peptide. To measure whether protective immunity could be achieved by vaccination with μ EBs, splenocytes were isolated from mice vaccinated with OVA protein, SIINFEKL peptide, SIINFEKL-presenting mBDMCs, or SIINFEKL-presenting mBMDC-derived μ EBs. Whether the isolated splenocytes were cytotoxic to OVA-presenting EL4 cells (E.G7-OVA) *ex vivo* was assessed at effector to target (E:T) ratio 50:1 (**Figure 4.8**). Percent viability of E.G7-OVA cells decreased as E:T ratio increased for those groups treated with OVA protein, BMDCs, and μ EBs, indicating that protective immunity was achieved. At the relatively low E:T ratios assessed in this study, it is consistent with the

literature that no statistical significance would be expected between protein, cell, and μ EBs-treated groups.¹¹¹ In order to assess any benefits of μ EBs *in vivo*, a tumor challenge study was completed.

4.3.5 Protective immunity in tumor challenge study by μ EBs vaccination

While μ EBs have a variety of benefits including ease of production, size and lack of whole-cell components, it was important to investigate their efficacy in generating protective immunity in a tumor challenge scenario. While mature BMDCs are known to induce protective immunity¹⁰², EVs produced by sulfhydryl blocking have not previously been assessed for this potential. Seven days after completion of a vaccination schedule (a single vaccination followed by a booster vaccination two weeks later), mice were

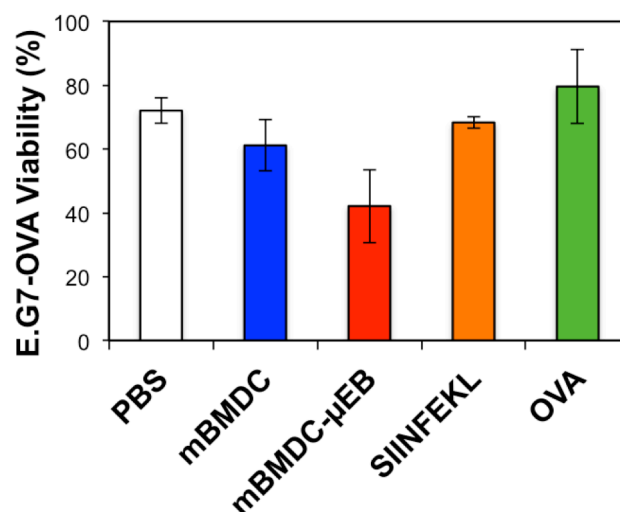


Figure 4.8. *In vivo* CTL activation with μ EBs. C57BL/6 mice (n=3) were vaccinated twice over two weeks with OVA protein, SIINFEKL peptide, SIINFEKL-presenting mBDMCs, or SIINFEKL-presenting mBMDC-derived μ EBs. Each group received vaccinations that were equivalent to a dosage of 100 μ L of 50 μ M SIINFEKL as quantified by CPRG assay. Seven days after the second injection, mice were sacrificed, spleen cells were isolated, and a CTL activation assay was completed at E:T ratio 50:1.

challenged with E.G7-OVA cells and tumor growth and weight was assessed over four weeks and survival was documented (**Figure 4.9a**). μ EBs were found to be as effective as BMDCs in generating protective immunity in a tumor challenge scenario; in combination with μ EBs' scalability, size, and cell-free aspect, this result indicates promise for μ EBs as effective cancer vaccines.

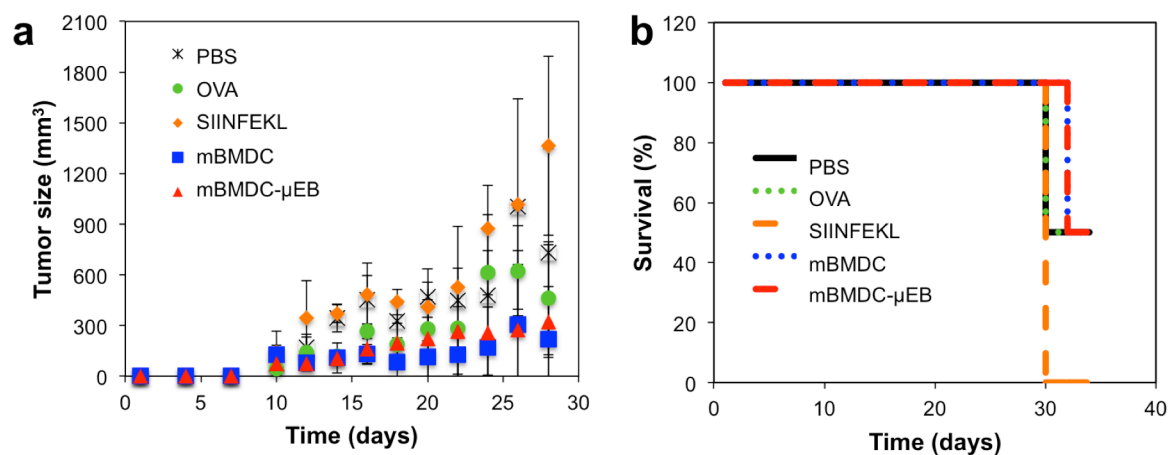


Figure 4.9. Protective immunity in tumor challenge post μ EBs vaccination. C57BL/6 mice (n=2) were vaccinated with DPBS, OVA protein, SIINFEKL peptide, SIINFEKL-presenting BDMCs, or SIINFEKL-presenting BMDC μ EBs. Vaccinations were given twice 14 days apart. 7 days after the second injection, mice were injected s.c. with 300,000 E.G7-OVA cells in the right flank, and (a) tumor growth and (b) survival were monitored over time.

Beyond reducing tumor growth, μ EBs also performed as well as BMDCs in increasing survival rates (**Figure 4.9b**). BMDC or μ EB treatment led to complete remission in half of the animals treated. These promising *in vivo* results indicate that μ EBs should be assessed on a larger-scale to determine whether their effect is statistically significant. The next steps in this research will involve repeating the *in vivo* study with 5 animals per group and including SIINFEKL-presenting imBDMCs and SIINFEKL-presenting imBMDC μ EBs as

study groups. The next phases of this work will aim to elucidate whether maturation of μ EBs is indeed critical for achieving long-term cancer immunity.

Similarly to our study, pilot studies in the clinic have shown that immunotherapy using DCs loaded with peptide can lead to partial or complete tumor remission in various forms of cancer.¹¹²⁻¹¹⁵ However, studies with naturally-produced EVs have not shown as promising results^{106,116}, which may be due to lack of control over size and maturation properties of the vesicles. The method described for producing μ EBs will enable control over size and maturation and has the potential to lead to improved therapeutic outcomes.

4.4 Summary

Antigen-presenting μ EBs were produced from cells at a desired maturation state by sulfhydryl-blocking and performed as well as whole cell therapy in terms of slowing tumor growth and improving survival outcomes. Immunotherapy with μ EBs is safer and a promising alternative to immunotherapy with whole cells. This pilot study will be continued in the Kwon laboratory to include larger-scale assessment of μ EBs' efficacy *in vivo*.

CHAPTER 5: Conclusions and future directions

Parts of this chapter have been adapted from:

Ingato, D., Lee, J.U., Sim, S.J., Kwon, Y.J. (2016), Good things come in small packages:

Overcoming challenges to harness extracellular vesicles for therapeutic delivery. J Control

Release, 241: 174-185.

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5.1 Conclusions

In this dissertation, a method for controlling EB production by sulfhydryl blocking reagents is explored. This method led to an order of magnitude increase in EB production as characterized by protein quantification assay, and production was achieved in a fraction of the time required for natural production of EBs. Furthermore, EBs produced by sulfhydryl blocking were assessed for applications in chemotherapeutic delivery and immunotherapy. Tumor growth was slowed in tumor-challenged mice treated with chemotherapeutic-loaded, nano-sized EBs. Antigen-presenting micro-sized EBs activated T cells *in vitro* and cytotoxic T lymphocytes *in vivo* leading to improved survival outcomes in tumor-challenged mice. This technology has the potential to enable more rapid progression of EB-based therapeutics to clinical trials.

5.2 Future directions

5.2.1 Applications in therapeutic delivery

EVs also hold promise as delivery carriers for gene therapy. In 2007, Valadi et al.²³ established that exosomes were involved in the exchange of mRNAs and miRNAs between cells. Additionally, the mRNA and miRNA was functional in its new location even if the target cell was of another species. From this starting point, various miRNAs and other forms of RNA including siRNA were shown to be transported by EVs.¹¹⁷⁻¹²⁰ The first demonstration that exosomes could be used as carriers to deliver exogenous siRNA came in 2011. Alvarez-Erviti et al.¹⁴ loaded dendritic cell exosomes with siRNA *via* electroporation and demonstrated knockdown of BACE1, an Alzheimer's target, in the brains of mice. To improve RNA loading into EVs, Hung and Leonard¹²¹ developed an EV loading protein fused with RNA.

Protein delivery is another application of EV technology. Shimoda et al.¹²² demonstrated that exosomes derived from CagA-expressing cells contain virulence factor CagA. Furthermore, the exosomes were capable of delivering the CagA to gastric epithelial cells. This study was among the first promising indications that EVs can be used to deliver proteins to target cells. Aspe et al.¹²³ also demonstrated protein delivery *via* exosomes; melanoma-derived exosomes were able to deliver Survivin to pancreatic carcinoma cells.

5.2.2 Parent cell selection

Interest in cancer associated EVs has led to a surge of research in the field, some of which has indicated that EVs from malignant cells contribute to metastasis. Skog et al.⁸⁵ showed that glioblastoma microvesicles carrying RNA and angiogenic proteins such as

angiogenin, VEGF and TIMP-2 aid in metastasis and angiogenesis. Contrastingly, Baj-Kryzworzeka et al.³⁸ determined that tumor cell-derived microvesicles transporting vascular endothelial growth factor, hepatocyte growth factor and interleukin-8 had antiapoptotic effects on monocytes; this indicates that cancer-derived vesicles actually aid in macrophage survival. Further research is expected to shed new light on the role of EVs in cancer progression. However, these studies indicate that the intrinsic biological functions of EVs must be accounted for when designing therapeutic carriers and that careful parent cell selection is necessary to avoid producing EVs that contribute to metastasis.

5.2.3 Isolation and purification

Conventional strategies for EV isolation are mostly based on size and buoyant density. The most common strategy used for isolating EVs is ultracentrifugation, a method that includes performing a sequence of centrifugation steps reaching speeds often greater than 100,000g. In the first step, centrifugation at 1000g removes cells including dead cells and relatively large cell debris. Next, ultracentrifugation steps reaching speeds near 100,000g concentrate the EVs.^{124,125} Although widely used for EV isolation, centrifugation is time consuming (>4 hours for standard protocols), yields poor EV recovery (5~25%)^{126,127}, has low specificity (co-purification with non EV debris), and requires skilled technicians and expensive equipment.¹²⁸ Density-gradient separation techniques, such as adding a sucrose gradient centrifugation step, improve the purity and the recovery rate of extracted EVs compared to differential centrifugation.¹²⁴ While density-gradient separation strategies can lead to higher purity and improved recovery rate,^{124,129} they are less time

efficient due to complicated sample processing. Also, these techniques require the same ultracentrifugation equipment and may be impractical for many clinical applications.^{124,130}

Unlike conventional strategies that isolate and purify EVs based on their size and buoyant density, immunoaffinity-based capture strategies are based on the highly specific interaction of a target with its antibody associated ligand. The highly selective binding of antibodies to specific targets offers advantages compared to conventional separation techniques including reduced isolation times, preservation of protein activity by gentle elution conditions and increased purification efficiency.^{131,132} By performing immunoaffinity based isolation using a microfluidic device modified with biotinylated anti-CD63 (a common exosomal marker), Chen et al.¹³³ and Kanwar et al.¹³⁴ demonstrated fast (~1h) and specific isolation of EVs from cell culture media and serum samples. He et al.¹³⁵ reported a cascading microfluidic device for the purpose of combining specific immunoisolation with targeted protein analysis of circulating exosomes from patients' plasma. The on-chip isolation with antibody-labeled magnetic beads (immunomagnetic method) allows for capture and enrichment of EVs due to the large surface area of the beads. This isolation strategy takes less than 1.5 hours and requires plasma sample volumes as low as 30 μ L. Compared to other isolation methods that rely on physical properties, immunoaffinity-based capture strategies are able to attain higher purity of isolated EVs. However, they also result in a significantly reduced yield of vesicles and damage to captured vesicles. All in all, immunoaffinity-based isolation strategies coupled with a downstream analysis technique are a potent method of characterization that can be applied in clinical practice.

In summary, improving methods for isolation and purification of EVs is a significant challenge.^{136,137} In the last few years, new devices have emerged to scale microfluidics to the nanoscale for EV isolation. These devices, along with conventional methods, have great potential to improve EV isolation by increasing the purity of isolated EV samples and reducing isolation time and reagent volume. However, most strategies for isolating EVs using nanoscale devices have challenges such as off-chip steps for sample preparation including reagent mixing or plasma extraction^{131,134}, and future work will need to be done to enable cost- and time-efficient large-scale isolation and purification of EVs.

5.2.4 Improvement of therapeutic loading

Improving loading of therapeutics into EVs, leading to fewer EVs necessary for treatment, could circumvent production issues. EVs naturally carry RNAs such as miRNA throughout the bloodstream.¹³⁸ Therefore, RNA-containing EVs may be obtained without further loading steps. However, efficient loading of EVs with RNA of interest can be more effectively achieved by first loading the parent cell. In 2011, Akao et al.¹¹⁷ demonstrated that macrophages transfected with miR-143 secrete vesicles containing the miRNA at an entrapment efficiency of ~0.20-0.25%. Small molecules have also been loaded into EVs this way. Pascucci et al.⁶⁸ produced microvesicles containing Paclitaxel from mesenchymal stromal cells loaded with the drug.

Direct EV loading is much more common in the literature and involves loading EVs after isolation from the parent cells. In the following subsections, a broad range of passive and active loading strategies are described. Although these strategies require additional

purification processes in comparison to loading EVs *via* their parent cells, they often produce more efficient loading outcomes.

Passive loading is the simplest method of introducing a therapeutic of interest into EVs. The strategy involves incubating the isolated EVs with the therapeutic and then purifying the EVs post-loading. Sun et al.⁶⁶ demonstrated passive loading of exosomes incubated with curcumin. Incubation of exosomes with curcumin for 5 minutes produced a loading rate of 2.9 g curcumin per 1 g exosomes and allowed for delayed release *in vivo*. Recently, Saari et al.¹³⁹ described passive loading of prostate cancer cell-derived exosomes with Paclitaxel; 9.2% of the drug was loaded after a one-hour incubation at room temperature.

Active loading refers to strategies that enable more efficient penetration of therapeutic through the lipid bilayer than exclusive incubation. Electroporation, which involves increasing the permeability of the EV membrane by applying electric pulses, is perhaps the most common active loading strategy applied to EVs. Hood et al.¹⁴⁰ applied single-pulse electroporation to exosomes to load RNA and demonstrated a significant loading enhancement compared to passive loading. Wahlgren et al.³⁷ optimized electroporation for loading RNA into exosomes. They reported the optimized conditions as 150V/100 μ F.

Recently, Haney et al.¹⁴¹ compared several other methods of active loading: permeabilization with saponin, freeze-thaw cycles, sonication, and extrusion. Sonication of exosomes in the presence of the therapeutic catalase proved to be the best formulation with the highest loading efficiency of ~ 200 μ g catalase per mg exosomes and slowest

release. To preserve the exosome structure, sonication was completed at 20% power with brief pulses of 4 seconds each.

Future work should focus on the production of EVs, both NIBS and μ EBs, from cells that have been pre-loaded with the cargo of interest. Studies have shown that giant plasma membrane vesicles produced by sulfhydryl blocking contain cytoplasmic content from their parent cell.⁵⁵ To ensure effective loading of EVs, future studies should aim to load NIBS and μ EBs with therapeutics that are localized in the cytoplasm of the parent cell.

5.2.5 Quality control

Before EVs can be seriously considered for clinical trials, quality control parameters must be met. Since the field is in its infancy, these parameters are still being debated. However, they will most certainly include standards describing the contents and composition of therapeutic vesicles.

Studies have shown that both exosomes and microvesicles contain transcription factors and RNAs from their parent cells.^{35,36,142} However, their content is not always representative of that of the cells from which they were derived.²³ EVs serve as a means to transport intracellular content between cells, and may be enriched in certain proteins and RNAs by a means that is not fully understood.

Known to express certain surface markers, EVs also possess biological functions associated with their membrane composition. In general, exosomes have been characterized by enriched levels of transmembrane proteins, such as CD9 and CD63, as well as integral membrane proteins, such as Flotillin-1.^{142,143} Additionally, exosomes have surface molecules that are specific to the cell type and activation state from which they

were derived. For example, cancer exosomes express NKG2D ligand, which binds T and B cells in a form of immune evasion.⁴¹ Although the composition of exosomal surface markers is dependent on the parent cell, it is often quite different from the composition of the plasma membrane.¹⁸ Contrastingly, microvesicles maintain the surface composition of the plasma membrane because they are formed through budding of the plasma membrane.¹⁴⁴

The most intriguing aspects of using EVs for therapeutic delivery are the possibilities of innate targeting, immune activation and immune evasion. The literature has described exosomes that specifically target cell types depending on their source⁶⁶, but further work is necessary to explicate the mechanism by which these interactions occur. Obregon et al.⁷⁷ demonstrated EV immune activation in 2006. Lipopolysaccharide activated dendritic cell-derived microvesicles were able to prime T cells *via* transfer to resting dendritic cells.^{145,146} Shortly after, EVs derived from cells with MHC markers were shown to express MHC class I and class II proteins.¹⁴⁷ MHC molecules enable EVs to transport material in the extracellular space without stimulating an immune response.

If EVs are to make progress as therapeutic delivery agents, future work should provide insight into the intrinsic functions of EVs and how these functions are related to their composition. With sufficient understanding of their biological functions, EVs can be harnessed to target specific cell lines, activate the immune system to an antigen, and/or navigate the extracellular space without initiating immune response.

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