Design and Characterization of a Stealth Immunoliposome Encapsulating a Large Molecule Biotherapeutic (Antibody)

Design and Characterization of a Stealth Immunoliposome Encapsulating a Large Molecule Biotherapeutic (Antibody)

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Abstract

This thesis project will endeavor to solve the obstacles around synthesis of Immunoliposomes loaded with an antibody therapeutic. Most steps in the preparation of liposomes in most of the methods include harsh conditions that are not amenable for antibody therapeutic entrapment. These methods utilize high concentrations of organic solvents (chloroform and/or methanol) which, if in contact with an antibody at high concentrations would not provide an optimal environment for the antibodies' tertiary structure. Additionally, preparation methods usually employ physical processes such as high-speed centrifugation, sonication, milling and emulsification in an attempt to dissolve lipid compositions in organic solvents, all of which if not optimized could render the biotherapeutic inactive. This study primarily focuses on preparation of liposomes using the Dry Film Hydration method. Multiple lipids were mixed together (ratio amounts based on the molecular weights) in absolute ethanol to make a homogeneous mixture. The mixture was first evaluated on the Nanoassemblr platform (Precision Nanosystems), which enabled small scale controlled, bottom-up, and assembly of liposomes. The mixing to get a homogenous preparation is done on cartridges that allow millisecond mixing of nanoparticle components at the nanolitre scale. This technology is user friendly and allows a quick method to evaluate different lipid compositions, different flow ratios and rates while characterizing liposome size and size distribution in order to determine the best lipid mixture/composition yielding liposomes within the range of requirement for

your purposes. Once the Lipid compositions were selected, larger preparations of about 5-20mL were prepared and a thin dry film/cake was accomplished using the rotary evaporation system in vacuum. The film allowed to dry in vacuum after which it was hydrated with an antibody biotherapeutic dissolved in an aqueous medium. Further sizing of liposomes was accomplished via bath sonication and filtration of the liposomes to remove aggregated or liposome debris was done using Tangential Flow Filtration (TFF), which filters liposomes based on a cutoff membrane at a specific KiloDalton (KDa). Using Dynamic Light Scattering technology (DLS), it was realized that liposomes of distinct sizes could be synthesized ranging from 200nm – 1000nm. Sizing by sonication on the water bath and then filtration via TFF especially improved the quality of the preps ensuring a more monodispersed population of liposomes. Sonication yielded uniformly monodispersed liposomes up to a certain point upon which prolonged sonication affected the dispersity and stability of the ensuing liposomes. To prepare the targeting mAb for conjugation on the surface of liposomes in order to make Immunoliposomes, a monoclonal antibody was prepared by digesting it to make $F(Ab)'_2$ using a commercially available kit. The targeting mAb were digested and thiolated to reduce and expose disulfide bonds, which were later, used to conjugate onto liposomes bearing chemically modified lipids. To measure biotherapeutic encapsulation efficiency, liposomes were treated with TritonX-100 to rupture them followed by an ELISA method to detect and quantify amounts of biotherapeutic after TritonX-100 treatment. Conjugation of targeting mAbs was also characterized using an ELISA method for detection of the targeting antibody in modified liposomes incubated with the thiolated mAb.

Dedication

This work is a dedication to my parents; my dad Julius Njenga Kuria and my mom Margaret Mumbi Njenga. You took the sacrifice and denied yourselves plenty to see my siblings and I through many financially tough years of high school, especially, those couple of years where three of us were all in school at the same time yet you never quit! You never had much, yet much was demanded of you, you took the sacrifice and you did it for us! This is for you, I am forever grateful!

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A special thank you to my darling wife, Hellen Kuria and my two sons Jayson and Jaylen Kuria for the hard sacrifices you have made throughout the course of this project. You have allowed me to take time away from your lives to accomplish this project without any complains. You also sacrificed many nights of your sleep to stay up with me so I can continue writing and you pushed me to stay on course with this project. I could not have done this without you. I am forever thankful.

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Chapter I

Introduction

Neurological Disorders and Challenges with Current Treatment Approaches

The world population is getting older and as such, associated chronic old-age related diseases of the Central Nervous System (CNS) such as Alzheimer's disease (AD) and Parkinson's disease (PD) are becoming an epidemic and therefore a global burden. These diseases affect older adults disproportionately contributing to disability, diminished quality of life and increased healthcare costs. With the looming "baby boom effect", the number of people aged 65 and over will double in context to the entire global population from 7% to 16% within the time period in the year 2000 to 2050, which constitutes an extra billion elderly individuals altogether (The WHO; Center for health development 2008) (Fig. 1). These diseases not only affect the aging but also the rest of the population. They cause distress among family members and decrease their earning potential due to lost wages as they care for their elderly relatives.

AD and PD take the greatest proportion of old-age associated neurodegenerative diseases of the CNS. AD is a progressive disorder that presents with pathological hallmarks of extracellular amyloid-β (Aβ) plaques and neurofibrillary tangles of intraneuronal hyperphosphorylated tau protein (Gao, Pang and Jiang, 2013). As the leading cause of dementia, AD currently affects more than 24 million people worldwide and is projected to affect 115 million people by 2050 (Gao et al., 2013) . With the

staggering number of potential patients projected within the next few decades, research and development in the field of neurodegenerative disorder therapeutics requires new approaches that will ensure faster development of drugs and make them available to patients sooner. Aggregations of Aβ and tau proteins are both neurotoxic and are the most important instigators of dementia.

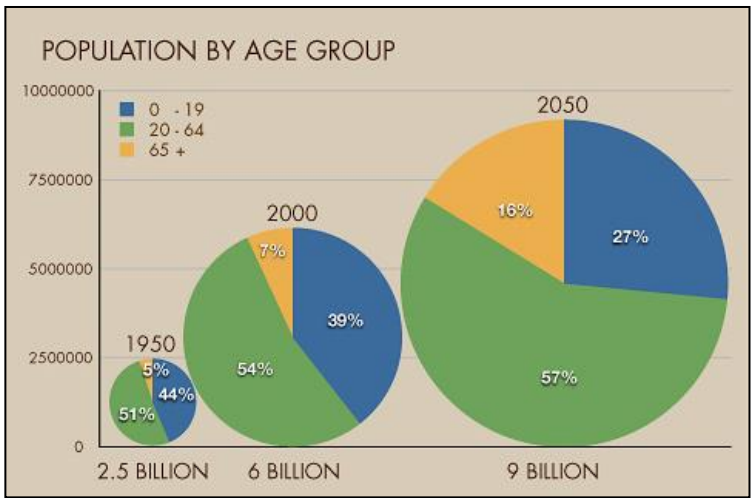


Figure 1: Schematic pie chart showing comparison of aging demographics from 1950-2050 (100 yrs. Source: World Health Organization (2008).

The most common treatment for AD is inhibition of Aβ plaque formation via intracerebral injection of an anti-Aβ fusion antibody (Gao et al., 2013). However, the efficacy of the fusion protein has shown to be overshadowed by poor stability and the presence of immunogenicity. Encapsulating anti-Aβ therapeutic fusion antibodies in nano-therapeutics could potentially increase their bioavailability, extend their stability in circulating blood as well as decreased their immunogenicity profile. As the second most common neurodegenerative disorder, PD affects 1–2% of the population over the age of

65 (Gao et al., 2013). PD is characterized by selective loss of dopaminergic neurons in the substantia nigra and brainstem, accumulation of α-synuclein aggregates, leading to difficulty controlling movement. Glial-derived neurotrophic factor (GDNF) is a potential therapeutic for PD due to its neuroprotective effect. However, the application of GDNF is limited by its poor penetration through the Blood Brain Barrier (BBB). According to Gao *et. al.*, a monoclonal antibody directed against the Transferrin Receptor TfR (cTfR mAb) was fused with GDNF to enable the protein to penetrate the BBB which, enabled brain uptake of roughly 3.1% of the injected dose per gram of brain tissue. However, there was rapid blood clearance, which became the limiting factor for the application of the fusion protein (Gao et al., 2013). Taken together, existing therapies for these debilitating diseases only treat the symptoms and therefore not effective in reducing disease burden therefore posing a big global burden and constituting a global highly unmet need.

These challenges and obstacles encountered with treating CNS diseases and disorders are not because there is no advancement in development of new drugs but rather because the brain is a complex organ (Mohammad, et al., 2005). Very few pharmaceutical companies are willing to take the risk in investments granted the rate of failure for Food and Drug Administration (FDA) approval. Although numerous compounds undergo the drug discovery and development process, very few make it through the pipeline and ultimately to the patients. Despite the challenges around understanding the complexity of the brain, CNS diseases are becoming more and more challenging to treat owing to the slow pace of drug approvals by the FDA. Furthermore, the chances of good clinical outcomes compared to all other compounds combined (Gaffney, 2014) are dismal. According to a study carried out by Tufts Center for the

study of drug development, of all compounds that were in pharma and biotech pipelines from 1995-2007, only 6.2% of CNS compounds that entered Phase I testing were eventually approved by the FDA compared to 13.3% of all other drugs (Gaffney, 2014) combined (Fig. 2). According to that study, CNS drugs had lower success rates during every single phase of the clinical development process, with marked differences in Phase II and Phase III testing and needed to wait an average of 19.3 months for approval decision compared to just 14.7 months for all other drugs over the 1999-2013 period (Gaffney, 2014) (Fig. 2). The low success rate for the approval of these drugs is attributable to a variety of factors including the complexity of the brain and the liability of CNS drugs to cause CNS side effects. Additionally, the requirement of the CNS therapies to cross the BBB is the single most common hindrance for development of new biotherapeutics for CNS disorders among many others (Gaffney, 2014).

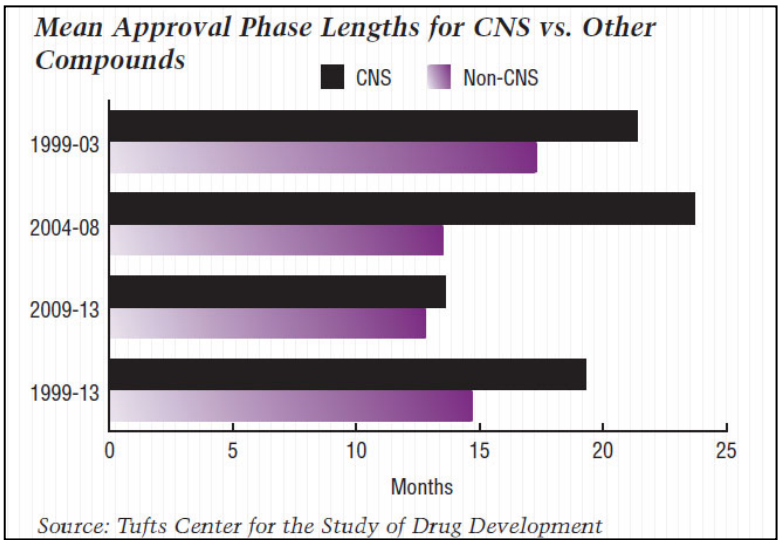


Figure 2: A graphical representation showing the average approval ratings by time (months) of common conventional pharmaceutics compared to CNS-based therapeutics. Source: Gaffney (2014). Pharm. Res.

Nanotechnology is however changing the landscape over traditional thought-process of

treatment approaches and intervention using cutting-edge science aimed at either improving the current existing methods or entirely replacing the traditional methods (Desai, 2012). In particular, nanoparticles offer the ability to penetrate the BBB when specific ligands are attached to their surface, an attribute that is by far the strongest advantage for their case in CNS therapeutics. As far as drug delivery using nanoparticles is concerned, their most applicability is in cancer therapeutics and therapies for neurodegenerative disorders where conventional therapeutic approaches have failed to make an impact (Desai, 2012). However, in order for any of these nano-drugs to have any impact in medicine, a lot of work still needs to be done to show that they are clinically viable treatment approaches with the appropriate therapeutic attributes and outcomes. They need to show appropriate potency, specificity to their targets, metabolic stability, highly absorbability, a rather appropriate toxicity profile, and can be easily formulated in a reproducible manner (Desai, 2012). Granted the challenges behind conventional existing therapeutics and specifically in cancer and neurodegenerative diseases, there is high-unmet medical need to utilize substitute methods that are more effective and amenable for patient treatment.

The Blood Brain Barrier

The CNS, which consists of the brain and spinal cord, is protected by the BBB, a barrier that is formed by tight junctions between cerebral capillary endothelial cells and is a critical regulator of the brain's homeostasis (Nau, Sörgel, Eiffert, 2010). It restricts free penetration of foreign toxic substances and is almost impenetrable. Despite its critical

role and maintenance of a homeostatic environment, the BBB also becomes a persistent obstacle in efforts to deliver therapeutic agents into the brain, causing a great impediment in treatment of CNS disease (Nau, 2010). Paul Ehrlich was the first person to propose on the existence of the BBB when he performed the first experiments by injecting anilin dyes into the blood of experimental animals and noticed that all organs with the exception of the brain were stained. Later on, Ehrlich's student Edwin Goldmann injected trypan blue into experimental animals and likewise noticed that the choroid plexus and the dura mater were stained but the dye did not substantially enter the cerebrospinal fluid (CSF) (Nau, 2010). Conversely, after the direct injection of trypan blue into the CSF, the brain and spinal cord were stained indicating the absence of a tight diffusional barrier between CSF and brain tissue. These studies led to the current understanding of the BBB as a specialized system of cells that has dual function as a natural barrier and gatekeeper for the brain as well as a carrier function (Nau, 2010).

The anatomical structure and physiology of the BBB

The BBB consists of a complex network of endothelial cells of the blood vessels in the brain, the choroid plexus epithelium, which is the border between the blood and ventricular cerebrospinal fluid and the arachnoid epithelium, which separates the blood from the subarachnoid CSF (Loureiro, Gomes, Coelho, Pereira, Rocha, 2014). These cells are responsible for the different functions such as maintenance of neuronal microenvironment, tissue homeostasis etc. As a specialized network of complex cells, it lacks the porosity seen in other tissues to allow rapid movement of small molecules and

nutrients across into organ interstitial fluid from circulation (Loureiro, 2014). The BBB as a Carrier: As a carrier, the BBB helps nutrients such as amino acids and neuropeptides to get across into the deep brain through specific receptors (Fig. 3). This barrier is not a passive anatomical lipid phase membrane, but rather a dynamic interface containing both physical and metabolic transporter components (Loureiro, 2014). Only small lipid-soluble molecules (about 400Da in size or smaller) and blood gases like oxygen and carbon dioxide can diffuse passively through the BBB while polar nutrients such as glucose and amino acids require specific protein carriers in order to reach the brain (Loureiro, 2014). Different endogenous transport systems are expressed at the BBB surface (Fig. 3) and are responsible for the transport of essential hydrophilic macromolecules to the brain (Loureiro, 2014).

Hydrophilic essential macromolecules such as proteins are transferred into the deep brain across the BBB by endocytosis in vesicles, which can either be specific via receptor-mediated transcytosis (RMT) or less specific via adsorptive-mediated transcytosis (AMT) (Loureiro, 2014). Large molecules like transferrin or insulin are usually transported via RMT (Fig. 3D), using specialized ligand specific receptor systems on the surface of brain endothelial cells (Loureiro, 2014). However, small proteins such as albumin are transported via AMT (Fig. 3E), where their transport is triggered by electrostatic interactions between positively charged proteins and negatively regions of the membrane surface of brain endothelial cells (Loureiro, 2014). The lack of penetrance due to the defense mechanisms around the BBB causes poor drug bioavailability in the CNS and insufficient cellular uptake and subsequently lack of therapeutically relevant concentrations that warrant a favorable outcome (Loureiro, 2014). Incidentally, most

therapeutic agents are eliminated in peripheral blood before reaching the CNS via immune system clearance further making it even more challenging to reach therapeutically relevant concentrations.

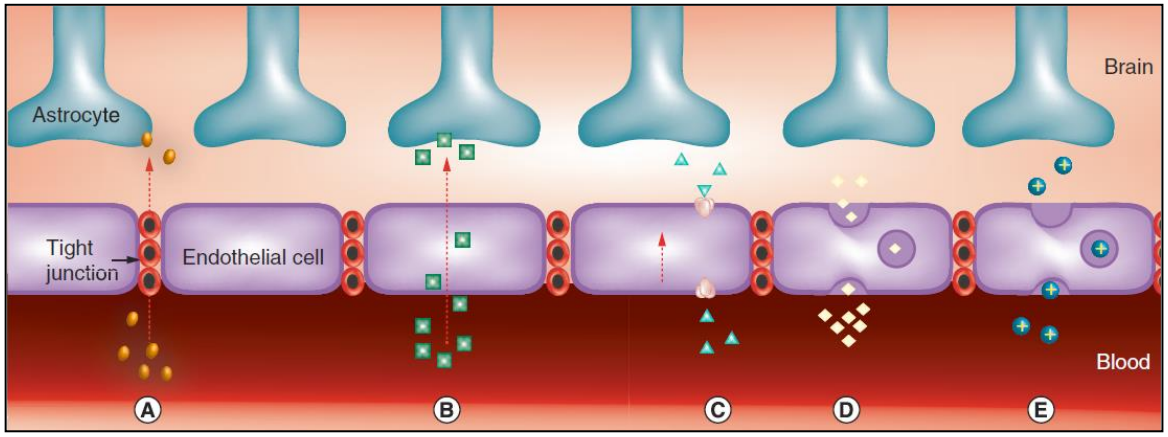


Figure 3: An illustration showing transport of molecules through the BBB. A: Para cellular aqueous pathway used by water soluble molecules; B: Transcellular pathway for lipophilic molecules; C: Membrane transport used by molecules e.g. glucose and Amino Acids; D: RMT of macromolecules like Insulin; E: AMT of cationized plasmatic proteins. Source: Loureiro et. al.(2014). Nanomedicine.

Even though all these processes are very critical and important to maintain homeostasis in the CNS in normal circumstances, they present a challenge and a major hindrance from a drug delivery perspective, making the BBB one of the single most impeding obstacles to overcome in treatment of CNS disorders, despite its critical function protecting the brain in normal circumstances. The BBB as a barrier: An effective barrier, the BBB acts as the interface between the blood of the brain microvasculature and the brain tissue and is crucial for achieving normal functions of the CNS (Wolff, Antfolk, Brodin, Tenje, 2015). Small lipophilic gases, such as O_2 and CO_2 may diffuse

freely across the BBB, but tight junctions restrict transport of hydrophilic molecules (Wolff, 2015). The barrier is tight for polar compounds because of their low lipid permeability, leaving only very small polar molecules to cross through (Wolff, 2015) (Fig. 4).

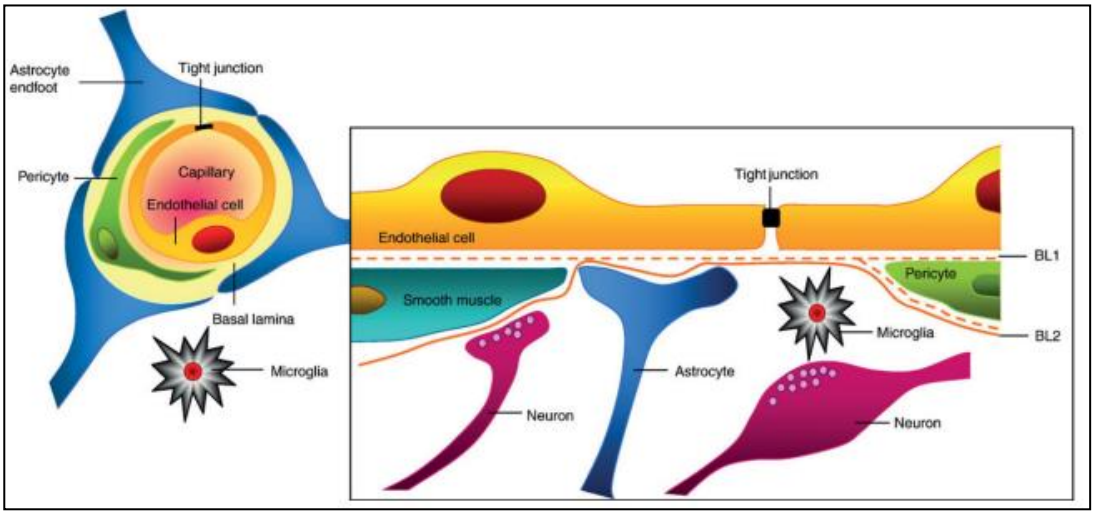


Figure 4: The schematic shows the neurovascular unit showing the barrier organization of the BBB with tight junctions, basal lamina (BL1/2) and various cells that help maintain a homeostatic microenvironment. Source: Wolff et. al.(2015). Pharm Sci.

The barrier function is achieved through a four-fold defense mechanism; 1) The paracellular component formed by interendothelial junctions restricts free movement of water soluble compounds between two adjacent cells. 2) The transcellular barrier is made possible by the low level of endocytosis and transcytosis characteristic for brain endothelial cells and inhibits substances through the cytoplasm. 3) The enzymatic barrier consisting of such complex enzymes as acetylcholinesterase, alkaline phosphatase, gamma-glutamyl transpeptidase, monoamine oxidases and other drug metabolizing

enzymes is capable of degrading different chemical compounds. 4) The cerebral endothelium expresses a large number of efflux transporters, which act as pumps to flush compounds out of the CNS periphery (Wilhelm, Fazakas, Krizbai, 2011). All these defense mechanisms help in blocking harmful substances from entering the CNS while only allowing in necessary nutrients.

In-vitro models of the BBB

Currently, there are experimental models of the BBB, but all are limited in one way or another thus allowing only basic research on the BBB and its interactions with potential drug candidates. Most are non-human or *in-vitro* systems that do not recapitulate physiological CNS microenvironments further complicating research on about how the BBB functions and the potentiality to design therapeutics for CNS treatments. Primary Cells and Cell Lines: There are in-vitro models such as the Mardin-Darby canine kidney (MDCK) which have good paracellular permeability but do not quite mimic brain endothelial cells and do not have similar transporter expression patterns that may determine brain penetration of drugs (Wolff, 2015; Wilhelm, 2011). Human umbilical endothelial cells (HUVECs) have also been proposed and used as BBB models but the downside is that even though they are human they are not of cerebral origin (Wolff, 2015; Wilhelm, 2011). Rat and mice offer great advantages since they are more characterized and can be engineered to create either transgenic (over expression) or knockouts (no expression) models but due to their small size, relatively low amounts of brain endothelial cells can be harvested from them (Wolff, 2015; Wilhelm, 2011).

The RBE4 cell line derived by transfection of rat brain microvessel endothelial cells with a plasmid containing the E1A adenovirus gene is among the best characterized brain endothelial cell line. It has shown to retain and maintain most of BBB properties with good expression of most BBB components. This cell line has been used successfully in brain endothelial cell signaling studies as well as P-glycoprotein regulation studies and permeability studies (Wolff, 2015; Wilhelm, 2011). Although very useful in enlightening on the basic science and research of the BBB, the fact that they are non-human and cell lines do not recapitulate physiological CNS conditions only allows minimal studies.

Microfluidic Flow Systems: In addition to cell line models, the importance of flow in the brain microenvironment has initiated development of new technologies that are better and capable of mimicking the physiological function and response of the brain micro-capillaries (Wolff, 2015; Wilhelm, 2011). Two major designs that are based on different approaches are available.

The Dynamic *in-vitro* BBB model (DIV-BBB) makes use of artificial capillary-like structural supports for co-culturing Endothelial Cells (ECs) and astrocytes and allows for the presence of intraluminal flow (Naik and Cucullo, 2012) (Fig. 5). This system is more superior and novel having been initially exploited for mass production of manufacturing bioreactors (Naik, 2012). It allows brain microvascular ECs (animal or human, both primary cultures and cell lines) to be co-cultured with astrocytes (juxtaposed to each other) under the exposure of quasi-physiological pulsatile laminar shear stress (Naik, 2012). This model allows for formation of a BBB that resembles the in situ both functionally and anatomically. The DIV-BBB apparatus is represented by a bundle of microporous pronectin-coated polypropylene hollow fibers, which enable co-culturing of

brain and non-brain vascular ECs from various origins such as HUVECs with glial cells (Naik, 2012). These glial cells are seeded alongside the ECs on the outer surface of the same hollow fibers and artificial capillaries are connected to a media reservoir through a gas-permeable (e.g., silicon) tubing system for the exchange of O_2 and CO_2 (Fig 5).

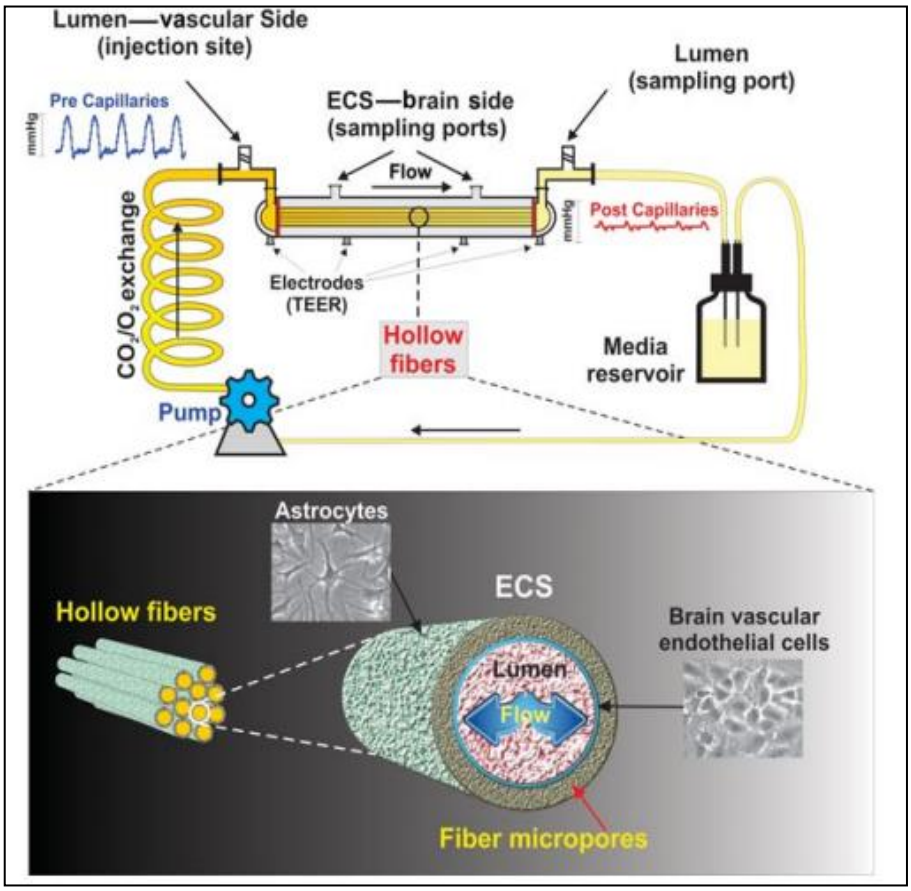


Figure 5: A schematic representation showing the set-up of the DIV-BBB with the co-culture chamber illustrated in detail, capillary system, pulsatile pump and gas exchange system. Source: Naik et. al.(2012). Pharm Sci.

A pulsatile pump generates the intraluminal flow, which can be regulated to produce (based on the diameter of the hollow fibers and the viscosity of the medium) intraluminal pressure physiologically comparable to that observed in capillaries *in-vivo* (Naik, 2012). The same computer controlled pump can be used to reproduce the rheological features of other vasculature (e.g. venules and large arteries), thus further expanding the usability of this system in vascular research (Naik, 2012). In addition, this system allows for circulation of blood cells, which enables to study a number of cerebrovascular/systemic pathologies related to rheological impairments and/or inflammation including hypoperfusion, cerebral ischemia and brain edema (Naik, 2012). The DIV-BBB can be established using primary or immortalized cell lines and allows for the inclusion of other relevant cell types (Naik, 2012).

Among its limitations, its design does not allow for visualization of the intraluminal compartment to assess morphological and/or phenotypic changes of the vascular endothelium. Also, in contrast to conventional static BBB models (e.g., Transwell), this apparatus is not designed for a high-throughput pharmaceutical study, and the technical skills/time required to establish the system is significantly higher (Naik, 2012). Cell characterization is possible but limited because it requires harvesting the cells from either compartment, an invasive procedure that can alter a number of morphological and physiological characteristics of the cells (Naik, 2012). Additionally, the initial cell load requirement to establish the DIV-BBB model is quite significant, (more than a million cells) especially if primary cultures are to be used. Given the limitations of all these systems, it still remains a challenge to study the BBB using in-vitro models as there isn't a single model that could directly recapitulate the physiological microenvironment of the

human BBB. More studies and advances in this field are needed in order to make an impact that will translate into treatments that are effective enough for the eagerly waiting patients.

BBB Penetration and Therapeutic Implications

Efforts to penetrate the BBB have been made including direct catheter insertion, delivery by convection, changing the drug agent into a lipophilic substance or attaching the drug to a carrier that can initiate penetration and transport through the BBB. Additionally, other methods have interrogated the permeability of the BBB through injection of hypertonic solutions into the carotid artery to temporarily alter and disrupt BBB permeability (Kinoshita, McDannold, Jolesz, and Hynynen, 2006). The downside to these methods is that most of them are quite invasive and could lead to irreversible gross effects to the BBB contradicting the value of the therapy.

MRI-Guided Focused Ultrasound: Kinoshita et al. demonstrated a novel technology for BBB penetration using ultrasound energy and Magnetic Resonance Imaging (MRI) to facilitate BBB disruption. The combined use of focused ultrasound and MRI facilitates image-guided target planning and real-time temperature mapping during sonications of various brain tumors as demonstrated in numerous clinical trials (Kinoshita, 2006). Using gas-bubble based ultrasound contrast agents in conjunction with ultrasound exposure, ultrasound can cause bio-effects such as temporary changes in cell membrane permeability to cause disruption of the BBB (Kinoshita, 2006). The group showed that

intravenously administered dopamine D_4 receptor-targeting antibody crossed the BBB and recognized its antigens and via MRI was able to monitor the level and extent of BBB disruption (Kinoshita, 2006). The issue with this novel technology though is its implementation in human studies. While this method shows promise on the pre-clinical animal study stage, it has limitations in humans due to its invasiveness, signal attenuation and distortion from the skull as it needs implantation in an opening to directly apply energy to the brain (Kinoshita, 2006; Azad, Pan, Connolly, Remington, Wilson and Grant, 2015). Furthermore, like most CNS biotherapeutic agents being administered via IV, bioavailability in the CNS becomes an issue and therefore lacks clinical relevance.

Osmotic Disruption: The concept of osmotic disruption involves delivery of a hyperosmotic agent causing water to egress from endothelial cells, which results in shrinkage of endothelial cells and thus dysfunction of tight junctions (Azad, 2015). This dysfunction leads to permeability of the BBB allowing for a small therapeutic window of several hours in which the biotherapeutic can be administered (Azad, 2015). Mannitol is a commonly use disruptor of the BBB via this method which several studies claim increases the concentration of therapeutic agents in the brain by 90-fold (Azad, 2015). The down side with this method is that the permeability of the BBB is non-selective which becomes problematic as it raises concerns of toxicity to the CNS.

Bradykinin Administration: Administration of bradykinins has been shown to upregulate caveolin-1 and caveolin-2 at the BBB, upregulation of which increases brain endothelial cell permeability (Azad, 2015) . This method's central limitation is that the

effect of the upregulation and thus cell permeability is exceedingly transient therefore showing minimal therapeutic benefits.

Radiation-Induced Disruption: The use of radiation therapy to induce DNA damage hence cell death has shown utility in recent studies to induce selective disruption of the BBB (Azad, 2015). Both animal and human studies have demonstrated that radiation therapy can induce focal BBB disruption with minimal effects on normal vasculature (Azad, 2015). The down side to this method is obviously DNA damage, which is irreversible and hence may cause unwanted gross effects (Azad, 2015).

Convection-Enhanced Delivery (CED): First described in 1994 by Bobo et al., it involves use of surgically implanted catheters that enable continuous delivery of chemotherapy directly into the tumor through positive pressure micro perfusion (Azad, 2015). In some cases, nanoparticles are delivered via CED, which presents challenges due to inability to have clearance strategies for these nanoparticles. In addition, this technique has major drawbacks in terms of being extremely invasive and limited drug/biotherapeutic distribution due to backflow. Its safety and efficacy profiles are yet to be determined (Azad, 2015).

Viral-mediated circumvention: This involves the use of viral vectors to deliver therapeutics in brain cancer treatment. Mostly used for brain tumors that have acquired chemotherapy and drug resistance, its strategy is to target tumors via cell surface receptors and use virus replication derivatives to combat tumor growth (Kinoshita, 2006; Azad, 2015). The value of using viral entities is primarily because viruses are small and therefore can enable penetration across the BBB. *In-vivo* studies with the measles virus

demonstrated a cytopathic effect on glioma stem cells and prolonging survival in a mouse model (Azad, 2015). Additionally, Toca 511, a retroviral replicating vector has been shown to safely deliver cytosine deaminase gene and improve survival for glioblastoma models *in-vivo* (Azad, 2015). This technique shows promise especially when used in conjunction with radiation therapy but carries a certain level of risk associated with the virulence ability of using viruses or viral entities in the CNS (Azad, 2015).

Carrier Molecules: Additional strategies for BBB penetration and drug delivery into the CNS involve the use of carrier molecules to transport drugs across the BBB. These carriers have evolved over time and allowed the use of surface modifications to optimize transport and targeting capabilities. Various nanoparticle carrier systems exist including but not limited to dendrimers, fullerenes, nanoshells, carbon nanotubes, quantum dots, polymeric micelles and liposomes. Polymeric nanoparticles such as poly(lactic-coglycolic acid) and dendrimers have been studied at length in context to brain tumor treatments (Azad, 2015). Nanotherapeutic approaches also have used magnetic therapy to localize drug-carrying molecules whereby a carrier molecule with iron residues is guided to the tumor location with an external magnetic field. Although these approaches present challenges in accurately monitoring dosage as well as systemic toxicological effects due to lack of clearance from the brain, they offer promise in drug delivery.

Liposomal Delivery: By far, this delivery method shows the greatest promise in terms of delivery of biotherapeutics into the CNS space. In fact, no other delivery tool would be as applicable in this space without thinking of detrimental contradicting effects elicited by the delivery process or off-target toxicities. For instance, none of the metallic

or carbon-based nanoparticles could be used effectively due to toxicity issues and lack of clearance mechanisms. Direct intracerebral implantation by means of a viral vector, such as adenovirus or herpes simplex virus also provokes an inflammatory response and demyelination in many animals and virtually all humans (Schnyder and Huwyler, 2005). For liposomes, the profile is completely different granted that they are fully biocompatible, fully biodegradable and their controllable size opens up the potential for penetrating various biological barriers within the body, especially the BBB which may open new ways of thinking in context to drug delivery into the brain. Liposomal formulations show promise in terms of efficiency (prolonged circulation half-life and therefore more accessibility into the brain), specificity to target via a targeting component (increasing concentration in the microenvironment), biocompatibility and biodegradability (Schnyder, 2005).

Nanotechnology and Nano-therapeutics in Modern Medicine

Modern medicine has taken huge strides in evolvement and more so in clinical drug delivery of therapeutic drug molecules. The application of nanotechnology in medicine, also known as nanomedicine, offers the prospect of powerful new tools for the treatment of human diseases and the improvement of human biological systems using nanotechnology to achieve medical benefit. The ultimate goal of this new field is to improve the process of diagnosing, treating, and preventing disease and traumatic injury, relieving pain and preserving human health, using molecular tools and molecular knowledge of the human body (Freitas, 2002; Zhang, Gu, Chan, Wang, Langer and

Farokhzad, 2008). Nanotechnology is a developing field that has been slowly evolving but has recently taken huge leaps forward within the last decade and that holds promise to developing effective treatments for many unmet medical needs. It is involved with production and manufacture of such entities as nano materials, nano tools, nano robots as well as nano devices. Applications with these different entities can range from health and medicine, electronics, transportation, energy and environment, space exploration etc (Freitas, 2002; Zhang, 2008).

In definition, Nanotechnology is the understanding and control of matter generally in the 1–100nm dimension range (Zhang, 2008) (Fig. 6). The nano scale is the place where properties of most common things are determined just above the scale of an atom, and where objects have at least one dimension (height, length, depth) that measures between 1 and 999 nanometers (1-999 nm) (Zhang, 2008). Engineered objects at this scale, also known as nanoparticles take on unique and novel properties that cannot be achieved at the bulk scale (Zhang, 2008). Development of nanotechnology started around 1958 and the various stages of development have been evolving steadily ever since with the last 20 years seeing the most exponential growth especially in pharmaceutical sciences. During the 1980s, three liposome-dedicated US start-up companies (Vestar in Pasadena, CA, The Liposome Company in Princeton, NJ, and Liposome Technology Inc., in Menlo Park, CA,) were the first in developing three different liposomal anthracycline formulations (Weissig, Pettinger and Murdock, 2014). Liposome technology emerged as the leader in this field, which led to the US Food and Drug Administration (FDA) approval of the first “nanodrug” in 1995 called Doxil® (Weissig, 2014).

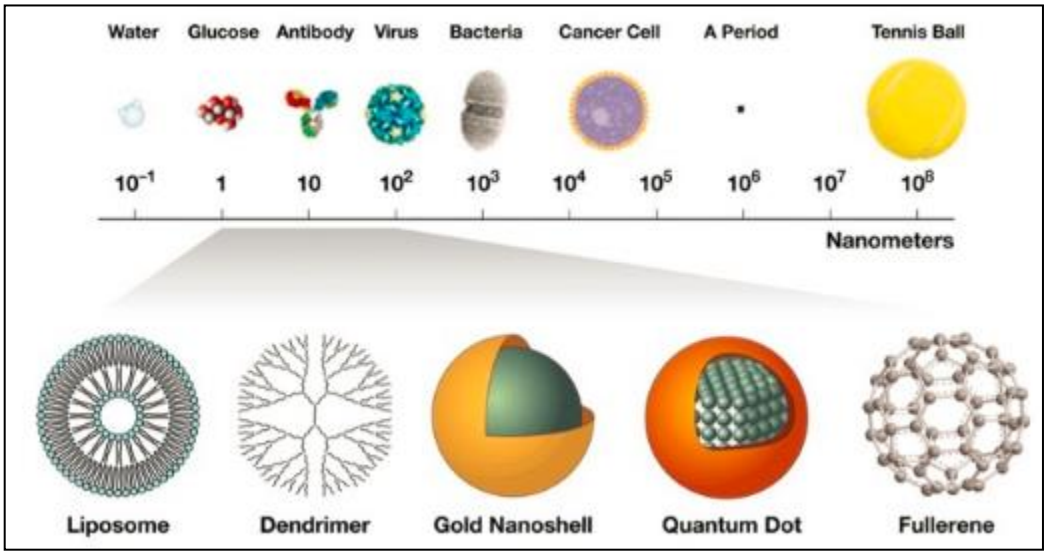


Figure 6: A schematic illustration showing the size scale of nanoparticles in comparison to other biomolecules. Source: National Cancer Institute (2014).

The concept of "nano" arose from emerging nanoscience and technology at the height of its abrupt growth in late 1990s to early 2000s (Table 1), and quickly became mainstream within the pharmaceutical science community. Colloidal gold, a traditional alchemical preparation, was re-invented into gold nanoparticles, and colloidal drug-delivery systems re-emerged as nanosystems of nanodrug delivery (Weissig, 2014). Based on major advances in nanoscale material science, the National Institute of Health (NIH) started the National Nanotechnology Initiative (NNI) in the year 2000 as a federal government program in order to promote nanoscience-related research and development (Weissig, 2014) (Table 1). As of January 2012, a comprehensive analysis of the worldwide state of investigational and approved nanomedicine products identified 67 commercialized nanodevices and 33 marketed nanotherapeutics. A total of 25 devices

and 122 therapeutics currently in development accounted for 789 ongoing clinical trials (Weissig, 2014).

Table 1: A summary showing some of the major developments in the nanotechnology industry beginning from inception to the most recent advances as of 2011.

Year	Selected Major Developments in nanotechnology
1959	R. Feynman initiated thought process
1974	The term nanotechnology was used by Taniguchi for the first time.
1981	IBM Scanning Tunneling Microscope
1985	"Bucky Ball"
1986	First book on nanotechnology Engines of Creation published by K. Eric Drexler, Atomic Force Microscope
1989	IBM logo was made with individual atoms
1991	S. Iijima discovered Carbon Nano tube for the first time.
1999	1st nano medicine book by R. Freitas "Nano medicine" was published
2000	For the first time National Nanotechnology Initiative was launched
2001	For developing theory of nanometer-scale electronic devices and for synthesis and characterization of carbon nanotubes and nano wires, Feynman Prize in Nanotechnology was awarded
2002	Feynman Prize in Nanotechnology was awarded for using DNA to enable the self-assembly of new structures and for advancing our ability to model molecular machine systems.
2003	Feynman Prize in Nanotechnology was awarded for modeling the molecular and electronic structures of new materials and for integrating single molecule biological motors with nano-scale silicon devices.
2004	First policy conference on advanced nanotech was held. First center for nano mechanical systems was established, Feynman Prize in Nanotechnology was awarded for designing stable protein structures and for constructing a novel enzyme with an altered function.
2005-2010	3D Nano systems like robotics, 3D networking and active nano products that change their state during use were prepared.
2011	Era of molecular nano technology started

Note 1: Retrieved from Nikalje et. al.(2015). Med. Chem.

The Case for Nanomedicine

An obvious advantage of nanotechnology is the ability to manipulate the size of the resulting entities thereby allowing different modalities that could be used for a wide variety of applications. Nanomedicine offers the promise of revolutionizing different treatment approaches where conventional therapeutics have not been effective. It makes early detection and prevention, improved diagnosis, proper treatment and follow-up of diseases possible, using nano materials, nano electronic biosensors, nano robots and nanoparticles (De Jong, and Borm, 2008). The therapeutic properties of either existing drugs or new drug entities can be improved by proper designing of drug delivery systems thus making failed therapeutics effective and safe for the patients.

Nanomedicine offers several inherent advantages versus traditional conventional drug therapies. Delivery methods utilizing nanotechnology enable many drugs to be more soluble and biocompatible, increases absorption and half-lives, and simplify treatment plans. The goal of research using nano-drug delivery include; more specific drug targeting and delivery, reduction in toxicity while maintaining therapeutic effects, greater safety profile and biocompatibility (De Jong, 2008). Ultimately, this result in lower administration dosage and more directed administration of therapeutics, thus creating a safer and more effective treatment option that alleviates all concerns pertaining to toxicities in healthy tissue (De Jong, 2008). An added advantage in developing nano-therapeutics over traditional therapeutics is the pace at which nano-therapeutics are being developed through a pipeline from inception to the market. With such a high unmet need,

patients are eagerly waiting for therapies over a long period of time with many dying within that waiting period. On average, it takes a pharmaceutical company 16 years to take one drug through the discovery and development pipeline from candidate selection to approval with an average cost of $500M - $1B yet the success rate is miniscule (McNeil, 2005) (Fig. 7). Out of the 5,000 – 10,000 compounds that enter the high throughput screening stage, only one compound successfully attains FDA approval resulting in wasted time, revenues and a prolonged period to get the compound to patients who really need treatment (McNeil, 2005) (Fig. 7).

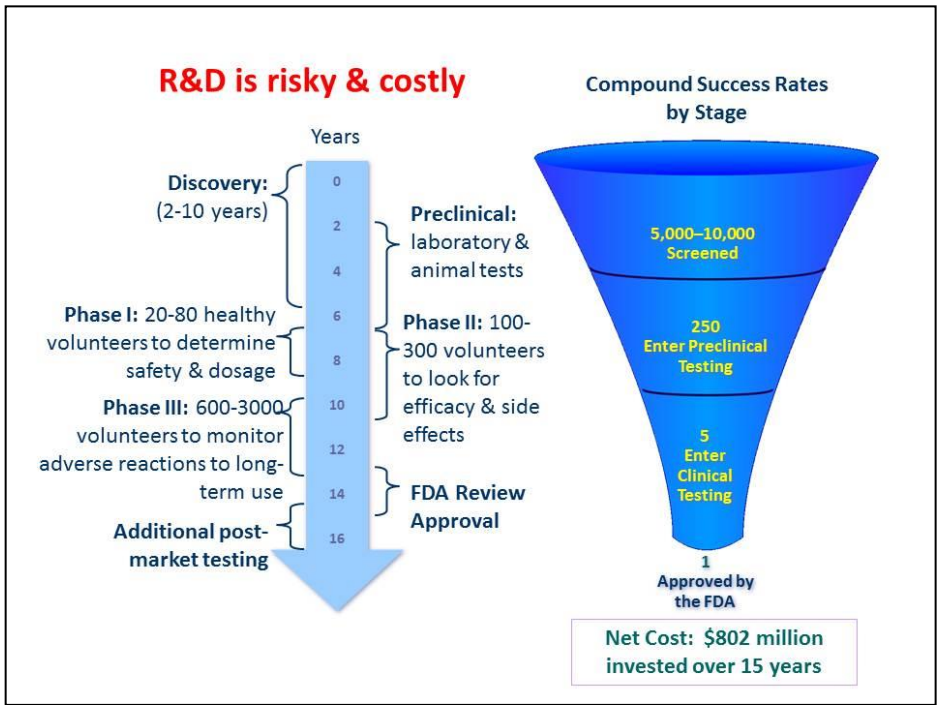


Figure 7: A schematic Illustration of the drug discovery and development process for the conventional traditional pharmaceutics. Source: BartsMS blog (2015).

Most of the potential lead candidates will likely fail this process due to factors such as lack of solubility therefore no absorption, a poor distribution profile, poor metabolism and lack of excretion therefore causing systemic toxicities (McNeil, 2005). Nanotechnology however has the potential to change the tide and revolutionize the industry by enabling compounds that would otherwise fail FDA approval to go through the process and pass the test. The ability to run high throughput screening during lead candidate selection eliminates the tedious complicated process of trial and error in discovery research which takes approximately 2-10 years (McNeil, 2005) (Fig 8).

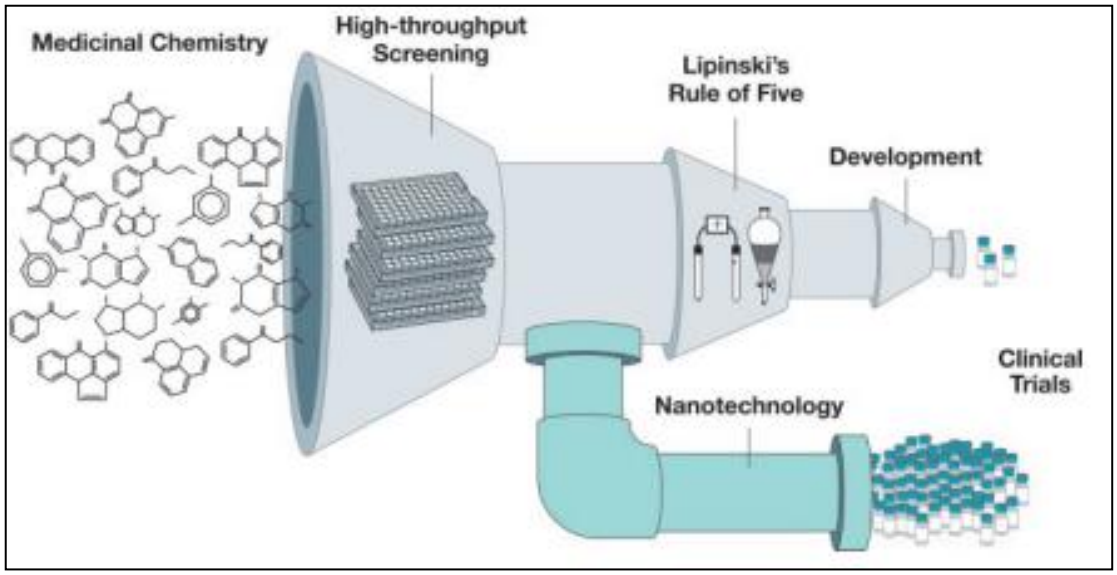


Figure 8: A schematic diagram showing how nanotechnology could change the landscape of drug approval process by allowing most of the drugs that could have otherwise failed FDA approval into safe and efficacious drugs available for clinical trials. Source: McNeil (2005) Leuk. Bio.

High throughput alone is projected to cut discovery time by half therefore ensuring that therapeutics get through the pipeline faster, saving revenues while at the same time ensuring that drugs get to the patients fast enough to alleviate the high unmet medical

needs (McNeil, 2005) (Fig 8). This attribute alone is projected to cut discovery time by half therefore ensuring that therapeutics get through the pipeline faster, saving revenues while at the same time ensuring that drugs get to the patients fast enough to alleviate the high unmet medical needs (McNeil, 2005) (Fig 8). The concept of encapsulating insoluble therapeutics in or on soluble nano-carriers allows favorable outcomes with clinical benefit whereby the therapeutics maintain a good profile in terms of a drug's pharmacokinetics in the human body, including their absorption, distribution, metabolism, and excretion (ADME). These attributes therefore enable progression of therapeutics that would otherwise fail FDA approval through the pipeline.

Nanoparticles for drug delivery

Nanoparticles are generally smaller than most human cells (10,000 –20,000 nm in diameter) and organelles and similar in size to large biological macromolecules such as glucose, enzymes, antibodies and receptors (De Jong, 2008; McNeil, 2005). These complex nanoscale systems consist of at least two components, one of which is the pharmaceutically viable active ingredient (chemically or biologically active) and the carrier function. Alternatively, the drug itself could have both attributes and in such case then the active drug itself would also be its own carrier. Some of the many reasons for these systems' attractiveness in medicine is based on their unique properties such as surface to mass ratio (that is large and controllable unlike that of other particles) and their quantum properties (Nikalje, 2015). These properties confer them the ability to adsorb and carry other compounds such as drugs, probes and proteins due to their relatively large

surface area. They also provide greater bioavailability due to their specificity to the target therefore allowing the use of smaller drug doses yet better efficacy with the least amount of toxicity (Nikalje, 2015). They are small enough to evade immune clearance (mononuclear phagocytic processing or enzymatic degradation), yet large enough to avoid rapid renal filtration. They can easily move across cell membranes as well as interact well with biomolecules (Nikalje, 2015). Overall, their success in nanomedicine drug delivery is based upon three facts: 1) efficient encapsulation of the drugs 2) successful delivery of drugs to the target and 3) successful release of the drug (Nikalje, 2015). These attributes ensure enhanced delivery and reduced toxicity of the free drug to non-target organs due to an increase in therapeutic index, the margin between the doses resulting in therapeutic efficacy (e.g., tumor cell death) (Nikalje, 2015). The main issues however in search for appropriate carriers as drug delivery systems pertain to design requirements from new materials. They include such attributes as drug incorporation and release, formulation stability and shelf life, biocompatibility, bio-distribution and targeting and finally, functionality (Nikalje, 2015). These factors are critical in drug delivery but the ultimate endpoint depends on the intended applications. Nanoparticles open up a new arena of powerful tools and approaches all geared towards the treatment of human diseases and the augmentation of human biological systems due to their interaction with system biomolecules on a tissue as well as cellular level.

Nanoparticle design Evolution and Requirements have evolved with the field and in parallel with studies investigating their responses on biological systems. Material design evolved whenever the effect of size, shape, or surface charge was further interrogated. Overall, three generations of nanoparticles have been engineered for biomedical

applications. The first-generation consisted of novel nanomaterials functionalized with basic surface chemistries to assess biocompatibility and toxicity (Albanese, Tang and Chan, 2012) (Fig. 9). The focus of these materials was to determine the effects of the most basic prerequisites for design requirements in context to drug delivery, which include size, shape and surface charge (Albanese, 2012). However, most of these studies included experiments in serum-free media or did not account for serum-protein interactions with their nanomaterials and did not interrogate clearance from peripheral blood, hence explaining their rapid clearance from circulation (Albanese, 2012).

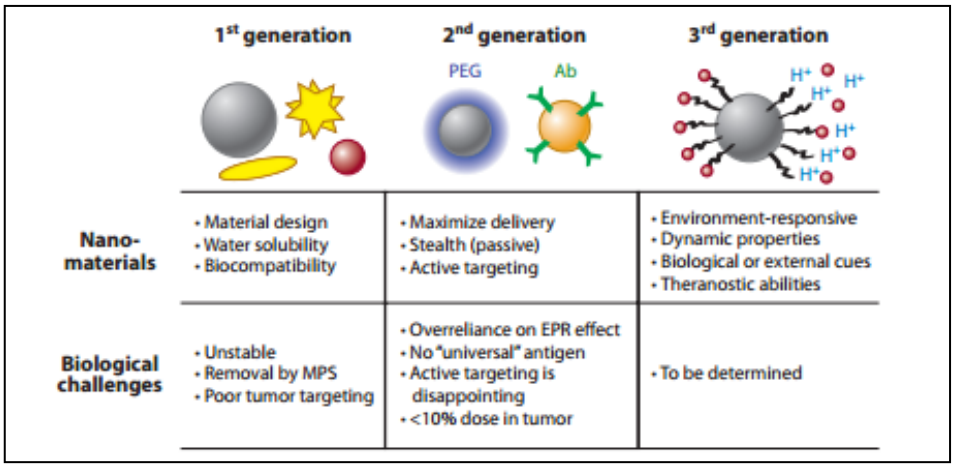


Figure 9: Schematic diagram showing the 3 generations of nanoparticles. Source: Albanese et al.(2012). Annual Rev. Biomed. Eng.

Second-generation nanomaterials emerged with optimized surface chemistries that improved stability and targeting in biological systems (Albanese, 2012) (Fig. 9). Surface chemistry modifications or functionalization enables evasion from immune clearance as well as increased the nanoparticles' blood circulation half-life. These attributes categorize second-generation nanomaterials by two important design characteristics; stealth and

active targeting (Albanese, 2012). The goal of stealth properties is to maximize blood circulation half-life to ensure that a nanoparticle remains in circulation long enough thus increasing its chances of entering the target microenvironment. Several studies have utilized polyethylene glycol (PEG) to show the increased half-life of nanomaterials by simply adding the polymer on nanoparticle surface. By so doing, PEG is able to act as "brush" against cellular components that would otherwise recognize and flag nanoparticles for immune destruction. PEG also acts as a protective barrier by keeping the nanoparticles from active interactions with serum proteins and enzymes that would otherwise digest and compromise nanoparticle and the encapsulated drug stability. Alternative stealth molecules such as serum proteins, lipids and silica have also been investigated, but PEG remains the most widely used approach (Albanese, 2012). Targeting ligands or moieties are used to potentially increase specificity for the target thereby increasing the therapeutic efficacy. The third-generation nanomaterials shifted the paradigm of design from stable nanomaterials to "intelligent" environment-responsive systems that could further improve targeted compound delivery (Albanese, 2012) (Fig. 9).

Effect of Size on nanoparticles (First-Gen): Among the most basic prerequisites for design requirements of nanoparticles for drug delivery is size. While large nanoparticles (> 150nm) may be desirable in drug delivery for increasing loading efficiency or increasing surface area to enable surface modifications, they are problematic as they tend to accumulate in the Liver, Lungs and Spleen (De Jong, 2008; Blanco, Shen and Ferrari, 2015) (Fig 10). On the other hand, small nanoparticles (< 20nm) can egress the blood

vessel walls and enter into tissue or are highly filtered out by the kidneys (Blanco, 2015) (Fig 10).

Effect of Shape and Charge (First-Gen): The same accumulation phenomenon also applies when cylindrical, disc-shaped and positively charged nanoparticles are used (Blanco, 2015) (Fig 10). Again, whatever the intended case application might be, each of these attributes might be exploited as an advantage or seen as a challenge.

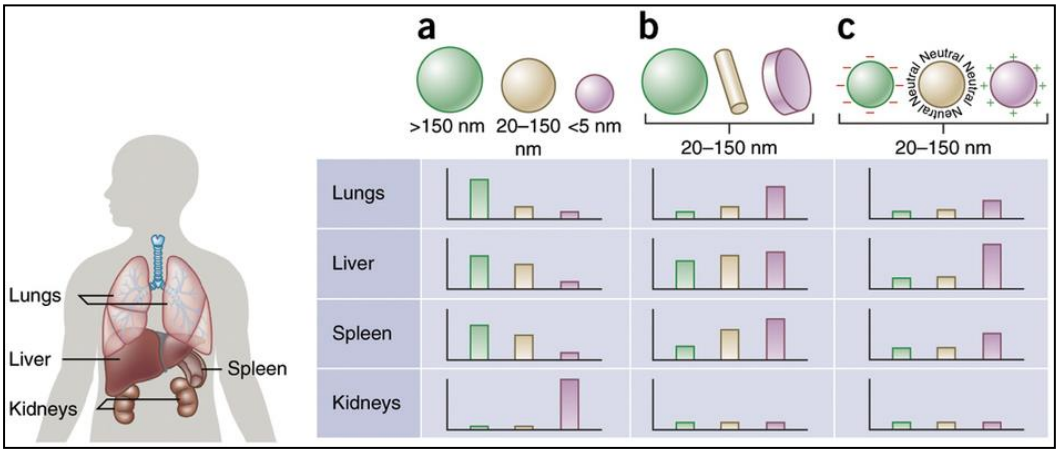


Figure 10: A schematic illustration of the effect of nanoparticle size, shape and charge on biodistribution and accumulation. Source: Blanco et. al.(2015). Nat. Biotech.

Interestingly, the attributes that seem undesirable in certain applications can alternatively be exploited to deliver drugs to specific intended sites. For instance, while it may be considered that small nanoparticle egression from circulation is a challenge, imaging technologies could potentially take advantage of that attribute using magnetic nanoparticles that can image metastatic lesions in lymph nodes because of their ability to exit the systemic circulation through the permeable vascular epithelium (McNeil, 2005; Blanco, 2015). Likewise, while positively charged nanoparticles have a tendency to be

accumulated in the liver (Fig. 10), the same could be exploited if the application is liver targeting to deliver therapeutics for liver disease such as liver metastasis or hepatitis (McNeil, 2005; Blanco, 2015).

Surface chemistry modifications or Functionalization (Second-Gen): Recognition components or targeting molecules ensure that once the nanoparticle enters circulation, it does not float blindly without a specific destination. The recognition component (moiety/flag/receptor) allows the nanoparticle to recognize specific elements at the intended destination thereby making it very specific to its intended target. This requirement is crucial in homing the molecule where its required and therefore minimizing off-target-associated toxicity and it also helps in increasing doses of the therapeutic by concentrating most of the nanoparticles on the target microenvironment (McNeil, 2005; Blanco, 2015). This component can be a humanized monoclonal antibody that has high specificity and affinity to the expressed receptor on the target site thereby ensuring that the nano-particle only targets that receptor and that there is no cross-reactivity to any other receptor (McNeil, 2005; Blanco, 2015) (Fig.11). Detection components or image contrast agents (Fig. 11) are useful but only in specific cases where the nanoparticle requires tracking via imaging tools or specific probes but not useful if appropriate studies have been conducted to establish that the nanoparticles reach their intended destination. This component is specifically important in cancer treatment or in administration of other toxic payloads in tissue to ensure that before off-loading the toxic drug onto tissue, all the administered drug has reached its intended destination (McNeil, 2005; Blanco, 2015). Immune-evasion or stealth components ensure that once the nanoparticle enters circulation, the immune system does not tag or flag the nanoparticles

for phagocytic destruction or enzymatic degradation and neither does it cause an immune response that would contradict treatment benefits.

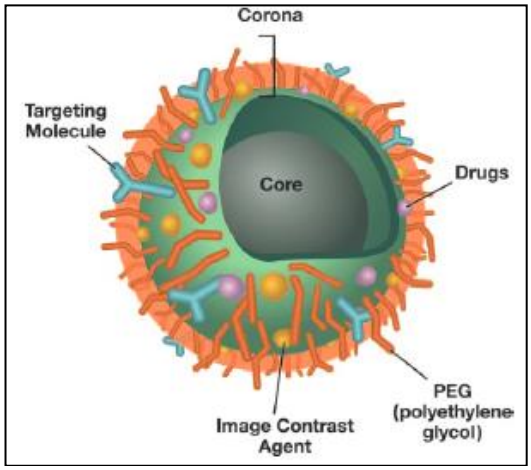


Figure 11: An adaptation illustrating different surface chemistries on a second generation nanoparticle to make it stealth (PEG), specific (Targeting molecule) and trackable (Image contrast Agent). Source: McNeil (2005). Leuk. Bio.

Evasion from an immune attack is critical as it ensures prolonged blood circulation half-life. Although several approaches have been made using different molecules such as silica and lipids, Human Serum Albumin (HSA) and PEG remains the mostly widely used molecules (McNeil, 2005; Blanco, 2015). While HSA allows nanoparticles to pass through and co-exist in circulation with immune cells and components while masquerading as a host molecule without being recognized by the immune system, PEG acts as a "brush" to "brush off" any immune components that may interact with the nanoparticles thereby avoiding active interaction with immune components (McNeil, 2005; Blanco, 2015) (Fig. 11). An extra-added advantage of PEG is that it prolongs circulation half-life of the nanoparticles thereby allowing the nanoparticles the

opportunity to reach the intended target before being recycled or metabolized, as would most host proteins in circulation (McNeil, 2005; Blanco, 2015). Taken together, design requirements are critical and cannot be ignored in the early planning of studies as they dictate the specific type and composition of nanoparticle to be used for the specific application in drug delivery.

Types of Nanoparticles

Many types of nanoparticles exist and are used for various applications including drug delivery, hyperthermia therapy (e.g. application of localized excessive heat to burn a tumor), optical imaging via incorporation of tracking tags and as contrasting agents for MRI. They can be differentiated based on composition, structure or properties; however, given their heterogeneity it is difficult to have pure compositions (Ageitos, Chuah, ang Numata, 2016). Depending on the type of nanoparticle, cargoes can interact with nanoparticles either covalently, non-covalently (electrostatic or hydrophobic interactions, hydrogen bonding) or can be physically entrapped in the matrix (Ageitos, 2016). Different source materials are used to engineer nanoparticles each offering specific benefits in context to the intended application. Source materials may be of biological origin like phospholipids, lipids, lactic acid, dextran, chitosan, or have chemical characteristics like various polymers, carbon, silica, and metals (De Jong, 2008; Ageitos, 2016). Additionally, based on the applied nanochemistry and the source materials, many types can be engineered of different shapes, sizes and surface charge including, Carbon Nanotubes (CNTs), Fullerenes, Nanopeapods, Nanocups, Quantum Dots, Gold

Nanoparticles, Iron-oxide nanoparticles, Polymeric nanoparticles, Polymeric micelles, Dendrimers, Nanowires, Liposomes and Virosomes (Ageitos, 2016) (Fig. 12).

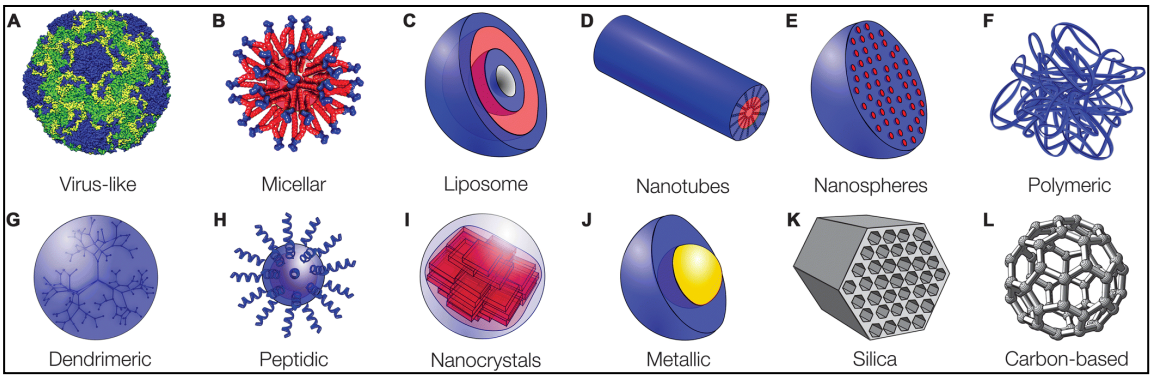


Figure 12: An illustration showing schematic diagrams of different types of nanoparticles based on source material and loading efficiencies. Different nanoparticles entrap cargo either in the core, between layers, on the surface etc. Source: Ageitos et al.(2016). Royal Society Chem.

Virosomes or Virus-like Nanoparticles (VNPs) (Fig 12A): Amazingly for some nanoparticles, instead of using "top down" or "bottom up" approaches to make synthetic nanomaterials using certain source materials, hybrid delivery systems have been utilized. Specific molecules or organisms in nature can be exploited or "hijacked" to make stealth nanocarriers for specific delivery. Virosomes are non-infectious nano-sized pathogens (10–200nm) that would naturally deliver their genetic material into a determinate cell or organ, but can be exploited to deliver drug (instead of their genome) in a non-infectious fashion (Ageitos, 2016). They are mainly composed of proteinaceous materials (capsid) that have provided the basis for the development of drug and gene nanocarriers (Ageitos, 2016). The key to their utility is because viruses have evolved well enough for centuries allowing them the trickery to evade the immune system and therefore infect certain

specific cells or organs. The fact that they already have specificity to specific components of the cells and other biological systems combined with evasion of immune attack make them effective tools for specific delivery. VNPs lack endogenous genetic material and thus their inability to replicate or to alter the host genome. They can be formed by self-assembly of capsid proteins over a functionalized inorganic nanoparticle core into well-characterized monodisperse structures (Ageitos, 2016). The capsid proteins can further be engineered and modified with different chemicals and proteins to promote specific targeting and improve their penetration properties.

Metallic Nanoparticles (Fig 12J): Metallic nanoparticles are heavily utilized in biomedical sciences because they can be prepared and surface-functionalized in many different ways (Ageitos, 2016). These nanoparticles are used in diagnostics as well as for drug and gene delivery because of the ability to be easily tunable over a broad range of sizes and shapes (Ageitos, 2016). They usually are composed of gold, platinum, titanium dioxide, copper, iron oxides or maghemite and can be functionalized via thiol-metal chemistry (Ageitos, 2016). Metallic nanoparticles can combine properties such as surface plasmon resonance, magnetism, or anti-oxidant capabilities. Among the most used in drug delivery applications are the colloidal gold nanoparticles, given that cells can internalize them without apparent cytotoxicity (Ageitos, 2016). In fact, shelling or coating nanoparticles made from other metals with gold have shown to reduce their cytotoxicity while increasing their stability. Gold nanoparticles are one of the most successful inorganic carriers in oncology therapeutics given their ability in the thermal ablation of tumors although those with quantum sizes (1.5nm diameter) have shown toxicity (Ageitos, 2016). Unfortunately, the toxicity profile observed at these sizes is

because of their ability to penetrate into the cellular nucleus and bind irreversibly to DNA (Ageitos, 2016). Moreover, most metallic nanoparticles have shown the initiation of production of reactive oxygen species (ROS) and oxidative stress, though ubiquitously (Ageitos, 2016). Taken together, the safety of the use of metallic NPs in vivo is hotly under debate, considering that divalent cations and heavy metals are toxic, causing deleterious irreversible damage and thus contradicting the value of their application in drug delivery.

Carbon-based Nanoparticles (CNPs) (Fig.12L) They include such CNPs as Fullerenes and Nanotubes, which are hollow, carbon-based cage-like NPs that have been employed for drug and gene delivery (Ageitos, 2016). These materials are constructed within a sheet of graphene arranged into small cylindrical or spherical structures. They can be formed using single or multi-walled graphene sheets and have different sizes and diameters depending on the synthesis conditions (Ageitos, 2016). Unmodified CNPs are insoluble, therefore requiring modification of their surface to improve solubility and reduce cytotoxicity (Ageitos, 2016). CNPs can be functionalized by the addition of compatible functional groups for the delivery of various biomolecular cargoes (Ageitos, 2016).The efficiency of their transport across cells is related to their hydrophobic nature, which allows penetration without damaging the cellular (Ageitos, 2016). However, these carriers have been reported to cause cellular apoptosis due to ROS production in the mitochondria and, similar to other fullerene compounds, they reduce the systemic immune response (Ageitos, 2016).

Silica Nanoparticles or Silicon dioxide NPs (Fig. 12K) are multifunctional structures available in micro- or mesoporous forms suitable for the encapsulation of various cargoes. Their low cytotoxicity and easy derivatization with different surface chemistries make them versatile tools for cargo delivery with excellent physicochemical stability (Ageitos, 2016). Silica NPs can be prepared as hollow or multichannel structures, which can be used for drug/gene cargoes while the mesoporous version of NPs present a high surface area and large pore volumes for functionalization (Ageitos, 2016). They have shown cytotoxicity caused by ROS generation, which could be correlated with size and surface charge (Ageitos, 2016).

Dendrimers (Fig 12G), are radially hyper-branched polymers with regular repeat units. They are attractive systems for drug delivery due to their highly defined dispersity, nanometer size range, spheroid-like shape and multi-functionality. Albeit easy to make, they have multiple copies of functional groups on their surface, which enables derivatization for biological recognition processes (Ageitos, 2016). Unfortunately, dendrimers are reported to cause hematological toxicity especially in non-functionalized version (Ageitos, 2016). Examples of typical dendrimers include poly(propyleneimine) (PPI), poly(amido amine) (PAMAM), poly(2,2-bis(hydroxymethyl)propionic acid (bis-MPA), poly(glycerol-succinic acid) (PGLSA-OH) or epsilon derivatives of PLL (Ageitos, 2016).

Peptidic NPs (Fig 12H), are based on the use of peptide sequences that promote cellular internalization known as cell-penetrating peptides (CPPs) or protein transduction domains (Ageitos, 2016). They are short peptides (6–30 amino acids) that are able to

cross the cellular membrane for intracellular trafficking of cargoes and have been postulated to have strong affinity for lipid bilayers hence their ability to be internalized by cells (Ageitos, 2016). Similar to other NPs, CPPs can be covalently linked to the cargo, forming a conjugate that promotes transport and internalization of the complexes via cellular pathways (Ageitos, 2016). Their downside though is that this covalent modification may alter the biological activity of cargoes and therefore alter their intended biological function (Ageitos, 2016).

Nanocrystals (Fig 12I), are associations of molecules in a crystalline form, composed of pure drug with only a thin coating of surfactants. Drug nanocrystals can be generated by "bottom-up" (intermolecular association) or "top-down" (milling of crystals) technologies (Ageitos, 2016). These NPs are composed of 100% drug without the addition of carrier materials such as polymeric NPs and have been more studied for material science than for drug delivery, given that not all therapeutic compounds can be easily crystallized (Ageitos, 2016). They are however the tool of choice for the oral administration of drugs, since their nano-scale size improves drug solubility and dissolution rate as well as increasing adhesion to the intestinal wall and capillary uptake.

Micelles (Fig 12B), are lipid-based carriers that enable transport of hydrophilic compounds and are more favorable in biological aqueous conditions. Hydrophobic cargoes can be transported by amphipathic NPs forming the classical core–shell carriers (Ageitos, 2016). Their hydrophilic part is exposed to the medium while forming a hydrophobic core. These NPs are particularly used in cancer therapy where encapsulation of chemotherapeutic agents has shown to reduce their cytotoxic effect and increase their

effectiveness. These NPs show good physical stability and biocompatibility although they have a short half-life in vivo and are removed from the blood circulation by the reticulo-endothelial system, particularly in the liver and the spleen (Ageitos, 2016).

Polymeric nanoparticles (PNPs) (Fig 12F): PNPs can be categorized into either non-biodegradable and/or biodegradable polymers. Biodegradable polymers, which are widely used for drug delivery can be divided into two groups, namely biopolymers (protein, peptide and polysaccharide) and synthetic polymers poly(lactic acid) (PLA) and poly(ε-carpolactone) (PCL) (Ageitos, 2016). Biodegradable synthetic PNPs comprise poly(glycolic acid) (PLA), poly(lactic-glycolic acid) (PLGA),4 poly(methyl methacrylate) (PMMA), or poly(L-glutamic acid) (PGA) (Ageitos, 2016) etc. Biodegradable PNPs have emerged as very useful tools in drug delivery due to their attractive properties including that they are biodegradable, biocompatible with biological systems and are very tunable for controlled drug delivery.

In fact, apart from liposomes which have seen multiple products get FDA approval and a long list still in clinical trials, biodegradable PNPs are the closest nanoparticles to liposomes. Combined together, liposomal drugs and polymer–drug conjugates are two dominant classes, accounting for more than 80% of the total amount clinically approved nanoparticle-based therapeutics (Zhang, 2008). These PNPs have the ability to be layered in “onion-like” layers that degrade at different rates to allow controlled drug release. This aspect is possible because the choices of polymers for the NP formation dictates a different degradation rate under selected conditions thereby allowing formation of PNP layers with distinct degradation rates. These different degradation rates are incorporated

into the synthesis process by making several layers of different polymers on which the drug is entrapped at either the same or different concentrations. This attribute is especially important in cancer therapeutics where several doses of different strengths may be required to ensure complete tumor destruction.

Non-degradable PNPs, including N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) and poly(vinylpirrolidone) (PVP) etc. do not induce significant cytotoxicity within biological systems (Ageitos, 2016) and can be used as an alternative. Natural polymers including albumin, silk, chitosan, and heparin have also been employed for the delivery of oligonucleotides, proteins, and drugs. The high versatility of these PNPs allows countless numbers of possible combinations hence even better versions of PNPs. PNPs can be conveniently prepared either from preformed polymers or by direct polymerization of monomers using classical polymerization or polyreactions (Rao and Geckeler, 2011). Methods like solvent evaporation, salting-out, and dialysis can be utilized for the preparation of PNPs from preformed polymers. A new method, the supercritical fluid technology, involves the rapid expansion of supercritical solutions or rapid expansion of supercritical solutions into liquid solvent for preparation of PNPs from preformed polymers. This method is especially amenable for preparation of NPs for drug delivery because it is free of surfactants or organic solvents which is usually the case in the preparation of most NPs.

Liposomes as Nanocarriers for Drug Delivery of Biotherapeutics

Liposomes are spherical-enclosed lipid vesicles with a bilayered membrane structure composed of natural or synthetic amphiphilic lipid molecules. They are easy to synthesize or prepare and can readily be loaded with a therapeutic agent encapsulated into or on them. Lipids used to make them are phospholipids that form a cell membrane-like phospholipid bilayer structure. They contain a hydrophilic phase inside the core and a lipophilic phase between the layers, and can therefore entrap both hydrophilic and hydrophobic loads at the same time. Liposomes have been widely used as pharmaceutical carriers in the last decade because of their unique and attractive abilities. They are considered non-toxic, biocompatible and fully biodegradable nanocarriers, hence their attractiveness. Among the cited attributes that make them especially suited for drug delivery include such attributes as encapsulation of both hydrophilic and hydrophobic therapeutic agents with high efficiency and protection of encapsulated drugs from undesired effects of physiological conditions. Furthermore, they can be functionalized with specific ligands (antibodies) that can target specific cells, tissues, and organs of interest, and can be coated with inert and biocompatible polymers like PEG prolonging their circulation half-life in vivo. Taken together, they form desired formulations with needed composition, size and surface charge among other properties (Loureiro, 2014; Zhang, 2008; Sercombe, Veerati, Moheimani, Wu, Sood and Hua, 2015) (Fig 13).

Overall, there are four different kinds of liposomes. They include conventional liposomes, PEGylated liposomes, Ligand-targeted liposomes and a combination of all the

three versions (Fig 13). Conventional liposomes were the first generation of liposomes to be developed. They consist of a lipid bilayer that can be composed of cationic, anionic, or neutral (phospho) lipids and cholesterol, which encloses an aqueous space. Research on the clinical potential of conventional liposomes began in the 1980s, whereby liposomal delivery proved useful for improving the therapeutic index of encapsulated drugs, such as doxorubicin and amphotericin (Sercombe, 2015).

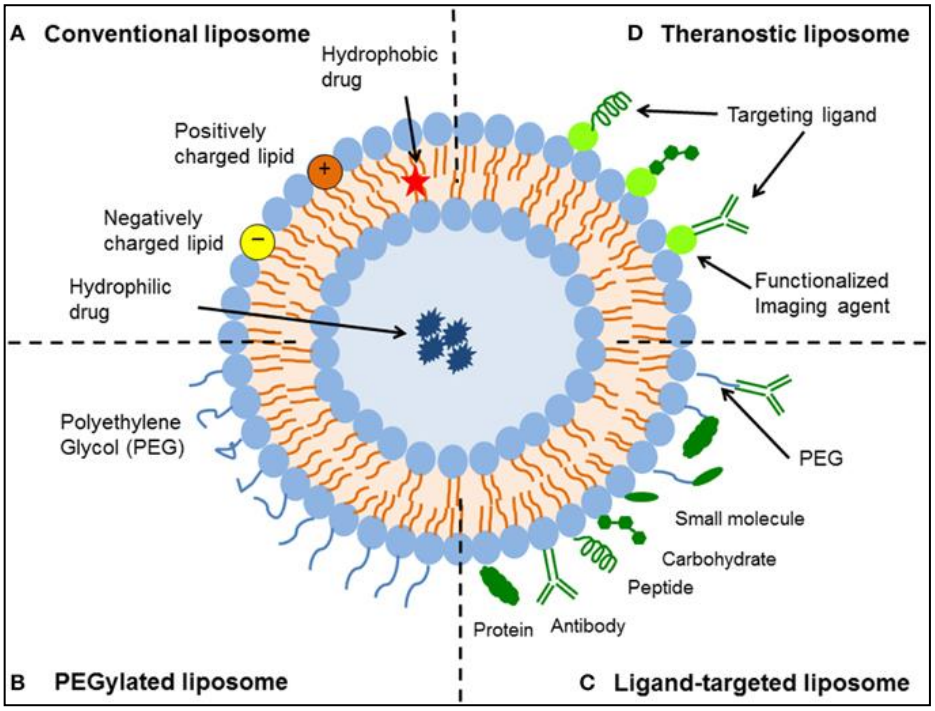


Figure 13: Schematic diagram showing the evolution of liposomes based on surface chemistries. Source: Sercombe et. al.(2015). Pharmacol.

When first developed, the idea was to reduce toxicity of compounds in vivo, through modification of pharmacokinetics and biodistribution to enhance drug delivery to diseased tissue in comparison to free drug. However, it was fast discovered that they were prone to rapid elimination from the bloodstream via opsonization and uptake by fixed

macrophages of the reticuloendothelial system (RES), mainly in the liver and spleen, therefore limiting their therapeutic efficacy (Sercombe, 2015). To improve liposome stability and enhance blood circulation half-life, PEG was utilized to make Sterically-Stabilized Liposomes (stealth) (Fig 13). This aspect reduced the elimination of drugs by prolonging blood circulation half-life with reported half-lives varying from 2 to 24 h in rodents (mice and rats) and as high as 45 to 90 h in humans, depending on the particle size and the characteristics of the coating polymer (Loureiro, 2014; Sercombe, 2015).

With further evolution of liposomal formulations, Ligand-Targeted Liposomes emerged and have since changed the landscape of drug delivery due to their vast potential for site-specific drug delivery (Sercombe, 2015). These liposomes can deliver drugs to designated cell types or organs in vivo, which selectively express or over-express specific ligands (e.g., receptors or cell adhesion molecules) at the site of disease making them highly effective with very minimal toxicity. Many types of ligands are available, including antibodies, peptides/proteins and carbohydrates (Fig 13). The coupling of antibodies, particularly monoclonal antibodies (mAbs), to create Immunoliposomes represents one of the more versatile ligands that can be affixed to liposome surfaces due to their stability and higher binding avidity because of the presence of two binding sites on each molecule.

When first introduced, Immunoliposomes were limited in terms of in vivo performance, due to poor pharmacokinetics and immunogenicity, thereby hindering their use in humans (Sercombe, 2015). This hurdle was solved by introduction of yet more complex liposomes, Theranostic Liposomes, which combine attributes of the Sterically-

Stabilized Liposomes and those of Ligand-Targeted Liposomes thereby introducing highly specific and stealth liposomes with better blood circulation half-life (Sercombe, 2015).

Even though it is still widely debatable as to whether stealth Immunoliposomes have therapeutic advantages over bare liposomes without the targeting ligands, a number of studies have demonstrated enhanced uptake and efficacy of ligand-targeted liposomes in diseased tissue in comparison to non-targeted liposomes in vivo. For example, folate-conjugated liposomes showed enhanced biodistribution in folate-expressing tumors in a murine model over non-conjugated liposomes (Sercombe, 2015). Additionally, ICAM-1 mAb conjugated loperamide-encapsulated liposomes, demonstrated increased efficacy and localization of the targeted nanoparticles to peripheral inflammatory tissue in a rodent model over non-targeted liposomes (Sercombe, 2015).

Doxil was the first liposomal-based formulation drug that was approved by the FDA for treatment of AIDS associated with Kaposi's sarcoma in 1995 (Northfelt, Dezube, Thommes, Miller, Fischl, Friedman, Kaplan, Du, Mamelok and Henry, 1998). By encapsulating doxorubicin (a widely used anticancer chemotherapeutic drug) into stealth liposome carriers comprised of hydrogenated soy phosphatidylcholine, cholesterol, and PEGylated phosphoethanolamine, Doxil dramatically prolonged doxorubicin circulation half-life and enhanced drug deposition in the tumor tissue (Northfelt, 1998). To date many liposomal formulations have been explored and studied extensively in the pharmaceutical sciences industry for various indications and have been marketed to treat various diseases (Zhang, 2008). Additionally various formulations are either in clinical

trials (Zhang, 2008) or in preclinical development (Zhang, 2008). Albeit all the successes in various indications, none have yet made an impact in CNS therapy and none seems to be in the horizon to treat CNS indications. This is partly associated with challenges associated with the BBB and the potential pitfalls that could lead to a high failure rate in therapeutics targeting this space. In context to liposomal delivery in CNS, a few attempts have been made but only with additional BBB disruption procedures to assist liposomal penetration across the BBB (Joshi, Singh, Wang, Chaudhuri, Ellis, Bruce, Bigio and Straubingere, 2014).

A few studies have investigated brain tissue uptake of liposomes after a short exposure following intra-arterial (IA) injections (Joshi, 2014). In another study, it was reported that there was 15-fold greater uptake of magnetically targeted cationic liposomes after IA injection, compared to intravenous injection (Joshi, 2014). Similar advantages of cationic liposomal preparations were observed during IA injection particularly when injected during transient cerebral hypoperfusion (TCH) (Joshi, 2014). To date, no study has yet investigated the role of nanoparticle surface charge in relation to deposition efficacy following IA delivery, or the effect of BBB disruption regimens that are under preclinical investigation for enhancing brain delivery (Joshi, 2014). In a separate rabbit model study, effects of concurrent disruption of the BBB upon brain deposition of a liposomal formulation of mitoxantrone that was optimized for systemic delivery following Intravenous (IV) administration was investigated (Joshi, 2014). However, that formulation, which was coated with PEG to confer extended blood circulation time, did not undergo appreciable deposition during IA delivery. In addition, it appeared that intra-carotid mannitol resulted in erratic and incomplete disruption of the BBB (Joshi, 2014).

For drug delivery into CNS to be considered viable and have a chance at clinical trials, any of these methods requiring BBB disruption will not hold as BBB disruption may cause exaggerated gross events thereby contradicting the value of treatment.

In another rat glioma model study, it was found that the surface charge of liposomes is a significant factor for deposition within the brain. The beneficial effect was noted independent of techniques disrupting BBB permeability, offering a safer and simpler method of administration (Azad, 2015; Joshi, 2014). Other studies have added compounds such as wheat germ agglutinin (WGA) to the liposome surface which aids in adsorptive endocytosis in the BBB, as this glycoprotein binds to negatively charged residues in the epithelial membrane (Azad, 2015; Joshi, 2014). Liposomes modified with WGA have been shown to reliably target glioma tumors both in vitro and in vivo, offering a possible area of research for glioblastoma treatment (Azad, 2015; Joshi, 2014). Overall, liposomal delivery without disrupting BBB permeability offers a new avenue for delivery of therapeutics into the CNS and could be the only saving hope for treatment of neurodegenerative diseases such as AD and PD. This method would be a far much better alternative than existing invasive procedures or conventional approaches, which have not been effective especially in treatment of these two debilitating diseases.

One of the biggest benefit and advantage of using liposomes is that they have already been approved for human use (Kraft, Freeling, Wang and Ho, 2014) and therefore it would not be an entirely new entity entering the clinic for human treatment. Of all the 15 liposome and lipid-based drug formulations approved for human use with other select candidates already in phase I/II/III clinical trials, interestingly, none of the FDA-

approved liposome or lipid formulations can actively target a specific site for specific and/or controlled drug delivery and release (Kraft, 2014). The fact that this space is unique and untouched creates a big incentive for the pharmaceutical industry in terms of unrealized revenues. It also allows a possibility of renewal of patents as well as filing new patents of failed drugs under new delivery mechanisms, which have the potential to become blockbuster drugs with huge financial gains for the industry.

Challenges/Limitations: Of the cited limitations for use of these delivery methods, one of the major setbacks for development and manufacturing of drugs in this category entails preparation methods. The synthesis and preparation of the liposomes poses a challenge when it comes to encapsulation of biotherapeutics from a therapeutic drug standpoint. The process involves use of organic solvents to dissolve lipid compositions and these solvents are objectionable in preparation of medicinal liposomes because of potential side effects to humans due to residual quantities of the solvents (Karn, Cho, Park H., Park J. and Hwang, 2013). Furthermore, the organic solvents could potentially render the biotherapeutic inactive thereby affecting expected clinical outcomes. In almost all cases, liposomal formulations use organic solvents due to the mixed lipid compositions to enhance solubility of the lipids into a homogeneous mixture (Karn, 2013), which improves liposomal stability and therefore better encapsulation efficiency.

Few methods have endeavored to substitute organic solvents with other components such as supercritical fluids (SCFs) but the results haven't been successful because the end product has yielded large liposomes (>1000 nm) that are unstable and that have high potential for accumulation in the kidney, liver and spleen due to size (Karn, 2013).

Although most encapsulation studies have been attempted in chemically active non-biological entities, successful encapsulation of active biotherapeutics (e.g. antibodies) for clinical use remains a challenge (Karn, 2013). This is due to the heavy reliance on organic solvent mixtures during the preparation process, which could contradict the clinical plan due to either potential to introduce organics in patients and therefore expose them to risk of toxic organics or the potential to ruin the biotherapeutic's native state and therefore its biological function or ability to be a therapeutic. Whether a biotherapeutic loaded in the core of the liposome or used as a tageting molecule for specific delivery, such conditions have potential to cause mis-folding of the native protein thereby altering its structure and therefore its biological activity. Identification of the right conditions is therefore essential to achieving success.

Monoclonal Antibody (mAbs):

Antibodies also known as immunoglobulins (Igs) are proteins produced by the plasma cells of the immune system to specifically bind foreign micro-organisms and render them inactive. There are five different classes of Igs (IgA, IgD, IgE, IgG and IgM) (Fig 14) and among them, the IgG is the most predominant comprising about 80% of all Igs in human serum (Wang W., Wang E. and Balthasar, 2008). Each antibody consists of four polypeptides; two heavy chains and two light chains joined to form a "Y" shaped molecule (The Biology Project: Antibody Structure; The University of Arizona, 2000) (Fig 14).

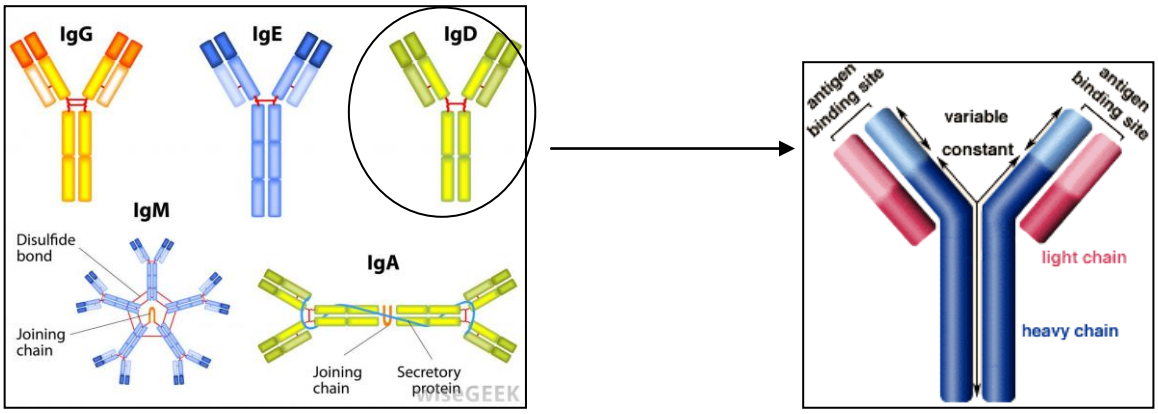


Figure 14: (Left) A schematic illustration of the different classes of antibodies based on structure. Adapted from: Shutterstock (2003-2017). (Right) A schematic diagram showing the structure of an antibody. Source: The Biology Project: (2000).

The amino acid sequence in the tips of the "Y" region (variable/hypervariable region) varies greatly among the different antibody classes. The variable region includes the ends of the light and heavy chains. This region, composed of 110-130 amino acids, give the antibody its specificity for binding antigen (The Biology Project: Antibody Structure; The University of Arizona, 2000). Treating the antibody with a protease can cleave this region, producing Fab fragments of antigen binding that include the variable ends of an antibody (The Biology Project: Antibody Structure; The University of Arizona, 2000). The constant regions on the other hand determine the mechanism used to destroy an antigen and are the basis for the different antibody classes and they also determine an antibody's immune function.

Antibodies have long being used for therapeutic purposes (immunotherapy) to specifically bind to cells or proteins for either inhibitory or stimulatory purposes. Due to their potency and high affinity to proteins, they can effectively bind to abnormal proteins

or the excess accumulation of normal proteins, as is the case of proteinopathies (Southwell and Patterson, 2010). In particular, most treatment approaches for the treatment of AD and PD involve using fusion antibodies directed against Aβ and Tau for AD and Anti-α-synuclein for PD (Southwell, 2010). This work started when it was discovered back in 1996 that anti- Aβ antibodies can dissolve Aβ aggregates in vitro and prevent aggregation of monomers (Southwell, 2010). Numerous mAbs are already in the market to treat various autoimmune diseases such as Multiple Sclerosis, Crohn's Disease, Rheumatoid Arthritis etc. Immunotherapy possesses several desirable pharmacological characteristics that make it attractive for CNS therapy (Southwell, 2010). First, antibodies possess very long serum half-lives and are hydrophilic making them easily soluble and they are not easily eliminated in peripheral blood (Wang, 2008; Southwell, 2010). Second, they are biocompatible to the human body and therefore do not elicit immune clearance by the macrophages (Wang, 2008; Southwell, 2010). They are very potent given their specificity, affinity to their targets and have very limited off-target and therefore evoke no toxicity in the body (Wang, 2008; Southwell, 2010). Lastly, they are easily degradable without the need of a complex process to clear and eliminate them out of the body (Wang, 2008; Southwell, 2010).

Albeit being very effective in CNS therapy approaches, their bioavailability in the CNS without the aid of a nanocarrier is very poor. When naked mAbs are used to target the CNS, most are unable to get access to the brain owing to their size (150kD) which restricts their penetration across the BBB (Loureiro, 2014; Southwell, 2010). In addition to restriction by the BBB, the few mAbs that make it across the BBB (~0.1% of total concentration) have a much shortened accumulation time in the brain due to a very

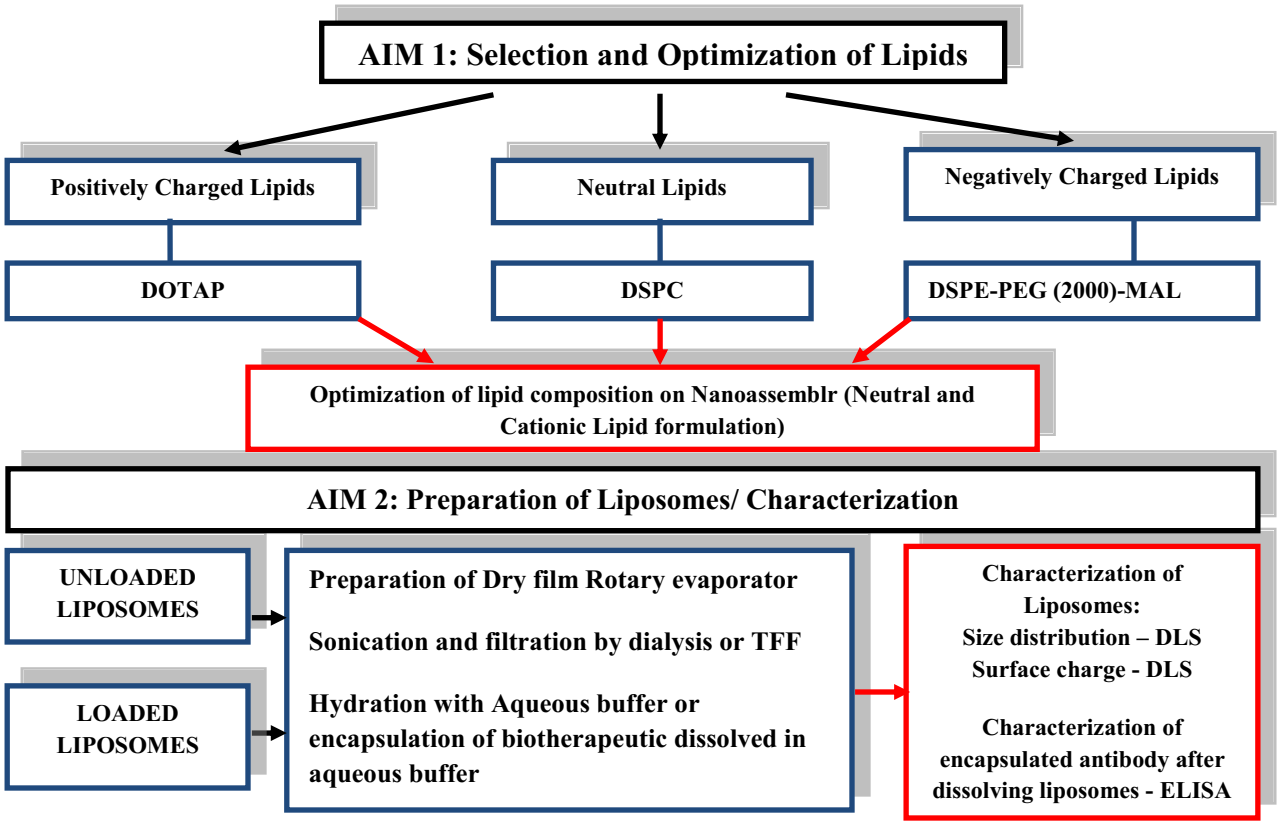
effective efflux system present in the brain to eradicate components that are otherwise not meant to be in the brain (Loureiro, 2014; Southwell, 2010). These hurdles therefore make it very challenging to reach an efficacious dose that would warrant a good clinical outcome despite their effectiveness. Combined with Theranostic Immunoliposomes, these hurdles could be eliminated since targeted stealth immunoliposomes have shown good promise in terms of bioavailability to the brain. The fact that antibodies are stable, have high specificity for their targets and have high avidity due to their dual binding epitope makes them very effective targeting ligands for targeted delivery.

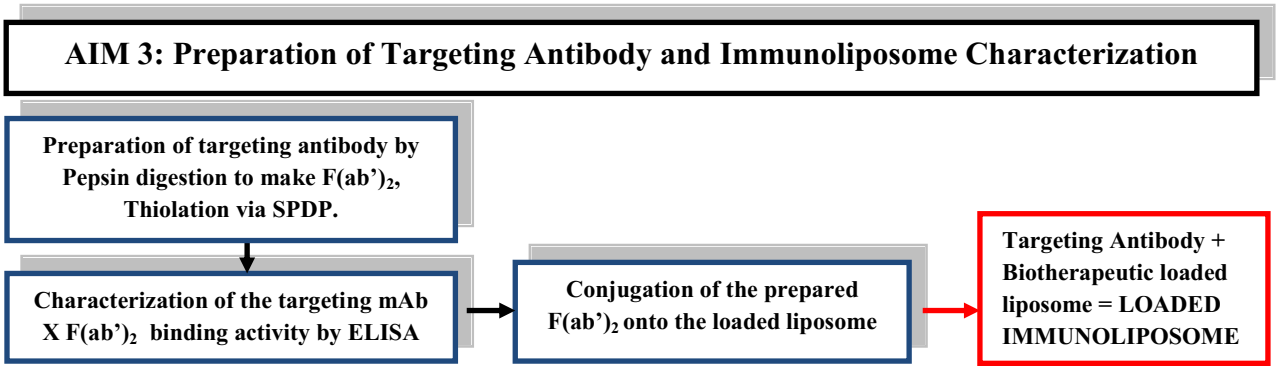
Chapter II

Research Methods and Materials

This section emphasizes and describes the research methods including the design of the project with key steps shown in the schematic of the design workflow. Various analytical methods were used for feasibility, formulation of liposomes and characterization of the liposomes. A systematic method was used for selection of lipids on the Nanoassemblr followed by preparation of liposomal preparations using the rotary evaporator and finally conjugation of targeting antibody onto the surface of the liposomes.

Research Design Workflow





Experiment Approach and General Considerations

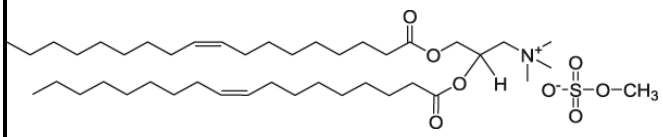
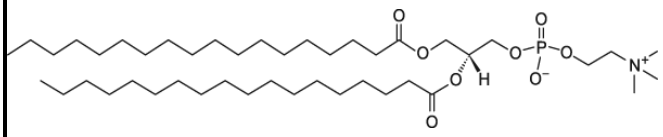
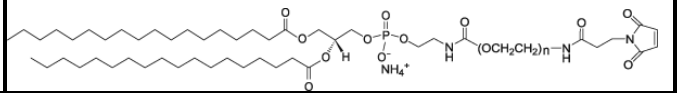
The depth of this work did not include screening different lipids in each class as that would have been time consuming and not possible to accomplish the thesis study within the allotted time. The work therefore includes lipids that have been tested in liposome formulations before and are known to produce good quality of liposomes. In particular, this work was lead and influenced by the work of three research papers. Maruyama *et.al.,* developed Fab'-PEG Immunoliposomes that were 100-130nm using 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), Cholesterol (CHOL) and dipalmitoyl-phosphatidylethanolamine N-[maleimide(polyethylene glycol)-2000 (DPPE-PEG-Mal) at molar ratio of DSPC/CHOL/ DPPE-PEG-Mal (2:1:0.2) (Kazuo, Nobuya, Toshiaki, Kazuhiro and Motoharu, 1997). They incorporated a modified lipid with a Maleimidyl terminal group into the liposome formulation that enable chemistries with the antibody Fab' fragment. When an antibody is digested into Fab' using enzymes, Thiol residues are exposed that allow conjugation into Maleimide terminals of the PEG without affecting the antigen binding activity of the antibody. The use of a Fab' fragment instead of a whole antibody allows 1) exposure of Thio residues for conjugation 2) increased antibody

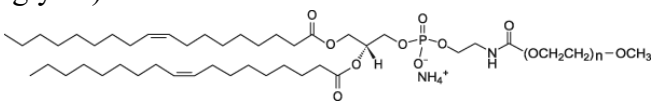
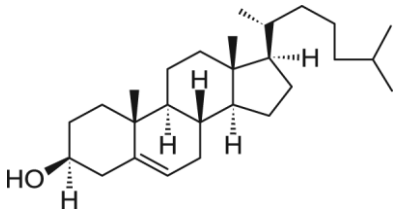
density on the liposomal surface and 3) potentially eliminates unwarranted activity of the Fc portion of the antibody such as Fc mediated effector functions on immune cells (Kazuo, 1997). In a separate study, another group used egg phosphatidylcholine (PC), Cholesterol (CHOL) and N-(3-(2-pyridyldithio)-propionate)-phosphatidylethanolamine (PDP-PE) at molar ratios of PC/CHOL/PDP-PE (12/6/1) in chloroform (Mercadal, Carrion, Domingo, Petriz, Garcia and De Madariaga, 1998) to make immunoliposomes with a MY-10 mAb against CD34+ cells. The mAb was conjugated with Thiolation using N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) and dithiothreitol (DTT). In a follow up study (Mercadal, Domingo, Petriz, Garcia and De Madariaga, 2000), they developed immunoliposomes using the same SPDP method for conjugation of the mAb for ex vivo applications. In this study though, they replaced the PDP-PE with PDP-PEG-PE to exploit he PEG/SPDP linkage chemistries (Mercadal, 2000). In addition to the cited works on liposome formulations, various groups have also summarized different approaches on liposome preparation using the Dry Film Hydration method (Samad, Sultana, and Aqil, 2007; Dua, Rana and Bhandari 2012). These methods of liposome formulation using the dry film hydration-rehydration methods were referenced and applied during formulation of liposomes for this project. On methods of conjugation of antibody ligands to liposomes using different chemistries, this thesis project was influenced by two groups that have previously done thorough studies on chemistries for attaching ligands to liposomes (Nobs, Buchegger, Gurny and Allemann, 2004; Ansell, Harasym, Tardi, Buchkowsky, Bally and Cullis, 2000).

Materials, Equipment/Platforms and Reagents

Lipids: Selection of Lipids for the experiments was influenced by the cited works and optimized to determine optimum molar ratios. All the lipids and PEG illustrated on the table below were purchased from Avanti Polar Lipids.

Table 2: The table below shows the lipids selected for liposomal formulation in this thesis project including their charge, source information and chemical structures.

Lipid Charge	Acronym/ Source and other Pertinent Information	Reagent Full Name/ Structure
Cationic	**DOTAP** Avanti Polar Lipids; DOTAP Chloride salt; MW: 698.55; Cat #: 252042; Lot # 181TAP-156; storage -20C or below	1,2-Dioleoyl-3-Trim ethylammonium Propane 
Neutral	**DSPC** Avanti Polar Lipids; MW: 790.15; Cat #: 252042; Lot # 180PC-147; storage -20C or below.	1,2-distearoyl-sn-glycero-3-phosphocholine. 
Anionic	**DSPE-PEG (2000)-Maleimide or Carboxy** Avanti Polar Lipids; MW: 2941.64 [Mal] / 2849.54 [Carboxy];	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide/Carboxy(polyethylene glycol)-2000] 

	Cat #: 474922-22; Lot # 180PC-147; storage -20C or below	
N/A	**PEG-PE (2000)** Avanti Polar Lipids; MW: 2693.32; Lot # 140PEGPE-40; storage -20C or below	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 
Neutral	**Cholesterol** Avanti Polar Lipids; 700100P; MW: 386.66; Lot # PCHOL-17; storage -20C or below	Cholesterol (Plant Derived) 

Antibodies and their processing accessories: Both the targeting monoclonal antibody (mAb X) and the monoclonal antibody biotherapeutic (mAb Y) were donated by the Translational Department at Biogen Inc. Antibody purification kits and buffer exchange desalting columns were purchased from Thermo Scientific. The SPDP cross-linker was purchased from Thermo Scientific. The $F(ab')_2$ preparation kit was purchased from Thermo Scientific. DTT was purchased from Sigma.

Buffers and Reagent Preparations: The following buffers on the table below were prepared at Biogen Inc.

Table 3: List of buffers made at Biogen Inc. for this thesis study

1.	Phosphate Buffered Saline with EDTA (PBS-EDTA): 100mM Sodium Phosphate, 150mM NaCl, 1 mM EDTA, 0.02% Sodium Azide pH 7.5
2.	Citrate Buffer: 0.05M Sodium Citrate, 0.05M Sodium Phosphate, 0.05M Sodium Chloride pH 7.0/pH 5.5
3.	DTT Solution: 2.5M DTT in 0.2 M Sodium Acetate pH 5.5
4.	20 mM SPDP Solution: 2mg SPDP into 320uL DMSO
5.	Phosphate Buffered Saline with EDTA (PBS-EDTA): 20mM Sodium Phosphate, 150mM NaCl, 1 mM EDTA, 0.02% Sodium Azide pH 7.5
6.	Acetate Buffer: 100mM Sodium Acetate Buffer, 100mM NaCl, pH 4.5

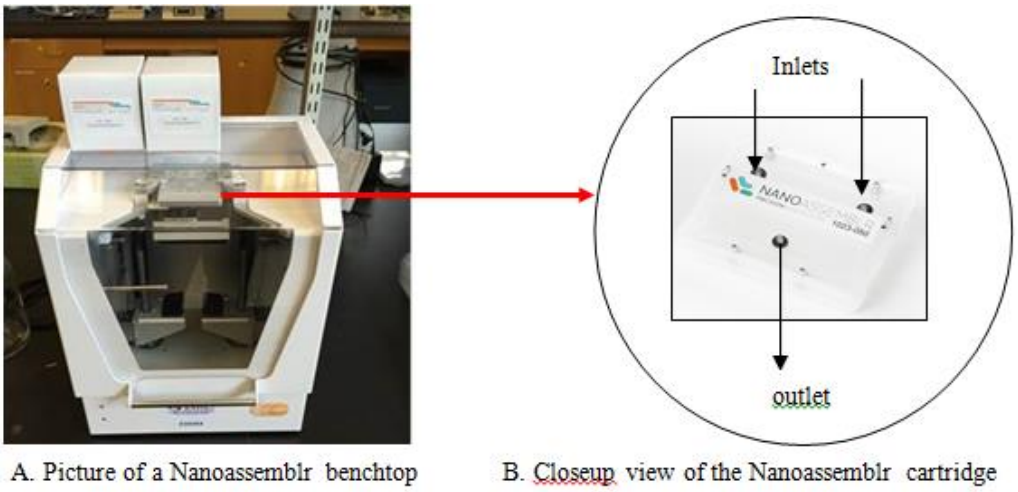
Equipment and Platforms

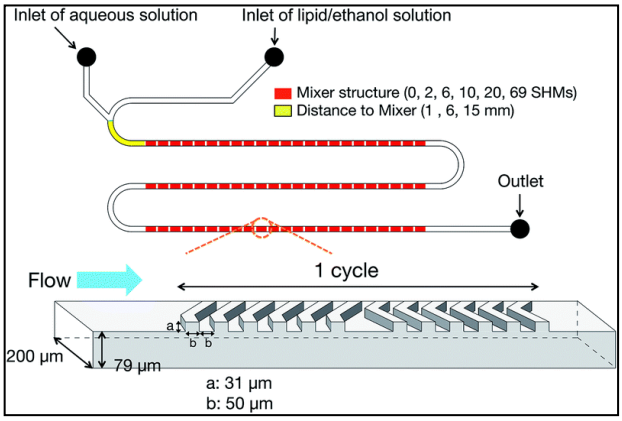
All the equipment listed below are property of the Analytical Development Department at Biogen Inc. The following equipment platforms were used throughout the course of this thesis project.

The Nanoassemblr Benchtop Platform (Precision Nanosystems):

This Platform, is a small formulation system that allows small-scale formulations (1-20 mL) per batch with ease-of-use nanoparticle formulation. With proprietary microfluidics, the technology enables controlled, bottom-up, molecular self-assembly of nanoparticles through millisecond mixing of components at nanolitre scale (Fig. 15). To set up the instrument, a lipid composition is dissolved in an organic solvent (ethanol) and put in a syringe. An aqueous buffer (PBS) is put in another syringe and then both are inserted on the inlet ports of the cartridge by screwing them in (Fig. 15). Using the precision nanosystem software, lipid compositions are optimized accordingly by adjusting

parameters such as organic to aqueous mixing ratios, flow rates, volumes etc. these parameters can be used to fine tune liposome size or titrate out organic solvents.





C.

Figure 15: A: Nanoassembler benchtop platform with location of the cartridge. Source: Precision Nanosystems (2016). B: Components of the Nanoassemblr cartridge showing inlets for the organic and aqueous phases. Source: Precision Nanosystems (2016). C: An illustration of the concept behind the nanoassemblr micro-mixing cartridge featuring microfluidic staggered herringbone micromixers. Source: Maekia et al. (2015). RSC Adv.

Zetasizer Nano (Malvern Instruments, Model ZSP):

The principle is based on fine particles and molecules in solution being in Brownian motion (constant random thermal motion) and therefore diffusing at a speed related to their size, with smaller particles diffusing faster than larger particles. The speed of Brownian motion is also determined by the temperature, and therefore precision temperature control is essential for accurate size measurement. A diluted sample is loaded on a cuvette and placed on the cuvette portal (Fig. 16). To measure the diffusion speed, a speckle pattern is produced by illuminating the sample with a laser and a beam is focused at a speficic angle through the sample using a mirror and lense (Fig. 16). The scattering intensity at a specific angle fluctuates with time, and this is detected using a sensitive avalanche photodiode detector (APD) (Fig. 16). The intensity changes are analyzed with a digital auto-correlator which generates a correlation function as a representation of the particle size and the particle size distribution (Fig. 16).

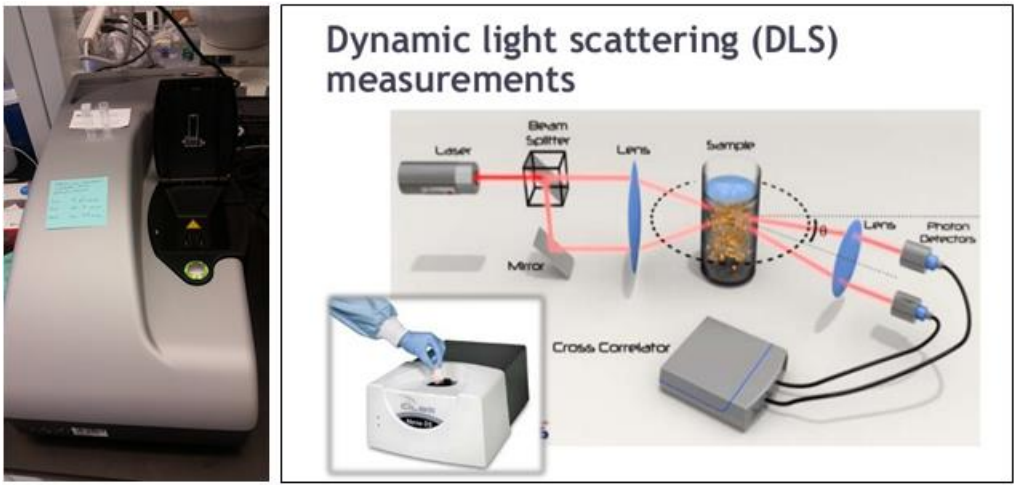


Figure 16: Picture taken from the zetasizer nano (ZSP) used in this study. Source: Biogen Inc (2016). (Right) schematic illustration depicting the principle of DLS. Source: In ® Slide Share (2015)

Tangential Flow Filtration (TFF) (Spectrum Labs, Model KrosFlo): This technique works with the principle of barrier separations to differentiate components based on size. It utilizes a cutoff membrane that separates particles based on the membrane pores (Fig. 17). Components larger than the membrane pore are quantitatively held back by the membrane while smaller components pass through the membrane structure along with the permeate. In this way, the feed stream passes parallel to the membrane face as one portion passes through the membrane (permeate) while the remainder (retentate) is recirculated back to the feed reservoir (Fig. 17). Although there are other methods for driving the separation process such as electric charge (e.g. caustic-chlorine cells) and diffusion (e.g. dialysis and oxygenation devices), Spectrum hollow fiber membrane modules are designed for applications in which pressure is the driving force (Fig. 17).

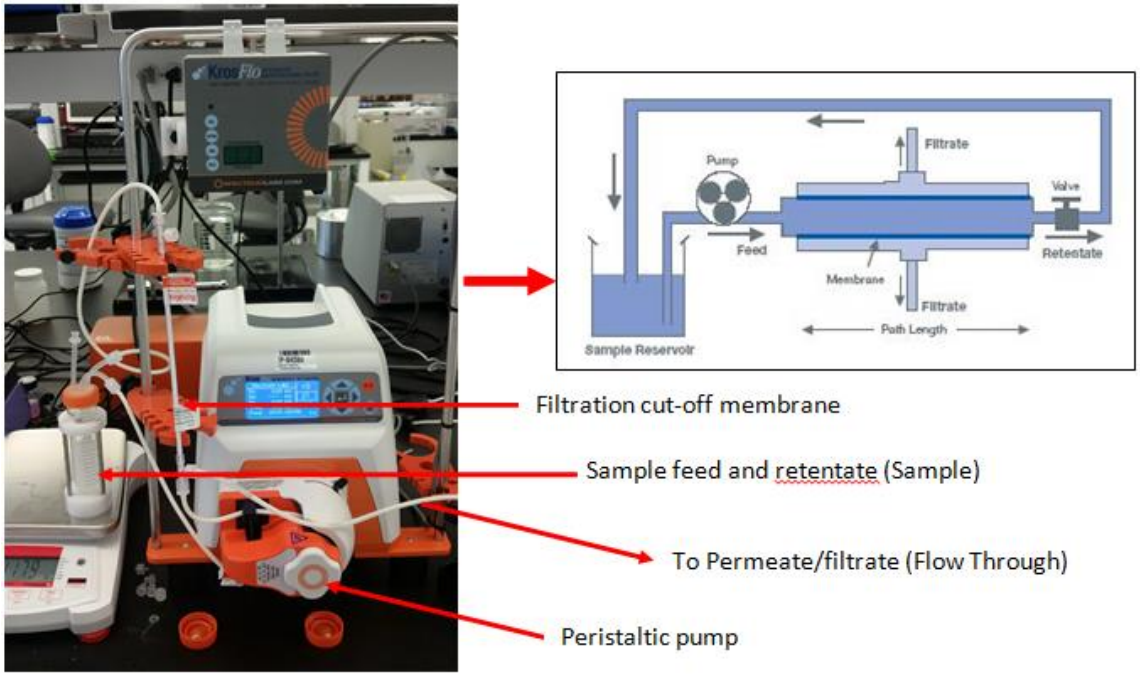


Figure 17: (Left) A picture of the TFF system that was used in this study (Source: Biogen Inc.). (Right) A schematic illustration of the filtration system depicting direction of flow from the sample feed, through the membrane and into either the filtrate vessel or back into the sample feed. Source: Schwartz et. Pall Corporation.

Rotary Evaporator (Buchi Rotavap, Model R-124): The rotary evaporator (rotavap) is a distillation system that works by heating the liposome formulation dissolved in an organic solvent under low pressure (vacuum) causing the organic solvents to evaporate at a lower temperature than normal and condensing them in a separate container (Fig. 18).

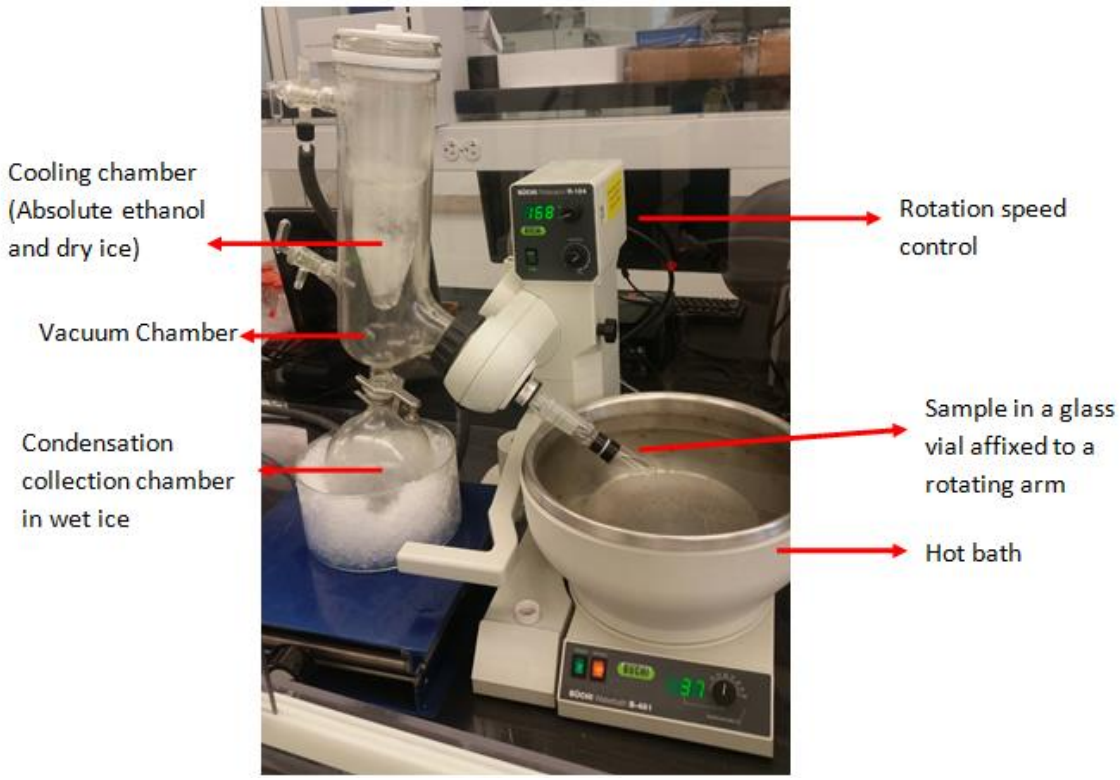


Figure 18: A picture showing set up of the rotary evaporator used in this study Source: Biogen Inc. (2016).

The rotary evaporator (rotavap) is a distillation system that works by heating the liposome formulation dissolved in an organic solvent under low pressure (vacuum) causing the organic solvents to evaporate at a lower temperature than normal and condensing them in a separate container (Fig. 18). This leaves the lipid formulation as a dry, thin layer on the walls of the source vessel, which can later be rehydrated with an aqueous phase containing the cargo to be encapsulated in the liposomes. The sample is affixed to a rotating arm to ensure uniform distribution of heat across the sample, which also allows submersion of the sample vial into a hot water bath (Fig. 18). Once heated, the sample vapor is quickly condensed and caught on a separate chamber in wet ice.

Bath Sonicator (VWR):

The concept is based on introducing high power ultrasound into a liquid medium, which creates alternating high and low-pressure cycles with rates depending on the frequency. The sound waves are transmitted in the fluid upon which during the low-pressure cycle, high-intensity ultrasonic waves create small vacuum bubbles or voids in the liquid. When the bubbles attain a volume at which they can no longer absorb energy, they collapse during a high-pressure cycle thereby enabling sizing specific size of particles at a particular wavelength. This concept is based on time as prolonged exposure continues collapsing particles in the next energy frequency. This instrument was for liposomal formulations to size them to a specific target size range and to determine effects of different sonication parameters on liposome size.

Nanodrop (Thermo Scientific, Model nd-8000):

The Nanodrop is a spectrophotometer that utilizes fiber optics technology to measure protein or nucleic acid concentration in a sample by allowing the sample to bridge two fiber optic ends (between pedestals). A xenon lamp flashes light across eight samples sequentially and finally the spectrometer utilizes a CCD array to analyze the light that passes through the sample. Data is derived as a spectrum of absorbance at 280nm for a purified protein, which is calculated as the concentration (mg/ml). This instrument was used for protein concentration check during pepsin digest of the targeting mAb X.

SpectraMax Plus 384 Microplate Reader (Molecular Devices, Model 340PC):

The SpectraMax Plus is a plate based spectrophotometer that uses almost the same concept as the nanodrop but instead uses a colorimetric technology to determine

absorbance of a certain protein concentration based on the intensity of the color change. This instrument was used for ELISA assays for determination of antibody concentration (both targeting and encapsulated) before and after processing.

Dialysis:

Dialysis is a simple method based on selective separation of molecules via diffusion across a semi permeable membrane to separate based on size. It is used in a variety of applications including buffer exchange, desalting, removal of labeling reagents. Dialysis was used in initial small scale preparations to remove or buffer exchange liposome formulations off of absolute ethanol.

Experimental Procedures and Methods:

Aim 1: Optimization of Lipid compositions on the Nanoassemblr Benchtop Platform:

The lipid molar ratios were selected based on previous work by Maruyama *et.al.,* (1997). Before dissolving lipids in organic solvents to inject into the Nanoassemblr benchtop platform, calculations were done to determine the Mass (mg) of each lipid to be added in order to determine ratios of lipid compositions. The following formula was used to determine the mass in grams of each lipid.

Equation 1: An equation used to calculate the Mass (g) of each lipid needed to achieve the appropriate molar ratios

Mass (g) = Concentration (moles/L) X Volume (L) X Molecular Weight (g/mol)

Experiment 1: Preparation of a Neutral Liposome Formulation: Based on the formula above, a neutral liposome formulation was made with DSPC/CHOL/DSPE-PEG (2000)-Maleimide (molar ratios 2:1:0.2). The following table summarizes the masses of each formulation dissolved in absolute ethanol (Table 4).

Table 4: Lipid composition showing the weights of each lipid for neutral charge liposome formulation

Lipid	MW	Molar Ratio	Molarity (mM)	Mass Needed (mg)
Cholesterol	386.65	1	6.25	12.1
DSPC	790.15	2	12.5	49.4
DSPE-PEG (2000)Mal	2941.64	0.2	1.25	18.4
Total Molarity 20 mM			Total Volume 5 mL	

The lipids were all weighed out into the same glass vial. 5 ml of absolute ethanol was added into the lipid mixture and vortexed for about 10 minutes at low speed until all lipids dissolved in ethanol forming a milky suspension. The Nanoassemblr platform was set-up and the software settings were made according to the table below (Table 5).

Table 5: Software Settings for the neutral (DSPC/CHOL/DSPE-PEG(2000)-Mal) formulation

Samples	Total Volume	Flow Ratio (Aqueous: Organic)		Total Flow Rate
Prep 1	2.0 mL	3:1		12 mL/min
Prep 2	2.0 mL	1:1		12 mL/min
Software settings				
Aqueous (Left inlet)			**Organic (Right Inlet)**	
Samples	**Total Volume**	**Total Flow Rate**	**Volume**	**Total Flow Rate**
Prep 1	1.5 mL	9.0mL/min	0.5 mL	3.0mL/min
Prep 2	1.0 mL	12.0mL/min	1.0 mL	12.0mL/min

Two preparations were made out of the 5 mL total preparation that was aspirated (2 mL) into a 3ml syringe. On the Aqueous (Left inlet), 2.7 mL of Gibco ™ PBS pH 7.4(Thermo Scientific, Cat#: 10010023; Calcium and Magnesium free) was aspirated into the syringe and screwed into the left cartridge inlet. For the organic syringe, a total of the 2ml of the prepared neutral liposome formulation was aspirated and screwed into the right cartridge inlet. On the Nanosystem software, plungers were reset to recalibrate before starting each protocol. Both preparations were made back to back while collecting the sample at the end of each protocol. For one of the multiple formulations that were prepared using this protocol, dialysis was used to attempt to buffer exchange ethanol off the formulation to see if it would improve upon the prep quality.

Experiment 2: Preparation of a Cationic Liposome Formulation: Based on the formula for determination of Mass, a cationic liposome formulation was made with DOTAP/DSPC/CHOL/ DSPE-PEG (2000)-Maleimide/PEG-PE(2000) (molar ratios 4:2.5:11.5:0.25,0.75). The following table summarizes the masses weighed out of each formulation dissolved in absolute ethanol (Table 6).

Table 6**:** Lipid composition showing the weights of each lipid for cationic charged liposome formulation

Lipid	MW	Molar Ratio	Mass Needed (mg)
DOTAP	698.54	4	50.0
DSPC	790.15	2.5	13.4
DSPE-PEG (2000)Mal	2941.64	0.25	2.0
Cholesterol	386.65	11.5	31.8
PEG-PE(2000)	2693.29	0.75	14.5

The lipids were all weighed out into a glass vial and 10 ml of absolute ethanol was added into the lipid mixture and vortexed for about 10 minutes at low speed until all lipids dissolved in ethanol forming a milky suspension. With a wider variety of lipid compositions, this prep was used to investigate the effect of flow ratio and flow rates on liposome size and size distribution by incorporating 2 different flow ratios and making 3 alterations on the flow rate to come up with 6 different sample combinations (Table 7). The same protocol was followed as had been done for the neutral prep formulation only that this formulation utilized small volumes to accommodate syringe capacity.

Table 7: Software Settings for the cationic (DOTAP/DSPC/CHOL/DSPE-PEG(2000)-Mal/PEG-PE) formulation

Samples	Total Volume	Flow Ratio (Aqueous: Organic)	Total Flow Rate
Prep 1	1.0 mL	1:1	4 mL/min
Prep 2	1.0 mL	3:1	4 mL/min
Prep 3	1.0 mL	1:1	8 mL/min
Prep 4	1.0 mL	3:1	8 mL/min
Prep 5	1.0 mL	1:1	12 mL/min
Prep 6	1.0 mL	3:1	12 mL/min
	Start waste = 0.25mL; end waste = 0.05mL		

Sample Preparations for DLS Evaluation on the Zetasizer ZSP: Once the formulations have been completed on the Nanoassemblr, sample preparation to evaluate on the

Zetasizer only requires dilution (1:25 dilution) in PBS. Samples were diluted 1:25 in PBS and evaluated for size.

Aim 2: Formulation of Cationic Liposomes using the Dry Film Hydration method on the Rotary Evaporator:

Based on experiments from Aim 1, it was determined that the cationic liposome formulation was more ideal as it produced more reproducible liposome preps albeit different formulation preparations thereby indicating that the lipid composition was more ideal and that these liposomes had a better chance of being more stable over the neutral formulation composition. Going forward, all experiments followed the same protocol of weighing out and dissolving lipids in absolute ethanol as discussed in Experiment 2 of Aim1.

Experiment 1: Preparation of "empty" liposomes using the Dry Film Hydration Method:

As discussed in the equipment section, the Dry film Hydration method is a distillation system that works by heating the liposome formulation to evaporate organic solvents from a liposome preparation, usually a at a lower temperature than normal. The process leaves the lipid formulation as a dry, thin layer on the walls of the source vessel, which can later be rehydrated with an Aqueous with or without the cargo. In this experiment, no cargo was included as it represents test system to determine the quality of the liposome formulation before encapsulating any cargo.

Figure 19: Picture of the rotavap set-up showing set-up conditions for process improvement. Source: Biogen Inc. (2016).

To start the experiment, lipid compositions were weighed out (Table 6) and dissolved in 10ml absolute ethanol. The rotavap was set up as depicted in the pictures below (Fig. 19) with dry ice and ethanol put in the cooling chamber and the condensation chamber lowered into a wet ice bath. The hot water bath temperature was set at 37^{o}C and oscillations at 168 RPM (Fig. 19). It was later discovered that the evaporation was going very slow and few adjustments were made to both the temperature and oscillations to run the evaporation at the rate of approximately 2 hours per 10 mL sample prep vial. It should be noted that these settings were used for all other subsequent experiments pertaining to the Dry Film Hydration method on the rotavap.

At the end of 2 hours, all the ethanol had evaporated from the formulation but the film was anyway left on the set – up for another hour under vacuum to ensure complete evaporation of the ethanol. At the end, a thin layer of dried lipid cake formed on the walls

of the glass vial. The vial was removed from the rotavap and stored immediately tightly shut at 2-8°C to prevent condensation inside the vial. The vial of dry film was later removed from 2-8°C and hydrated using 5 mL Hypure WFI quality water (GE; Cat # SH30221.10). The hydrated film was vortexed gently at low speed for 20 minutes until all the film was dissolved in PBS. For this experiment, water bath sonication was introduced to determine the effect of sonication on liposome size and dispersity. Before sonication a baseline sample was prepared (diluted 1:50 in water) and evaluated on the Zetasizer Nano. After that, multiple samples were collected in test tubes and a sonication time course was done up to 6 minutes. The first sample was sonicated for 3 minutes, measured on the Zetasizer followed by another 3 minute sonication for a total of 6 minutes of sonication. Another sample was collected and measured at 1 minute increments (same sample) with DLS measurements after each 1 minute sonication up to 6 minutes.

Experiment 2: Preparation of loaded liposomes using the Dry Film Hydration Method: After formulation of the empty liposomes, it was determined that Dry Film hydration method was an appropriate method of cationic liposome formulation. Based on data from Experiment 1, it was determined that this method is amenable for cationic liposomal formulation sine it was possible to get a good prep of formulation that was stable and uniformly dispersed. To that end, another experiment was set up to make a dry film on the Rotavap. This experiment was run with the same conditions and parameters as Experiment 1, upto the dry film step. The subsequent steps were continued as follows;

Sample Preparation: Prepare mAb Y for encapsulation into liposomes by diluting it down to a final working concentration of 10mg/mL in 10mL PBS pH 7.4.

mAb Y stock concentration = 20mg/mL

Dilute the antibody 1:4 in PBS: 5mL mAb Y into 5mL PBS pH 7.4 = 10.0mg/mL.

After preparation of the mAb for encapsulation, 10ml of the entire volume was added onto the dry film vial and vortexed gently at low speed for 10-15 minutes until all the lipids were dissolved into the buffer. This preparation is now a liposome formulation with the mAb Y encapsulated into its core. A small sample was collected and prepared (diluted 1:50 in Hypure water) for analysis on the Zetasizer. This sample served as the baseline measurement for subsequent operations on the loaded liposome formulation.

Experiment 3: Filtration of the loaded liposome preparation via TFF: The sample was then set up on the KrosFlo filtration system (Fig. 17) for filtration of loaded liposomes via Tangential flow. Filtration is important to filter out the excess un-encapsulted antibodies and only leave the loaded liposomes.

TFF set up: The following cut-off membrane for filtration was inserted into the apparatus; Cutoff membrane: Modified Polyether-Sulfone (mPES) membrane (Spectrum Labs; P/N: CO2-E500-05-N; Media/Rating- mPES/500KD; Surface Area:20 cm^2; Max. operation pressure: 30 PSI; S/N: 3285799-03/16-008).

The loaded liposomes was prepared by taking 5 mL of the liposome formulation and adding 10 mL of PBS ph 7.4 to make 15 mL in a 50 mL conical tube. The sample was then set up on the KrosFlo as depicted on the picture (Fig 17) and set to run at the flow

rate of 20ml/min. with filtrate collection. At the end of 1 hour, the sample feed had gone down from 15ml to about 7ml. More PBS was added to the sample to bring volume up to 15 ml again. A second cycle was set up to run at the same conditions. At the end of the cycle, the sample was down to 5 ml with about 10 ml of filtrate flow through. Both the sample and filtrate were saved and stored at 2-8C for next steps.

Experiment 4: An Enzyme Linked Immunosorbent Assay (ELISA) for loaded, filtered liposomes; quantitation of encapsulated mAb Y after dissolving liposomes in Triton X-100: Loaded liposome preparation for the ELISA assay: To enable quantitation of the encapsulated biotherapeutic on the ELISA assay, the liposomes were dissolved in 5%, 2%, 1%, 0.5%, 0.25%, 0.1% detergent (Triton X-100). For each of these detergent-treated samples, each sample was divided twice and diluted 1:500 or 1:1000 in assay buffer to get them within the quantitation range of the assay. In addition to the liposome formulations, the flow-through from TFF were treated the same way as the liposome formulations and run on the assay. Formulation Controls: Some formulation controls were also included in the assay. 1) controls to show if the Triton X-100 was effective in dissolving the liposomes were done by adding samples not treated with the detergent to contrast with the detergent treated samples. 2) A sample of the mAb Y that had not been encapsulated and therefore not treated with detergent was also included on the plate to determine if encapsulation or the detergent affected the binding activity of the biotherapeutic. See the ELISA plate layout to see the samples and layout on the plate.

A bridging ELISA method to measure Pharmacokinetic (PK) concentrations of the encapsulated therapeutic was set up for the following day. Basically, a costar high bind

clear Nunc plate was coated with the anti-mAb Y idiotype antibody in a coating buffer (Sodium Bicarbonate Coating Buffer; 0.1 M Sodium Bicarbonate pH 8.4; Biogen Inc. Buffer Prep group) and allowed to incubate overnight. The following day, the plate was washed once with wash buffer (1X Tris Buffered Saline (TBS) containing 0.05% Tween-20; Boston Bioproducts) and then blocked with block buffer (Tris-Sucrose; 0.1M Tris, pH 7.7, 20% Sucrose; Biogen Inc. Buffer Prep). The assay was set up with a calibration curve from which to backfit the the samples for concentration determination and assay controls to show that the ELISA assay is working were included. All samples were added on the plate following blocking and incubated to bind on the capture coating anti-mAb Y antibody. Plate was washed again and a detection antibody conjugated with an enzyme (Mouse anti-human IgG-Alkaline Phosphatase Conjugate) was added to detect the biotherapeutic mAb Y. the conjugate was incubated followed by washing the plate and substrate fro the enzyme (p-Nitrophenyl phosphate) was added. The plate was read in the microplate reader (SpectraMax Plus 384 Microplate Reader) using a kinetic read for 5 minutes set at a wavelength of 405 nm with a 1-minute delay.

Aim 3: Preparation of Targeting antibody by Pepsin digestion to create a $F(ab')_2$ fragment to be thiolated via SPDP and conjugated on the surface of liposomes:

A targeting antibody (mAb X) was pepsin digested to make a $F(ab')_2$ fragment using the Pierce™ $F(ab')_2$ Micro Preparation Kit (Product #44688). For antibody thiolation using SPDP crosslinkers the Thermo Scientific method (Product #21857) that exploits the exposed disulfide bonds on the $F(ab')_2$ fragment was used. At the end of these two protocols, Pepsin digestion to generate $F(ab')_2$ fragment of mAb X was characterized

using Gel electrophoresis and evaluating the size of the fragment in contrast to an undigested mAb. After SPDP modification, the same Thermo Scientic protocol was used to determine the level of SPDP modification on the $F(ab')_2$.

Chapter III

Results

This section details the findings of the various experiments carried out to select the lipid compositions, determine the flow ratios and flow rates of the formulations as well as all subsequent experiments carried out after selection of appropriate lipid compositions. In summary, lipid formulations were made without and with encapsulation of the biotherapeutic and then characterized on the DLS. Some of the praparations were filtered on the TFF with sonication to determine the effect of filtration after sonication on liposome size and polydispersity. Additionally, two flow ratios and 3 different flow rates were evaluated to see if any has effect on the final quality of the liposomal formulation preparations.

Aim 1: Evaluation of Nanoassemblr lipid formulations by DLS:

Two versions of formulations were prepared on the platform. One formulation attempted to interrogate lipid compositions for a neutral liposome formulation using three distinct lipids at different molar ratios while the other formulation attempted to formulate a cationic prep as described on the experiments section. All formulations were analyzed on the Zetasizer Nano ZSP for size in nanometers and for size distribution in terms of Polydispersity Index (PDI). The PDI is a measure of non-uniformity in a given sample calculated based on multiple readings of the same sample to determine the range of sizes in that specific sample. With the PDI being the measure of non-uniformity on a given

sample, the larger the PDI number as calculated by DLS, the more polydisperse the sample is hence considered a poor quality sample. A PDI of greater than 0.4 is considered broad therefore depicting that the sample quality is poor (broad range of sizes). On the contrast, a PDI below 0.4 depicts that the sample is monodispersed and of good quality.

Overall, both formulations yielded liposomes of varying sizes ranging from about 80nm – 500nm as measured on the Zetasizer Nano (Table 8 and 9). As for PDI, a few samples had considerably good PDI (<0.4) while most seemed to be polydispersed. In analysizing the data, it was evident that freshly prepared formulations were of good quality compared to formulations that had been prepared and stored at 2-8^{0}C for a period of about 12 days (Fig. 20), (Table 8). Based on the data, it is therefore preferable to use freshly prepared formulations until a conclusive stability program has been well established to determine the point at which the shelf life is affected. All fresh preparations that were prepared using a 1:1 flow ratio had good PDI below 0.4 (Fig 20). Additionally, a difference between the flow ratios of Aqueous to Organic streams show that the 3:1 (AQ:ORG) flow stream affects the overall quality of the formulation prep. All preparations that were made using the 3:1 flow ratio had considerably worse quality than those made with the 1:1 flow ratio (Fig 20). In one of the experiments, one preparation was made and divided up into 6 samples to investigate the effect of flow ratio and flow rate on formulation quality. The purpose of this experiment was attempting to investigate whether the volume per minute of the sample that passes through the microfluidic micro-mixing chamber has any effect on the overall quality of the liposomal formulation (Table 9). The flow rates (milliliter/ minute) that were tested include 4mL/min, 8mL/min and 12 mL/min with all flow rates being tested against both the 1:1

flow ratios and the 3:1 flow ratios (AQ:ORG) (Table 9). As evident from these results, there is no significant effect of the flow rate on liposomal formulation, meaning that regardless of the volume per minute passing through the microfluidic cartridge, the mixing is sufficient to give a good quality product. However, just as was seen in the neutral formulation, a difference between the flow ratios of Aqueous to Organic streams show that the 3:1 (AQ: ORG) flow stream affects the overall quality of the formulation preps (Fig 20 bottom panel). Although one can use whichever flow rate they choose, data suggests that only the 1:1 flow ratio (AQ: ORG) is amenable for preparations of good quality liposomes on the Nanoassemblr. This effect was not investigated in detail though the hypothetical explanation is that in more aqueous environment than organic, the lipids start to become unstable and most of what is measured might actually be aggregates and debris. This phenomenon explains why formulations are done in absolute organic solvents as these conditions help to keep the lipids solubilized and the phospholipids components tend to assume an intact conformation. Data on dialysis to remove excess traces of ethanol from the formulations showed poor polydispersity but is considered inconclusive. This is because the samples dialyzed for 2 days at room temperature and therefore the observed poor quality profile might actually be because of stability and not the dialysis process.

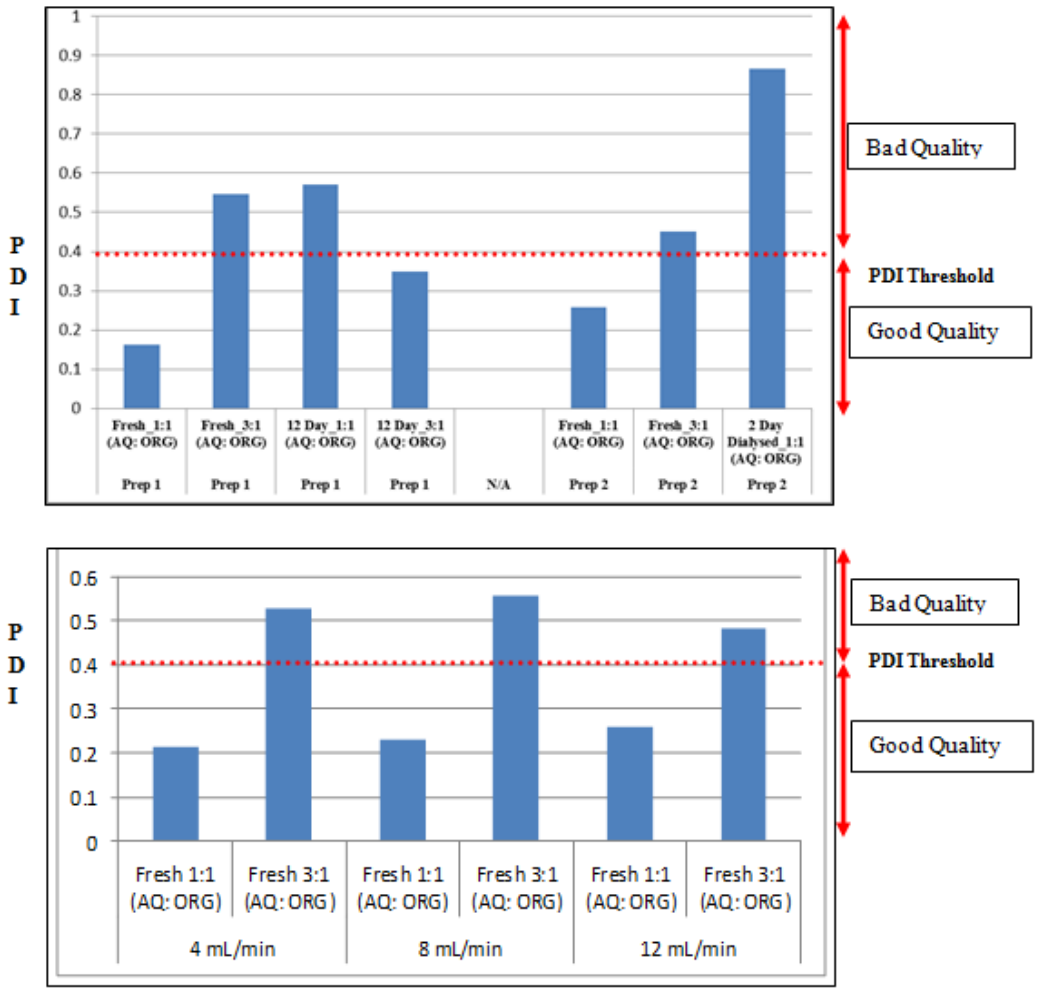


Figure 20: The top shows a neutral liposome formulation with polydispersity (non-uniformity) measured as PDI.

Table 8: Table of results showing two preparations of the Neutral formulation

Type	Sample Name	Measurement Date and Time	Z-Ave (d.nm)	PdI
Prep #1				
Size	DSPE-PEG-MAL/CHOL/DSPC 1:1 (AQ:ORG)_Fresh Prep_25May16	Wednesday, May 25, 2016 3:31:33 PM	**581.7**	**0.163**
Size	DSPE-PEG-MAL/CHOL/DSPC 1:1 (AQ:ORG)_Above Prep after 12 Days at 2-8^{o}C	Tuesday, June 07, 2016 11:48:09 AM	**452.6**	**0.570**
Size	DSPE-PEG-MAL/CHOL/DSPC 3:1 (AQ:ORG) _Fresh Prep_25May16	Wednesday, May 25, 2016 3:39:03 PM	**83.03**	**0.546**
Size	DSPE-PEG-MAL/CHOL/DSPC 3:1 (AQ:ORG)_Above Prep after 12 Days at 2-8^{o}C	Tuesday, June 07, 2016 11:30:19 AM	**429.9**	**0.348**
Prep # 2				
Size	DSPE-PEG-MAL/CHOL/DSPC 1:1 (AQ:ORG)_Fresh Prep 07Jun16	Tuesday, June 07, 2016 12:18:39 PM	**1134**	**0.258**
Size	DSPE-PEG-MAL/CHOL/DSPC 3:1 (AQ:ORG)_Fresh Prep 07Jun16	Tuesday, June 07, 2016 12:02:18 PM	**186**	**0.653**
Size	DSPE-PEG-MAL/CHOL/DSPC 1:1 (AQ:ORG)_Above Prep Dialysed 2 Days then measured	Thursday, June 09, 2016 3:17:39 PM	**1053**	**0.865**

Table 9: Table of results showing preparation of a cationic formulation

Type	Flow Ratio (Aq: Org.)	Flow Rate (mL/min)	Sample Name	Measurement Date and Time	Z-Ave (d.nm)	PdI
Size	1:1	4	DOTAP/DSPC/CHOL/PEG-DSPE-MAL/PEG2000	Wednesday, June 15, 2016 5:22:30 PM	**86.1**	**0.215**
Size	3:1	4	DOTAP/DSPC/CHOL/PEG-DSPE-MAL/PEG2000	Wednesday, June 15, 2016 5:25:59 PM	**81.28**	**0.527**
Size	1:1	8	DOTAP/DSPC/CHOL/PEG-DSPE-MAL/PEG2000	Wednesday, June 15, 2016 5:31:34 PM	**84.39**	**0.231**
Size	3:1	8	DOTAP/DSPC/CHOL/PEG-DSPE-MAL/PEG2000	Wednesday, June 15, 2016 5:35:31 PM	**97.22**	**0.557**
Size	1:1	12	DOTAP/DSPC/CHOL/PEG-DSPE-MAL/PEG2000	Wednesday, June 15, 2016 5:43:03 PM	**97.07**	**0.258**
Size	3:1	12	DOTAP/DSPC/CHOL/PEG-DSPE-MAL/PEG2000	Wednesday, June 15, 2016 5:48:44 PM	**74.64**	**0.482**

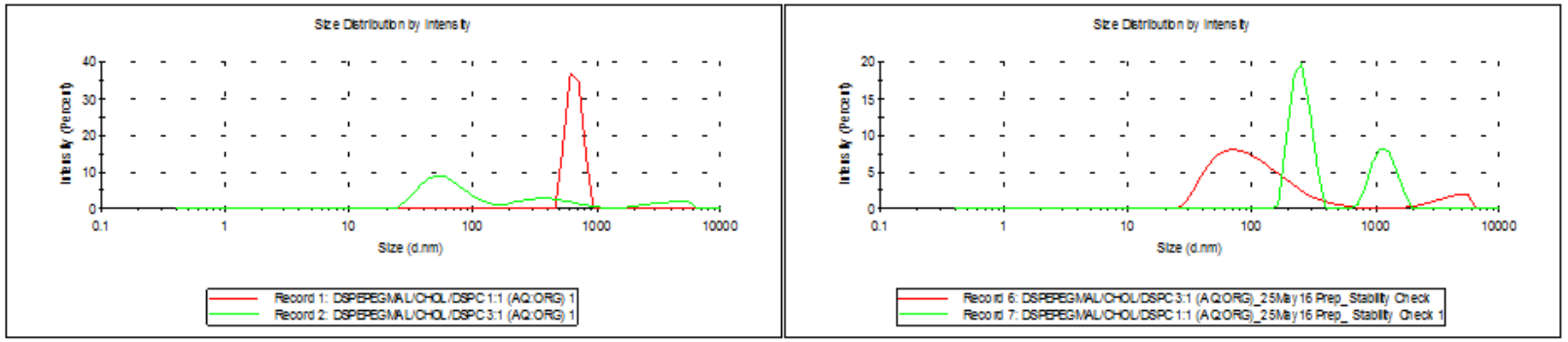


Figure 21: DLS spectrums showing size distribution intensity as measured on Zetasizer nano. Note: The spectrum colors are switched from the left to right panel. (Red on Left = green on right and vice versa)

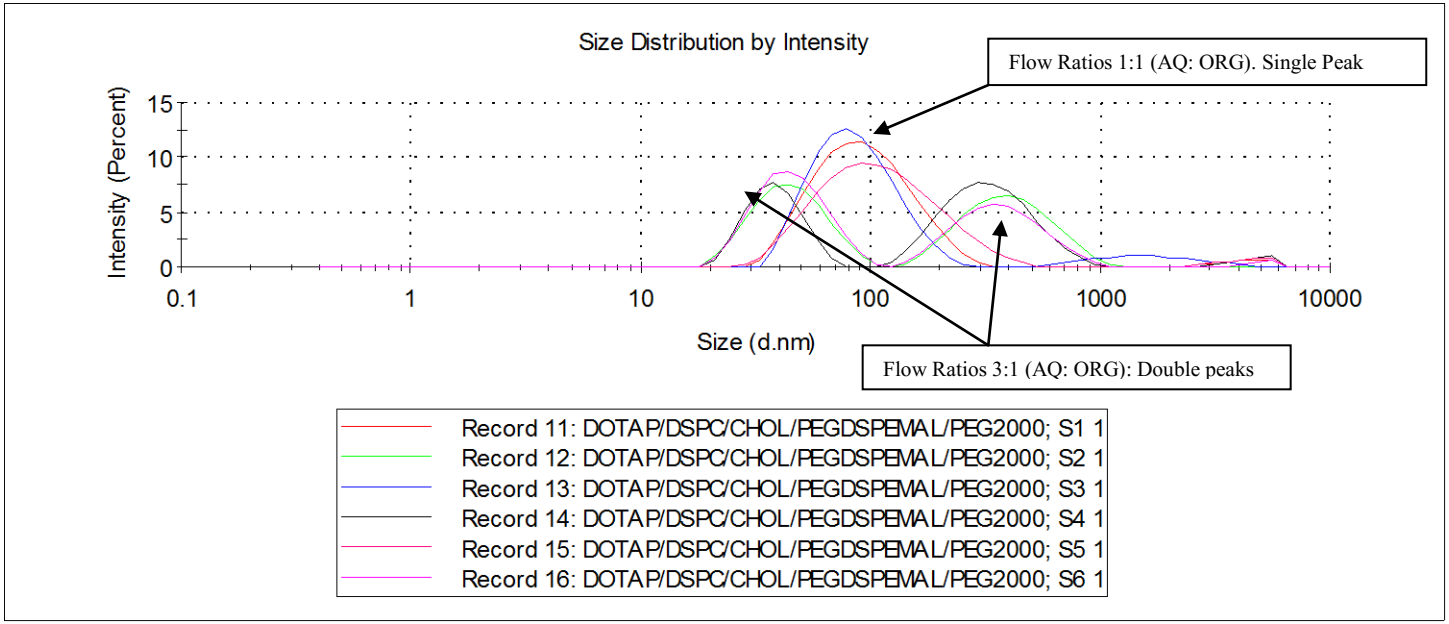


Figure 22: DLS spectrum showing size distribution intensity of the different flow ratios and flow rates. S1&S2 represent the 4mL/min flow rates, S3&S4 represent the 8mL/min flow rates while S5&S6 represent the 12mL/min flow rates.

DLS spectrums generated from the Zetasizer Nano showing neutral formulation of liposomes using two different flow ratios (Fig. 21). As the data shows, the difference in flow ratios has a big impact on size distribution intensity as measured on DLS. The 1:1 flow ratio generates one sharp peak (Fig. 21, left panel, red peak) while the 3:1 flow ratio has a very poor peak profile (Fig. 21, left panel, green peak). When the preparations were stored at 2-8^{0}C over a period of 12 days, both the 1:1 and the 3:1 flow ratios assumed poor peak profiles indicating that the preparations are not stable over a prolonged period of time (Fig 21, right panel). In the other experiment that compared different flow rates using both flow ratios, DLS spectrums show that the flow rates had no effect on size distribution intensity and instead the flow ratios had the biggest impact on size and polydispersity (Fig . 22). As can be seen on these spectrums, the 1:1 flow ratios generated one sharp peak that depicts a single population of liposomes while the 3:1 flow ratios generated two distinct peaks that indicate multiple populations. When the same 1:1 (AQ: ORG) was dialyzed over two days while buffer exchanging to remove traces of ethanol, the prep did not improve and instead appeared to get worse (Fig 20, top panel) (Table 8).

Aim 2: Preparation of Empty Cationic liposomes via Dry Film Hydration method on the Rotavap:

As explained on experiment section first a baseline sample was collected and measured without sonication. Another sample was prepared and sonicated for 3 minutes, measured on the Zetasizer followed by another 3-minute sonication for total of 6 minutes. Based on the data, sonication did not affect the size of nanoparticles significantly save for the one

sample that was sonicated for 3 minutes and then for another 3 minutes for a total of 6 minutes (Fig. 23).

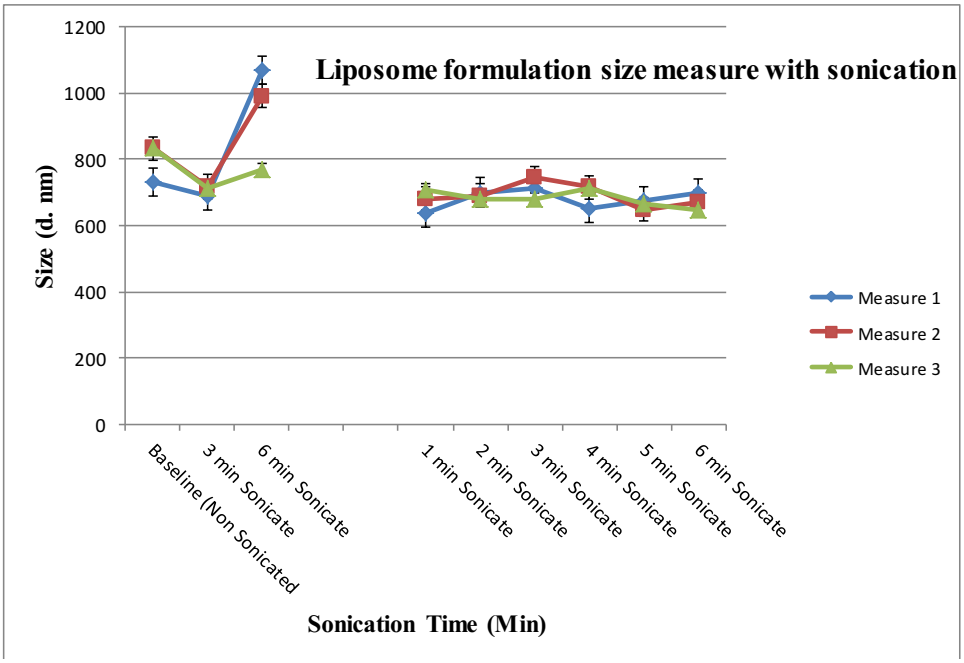


Figure 23: Line graph illustration of sonication on size of cationic liposomes.

Samples that were sonicated at 1-minute increments maintained similar size over the course of 6 minutes sonication time. The same can also be said of the polydispersity. The overall PDI for all samples was below the threshold (<0.4) save for the one sample at 6 minutes following the 3 minute sonication (Fig. 24). Overall, this preparation of "naked" un-loaded cationic liposomes was successful with overall good polydispersity although the hope was that sonication would be able to size the liposomes further into 200-400nm range. Based on these data, it is evident that these liposomes are stable and uniformly dispersed despite sonication up to 6 minutes.

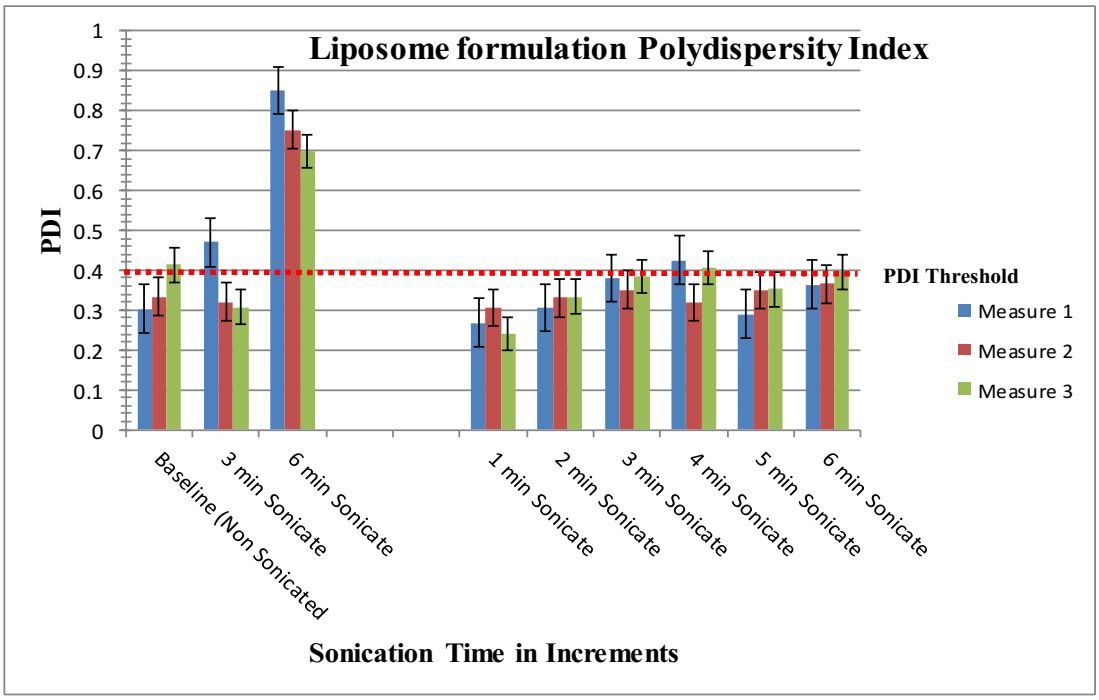


Figure 24: A bar graph illustrating sonication effect on PDI of cationic liposomes.

The DLS spectrums below also show that sonication did not affect the shift of the spectrum. In fact there is no significant difference between the baseline non-sonicated sample to the 6 minute sonicated sample (Fig. 25). Looking at both size distribution from the DLS spectrums (Fig. 25) or even the bar graph showing the distribution of the polydispersity index (Fig. 24), it is evident that sonication had only one instance when the sample had a very high PDI which was a result of prolonged sonication at a given time. Even still, that data is not quite conclusive as there is only an n of 1 without any comparator repeated experiment to confirm these results.

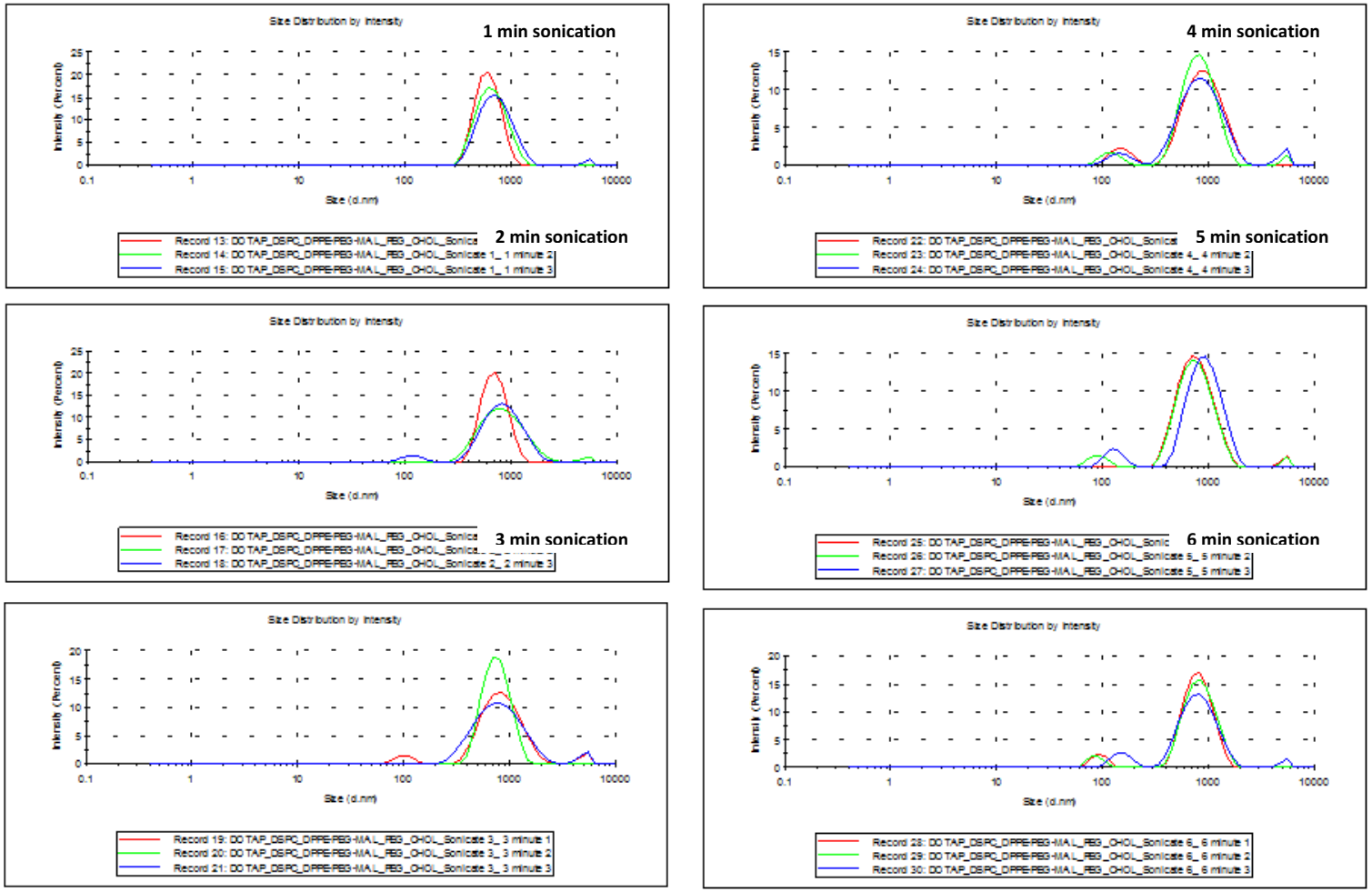


Figure 25: 3 panels show DLS spectrums after 1 minute up to 6-minute sonications. Overall, sonication did not have any significant effect on size distribution or any significant effect on sizing of liposomes by sonication

Experiment 2: Preparation of loaded Cationic liposomes via Dry Film Hydration method on the Rotavap:

Based on the results obtained from DLS for size and size polydispersity characterization, it is clear that both sonication and filtration had a huge effect on the size as well as polydispersity. According to the data, liposome samples that were not sonicated and those sonicated for only 1 minute had average size ranging from 750-1300nm. Sonication of the same preps with increments of 1 minute intervals sized the liposomes further to size range of about 450-530nm. At that point size didn't seem to change much going from 2 minutes to 6 minutes of sonication (Fig. 26 and Table 10). However, when the samples that had been sonicated for 6 minutes were filtered through a cutoff membrane on the KrosFlo TFF system, 2 separate measurements were taken confirming that these liposomes had attained the target size range of about 150-200nm (Fig. 26 and Table 10).

Table 10: Table illustrating DLS size measure of loaded liposomes

SIZE (nm)						
Sample ID	**Measure 1**	**Measure 2**	**Measure 3**	**Mean**	**SD**	**%CV**
Baseline	1271.0	1354.0	1266.0	**1297.0**	49.43	3.8
Non -Sonicated, TFF	765.5	767.7	806.3	**779.8**	22.95	2.9
1 min Sonicate, non TFF	878.4	859.3	846.2	**861.3**	16.19	1.9
2 min Sonicate, non TFF	661.8	524.7	404.0	**530.2**	128.99	24.3
3 min Sonicate, non TFF	608.7	436.0	368.0	**470.9**	124.09	26.4
4 min Sonicate, non TFF	552.1	489.1	424.6	**488.6**	63.75	13.0
5 min Sonicate, non TFF	535.1	430.6	429.5	**465.1**	60.65	13.0
6 min Sonicate, non TFF	555.0	409.3	577.9	**514.1**	91.45	17.8
6 min Sonicate, TFF-1	161.5	155.4	156.9	**157.9**	3.18	2.0
6 min Sonicate, TFF-2	162.7	163.5	159.4	**161.9**	2.17	1.3

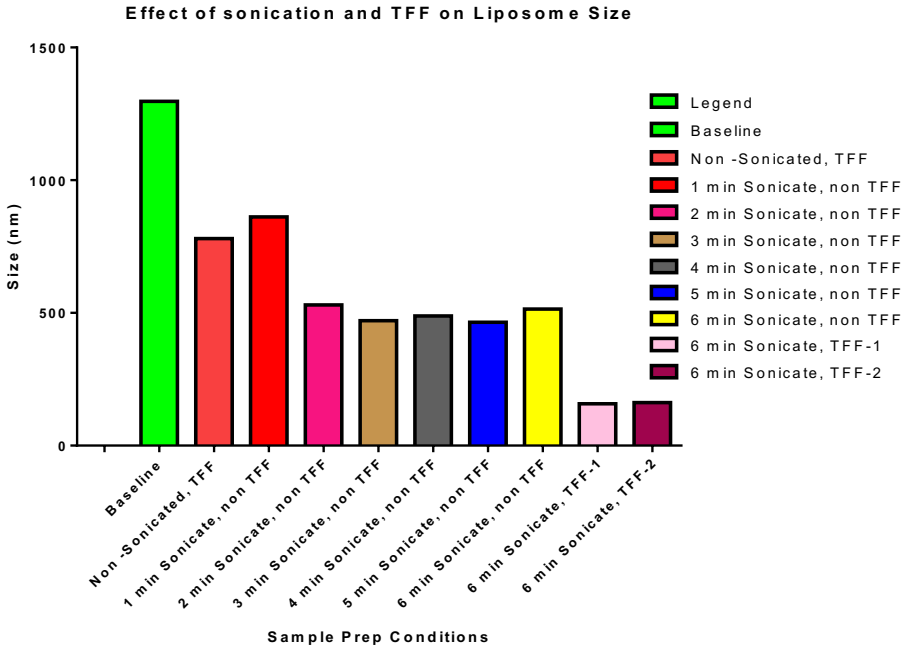


Figure 26: Bar graph depicting the effect of sonication and filtration via TFF on liposome size.

Overall, sonication had a very significant effect on sizing the liposomes and can be seen on the bar graph showing decreasing sizes with the increase in sonication time. Sonicating liposomes then filtering via TFF managed to bring liposome size from over 1000nm to less than 200nm. On liposome polydispersity, sonication seems to have caused a more non-uniform sample, which was expected based on the principle of sonication, but filtering the sample via TFF solves the polydispersity issue. Liposomes that had been sonicated for 6 minutes then filtered by TFF very good monodispersed spectrums on the Zetasizer Nano, which is also confirmed by the plotted data showing that filtration of the liposome preparation yielded liposomes that had a PDI below the threshold of 0.4 (Fig. 27 and Table 11).

Table 11: Table showing descriptive statistics on significance of measured DLS values for liposome polydispersity

PDI						
Sample ID	**Measure 1**	**Measure 2**	**Measure 3**	**Mean**	**SD**	**%CV**
Baseline	0.248	0.256	0.276	**0.260**	0.014	5.5
Non -Sonicated, TFF	0.312	0.297	0.352	**0.320**	0.028	8.9
1 min Sonicate, non TFF	0.519	0.562	0.426	**0.502**	0.070	13.8
2 min Sonicate, non TFF	0.521	0.86	1.000	**0.794**	0.246	31.0
3 min Sonicate, non TFF	0.664	0.99	1.000	**0.885**	0.191	21.6
4 min Sonicate, non TFF	0.55	0.682	0.829	**0.687**	0.140	20.3
5 min Sonicate, non TFF	0.616	0.853	0.853	**0.774**	0.137	17.7
6 min Sonicate, non TFF	0.555	0.931	0.707	**0.731**	0.189	25.9
6 min Sonicate, TFF	0.348	0.365	0.285	**0.333**	0.042	12.7
6 min Sonicate, TFF	0.295	0.36	0.305	**0.320**	0.035	10.9

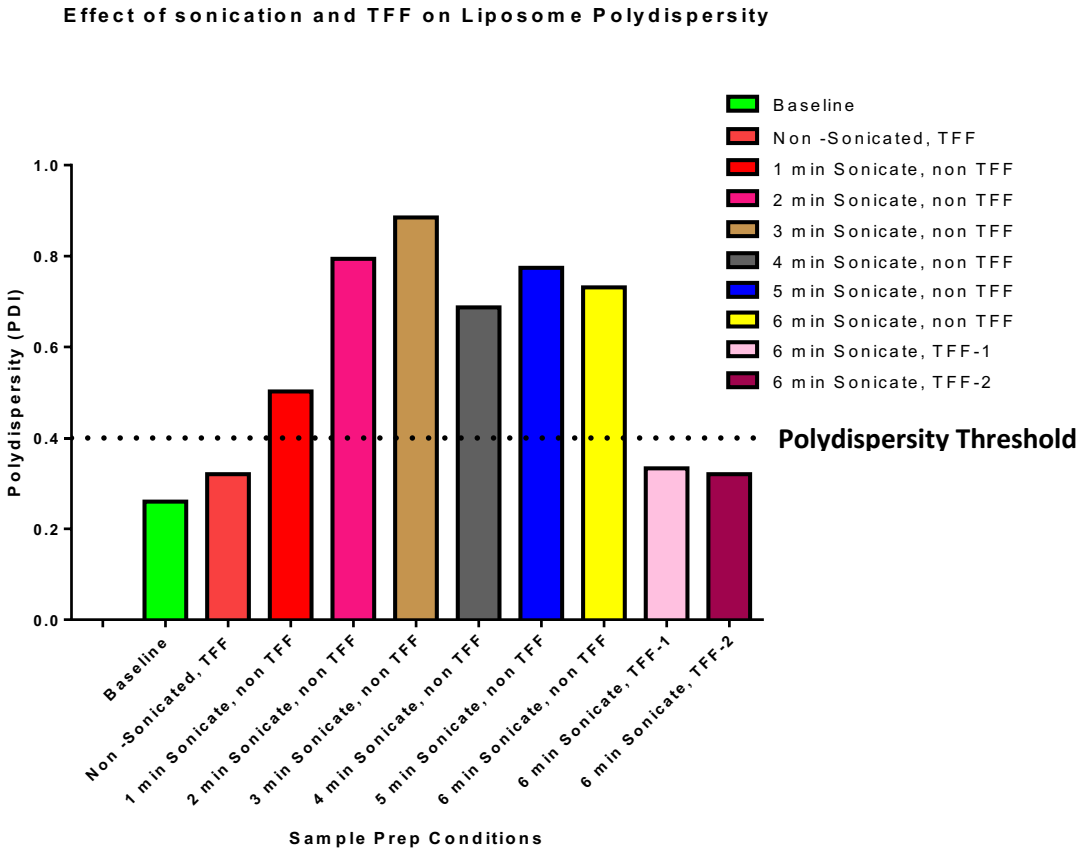


Figure 27: Bar graph illustrating effects of sonication and filtration on liposome polydispersity.

Experiment 3: Enzyme Linked Immunosorbent Assay (ELISA):

All assay controls and the calibrator for the ELISA assay performed satisfactorily as expected (Fig. 28 and Table 12). When liposomes were treated in detergent and run on an ELISA assay, there was very high signal showing that the encapsulation concentration was very high (Fig. 29). However, it is very clear that liposomes that had been treated with detergent then diluted 1:500 in assay buffer had a higher signal than the preparation not treated with detergent (Fig 29). This shows that there was more biotherapeutic available in Tx-100 treated samples which goes to indicate that the liposomes actually encapsulated the biotherapeutic and the detergent caused release of the biotherapeutic hence the higher signal (Fig. 29). Taken together, these experiments were successful but need just a few optimizations to have accuracy in quantitation.

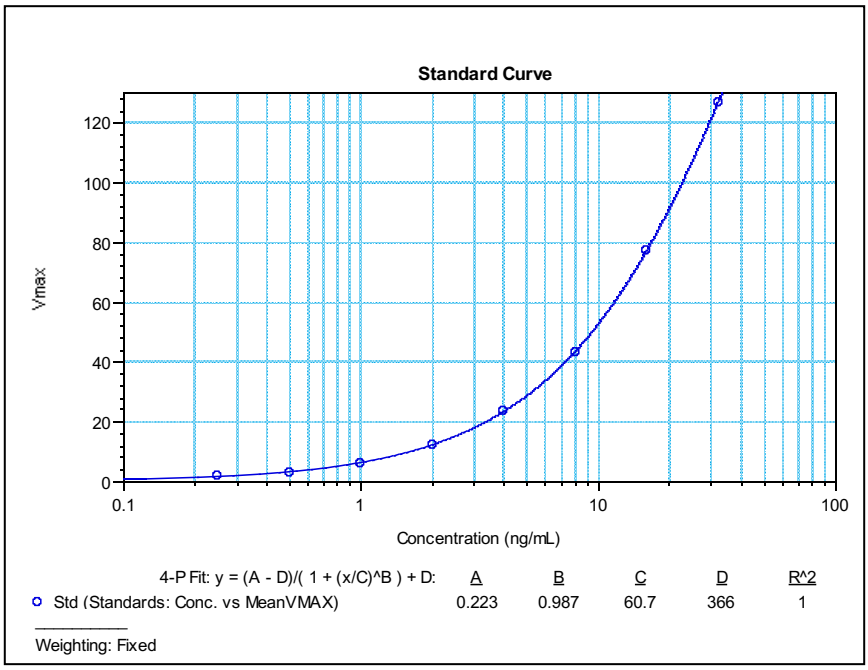


Figure 28: The ELISA calibrator shows that each standard curve point worked as expected.

Table 12: The table below shows that both assay controls and process controls worked as expected

Assay Controls	Conc.	MeanBackfit	%CV	Process Controls	Conc.	MeanBackfit	%CV
HQC01	20	24.041	0.7	**SA01 - mAb Y**	20	30.461	5.7
MQC01	5	6.026	1.6	**SA01 - mAb Y**	20	33.038	10.1
LQC01	0.625	0.415	0	**SA01 - mAb Y**	20	28.702	13.7

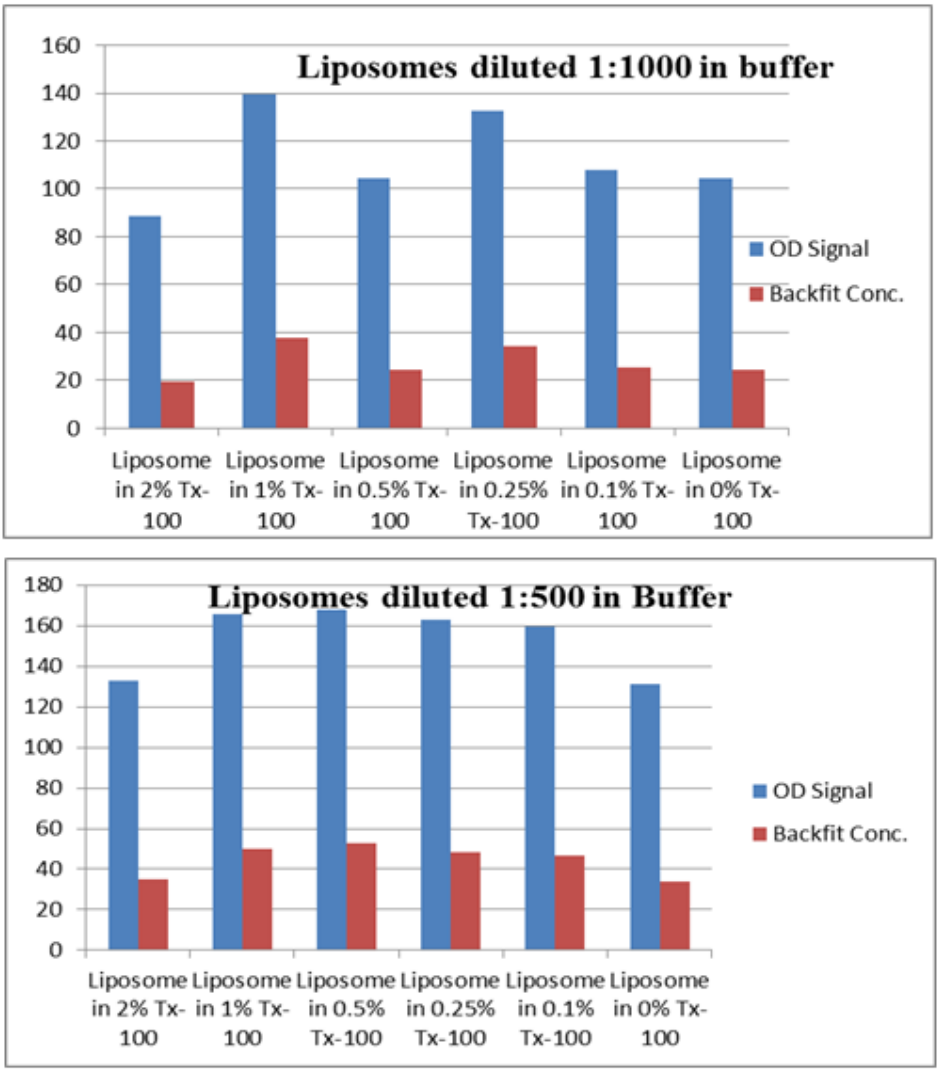


Figure 29: These bar graphs show the effect of detergent treatment of liposomes.

Overall, it was evident that liposomes treated with detergent had higher signal than liposomes not treated with detergent and although the fold change was not that significant, a difference can be observed therefore suggesting that the biotherapeutic was definitely encapsulated into the liposome.

Aim 3: Preparation of Targeting antibody by Pepsin digestion to create a F(ab')$_2$ fragment to be thiolated via SPDP and conjugated on the surface of liposomes.

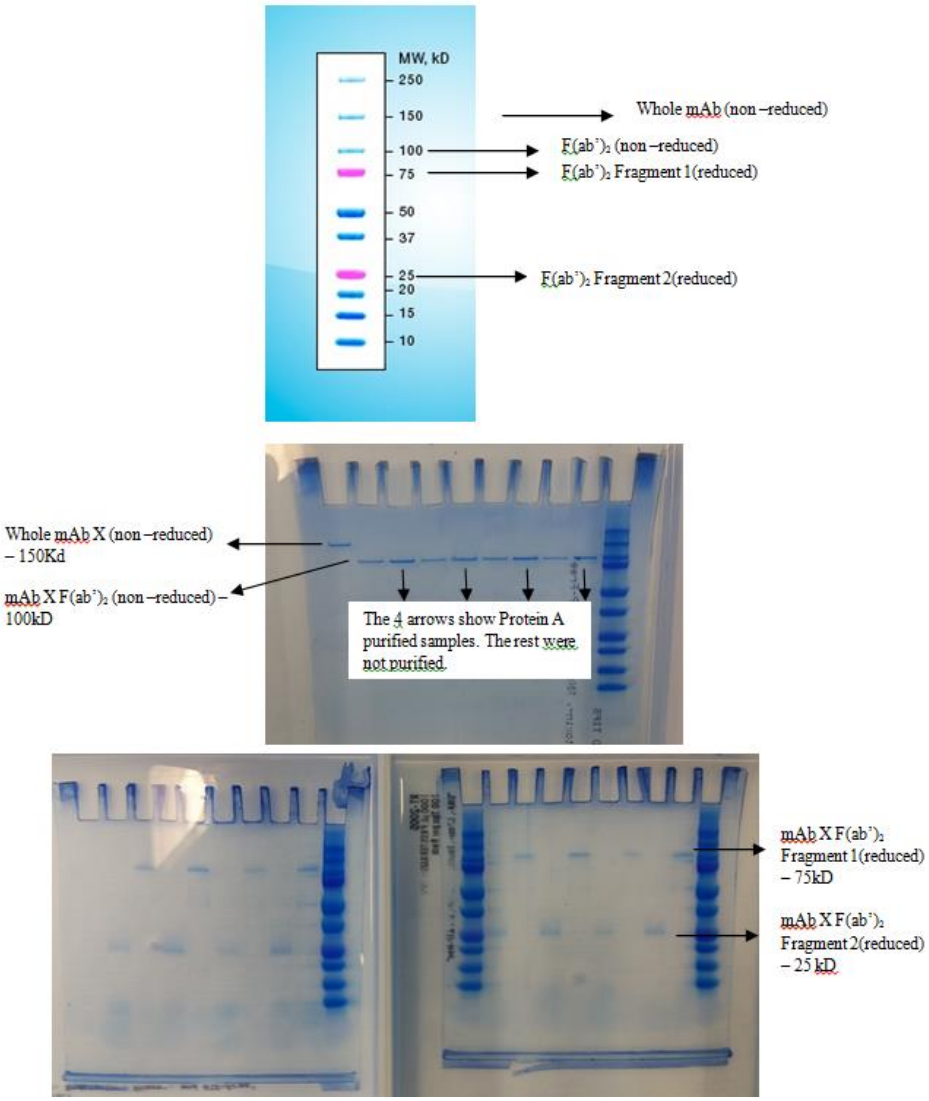


Figure 30: Illustration of level of pepsin digestion for the targeting mAb X.

Digestions were prepared for the mAb X targeting antibody. For each of the four digestions made, a sample was Protein A purified and the other was not Protein A purified making 8 total samples (Fig. 30). These samples were run alongside a non-digested whole mAb X and to confirm the digestion, the 8 total samples were run in non-reduced conditions and then they were reduced in β-mercaptoethanol (BME) (Fig. 30). According to these data, the pepsin digestion worked as separation of bands can be seen running in the appropriate expected molecular weights as depicted on the molecular weight ladder. Usually a whole antibody runs at 150KD, while a non-reduced $F(ab')_2$ fragment runs at 100kd (Fig. 30). When the F(ab')2 fragment is reduced in BME, two fragments form with one running around 75 kD and the other running around 25kD (Fig. 30). When the 4 purified F(ab')2 fragments were run on an ELISA, they generated equivalent signal as the whole antibody (Data not shown) suggesting that they had equivalent binding capacity as the whole mAb X. The fragments were later thiolated using SPDP and then reduced using DTT according to the Thermo Scientific protocol and based on their protocol on calculation of molar SPDP incorporation per protein, it was calculated that the amount of SPDP incorporated per purified F(ab')2 fragment was about 1.157 moles of SPDP per mole of protein. As for conjugation of mAb Y loaded liposomes with the prepared mAb X targeting antibody, 2 sets of data were generated on an ELISA assay but the data was inconclusive (data not shown) on whether the conjugation was efficient or not. The two sets of data generated contradicting results and therefore future work should focus on this last piece of the experiments to confirm and establish conjugation of biotherapeutic loaded liposomes with a targeting mAb.

Chapter V

Discussion

Liposomal drug formulations provide a very attractive avenue for novel drug delivery owing to their attractive properties that enable transport of drugs in organs that would otherwise not be "druggable" using conventional drug modalities. Diseases such as AD and PD present challenges in terms of treatment using biotherapeutic antibodies due to the poor bioavailability of these antibodies into the brain. Furthermore, the ability of some of these antibodies binding to off-target ligands or receptors presents a difficult situation due to off-target related adverse events thereby contradicting the value of treatment despite good efficacy in pre-clinical models of disease. The ability to encapsulate these efficacious antibodies into stealth liposomes presents a powerful method in which these therapies can be delivered to patients and enable delivery into the CNS space, one of the most difficult areas to deliver drugs due to the BBB. The process to make these liposomal formulations is however not trivial and requires numerous considerations to make the therapies amenable for clinical use. Of the many challenges associated with preparation techniques, the use of absolute organic solvents such as chloroform or ethanol may affect the final product due to potentiality to denature the biotherapeutic and hence make the biotherapeutic biologically inactive. Additionally, the inability to remove all traces of the organics could become problematic due to the potential adverse effects in patients. Given the shortfalls, it would therefore be advisable

to make liposomal formulations in mediums that can either extrude all the organics or perform titrations of the organics in aqueous phases to shift the ratio of the medium more towards aqueous than organic.

The latter method of organic titrations in aqueous phase mediums was one of the underlying aims of this thesis project. The initial experiments that were meant to interrogate lipid selection and composition also evaluated how shifting the ratio of the solubilizing medium more to an aqueous phase. The experiments investigated two different ratios of aqueous to organics where some of the preparations were made in 1:1 ratios of aqueous to organics and some preparations were made in 3:1 ratios of aqueous to organics. Data generated from these experiments suggest that the 3:1 flow ratio of aqueous to organic yielded liposomes that were of bad quality compared to flow ratios of 1:1 aqueous to organics (Fig. 20). The PDI, which measures the level of non-uniformity in a given liposomal formulation prep suggested that increasing the amount of the aqueous medium compared to the organic caused instability of the lipid composition resulting in a non-uniform preparation. The diverse populations of liposomes were either aggregated liposomes or liposomes that had broken apart leading to formation of debris or complexed aggregate of liposomes which when measured by DLS seemed as though there was a second population of liposomes. Even though the latter work continued with liposomes solubilized in absolute ethanol and prepared using the dry film method, characterization of the liposomes using an ELISA method demonstrated that the encapsulated biotherapeutic could be recovered successfully and in fact the biotherapeutic maintained its biological activity. The characterization of

immunoliposomes to determine level of antibody encapsulation and level of surface conjugation using the $F(ab')_2$ was not quite conclusive and may need focused attention to do additional experiments that will establish the hypothesis. Although there was promising data that came from the characterization of the encapsulated biotherapeutic, there is still the concern on whether the amount of detergent used could affect the biological activity of the antibody and therefore more optimization work is needed on that part to develop a better protocol that is more amenable for protein chemistry work.

Another challenge as pertains to characterization of the targeting surface antibody on the liposome is that another method may need to be evaluated for detection and quantitation of biotherapeutic loaded liposomes. This is because based on the orientation and size of the immunoliposome, a solution ELISA assay may not be conclusive because the detected signal or the lack thereof may not be a reflection of what is being measured in the solution ELISA assay. This opinion is based on the idea that binding and detection in such a assay is based on components being of the same size and molecular weight for efficient epitope binding. In this case, once liposomes are loaded with a biotherapeutic and then conjugated with another mAb, the size magnitude ratio between the liposome to be detected to the capture mAb coated on the plate shifts dramatically changing the potentiality of binding kinetics and therefore binding may not be optimal. Additionally, the presence of PEG on these formulations may also affect binding because the orientation of the antibody on the liposome may be skewed due to PEG molecules on the immunoliposome thereby obstructing binding pockets between the capture antibody to

the antibody on the liposome. This initial work shows promise but more work is needed to optimize several conditions.

Based on overall results, this thesis project was successful in many ways as exciting data was generated especially in liposome formulation. This project was able to establish different conditions that could be incorporated during liposome formulation to get an appropriate liposome formulation in terms of intended size and polydispersity of size distribution. More work is required to confirm about the targeting molecule conjugation on the surface of the molecule as well as recovery of the encapsulated biotherapeutic. Many processes and techniques used in this work were new to the analyst and therefore above many things, the learning process and experience gained throughout the course of this project are invaluable.

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