2016

The effect of macrophage phenotype and surface modification of liposomes on internalization

Lilusi Ma
Iowa State University

The effect of macrophage phenotype and surface modification of liposomes on internalization

by

Lilusi Ma

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER SCIENCE

Major: Materials Science and Engineering

Program of Study Committee:
Kaitlin Bratlie, Major Professor
Martin Thuo
Balaji Narasimhan

Iowa State University

Ames, Iowa

2016

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to thank my Major Professor, Dr. Bratlie, and my committee members, Dr. Thuo, and Dr. Narasimhan, for their guidance and support throughout the course of this research.

In addition, I would also like to thank my friends, and colleagues as well, Hannah Bygd and Lida Zhu for making my time at Iowa State University a wonderful experience. It is my honor to work together with these excellent PhD candidates.

This work was supported by the National Science Foundation under Grant No. CBET 1227867, the Roy J. Carver Charitable Trust Grant No. 13-4265, and the Mike and Denise Mack faculty fellowship to Dr.Bratlie.

ABSTRACT

The properties of nanoparticles play an important role in cellular uptake. In this study, we have investigated the effects of 14 arginine modifications on liposome characteristics such as size, zeta potential, the internalization of fluorescein with non-activated macrophages and changes in IC_{50} values on LPS-activated, IL-4 activated and non-activated macrophages. It is important to determine the influence of surface modifications of particle characteristics on internalization of different macrophages phenotypes. Here we demonstrated that modified surfaces impact particle internalization and this internalization is dependent on macrophage phenotype. Our data revealed that surface modifications alter the zeta potential and negligibly change particle size. Internalization is tunable through alterations in zeta potential and hydrophobicity. These findings demonstrated that targeted drug delivery to macrophages could be achieved by exploiting material parameter.

CHAPTER 1

INTRODUCTION

1.1 Targeted Drug Delivery

Drug delivery is a method or process of administering a pharmaceutical active compound to achieve a therapeutic effect in humans or animals[1]. In modern society, nanoparticle drug delivery systems play important roles in cancer treatment.[2] Cancer is already the second major cause of death in the US and the Agency for Healthcare research and Quality (AHRQ) estimates that the direct medical costs for cancer in the US in 2011 were $88.7 billion.[3]There are more than one billion case diagnosed annually and around 600,000 people will die from cancer this year, which is 1,600 deaths per day.[3] The goal of targeted drug delivery is to release the maximum amount of medication at the site of injury or disease and reduce the drug distribution in the whole body, thus reducing or eliminating off-target effects. To achieve this goal, the requirements for the drug delivery vehicle are: 1) a high drug loading capacity, 2) long circulation time, 3) appropriate release rates, and 4) non-specific interaction with the host immune system. [4]Nanoparticles coupled to ligands have the potential to achieve these requirements and be a "magic bullets"[5] to shoot the desired sites.

Passive delivery is commonly used in the clinic for targeting tumor sites. Due to the larger pore size of blood vessels supplying tumors, aggressive tumors inherently develop leaky vasculature. Increased retention resulting from poor lymphatic drainage in tumors of nanoparticles and permeation within the tumor region allow extravasation to enhance drug delivery effects at the tumor site.[6] This is called the enhanced permeability and retention (EPR) effect, which is the most common method of passive targeting. Tumor specificity is enhanced due to the differential accumulation of encapsulated nanoparticles, which is largely

influenced by particle size. Owing to the tight junctions of capillaries, passive targeting allows for increased drug concentration at the tumor site. [7]

There are various factors that influence passive targeting. Size, surface properties, molecule weight, and polymer or particle fabrication method all impact tumor uptake. Hydrophobic particles around 200 nm have shown higher plasma resident time, which enhances the EPR effect.[8] Many other factors have been reported from clinical studies such as surface charge, hydrophobicity, and immunogenicity [9] of the drug carriers; therefore, due to the complexity of the system, it is challenging to accurately predict material properties that will enhance tumor uptake.

To overcome the limitations of passive targeting, the idea of active targeting has been proposed, which involves the attachment of a ligand or molecule onto the surface of the drug delivery vehicle that can specifically bind to an overexpressed cell membrane receptor specific to the target cell.[2] Identification of specific tumor biomarkers is crucial for efficient active targeting with limited off target effects resulting from expression of receptors on healthy cells. It has been reported that active targeting improves the therapeutic index to tumors in mice.[10] The density of expressed targeted receptors on the cell surface is another determining factor for the success of active targeting. Increased specificity contributes to enhance antibody-mediated drug delivery, which is hindered by high cost and long production time.[11] Liposomal doxorubicin (Doxil®) coupled with a growth factor overexpressed by a breast tumor (ErbB2) demonstrated a faster and shaper regression in tumor volume.[12]Anticancer monoclonal antibody 2C5 (mAb 2C5) with nucleosome-restricted activity coupled with PEGylated Doxil remarkably improved accumulation in

Lewis lung carcinoma. Also, this targeted liposomal formulation enhances inhibition of tumor growth. [13]

Liposomes are promising vehicles and have various advantages as drug delivery systems. It is important to design efficient systems and improve therapeutic efficacy. Both active and passive targeting are crucial methods by which to optimize efficiency, as well as other characters such as biodistribution, toxicity, and circulations time.

1.2 Macrophages

Macrophages were the first described in 1884 and are a type of white blood cells, which differentiate from monotypes. [14] Macrophages are instrumental in both innate and adaptive immune responses .[15] Depending on the type of stimulus that actives macrophages, the cells can exist on a spectrum of phenotypes, the ends of which are the pro-inflammatory or classical activated M1 macrophages and anti-inflammatory or alternatively activated M2 macrophages. [16] M1 macrophages, also known as classically activated macrophages, are identified as CD64+CD80+ by flow cytometry.[17] Macrophages can be polarized towards an M1 phenotype through interferon-γ (IFN-γ), lipopolysaccharide (LPS), tumor necrosis factor α (TNF-α), or monocyte chemoattractant protein-1 (MCP-1).[18] Alternatively activated macrophages are CD11b+CD209+.[17] Interleukin-4 (IL-4), IL-10, IL13, glucocorticoids, or vitamin D3 contribute to M2 functional polarization.[19]

Macrophage internalization mechanisms are complex, and include macropinocytosis, endocytosis, and phagocytosis.[20] Macropinocytosis is the major uptake pathway for extracellular fluids, solutes, macro- or small particles.[21] Endocytosis is the general term to define the process for importing selected molecules, viruses, microorganisms, and nanometer sized particles. This process includes clathrin-dependent and independent receptor-mediated

endocytosis.[22] Receptor-mediated endocytosis is the most effective mechanism for nanoparticle delivery to cells. [23] Ligands decorating the surface of the nanoparticle could bind to cell receptors, resulting in membrane invagination.[79] Phagocytosis is a mechanism by which foreign particles such as dead cells, pathogens and digesting drug particles can enter cells. During the process of phagocytosis, the phagocyte-bound pathogen is surrounded by a phagocyte membrane and encapsulated in the phagosome, which is a membrane bound vesicle. [24] With the fusion of the phagosome and lysosome, the phagolysosomes digest internalized material.[25] However, the process of lysis of pathogenic microorganism leads to the generation of other toxic composition such as NO, hydrogen peroxide, and superoxide anion, which damage the healthy host cells. [26] The response of a phagocyte to a target can be multilayered, which is shown in Fig 1.1[25] The responses of effective clearance of pathogenic microorganisms are: 1) surface receptors detect the microorganism and engulf and kill them and 2) internalized targets are presented as antigens to T cells. [25]

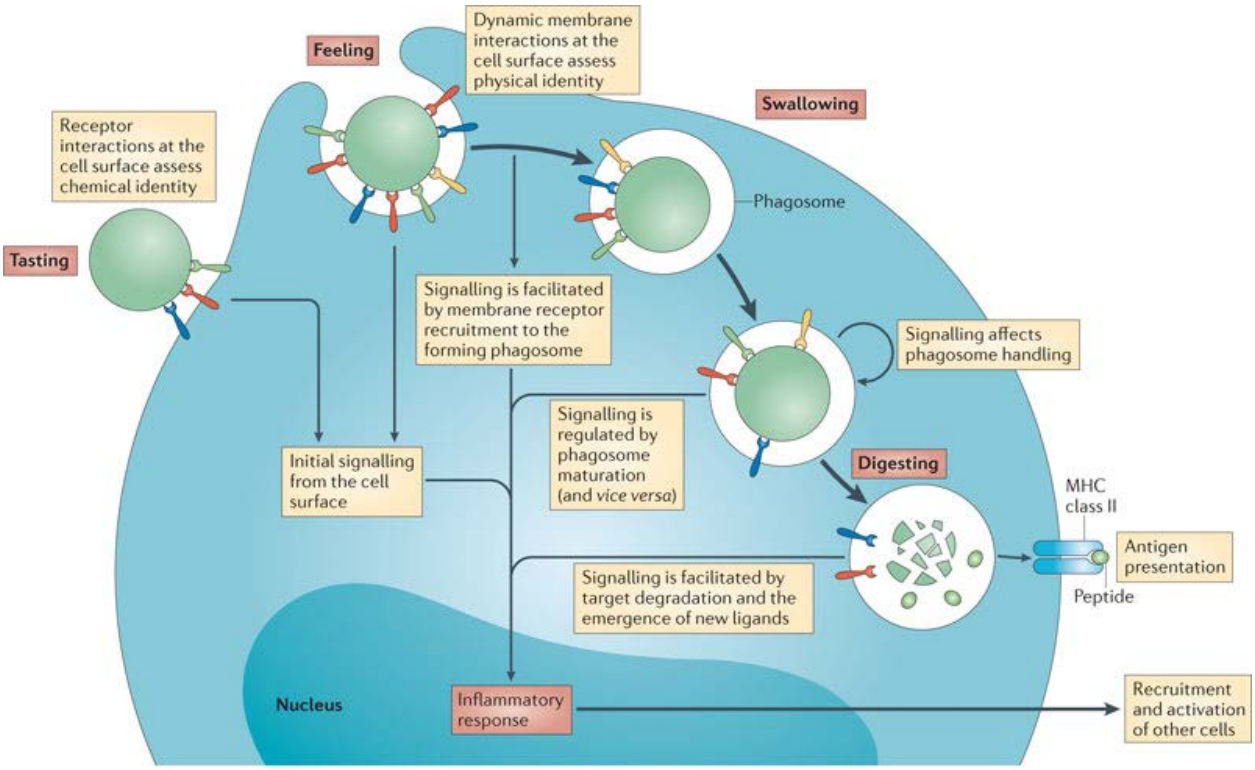


Figure1.1 Information processing at different stages of phagocytosis [25] As phagocytosis proceeds from the initial binding of a target to actin-dependent internalization and ultimately to degradation of the target in the phagolysosome, myeloid cells acquire information about the target through a variety of mechanisms. At the cell surface, receptors sample the chemical constituents of the particle and membrane dynamics facilitate an assessment of its physical properties. Additional information is gathered as the phagosome pinches off from the plasma membrane and as it matures through interactions with other intracellular compartments. Finally, the degradation of the target exposes ligands that were not previously accessible and releases ligands into the cytosol for detection by intracellular receptors. The information gathered by all of these processes is integrated to shape the ensuing immune response.

Although more receptors need to be identified, some receptors have been definitively shown to be mediate phagocytosis. Fc receptors could be used to recognize antibody-opsonized particles and initiate numerous signal pathways through two subtype receptors: activation receptors and inhibitory receptors.[27] However, most targets engage several receptors on the phagocytes' surface, making it hard to define the exact role of the individual receptors in internalization.

Macrophage reprograming plays an important role in cancer treatment and can offer potential antitumor activities. For health bodies, macrophages are a kind of protector, producing inflammatory mediators (cytokines and reactive oxygen species) and activating adaptive immune cells. [28] In the early stages of tumor growth, large quantities of inflammatory mediators are produced and a cancer-related inflammatory microenvironment is generated, [29] inducing tumor cell proliferation or genetic instabilities in the healthy cells. The early tumor development stage is characterized by an M1-like polarized inflammatory environment. [28] Production of IL-10, low levels of inflammatory cytokines, and reactive oxygen intermediates, along with poor antigen expressing abilities are characteristic of macrophages in mature tumors, which are considered to adopt a more M2-like phenotype. These cells are termed as tumor associated macrophages (TAMs). Thus, cancer could be considered a disease that is promoted by the permissive environment created by macrophages. Reprogramming the macrophages, from TAMs to M1-like phenotype, is a promising method to develop anti-tumor therapies.

1.3 Liposomes

Since the 1960s liposomes have been described and used as drug delivery vehicles for chemotherapeutics in cancer therapy. [2] Currently, liposomes are one of the most widely investigated drug delivery systems and are used in the clinic. [30,31] Seventeen liposomal drug formulations have been clinically approved and many more are in clinical trials. [32,33] Solutes carried by liposomes been extended to chelating agents [34], antibiotics [35], hormones[36], proteins [37], and anti-tumor drugs. [38]

Liposomes are artificially prepared spherical vesicles composed of a lamella phase lipid bilayer. The spherical structure allows liposomes to encapsulate a variety of types of

materials – both hydrophobic and hydrophilic drugs – within either the phospholipid bilayer or within the internal aqueous core, which makes them attractive drug delivery vehicles.

Size, surface polymeric coating, and surface charge are significant factors that influence the biological activates of liposomes. The size of liposome determines the biodistribution of the particles – in tumor vasculature or tumor interstitial space. For liposomes that passively target to the tumor site, liposomes are typically less than 200 nm in diameter. [8] Liposome can be coupled with active targeting ligands on their surfaces, releasing their payload near or within the tumor microenvironment. For larger particles, liposomes release the drugs within tumor capillaries. [39] Surface polymeric coatings enhance the stability of the particles and their resistance to clearance. [40] Polyetheylene glycol (PEG) is the most widely used polymer in liposomal drug delivery systems. PEG coated liposomal doxorubicin formulation has a half-life of 45 h [41] while non-PEG coated liposomes have a 2-3 h half-life. Surface charge is another factor that influences the clearance kinetics. Due to the effective process of ionic mediated interactions, cationic liposomes are widely used as DNA delivery. [42] Negatively and positively charged particles are taken up by cells to a larger extent than neutral particles.[3] Cationic liposomes are associated with efficient cellular delivery of drug cargoes and are routinely used in *in vitro* gene delivery applications. The positive charge particles have stronger electrostatic interactions with negatively charged cell membranes to enhance cell uptake.[43]

As a nanocarrier, surface modification involving various ligands impact the internalization of liposomes by cancer cells, Fig 1.2 shows the process of surface ligands target a cancer cell. The surface ligands promote the targeting efficiency and enhance the effects of cancer cell uptake drug. Liposomes coupled with triple-helical “peptide-

amphiphiles" (R1 (IV) 1263-1277 PA) exhibit increased stability compared to DPPC (dipalmitoylphosphatidylcholine) liposomal systems and have a higher loading capacity compare to unmodified liposomes. [12] Peptide HVGGSSV conjugated to liposomal doxorubicin has remarkable targeting ability and heightened cytotoxic effects. [44] Anti-HER2 PEGylated liposomes are preferentially internalized in $HER2^{+}$ breast cancer HER2 compared to non-targeted liposomal formulation. [45]

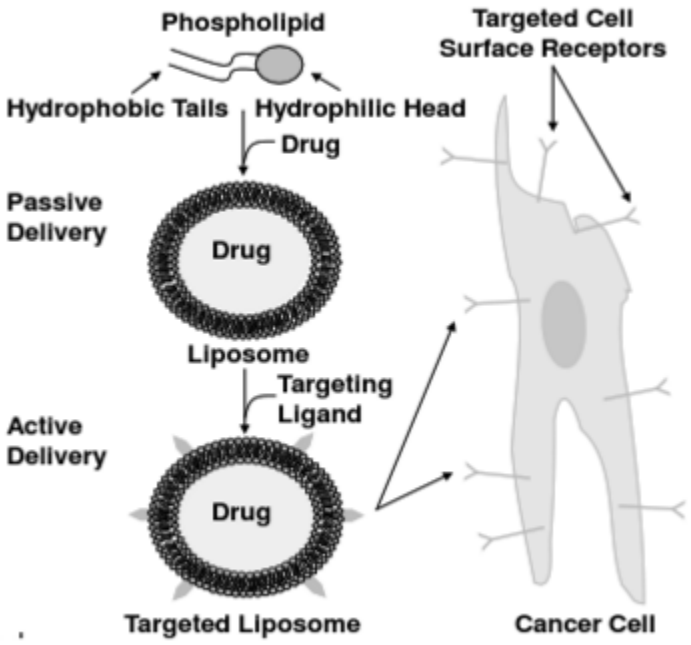


Figure1.2 Schematic depicting a liposome generated using phospholipids, followed by a liposome surface modified to contain a targeting ligand specific for upregulated cell surface receptors present on a cancer cell. [2]

Liposomes have been chosen as a therapeutic carrier owing to its advantages of: biocompatibility, preventing premature degradation of encapsulated cargo, entrapment of both hydrophilic and hydrophobic drugs, targeted delivery, site avoidance, and size and composition based tunable biodistribution.[46] Liposome structures are shown in Fig1.3. However, the major limitation of liposomes is the fast elimination from the blood steam and

recognition by the reticuloendothelial system.[47] Plasma proteins rapidly adsorb to liposomes and they can be eliminated through the mononuclear phagocytic system. Surface membranes coat with protein in a short time, which reduces the circulation time in blood. [48, 80]

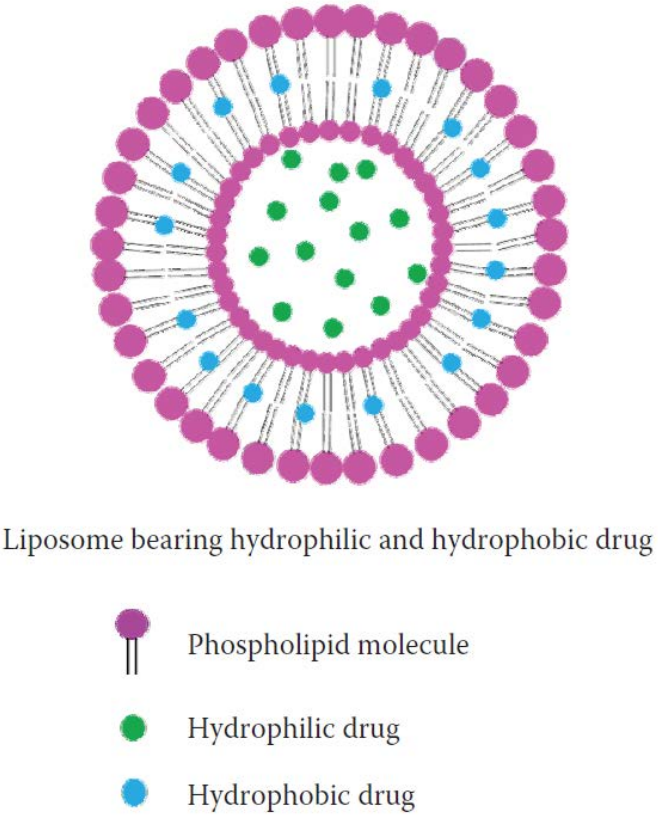


Figure1.3 The schematic representation of the structure of liposome [46]

Petros *et al*. [49] proposed that modifying the surface of liposomes with dysopsonins reduces recognition from complement components, such as the membrane attack complexes and inhibits phagocytosis from macrophages, neutrophils, and monocytes. Stealth liposomes have been coated with polymers to extend the circulation time such as PEG [41], poly(vinyl pyrrolidone) (PVP) [50], and amphiphilic polyacrylamide. [51]The blood circulation time has been increased and stability enhanced through these modifications. Combinatorial approaches have been utilized to extend circulation time of liposomes and improve targeted delivery of drug loaded liposomes through synergy. Xiong *et al.* [52] demonstrated that

doxorubicin encapsulated in RGD-modified PEGylated liposomes have higher splenic uptake and more effective inhibition in tumor growth than sterically stabilized liposomes encapsulating doxorubicin, while both formulations contribute to prolonged circulation time sand increased tumor accumulation. Cationic liposomes with PEG provide a greater colloidal stability of particles to enhance the circulation time and a lower zeta potential to prevent non-specific lung delivery. [42]

The US Food and Drug Administration (FDA) has approved diverse liposome formulations of the encapsulation of tumor therapeutic drugs. Liposomal formulations of doxorubicin have been developed for cancer treatment, which reduced cardiotoxic side effects and increased the therapeutic effects on the tumor. Doxil® was the first approved liposome formulation doxorubicin (Ben Venue Laboratories, Inc Bedford, OH) for chemotherapeutic treatment of refractory acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma. [53] Doxil® was the first generation of liposomal doxorubicin and reduces cardiotoxicity, neutropenia, vomiting, and alopecia. [53] Lipo-dox® (TTY Biopharm Company Ltd, Taipei Taiwan) is the second generation and prolongs the circulation half-time to 65 h. [53] The third and latest generation is ThermoDox® (Celsion Corporation,Lawrenceville, NJ), a thermosensitive liposomes, which releases entrapped drugs in the targeted area where the tissue temperature is evaluated. [54]

Liposomes are not only promising nanoparticle vehicles, but also have proven clinical success. Liposomes can be used to passively targeting cancer for treatment with chemotherapeutics. A growing number of studies are focusing on liposomes with surface modifications with targeting ligands and multiple functionalities, which could enhance the

internalization of chemotherapeutics by cancer cells, raise the rate of uptake of drugs, and control drug release.

CHAPTER 2

THE EFFECT OF MACROPHAGE PHENOTYPE AND SURFACE MODIFICATION OF LIPOSOMES ON INTERNALIZATION

The effects of surface modifications on liposomes using a library of arginine derivatives for improved drug delivery were examined. Encapsulating either fluorescein or doxorubicin, both unmodified liposome and modified liposomes were tested for their drug delivery properties and propensity for internalization with macrophages. The modified liposomes were characterized by dynamic light scattering (DLS) and zeta potential. The resulting liposomes were able to encapsulate doxorubicin with a loading efficiency greater than 90% and cumulative releases of less than 15% after 144 h. The internalization of these particles was examined by loading the liposomes with fluorescein or doxorubicin to test internalization through fluorescence level and half maximal inhibitory concentration (IC_{50}), respectively. Macrophages were activated with LPS or IL-4 to induce M1- or M2-like phenotypes. Naïve macrophages were also studied. Most modified liposomes enhanced the cytotoxicity of doxorubicin compared to unmodified liposomes. Macrophage phenotype was also observed to influence the cytotoxicity of the modified liposomes, with some modified liposomes enhancing the cytotoxicity in LPS stimulated macrophages and some enhancing IL-4 stimulated cells.

2.1 Introduction

Liposomes have been extensively used as biocompatible and biodegradable nanocarriers for a variety of applications extending from basic research to clinical uses. Currently, there are 17 different liposomal formulations that have been approved by US Food and Drug Administration (FDA) for clinical treatments and a large number of formulations are in clinical trials. [32] Doxil®, the first FDA approved liposomal formulation, was initially approved in 1995 for use in treatment of Kaposi's sarcoma. [55] DaunoXome®, a liposomal formulation daunorubicin, is in use as treatment of leukemia and blood tumors. Clinical reports have demonstrated that DaunoXome® results in less alopecia and neuropathy and tumor uptake is 10-fold higher compared to free drug. [53]. Compared to other nanoparticles, such as micelles, liposomes are unilamellar lipid bilayer nanoparticles and are larger than micelles, providing less drug leakage and enhanced encapsulation capability. [2] Liposomes are commonly delivered to tumor sites through passive targeting, exploiting the enhanced permeation and retention (EPR) effects of the tumor. [56] Liposomes also have the potential to actively target tumors through the tumor cell specific or maker targeting. PE38KDEL-loaded anti-HER2 PEGylated liposomes, possessing receptor-specific binding for HER2-overexpressing SK-BR3 cells, have shown more cytotoxic effects than non-targeted PEGylated liposomes. [45]. H2009.1 specific peptide conjugated to liposomal doxorubicin exploits multivalent peptides and liposomes to increase targeting to $\alpha_v\beta_{vi}$. [57]

Targeting drugs to macrophages have been reported through many methods, most of which are achieved through liposomes. [58,59] Macrophages change their phenotype due to the stimulus that activates macrophages. Pro-inflammatory or classical activated M1 macrophages [16] could be activated with interferon (IFN)-γ or lipopolysaccharide (LPS), while

anti-inflammatory or alternatively activated M2 macrophages could be activated by interleukin (IL)-4. [17] To more accurately describe the phenotype of the macrophages *in vitro*, the new nomenclature proposed by Murray *et al.* [60] will be used throughout the paper, which is based on the molecule used to activate the cells, for example M(LPS) and M(IL-4). Naïve cells will be denoted M (0). The plasticity of macrophages provides potential for macrophage reprogramming to synergistically act with chemotherapeutics. Tumor associated macrophages (TAMs) represent a subset of M2 macrophages, which can be reprogrammed cytotoxic M1 macrophages to prevent tumor growth. It has been reported that oligomannose-coated liposomes encapsulating 5-fluorourracil successfully controlled tumor growth via macrophages as cellular vehicles. [61]

Arginine plays an important role in immune responses, which is metabolized by two enzymes differently up-regulated in polarized macrophages: [18,62] nitric oxide synthase (NOS) and arginase. The former produces citrulline and reactive nitrogen intermediates in M1 macrophages, and the latter produces ornithine and urea in M2 macrophages. [18] Some arginine derivatives have been investigated and have shown therapeutic effects. Nitroarginine has neuroprotective effects in Alzheimer' disease, Parkinson's disease, and AIDS. [63] Acetylcarnitine, involved in membrane stabilization and repair, reduces protein oxidation and toxic fatty acid ethyl esters in different organs. [64]

In this study, we fabricated liposomes and modified their surfaces with 14 molecules chemically similar to arginine to investigate the effects of surface modifications on internalization by non-active macrophages. Also, we characterized the size and surface charge of both modified and unmodified liposomes. Finally, the liposomes were loaded with doxorubicin, an anti-cancer drug, and were examined for their loading efficiency and release

kinetics at pH 7.4. Changes in the IC_{50} of doxorubicin entrapped in the unmodified and modified liposomes was compared to that of free doxorubicin in M (LPS), M(IL-4), and M(0) macrophages.

2.2 Material And Methods

All materials were purchased through Sigma and were used as received, unless otherwise stated. Fresh deionized water (Milli-Q, Thermo Scientific Nanopure, Waltham, MA) was used throughout this study. Error bars indicated the standard deviation.

2.2.1 Liposome particle modification

In a 250 ml round-bottom flask, 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids, Inc., Alabaster, AL) (87.5mg) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, Inc.) (43.75mg) were dissolved in chloroform (15.75 ml) and rotary evaporated at 40°C for 5 mins. Then, the lipids were mixed in 15 ml phosphate buffered saline. (PBS, diluted from 10× solution to 0.1 M, pH 7.4, Fisher Scientific, Pittsburgh, PA) After dialyzing against Milli-Q water overnight, the liposome particles are freeze-dried by a lyophilizer (Labconco, 4.5L, Kansas City, MO). Fourteen different molecules (Figure2.1) were used to modify the liposomes. In a vial, 2 ml PBS, 10 mg/ml of lyophilized liposomes resuspended in PBS, 2 mg surface modifier, and 20 mg N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) were stirred overnight. This process repeated for all 14 surface modifiers. The particles were dialyzed overnight

against Milli-Q water and lyophilized.

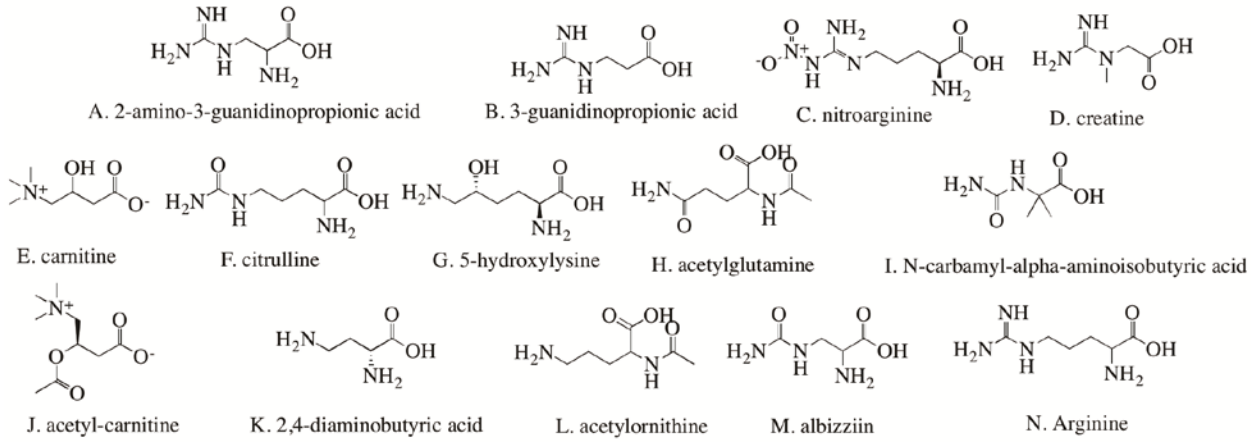


Figure2.1 Chemical structures of molecules used for the modification of liposomal particles. The arginine derivatives shown here are lettered for easier identification in experiments and discussion throughout the chapter**.**

2.2.2 Zeta potential and dynamic light scattering

Milli-Q water was adjusted to pH 7 with HCl or NaOH to ensure the ions in the water would not influence the results by interference with the liposomes. To 5 ml H_2O, 100 µl 1% w/v of particles were added and extruded through 100 nm polycarbonate membranes using an Avanti Mini-Extruder manual extruder (Avanti Polar Lipids, Inc.) 5 times for all 14 modified liposomes and unmodified liposomes. Zeta potential and particle size was measured with a Zetasizer Nano Z (Malvern).

2.2.3 Fluorescent particles and cellular uptake

To measure internalization of the liposomes by macrophages, 2mg of liposome particles were mixed with 1 ml FC (Fluorescein, 1 mg/mL in acetone). The liposomes were subsequently dried at 55 ℃ for 4 h and particles were resuspended in 2 ml PBS. The liposomal suspension was passed through a Sephadex G-50 column (Fisher Scientific) to remove the unencapsulated FC. RAW 264.7 cells (ATCC, Manassas, VA) were cultured at

37 ℃ with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM high glucose; Thermo Scientific) supplemented with 10% fetal bovine serum, 100 U/L penicillin, and 100 μg/L streptomycin to be referred to as complete media (CM). Cells (50,000 cells/well with 100 μL of media in each well, except negative control) were seeded in a black 96 well plate. After incubating for 24 h at 37°C, the media was carefully aspirated and then 200 μL liposomes resuspended in CM were added to the wells. These cells were either incubated at 37°C to measure internalization of the liposomes, or at 4°C for cold binding experiments.

To measure internalization of the particles, the cells were further incubated at 37°C for 4 h, the media aspirated, and 100 μL 0.25% trypan blue (Corning, Manassas,VA) was added to quench the extracellular fluorescence. The trypan blue was aspirated after 1 min and the fluorescence was measured at an excitation of 360 nm and an emission of 460 nm using a plate reader (BioTek Synergy HT Multidetection Microplate Reader, Winooski, VT).

Cold binding experiments were performed by incubating at 4°C for 4 h, aspirating the media, washing the plate with sterile PBS, and quenching with 100 μl 0.25% (w/v) trypan blue. The fluorescence was measured at 360/460 nm with the plate reader. Controls for both internalization and cold binding experiments consisted of the above experiments without cells and the above experiments without liposomes. Ten replicates were obtained for all modified liposomes.

2.2.4 Drug loading and release

Doxorubicin was used as a model drug for liposome loading and release. In 2 ml critic acid (150 nM, pH 4), 10 mg modified or unmodified liposome from section 2 was suspended and extruded 21 times using an Avanti Mini-Extruder manual extruder. The liposomes were neutralized to pH 7.4 with NaOH or HCl. Both doxorubicin solution (PBS,

10 mg/ml) and the extruded liposomes were heated to 65℃ for 10 minutes. Subsequently, 200 µl doxorubicin was added to the suspended liposomes and incubated at 65℃ for 45 minutes. The liposomes were centrifuged at 3000 rpm for 5 minutes and the supernatant was removed. To a 96 well plate, 50 µL of supernatant removed from the liposomes and 50 µL of PBS were added into each well. A standard curve was made through a serial dilution of 1mg/ml doxorubicin. The amount of unentrapped doxorubicin was analyzed by measuring the absorbance at 490 nm with a reference at 630 nm using a plate reader. Loading efficiency of the liposomes was calculated by

$$\text{Encapulation efficiency } (\%) = \frac{C_{total} - C_{sup}}{C_{total}} \times 100 \quad (1)$$

where C_{sup} is the concentration of doxorubicin in the supernatant and C_{total} is the concentration of doxorubicin added to the liposomes.

Liposomes loaded with doxorubicin were placed in a dialysis membrane, and placed in a beaker with 100 mL of PBS. The beaker was sealed and incubated at 37 ℃. Aliquots of 1 ml were collected periodically and assayed spectrophotometrically with a plate reader at an absorbance of 490 nm with a reference at 630 nm. After sample was removed, 1 ml PBS was added to maintain a constant volume.

2.2.5 IC_{50}

RAW 264.7 cells (50,000 cells/well in 100 µL CM in every well except the negative control) were seeded into a 96 well plate for 24 h. A serial dilution of liposomes loaded with doxorubicin was added to the plate. A positive control was conducted without particles. After incubating for 48 h at 37°C, the media was aspirated and 10 µL of 5 mg/mL methyl thiazol tetrazolium (MTT) and 100 µL CM were added to each well. The plate was incubated at

37°C for 2 h. A volume of 85 μL was aspirated from each well and 100 μL DMSO were added to dissolve the insoluble formazan crystals. The optical density at 540 nm and a reference of 690 nm were measured with a plate reader. Data was normalized to cells cultured without particles and 6 replicates were obtained for each experiment.

2.2.6 Statistics and data analysis

Statistical analysis was performed using XLSTAT statistical software. Statistical significance of the mean comparisons was determined by ANOVA. Differences were considered statistically significant for $p < 0.05$.

2.3 Results

2.3.1. Liposome characterization

Subtle differences between the different modifiers were reflected in zeta potential values derived from the electrophoretic mobility measurements under identical experimental conditions.[2] Zeta potential represents the relative surface charge of particles, which contributes to particle internalization. [1] Fig2.2 A shows the zeta potential of both modified and unmodified liposomes. Unmodified liposome have a zeta potential of -16.8 ± 0.8 mV，which is similar of other reports.[65,66] All modified liposomes were negatively charge over a wide range (from -8.9 to -33.9 mV), which may arise from the different functional groups of the modifiers.

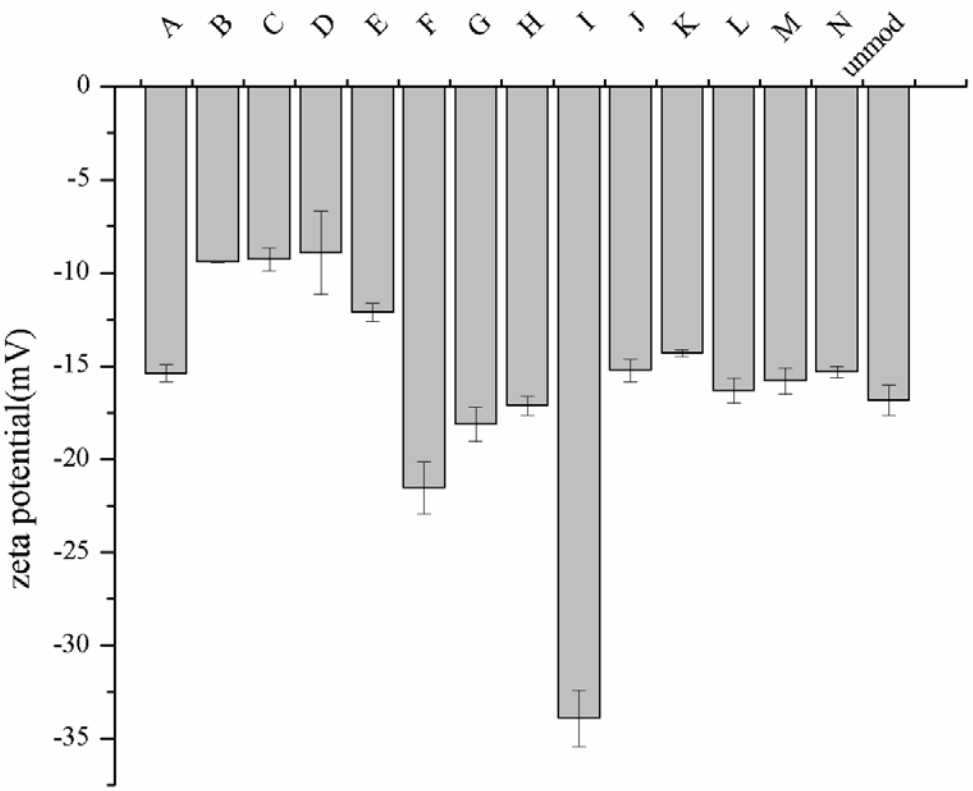
Nanoparticle size is a determining factor in drug delivery.[1] Through dynamic light scattering, the diameter of the nanoparticles was measured (Fig2.2 B). The unmodified liposome was found to be 96.3 ± 9.4 nm and the size of modified liposomes was measured to be ~100 nm, with the largest liposome being 108.8 ± 14.8 nm. The size of the unmodified

liposome matches the previous reports. [67,66] The polydispersity index (PDI) ranged from 0.096 to 0.167 for all of the particles.

2.3.2. Drug loading and release

A transmembrane pH gradient was employed as an active loading method to encapsulate doxorubicin in liposomes. [31] Through drug self-association and interaction with salts, doxorubicin precipitates

A



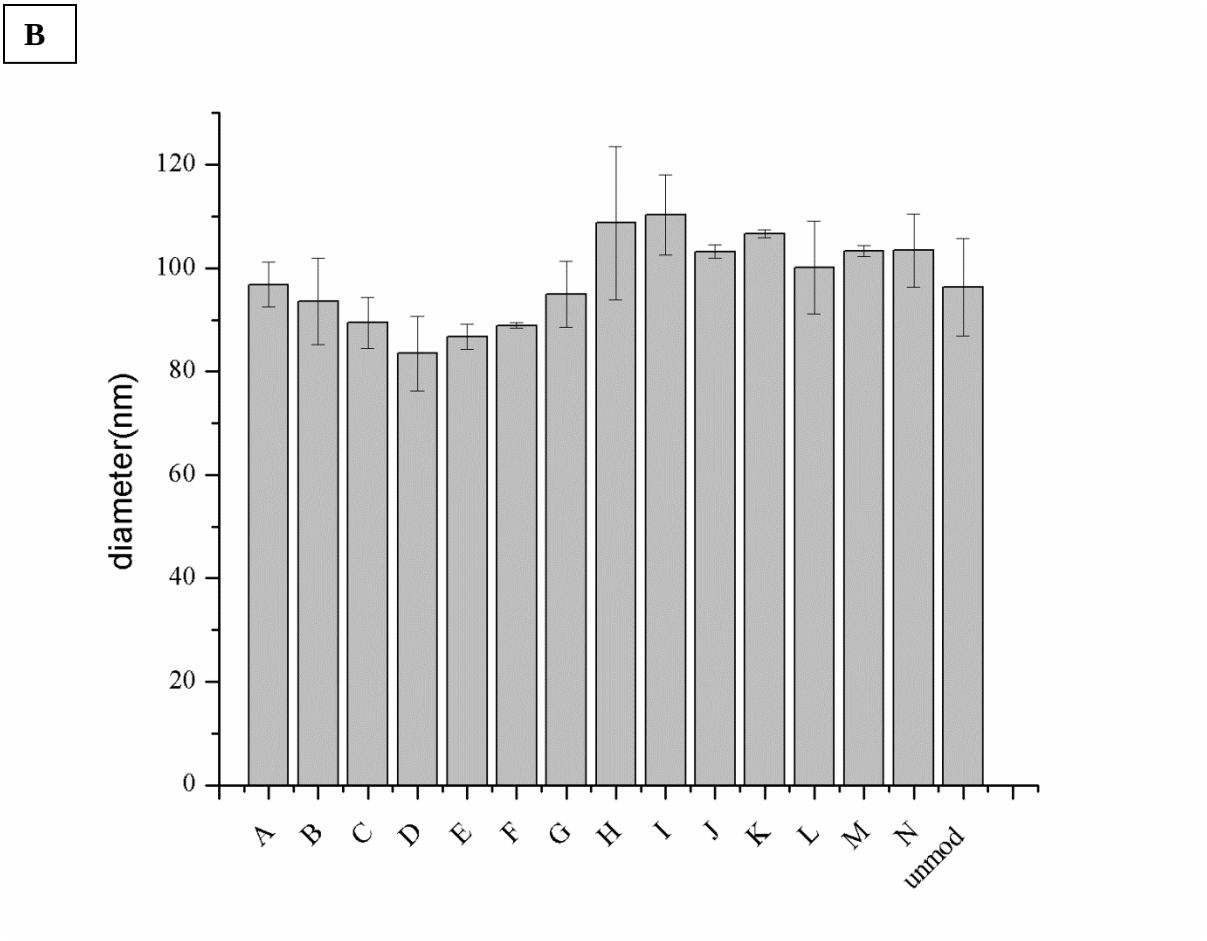


Figure2.2 Material characterization of modified liposome particles. (A) Zeta potential and (B) DLS measurements of the liposomes. Both measurements represent three replicates for each sample, all data are shown by mean value ± standard deviation.

in the aqueous core of liposome. [30] It is a direct method to encapsulate doxorubicin to achieve optimum efficiency and reduce costs. The loading efficiency (Fig2.3) shows that all liposomes have a loading efficiency of greater than 90%. Liposomes loaded with doxorubicin were incubated in PBS (pH 7.4) at 37 °C for 144 h to monitor drug release using the dialysis bag method. All of the liposomes released less than 15% of encapsulated doxorubicin after 144 h, as shown in Fig 2.4, which indicates sustained release capability. Modification D has a lower release percentage compared to the unmodified liposome ($p < 0.05$). According to the

statistical analysis, there are no differences among the modified and unmodified liposomes ($p > 0.05$), indicating a uniform encapsulation capability.

2.3.3. Particle internalization

The unmodified and modified liposomes were loaded with FC and were incubated with RAW 264.7 macrophages to measure particle internalization. A constant particle concentration (0.1mg/ml) was used throughout the study. After incubation with the cells, the fluorescence of FC was quenched with trypan blue. Fig 2.5 shows the fluorescence level of particle internalized with the macrophages. At 4 °C, all pathways for cell internalization are blocked. [68] The lack of fluorescence indicates that there is very little particle internalization or adsorption on the cell surface at 4 °C. At 37 °C there is a substantial increase in the fluorescence level, which demonstrates that the liposomes are internalized by the macrophages. The highest fluorescence value is for modification N, which is around 4 fold greater than the lowest modification, M. Particles A, C, D, G, H, K, and M are statistically similar to unmodified liposomes ($p > 0.05$) with all other modifications more favorably internalized compared to unmodified liposomes ($p < 0.05$).

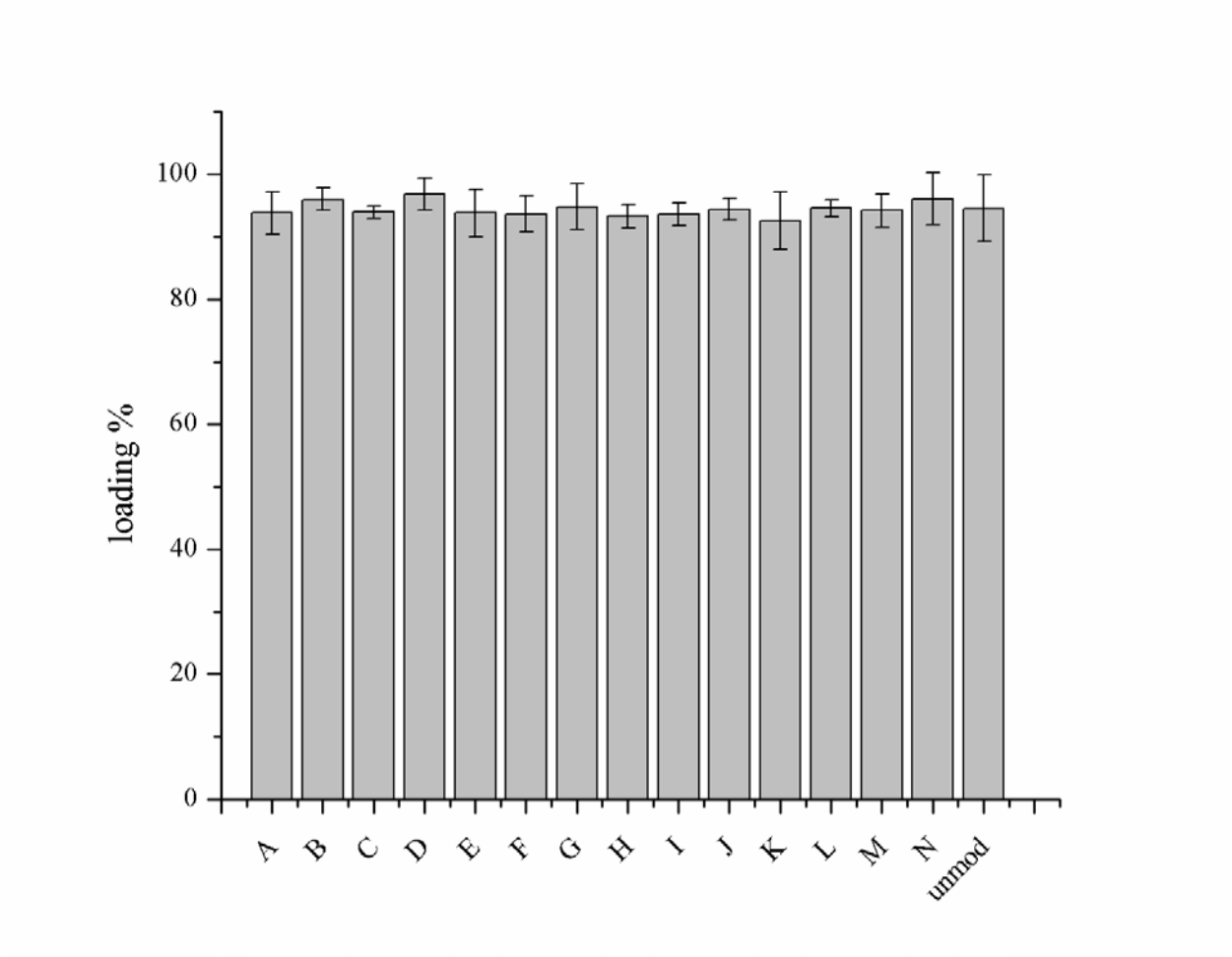


Figure2.3 Doxorubicin loading efficiency for unmodified and modified liposomes. Measurements represent three replicates for each sample; all data are shown by mean value ± standard deviation.

2.3.4. IC_{50}

IC_{50} is the half of maximum of inhibitor concentration, which is a measurement of the cytotoxic effects of liposomal delivery of doxorubicin. Macrophages stimulated with LPS or IL-4, along with naïve macrophages were incubated with the doxorubicin loaded liposomes for 48 h and their responses was calculated as a percentage of the cells not treated with the liposomes. [14] Sigmoidal dose-response curves were used to calculate IC_{50} for each macrophage condition and liposome modification, shown in equation 1:

$$y = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} \quad (1)$$

Where A_1 is the upper limit of the dose curve, A_2 is the lower limit, x_0 is the IC_{50}, and p is the steepness of the curve. M (LPS), M (IL-4), and M (0) macrophages were tested (Fig2.6).

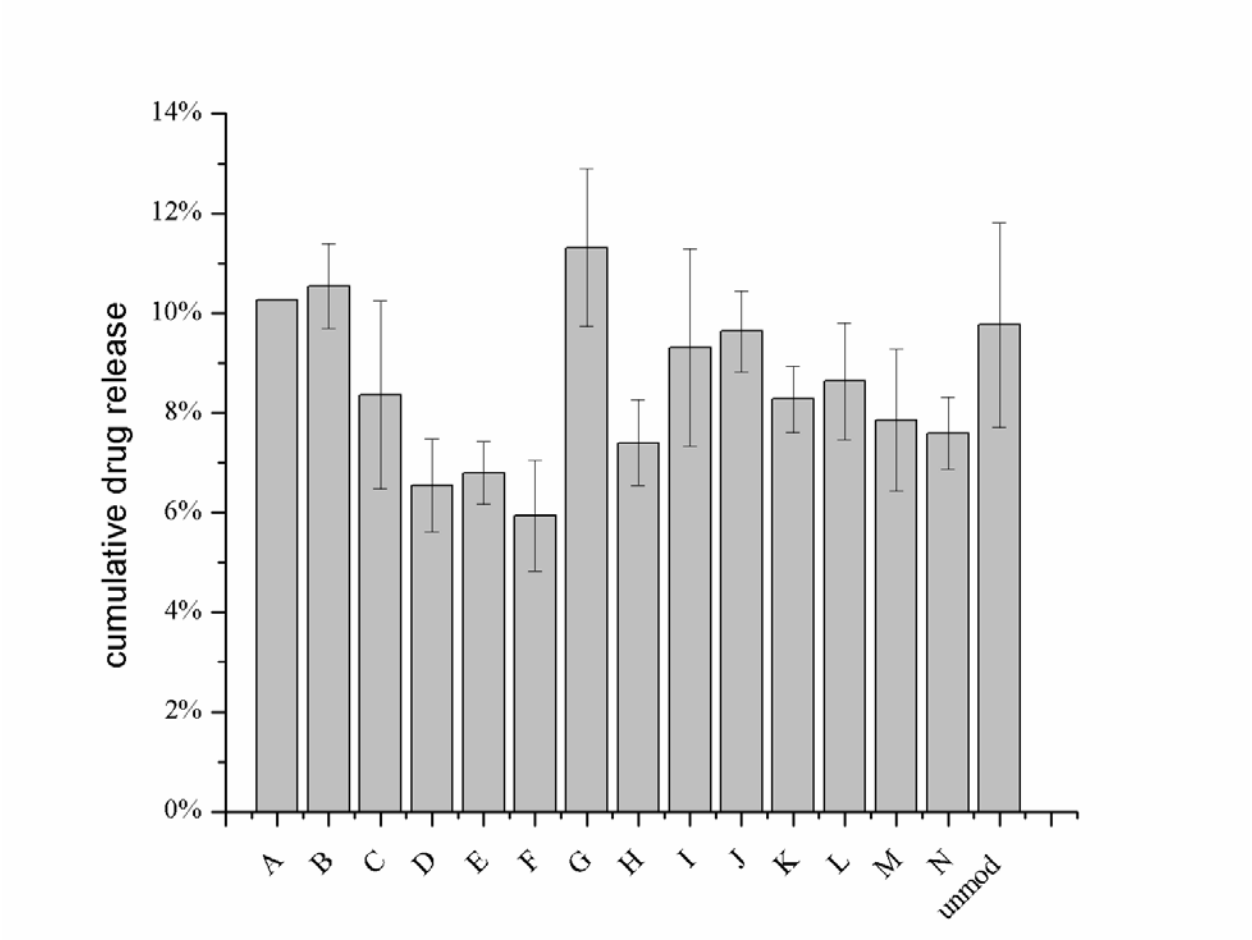


Figure2.4 Cumulative doxorubicin release from modified and unmodified liposomes at 144 h incubation in PBS.

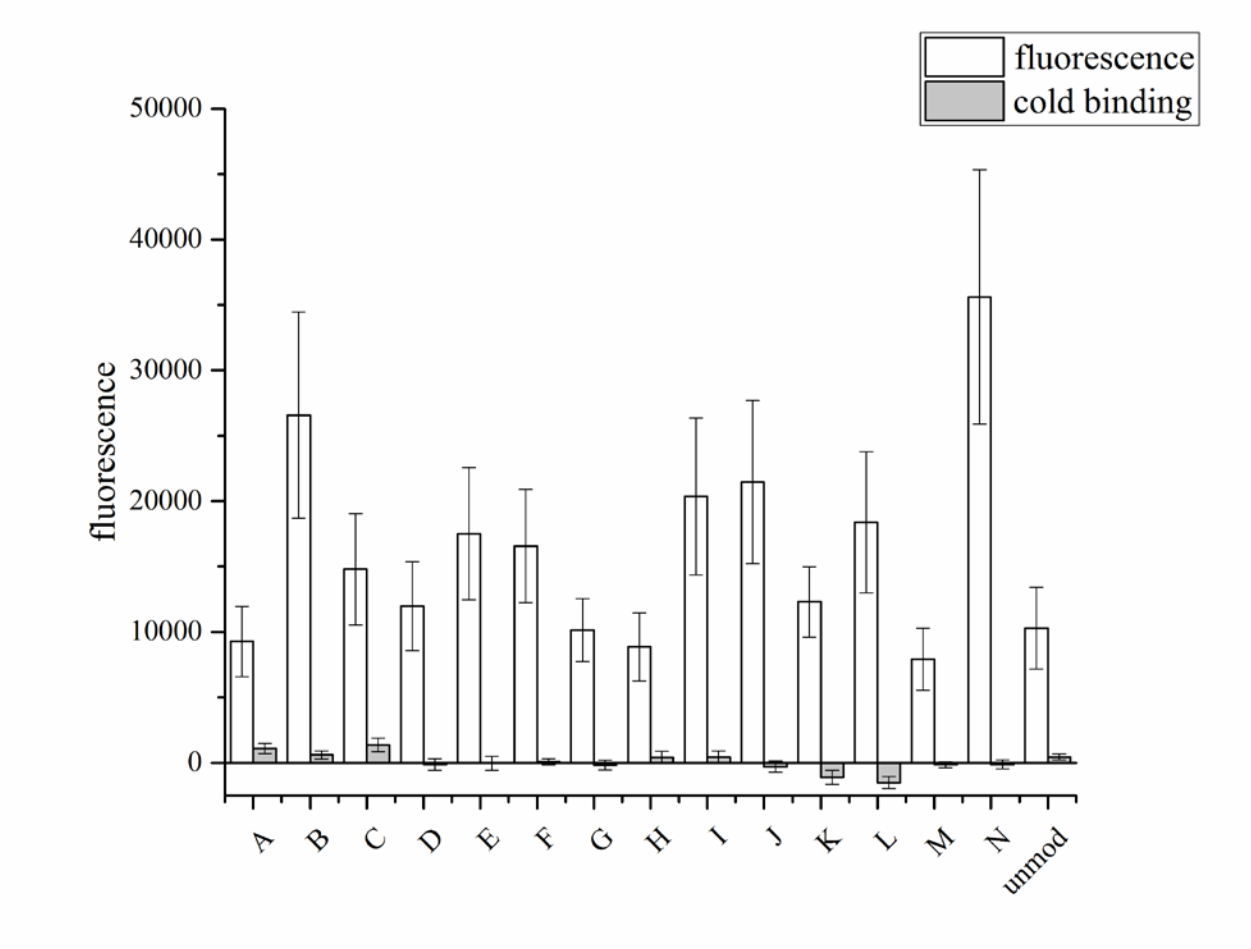
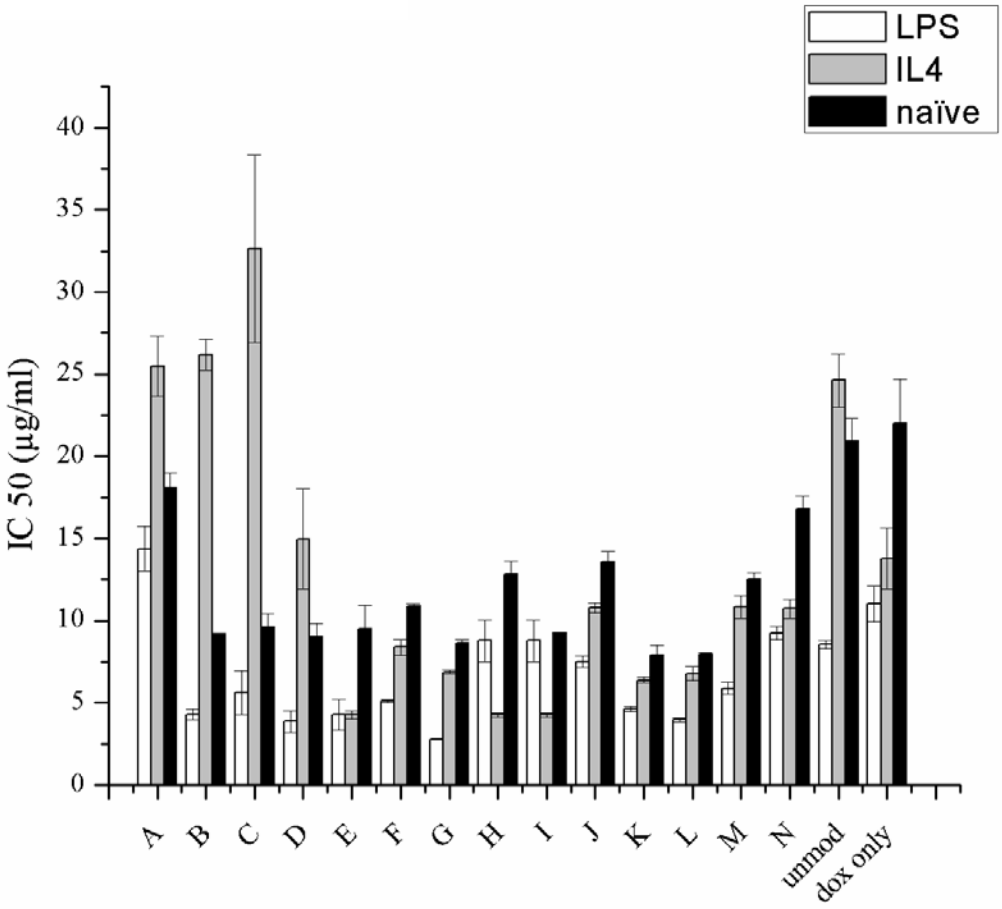


Figure2.5 Fluorescence level of FC loaded particles incubated with macrophages. Data presents the mean value of ten replicates for each sample ± standard deviation.

And according to Fig 2.6, surface modifications contribute to sharply decrease of IC_{50} compare to unmodified liposome for all macrophage phenotypes. Unmodified liposome has similar IC_{50} levels for M (IL-4) and M (0) and lower IC_{50} values for M (LPS). Lower IC_{50} values indicate higher cytotoxicity.

A


LPS
IL4
naïve
IC 50 (µg/ml)
0
5
10
15
20
25
30
35
40
A
B
C
D
E
F
G
H
I
J
K
L
M
N
unmod
dox only

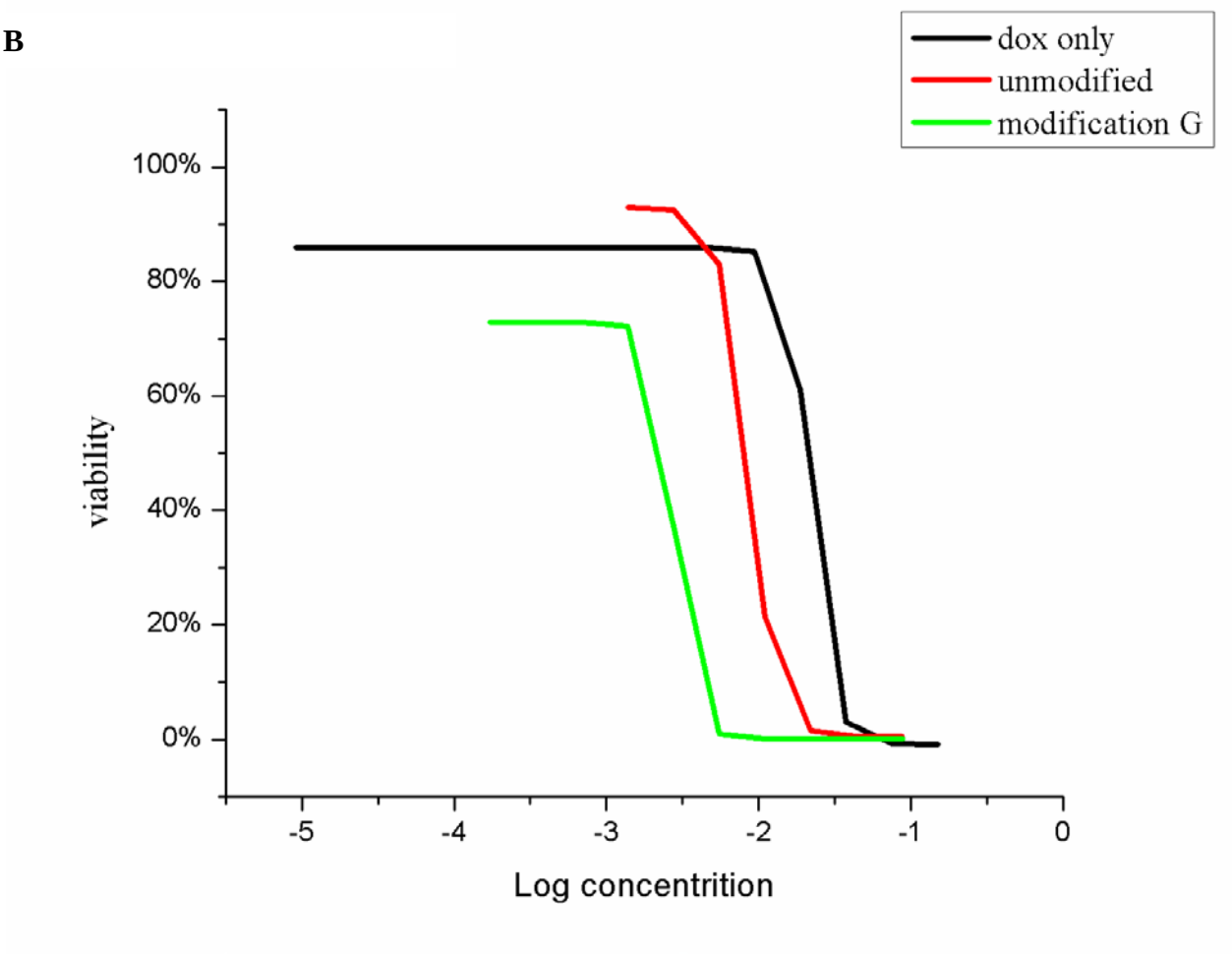


Figure2.6 IC $_{50}$ concentrations and dose-response curve by LPS or IL-4 or non-activated macrophages. (A) IC_{50}'s for modified liposomes, unmodified liposome loaded with doxorubicin and free doxorubicin by LPS or IL-4 activated or naïve macrophages. Data represents the mean value of three replicates for each sample ±standard deviation. (B) Dose-response curves for free doxorubicin, doxorubicin loaded in unmodified liposome and doxorubicin loaded in liposome G after incubating 48 h in the presence of IL-4 activated macrophages. Data represents the mean value of five replicates for each sample ±standard deviation.

Fig 2.6A and B show the IC_{50} and dose-response curves of modified liposome loaded with doxorubicin. For LPS activated macrophages, most of the liposomal doxorubicin formulations have more acute activity compared to the unmodified liposome (IC $_{50}$ <10 μg/ml). For IL-4 activated macrophages, unmodified liposome and modifications A, B, and C show weaker activities than unmodified liposomes, with IC_{50} values of 24.6, 25.5, 27.3, and

32.6 μg/ml, respectively, while modifications E through N show comparably acuter effects. Naïve macrophages exhibited higher IC_{50} in the presence of unmodified liposomes and modifications A and N compared to the modified liposomes ($p < 0.05$). All tested particles display a high toxicity, with the highest being modification C in the presence of IL-4 activated macrophages (IC_{50}= 32.6 μg/ml). From Fig2.6 IC_{50} values for most modifications were lower for LPS-activated macrophages than IL-4 activated macrophages, with the exception of modifications H and I and negligible differences between the two phenotypes for modification E. From the activated and non-activated data it could be concluded that doxorubicin loaded in modified liposomes alters the IC_{50} depending on the stimulation of macrophages and can be exploited to improve drug delivery.

2.4 Discussion

2.4.1 Liposome properties influenced by surface modification

It has been reported that liposome size and surface charge have a significant effect on potential targeting and encapsulation capability. [67,69] Modifications with the arginine derivatives in Fig 2.1 on the liposomes result in changes to the surface charge of liposomes. Unmodified and all of the modified liposomes explored here are negatively charged, which has been reported to have higher association effects with cells and more efficient delivery than neutral liposomes when encapsulating methotrexate-γ-aspartate. [58] Previous studies have reported that the extent of phagocytosis increases with increasing zeta potential, both negative and positive, and was lowest when the zeta potential was zero.[58] Several similarities between alterations in the zeta potential value and internalization with FC and IC_{50} values of encapsulated doxorubicin were observed. In internalization with FC, with the exceptions of

modifications B, F, J, and N, the internalization of the particles follows the zeta potential with more negative liposome surface charges corresponding to higher FC uptake. In the IC_{50} test, M(0) macrophages have increasing cytotoxicity with more negative zeta potential except in the case of medications C, G, H, I, and L. Attachment of liposomes to cell membranes is the first step of internalization, which is affected on surface changes of particles .[70] It has been suggested that there are cationic binding sites on cell surfaces that promote formation of clusters of negatively particles, owing to the repulsive interactions of the cell lipid membrane, thus improving internalization of negatively charged particles. [70] It is well known that charged particles become opsonized and enter cells through absorptive endocytosis. [71] Many positively charged liposomes exert toxic effects on cells, thus making negatively charged liposomes, such as those studied here, a more attractive carrier for drug delivery.

There were no significant differences in size between unmodified and modified liposomes after extrusion through 100 nm membranes, which provides uniform diameters and volumes. It has been reported that extrusion of liposomes could enhance the entrapment capability. [56] Small particle size (<200nm) is also known to enhance drug accumulation at the tumor site. [72] The high retention of doxorubicin in these liposomes demonstrates the strong and similar stability of particles due to the uniform liposome size (~100 nm), which has previously been demonstrated as a factor in high drug retention levels in liposomes. [67]The loading efficiency showed no significant differences across the library of modified liposomes. Size has been reported in many studies [52,45,73] as a crucial factor in altering pharmacokinetics due to the impact on tissue distribution and clearance. [45] These particles were kept around 100 nm to eliminate variables in characterizing drug release profiles and interactions with

cells. Taken all together, liposomal modifications with arginine derivatives have little influence on their size and drug loading ability.

2.4.2 Influence of macrophage phenotype on internalization and the efficacy of delivered doxorubicin

Macrophages play important roles in creating a permissive environment for tumors, thus targeting drugs to macrophages is an efficient method for drug delivery.[58] Targeting doxorubicin encapsulated in liposomes to macrophages is a method of improving the current passive targeting typically used with Doxil®, and increasing intracellular uptake is the main purpose for the future *in vivo* study. Doxorubicin is a widely and commonly used drug for cancer treatment. Doxorubicin can intercalate between DNA base pairs, which inhibits DNA and DNA-dependent RNA synthesis by template disordering and steric obstruction. [74] Another special character of doxorubicin is inducing the formation of covalent topoisomerase-DNA complex. When copying DNA, this capability could inhibit the religation portion of the ligation-religation reaction. Although doxorubicin contributions to the inhibition of tumor growth, cardiac toxicity is its main limitation. To ameliorate off-target effects, one effective approach is modifying the drug carrier, which could change the biodistribution of doxorubicin, leading to reduce the levels of the drug in healthy tissue.

Cell viability data shown in Fig 2.7 demonstrated cytocompatibility after 4 h incubation for both unmodified and modified particles. For LPS activated, IL-4 activated, and M(0) macrophages, viabilities are generally above 80%, indicating low toxicity of the particles. The phagocytic process of liposomes by macrophages starts with surface projections of lamellipodia, resulting from filaments. The lamellipodia become pseudopodia as cytoplasm flows into the lamellipodia, which allows the cell to engulf particulate. [58] The

complex steps of internalization between liposome and macrophage are 1) stable adsorption to the cell surface; 2) cellular uptake of intact vehicles; and 3) lysosomal degradation of the liposomes and their content.

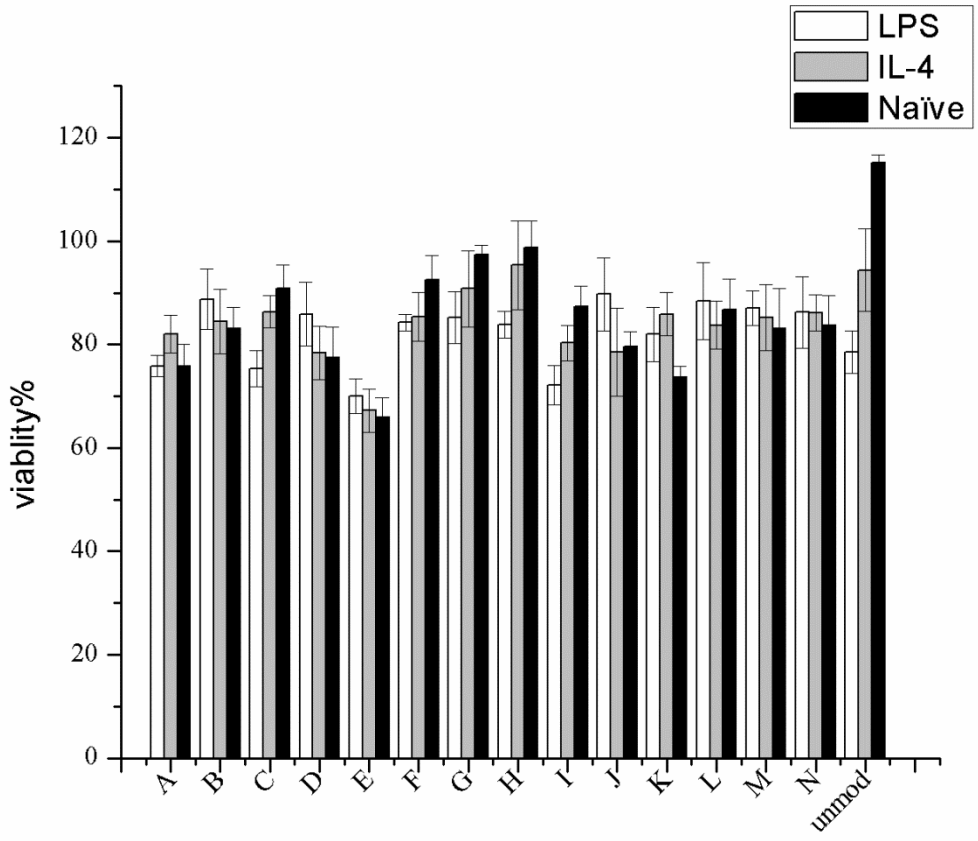


Figure2.7 Viability assay showing the cytotoxicity of all particles on LPS or IL-4 activated or non-activated RAW 264.7 cells. (Data represents the mean value of five replicates for each sample± standard deviation) (Taken from Hannah Bygd.)

Several mechanisms for endosomal escape have been proposed. The proton sponge effects, also known as the pH-buffering effect, is mediated by agents with a high buffering capacity and the flexibility to swell when protonated. [75] An extensive inflow of ions and

water induced by protonation into endosomal environment, results in cracking of the endosomal membrane and releasing the encapsulated contents. Poly (amido amine) s, which have protonated amine groups in their structure, contribute to a high buffer effect which results in increases in the osmotic pressure of the endosome and disrupts the endosomal membrane. [76] The flip-flop mechanism involves exchange of the liposomes in the endosomal wall and the delivered liposome, resulting in endosomal escape into cytosol. [77] Due to the electrostatic interaction between the cationic lipoplexes and the negatively charged lipids of the endosomal membrane, anionic lipids diffuse into the lipoplexes and form charge-neutralized ion pairs with cationic lipids. [78] Membrane fusion is the destabilization of the endosomal membrane due to fusogenic peptides, which is a significant process in cellular delivery and endocytosis. Haemeagglutin subnit HA2 of influenza virus change conformation when inside the endosomes due to the low pH environment and the highly conserved hydrophobic N-terminal region. This viral membrane fusion results in viral genome leakage to cytosol. [79] When liposomes interact with macrophages, the encapsulated drugs are taken by diffusing out of vehicles or directly “eaten” through lysosomal degradation. Escape from the liposome depends on the drug release rate from liposomes and macrophage permeability to the drugs. [52]

Figure 2.6A shows the IC_{50} values of modified liposomes on M(LPS), M(IL-4), and M(0) macrophages. The influence of polarization indicates macrophage phenotype is significant for internalizing liposomes. Lower IC_{50} levels demonstrate more acute activities of doxorubicin resulting from particle interactions with the cells. The different uptake levels may be influenced by different pathways for the different phenotypes. [28] From Fig 2.6 it is

obvious that M (LPS) have lower IC_{50} values, with the exception of modifications H and I, compared to M (IL-4) cells.

Although the internalization mechanisms of macrophages are complex, the results provides some clues to predict which modifications can be exploited to enhance toxicity of doxorubicin or improve selective delivery to specific macrophage phenotypes. The trends between the IC_{50} values of doxorubicin and internalization measured through entrapped FC exhibit many similarities. Internalization of FC loaded modifications are generally higher than unmodified liposome with the exception of modifications A, G, H, and M. Modification N is even four fold higher than unmodified liposome. In examining the IC_{50} values, the toxicity of doxorubicin is enhanced compared to unmodified liposomes with a few exceptions. For LPS activated macrophages, only modifications A and N are higher than unmodified liposomes ($p < 0.05$), while modification H and I are similar to unmodified liposomes. For IL-4 activated macrophages, modifications A, B, and C are higher than unmodified liposomes ($p < 0.05$). For non-activated macrophages, none of modifications are higher than unmodified liposomes. Thus, it is obvious that modifications to liposomes can enhance the cytotoxicity of encapsulated drugs. One possible explanation of difference between internalization of FC loaded liposomes and the IC_{50} values is the leakage and passive diffusion of loaded liposomes could be different for FC and doxorubicin. Doxorubicin loading efficiency has been shown in Fig 2.3, demonstrating the low drug release rate.

Hydrophobicity is another factor that leads to a difference in internalization. It has been shown that hydrophobic liposomes are more susceptible to phagocytosis than their hydrophilic counterparts. [58] Here, the IC_{50} values are remarkably influenced by

hydrophobicity. For modifications C, I, and L, which show mismatches between internalization of FC encapsulated liposomes and zeta potentials, these differences can be explained by hydrophobicity. All three modifications demonstrate higher surface tensions, resulting in increased hydrophobicity. Most modifications have lower IC_{50} values than unmodified liposomes when comparing similar macrophage activations, indicating improvement of cytotoxicity. This result shows that the arginine derivations improve liposomes' ability of taking up by macrophages and deliver drugs more efficiently and effectively.

2.5 Conclusions

Here, liposomes with surface modifications were studied for their ability to enhance drug delivery. Liposomes were modified with arginine derivatives. These modifications had no influence on particle size and drug loading efficiency. Differences were observed on the measured zeta potentials, which impacts internalization by macrophages. Cellular uptake of the liposomes was found to be dependent upon macrophage phenotype and surface modifications. There were also differences in trends between internalization of liposomal FC and the IC_{50} of liposomal doxorubicin, which were attributed to changes in the ability of doxorubicin to escape the endosome. This work demonstrates the importance of investigating how liposomes interact with different macrophage types and the ability to preferentially deliver drugs to specific macrophage phenotypes. The results claims that liposomes modified with arginine derivation are promising efficient nanoparticle vehicles for delivery to macrophages.

REFERENCES

1. Tiwari G, Tiwari R, Sriwastawa B, et al. Drug delivery systems: An updated review. *Int J Pharm Investig*. 2012;2(1):2-11. doi:10.4103/2230-973X.96920.

2. Popescu, Maria A., ed. Biotechnology in Agriculture, Industry and Medicine : Drug Delivery. New York, NY, USA: Nova, 2011. ProQuest ebrary. Web. 14 January 2016. Copyright © 2011. Nova. All rights reserved. 2016;(January).

3. Kelly C, Jefferies C, Cryan S-A. Targeted Liposomal Drug Delivery to Monocytes and Macrophages. *J Drug Deliv*. 2011;2011:1-11. doi:10.1155/2011/727241.

4. Fahmy TM, Fong PM, Goyal A, Saltzman WM. Targeted for drug delivery. *Mater Today*. 2005;8(8 SUPPL.):18-26. doi:10.1016/S1369-7021(05)71033-6.

5. Strebhardt K, Ullrich A. Paul Ehrlich ' s magic bullet concept : 100 years of progress. *Nat Rev cancer*. 2008;8(june):473-480.

6. Phillips MA, Gran ML, Peppas NA. Targeted nanodelivery of drugs and diagnostics. *Nano Today*. 2010;5(2):143-159. doi:10.1016/j.nantod.2010.03.003.

7. Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: Theory to practice. *Pharmacol Rev*. 2001;53(2):283-318. doi:VL - 53.

8. Cedervall T, Lynch I, Lindman S, et al. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proc Natl Acad Sci U S A*. 2007;104(7):2050-2055. doi:10.1073/pnas.0608582104.

9. Hall JB, Dobrovolskaia M a, Patri AK, McNeil SE. Characterization of nanoparticles for therapeutics. *Nanomedicine (Lond)*. 2007;2(6):789-803. doi:10.2217/17435889.2.6.789.

10. Moase EH, Qi W, Ishida T, et al. Anti-MUC-1 immunoliposomal doxorubicin in the treatment of murine models of metastatic breast cancer. *Biochim Biophys Acta - Biomembr*. 2001;1510(1-2):43-55. doi:10.1016/S0005-2736(00)00334-5.

11. Allen TM. Ligand-targeted therapeutics in anticancer therapy. *Nat Rev Cancer*. 2002;2(10):750-763. doi:10.1038/nrc903.

12. Rezler EM, Khan DR, Lauer-Fields J, Cudic M, Baronas-Lowell D, Fields GB. Targeted drug delivery utilizing protein-like molecular architecture. *J Am Chem Soc*. 2007;129(16):4961-4972. doi:10.1021/ja066929m.

13. Elbayoumi TA, Torchilin VP. Tumor-specific anti-nucleosome antibody improves therapeutic efficacy of doxorubicin-loaded long-circulating liposomes against primary and metastatic tumor in mice. *Mol Pharm*. 2009;6(1):246-254. doi:10.1021/mp8001528.

14. Stefater JA, Ren S, Lang RA, Duffield JS. Metchnikoff's policemen: Macrophages in development, homeostasis and regeneration. *Trends Mol Med*. 2011;17(12):743-752. doi:10.1016/j.molmed.2011.07.009.

15. Classen A, Lloberas J, Celada A. *Macrophage Activation: Classical versus Alternative.* Vol 531. 2009. doi:10.1007/978-1-59745-396-7_3.

16. Li AC, Glass CK. The macrophage foam cell as a target for therapeutic intervention. *Nat Med*. 2002;8(11):1235-1242. doi:10.1038/nm1102-1235.

17. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, Fantino E. Phenotypic, Functional and Plasticity Features of Classical and Alternatively Activated Human Macrophages. *Am J Respir Cell Mol Biol*. 2015;53(5):676-688. doi:10.1165/rcmb.2015-0012OC.

18. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677-686. doi:10.1016/j.it.2004.09.015.

19. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. 2002;23(11):549-555. doi:10.1016/S1471-4906(02)02302-5.

20. Poirier MG, Eroglu S, Marko JF. The bending rigidity of mitotic chromosomes. *Mol Biol Cell*. 2002;13(6):2170-2179. doi:10.1091/mbc.01.

21. Steinman BRM, Steinman BRM, Swanson J, Swanson J. Commentary The Endoeytie Activity of Dendritic Cells By Ralph M. Steinman* and Joel Swanson~. *Commentary*. 1995;182(August).

22. Decuzzi P, Ferrari M. The role of specific and non-specific interactions in receptor-mediated endocytosis of nanoparticles. *Biomaterials*. 2007;28(18):2915-2922. doi:10.1016/j.biomaterials.2007.02.013.

23. Mukherjee S, Ghosh RN, Maxfield FR. Endocytosis. *Physiol Rev*. 1997;77(3):759-803. http://www.ncbi.nlm.nih.gov/pubmed/9234965\ninternal-pdf://759.full-1477531393/759.full.pdf.

24. Moon H-G, Yang J, Zheng Y, Jin Y. miR-15a/16 regulates macrophage phagocytosis after bacterial infection. *J Immunol*. 2014;193(9):4558-4567. doi:10.4049/jimmunol.1401372.

25. Underhill DM, Goodridge HS. Information processing during phagocytosis. *Nat Rev Immunol*. 2012;12(7):492-502. doi:10.1038/nri3244.

26. Andrews T, Sullivan KE. Infections in Patients with Inherited Defects in Phagocytic Function. *Clin Microbiol Rev*. 2003;16(4):597-621. doi:10.1128/CMR.16.4.597-621.2003.

27. Nimmerjahn F, Ravetch J V. Fc?? receptors: Old friends and new family members. *Immunity*. 2006;24(1):19-28. doi:10.1016/j.immuni.2005.11.010.

28. Brown BN, Ratner BD, Goodman SB, Amar S, Badylak SF. Macrophage polarization: An opportunity for improved outcomes in biomaterials and regenerative medicine. *Biomaterials*. 2012;33(15):3792-3802. doi:10.1016/j.biomaterials.2012.02.034.

29. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature*. 2008;454(July):444. doi:10.1038/nature07205.

30. Lozano N, Al-Ahmady ZS, Beziere NS, Ntziachristos V, Kostarelos K. Monoclonal antibody-targeted PEGylated liposome-ICG encapsulating doxorubicin as a potential theranostic agent. *Int J Pharm*. 2015;482(1-2):2-10. doi:10.1016/j.ijpharm.2014.10.045.

31. Zhao C, Feng Q, Dou Z, et al. Local targeted therapy of liver metastasis from colon cancer by galactosylated liposome encapsulated with doxorubicin. *PLoS One*. 2013;8(9):e73860. doi:10.1371/journal.pone.0073860.

32. Ta T, Porter TM. Thermosensitive liposomes for localized delivery and triggered release of chemotherapy. *J Control Release*. 2013;169(1-2):112-125. doi:10.1016/j.jconrel.2013.03.036.

33. Darling-Hammond L. Teacher quality and student achievement: A review of state policy evidence. *Educ Policy Anal Arch*. 2000;8(5):761-769. doi:10.1038/sj.clp.

34. Publications O. United States Patent [191. 1978;79(1973):2-6.

35. Coating E, Of M, Coating AM. United States Patent [191. 1973.

36. Application F, Data P. ‘ United States Patent [191 Tamblyn. 1976.

37. Gregoriadis G, Ryman BE. Fate of protein-containing liposomes injected into rats. An approach to the treatment of storage diseases. *Eur J Biochem*. 1972;24(3):485-491. doi:10.1111/j.1432-1033.1972.tb19710.x.

38. Andresen TL, Jensen SS, Jørgensen K. Advanced strategies in liposomal cancer therapy: Problems and prospects of active and tumor specific drug release. *Prog Lipid Res*. 2005;44(1):68-97. doi:10.1016/j.plipres.2004.12.001.

39. Kong G, Anyarambhatla G, Petros WP, et al. Efficacy of Liposomes and Hyperthermia in a Human Tumor Xenograft Model : Importance of Triggered Drug Release Efficacy of Liposomes and Hyperthermia in a Human Tumor Xenograft Model : Importance of Triggered Drug Release 1. *Cancer Res*. 2000;60:6950-6957.

40. Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol Rev*. 1999;51(4):691-743. doi:VL - 51.

41. Klibanov AL, Maruyama K, Torchilin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett*. 1990;268(1):235-237. doi:10.1016/0014-5793(90)81016-H.

42. Nicolazzi C, Mignet N, De La Figuera N, et al. Anionic polyethyleneglycol lipids added to cationic lipoplexes increase their plasmatic circulation time. *J Control Release*. 2003;88(3):429-443. doi:10.1016/S0168-3659(03)00067-1.

43. Takano S, Aramaki Y, Tsuchiya S. Physicochemical properties of liposomes affecting apoptosis induced by cationic liposomes in macrophages. *Pharm Res*. 2003;20(7):962-968. doi:10.1023/A:1024441702398.

44. Lowery A, Onishko H, Hallahan DE, Han Z. Tumor-targeted delivery of liposome-encapsulated doxorubicin by use of a peptide that selectively binds to irradiated tumors. *J Control Release*. 2011;150(1):117-124. doi:10.1016/j.jconrel.2010.11.006.

45. Gao J, Zhong W, He J, et al. Tumor-targeted PE38KDEL delivery via PEGylated anti-HER2 immunoliposomes. *Int J Pharm*. 2009;374(1-2):145-152. doi:10.1016/j.ijpharm.2009.03.018.

46. Weiner N, Martin F, Riaz M. Liposomes as a Drug Delivery System. *Drug Dev Ind Pharm*. 1989;15(10):1523-1554. doi:10.3109/03639048909052502.

47. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov*. 2005;4(2):145-160. doi:10.1038/nrd1632.

48. Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc Natl Acad Sci U S A*. 1988;85(18):6949-6953. doi:10.1073/pnas.85.18.6949.

49. Petros R a, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. *Nat Rev Drug Discov*. 2010;9(8):615-627. doi:Doi 10.1038/Nrd2591.

50. Torchilin VP, Levchenko TS, Whiteman KR, et al. Amphiphilic poly-N-vinylpyrrolidones: Synthesis, properties and liposome surface modification. *Biomaterials*. 2001;22(22):3035-3044. doi:10.1016/S0142-9612(01)00050-3.

51. Lasic DD, Needham D. The “Stealth” Liposome: A Prototypical Biomaterial. *Chem Rev*. 1995;95(8):2601-2628. doi:10.1021/cr00040a001.

52. Xiong XB, Huang Y, Lu WL, et al. Intracellular delivery of doxorubicin with RGD-modified sterically stabilized liposomes for an improved antitumor efficacy: In vitro and in vivo. *J Pharm Sci*. 2005;94(8):1782-1793. doi:10.1002/jps.20397.

53. Press D. Clinical development of liposome-based drugs : formulation , characterization , and therapeutic efficacy. 2012:49-60.

54. Hossann M, Wang T, Wiggenhorn M, et al. Size of thermosensitive liposomes influences content release. *J Control Release*. 2010;147(3):436-443. doi:10.1016/j.jconrel.2010.08.013.

55. Wibroe PP, Ahmadvand D, Oghabian MA, Yaghmur A, Moghimi SM. An integrated assessment of morphology, size, and complement activation of the PEGylated liposomal doxorubicin products Doxil??, Caelyx??, DOXOrubicin, and SinaDoxosome. *J Control Release*. 2016;221:1-8. doi:10.1016/j.jconrel.2015.11.021.

56. Francisco S, Francisco S. PREPARATION OF LIPOSOMES OF DEFINED SIZE DISTRIBUTION BY EXTRUSION THROUGH POLYCARBONATE MEMBRANES Summary Liposomes of defined size and homogeneity have been prepared by sequential extrusion of the usual multilamellar vesicles through polycarbonate mem- . 1979;557.

57. Brown KC, Gray BP, Li S. From Phage Display to Nanoparticle Delivery: Functionalizing Liposomes with Multivalent Peptides Improves Targeting to a Cancer Biomarker. *Bioconjug Chem*. 2012. doi:10.1021/bc300498d.

58. Ahsan F, Rivas IP, Khan MA, Torres Su??rez AI. Targeting to macrophages: Role of physicochemical properties of particulate carriers - Liposomes and microspheres - On the phagocytosis by macrophages. *J Control Release*. 2002;79(1-3):29-40. doi:10.1016/S0168-3659(01)00549-1.

59. Rooijen N Van, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. 1994;174(1-2):83-93. doi:10.1016/0022-1759(94)90012-4.

60. Murray PJ, Allen JE, Biswas SK, et al. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*. 2014;41(1):14-20. doi:10.1016/j.immuni.2014.06.008.

61. Ikehara Y, Niwa T, Biao L, et al. A carbohydrate recognition-based drug delivery and controlled release system using intraperitoneal macrophages as a cellular vehicle. *Cancer Res*. 2006;66(17):8740-8748. doi:10.1158/0008-5472.CAN-06-0470.

62. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol*. 2005;5(8):641-654. doi:10.1038/nri1668.

63. Arimoto T, Bing G. Up-regulation of inducible nitric oxide synthase in the substantia nigra by lipopolysaccharide causes microglial activation and neurodegeneration. *Neurobiol Dis*. 2003;12(1):35-45. doi:10.1016/S0969-9961(02)00017-7.

64. Calabrese V, Scapagnini G, Ravagna A, et al. Disruption of Thiol Homeostasis and Nitrosative Stress in the Cerebrospinal Fluid of Patients with Active Multiple Sclerosis: Evidence for a Protective Role of Acetylcarnitine. *Neurochem Res*. 2003;28(9):1321-1328. doi:10.1023/A:1024984013069.

65. Ikonen M, Murtomäki L, Kontturi K. Microcalorimetric and zeta potential study on binding of drugs on liposomes. *Colloids Surfaces B Biointerfaces*. 2010;78(2):275-282. doi:10.1016/j.colsurfb.2010.03.017.

66. Abe K, Higashi K, Watabe K, et al. Effects of the PEG molecular weight of a PEG-lipid and cholesterol on PEG chain flexibility on liposome surfaces. *Colloids Surfaces A Physicochem Eng Asp*. 2015;474:63-70. doi:10.1016/j.colsurfa.2015.03.006.

67. Mirahmadi N, Babaei MH, Vali AM, Dadashzadeh S. Effect of liposome size on peritoneal retention and organ distribution after intraperitoneal injection in mice. *Int J Pharm*. 2010;383(1-2):7-13. doi:10.1016/j.ijpharm.2009.08.034.

68. Phases I. parasitenkunde with Macrophages in vitro : 1982:7-14.

69. ??alva E, Turan S ??zba??, Eren F, Akbu??a J. The enhancement of gene silencing efficiency with chitosan-coated liposome formulations of siRNAs targeting HIF-1?? and VEGF. *Int J Pharm*. 2015;478(1):147-154. doi:10.1016/j.ijpharm.2014.10.065.

70. Patil S, Sandberg A, Heckert E, Self W, Seal S. Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. *Biomaterials*. 2007;28(31):4600-4607. doi:10.1016/j.biomaterials.2007.07.029.

71. Epstein-Barash H, Gutman D, Markovsky E, et al. Physicochemical parameters affecting liposomal bisphosphonates bioactivity for restenosis therapy: Internalization, cell inhibition, activation of cytokines and complement, and mechanism of cell death. *J Control Release*. 2010;146(2):182-195. doi:10.1016/j.jconrel.2010.03.011.

72. Xu Z, Gu W, Huang J, et al. In vitro and in vivo evaluation of actively targetable nanoparticles for paclitaxel delivery. *Int J Pharm*. 2005;288(2):361-368. doi:10.1016/j.ijpharm.2004.10.009.

73. Gao JQ, Lv Q, Li LM, et al. Glioma targeting and blood-brain barrier penetration bydual-targeting doxorubincin liposomes. *Biomaterials*. 2013;34(22):5628-5639. doi:10.1016/j.biomaterials.2013.03.097.

74. [4] The Liposomal Formulation of Doxorubicin. 2005;391:71-97.

75. Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of biologicals. *J Control Release*. 2011;151(3):220-228. doi:10.1016/j.jconrel.2010.11.004.

76. Lin C, Engbersen JFJ. Effect of chemical functionalities in poly(amido amine)s for non-viral gene transfection. *J Control Release*. 2008;132(3):267-272. doi:10.1016/j.jconrel.2008.06.022.

77. Liang W, Lam JKW. Endosomal Escape Pathways for Non-Viral Nucleic Acid Delivery Systems. *Mol Regul Endocytosis*. 2012:429-456. doi:10.5772/46006.

78. Simões S, Slepushkin V, Pires P, Gaspar R, de Lima MP, Düzgüneş N. Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. *Gene Ther*. 1999;6(11):1798-1807. doi:10.1038/sj.gt.3301015.

79. Stegmann T. Membrane fusion mechanisms: the influenza hemagglutinin paradigm and its implications for intracellular fusion. *Traffic*. 2000;1(8):598-604. doi:10.1034/j.1600-0854.2000.010803.x.