Engineering a Proteoliposome Transporter to Capture Radioactive Cesium from Water

by

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ABSTRACT

Radioactive cesium (^{137}Cs), released from nuclear power plants and nuclear accidental releases, is a problem due to difficulties regarding its removal. Efforts have been focused on removing cesium and the remediation of the contaminated environment. Traditional treatment techniques include Prussian blue and nano zero-valent ion (nZVI) and nano-Fe/Cu particles to remove Cs from water; however, they are not efficient at removing Cs when present at low concentrations of about 10 parts-per-billion (ppb), typical of concentrations found in the radioactive contaminated sites.

The objective of this study was to develop an innovative and simple method to remove Cs^+ present at low concentrations by engineering a proteoliposome transporter composed of an uptake protein reconstituted into a liposome vesicle. To achieve this, the uptake protein, Kup, from *E. coli*, was isolated through protein extraction and purification procedures. The new and simple extraction methodology developed in this study was highly efficient and resulted in purified Kup at ~1 mg/mL. A new method was also developed to insert purified Kup protein into the bilayers of liposome vesicles. Finally, removal of CsCl (10 and 100 ppb) was demonstrated by spiking the constructed proteoliposome in lab-fortified water, followed by incubation and ultracentrifugation, and measuring Cs^+ with inductively coupled plasma mass spectrometry (ICP-MS).

The ICP-MS results from testing water contaminated with 100 ppb CsCl, revealed that adding 0.1 – 8 mL of Kup proteoliposome resulted in 0.29 – 12.7% Cs removal. Addition of 0.1 – 2 mL of proteoliposome to water contaminated with 10 ppb CsCl resulted

in 0.65 – 3.43% Cs removal. These removal efficiencies were greater than the control, liposome with no protein.

A linear relationship was observed between the amount of proteoliposome added to the contaminated water and removal percentage. Consequently, by adding more volumes of proteoliposome, removal can be simply improved. This suggests that with ~ 60-70 mL of proteoliposome, removal of about 90% can be achieved. The novel technique developed herein is a contribution to emerging technologies in the water and wastewater treatment industry.

DEDICATION

To my beloved parents, Zohreh and Alireza for all their endless love, effort, encouragement and sacrifices through every step of my life to help me reach what might seem unreachable

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CHAPTER 1

INTRODUCTION

Cesium is water-soluble and as an alkali Group I metal, has a chemical and biological behavior profile similar to potassium and sodium. One of the most important radioactive isotope of cesium is ^{137}Cs due to its high radioactivity and relatively long half-life time of 30.2 years (Bystrzejewska-Piotrowska & Urban, 2004,; Mosquera, Carvalho, Veiga, Mangia, & Anjos, 2006; Sangvanich, et al., 2010). Radioactive ^{137}Cs has been found in at least 23 of the 1,636 existing or former National Priorities List NPL sites identified by the U.S. Environmental Protection Agency (EPA). (ATSDR, 2004). These sites are being considered by the EPA for long-term federal cleanup actions.

Sources of exposure are primarily anthropogenic, including waste generated by nuclear power plants, nuclear power plant-related accidents, nuclear-powered satellites or submarines, explosion of nuclear weapons as well as low level radioactive waste from hospital and some research labs (Sung-Chan Jang, 2014; ATSDR, 2004; EPA, 2002). ^{137}Cs removal became an emerging issue after nuclear plant accidents in Chernobyl (1986) and Fukushima (2011) (Buesseler, et al., 2012; Nagy, Ming, Masataka, Masanobu, & Yusuke, 2012; Parajuli, et al., 2013; Ellen Tanudjaja, 2017).

Radioactive cesium has also been identified in soil, surface water and in many types of food including breast milk and pasteurized milk. Human cells can become damaged by emitted gamma radiation from exposure to ^{137}Cs via ingestion, inhalation, and/or dermal contact. (ATSDR, 2004; Thammawong, Opaprakasit, Tangboriboonrat, & Sreearunothai, 2013; Namiki, et al., 2012; Rosoff, Cohn, & Spencer, 1963). Other adverse effects from

exposure include medullar and reproductive function disorders, disruption of normal embryo development, misfunction of liver, kidney, and bladder, and cardiovascular disease and gastrointestinal distress (Sung-Chan Jang, 2014).

Commonly used methods for removing ^{137}Cs include Prussian blue (Sung-Chan Jang, 2014; Tomomi Koshiyama, 2018), nanoscale zero valent iron (nZVI) and nano-Fe/Cu particles (Shubair, Eljamal, Khalil, Tahara, & Matsunaga, 2018), phytoremediation (Entry, 1996; Neil Willey, 2006) and coagulation–flocculation–sedimentation (C-F-S) (Koji Kosaka, et al., 2012).

C-F-S is effective where turbidity is relatively high. In river water, Cs presents in particulate forms unlike ions in pond water that makes it difficult to remove Cs. Removal percentages of ^{137}Cs from pond water by coagulation and filtration are reported 6% and 0%, respectively (Koji Kosaka, et al., 2012).

Prussian blue, also known as ferric(III) hexacyanoferrate(II), is a dark blue pigment verified to be a low-cost adsorbent of high selectivity for cesium (Shokouhimehr, Soehnlen, Khitrin, Basu, & Huang, 2010). However, these adsorbents need to be coated on the surface of magnetic nanoparticles such as poly diallyldimethylammonium chloride (PDDA) coated iron oxide to be separated from the treated solution later by utilizing either a permanent or electro magnet (Bee, Massart, & Neveu, 1995; Laurent, et al., 2008; Sung-Chan Jang, 2014). (Sung-Chan Jang, 2014) tested 0.5 and 2 mg of Prussian blue-coated magnetic nanoparticles mixed with 10 mL of the aqueous solution of the radioactive cesium and reported cesium removal efficiency of more than 94% at a 0.2 mg/mL concentration. However, they have only tested radioactive activity of 90 Bq/g and considering 94%

removal, the final radioactivity of their tested sample would be about 5.4 Bq/g. The acceptable upper limit value set by Japan government for drinking water is 10 Bq/kg which is significantly less than 5.4 Bq/g (Hamasaki, Nakamichi, Teruya, & Shirahata, 2014). Hence, this study also needed to test lower concentration and radioactivity of Cs to see if these nanoparticles would also be efficient presence of low concentrations of cesium.

Nano zero-valent iron (nZVI) and nano-Fe/Cu particles are reported as promising materials for cesium removal from water. nZVI are synthesized following the method proposed by (Wang & Zhang, 1997); nano-Fe/Cu particles synthesis conditions are similar but with addition of $CuCl_2$ to ferric chloride before sodium borohydride injection. According to (Shubair, Eljamal, Khalil, Tahara, & Matsunaga, 2018), removal efficiency of 99% was reached for both nanoparticles at initial cesium concentration of 1 ppm at a dosage of 1 g-nanoparticles/L and 46.49% and 53.33% for nZVI and nano-Fe/Cu particles at dosage of 20 g-nanoparticles/L which is a pretty high dosage. In this study, surprisingly, the minimum cesium concentration that they have tested is 1 ppm, although, the actual level of cesium following a nuclear accidents like Chernobyl (Little, Alorkpa, Khan, Povazhnyi, & Vasiliev, 2018) is substantially lower (about 8-15 ppb). Thus, there is a need to develop a method that can be also applicable for removing cesium from the actual contaminated area.

Phytoremediation is suggested as a practical and economical method to remove radionuclides such as ^{137}Cs from the contaminated soil due to nuclear testing and nuclear reactor accidents. Radionuclides from contaminated soil accumulate in growing plants

inoculated with combination of mycorrhizal fungi and soil organic amendments, followed by the harvesting of the top portion of the plants (Entry, 1996; Neil Willey, 2006).

Here, we suggest a novel and economical method, a proteoliposome transporter composed of an uptake protein reconstituted into liposome, to capture ^{137}Cs present in low concentrations (10 - 100 ppb). We also revealed more about the behavior and uptake mechanisms of bacteria in the presence of nuclear waste contaminants. This work will subsequently contribute to emerging technologies in the water and wastewater treatment industry.

The membrane transporter (uptake protein) suggested here for ^{137}Cs removal is TrkD (or Kup) derived from *E. coli*. There are only a few reports on the kinetics of cesium uptake (Tomioka, Uchiyama, Yagi, & Fujii, 1995; Ella Zakharyan, 2001); it's been reported that the Kup system, the low affinity potassium transport system encoded by the TrkD gene in *E. coli*, is capable of high cesium accumulation (Tomioka, Uchiyama, Yagi, & Fujii, 1995). Kup encodes a protein of 622 amino acids with a molecular weight of 69.293 kDa (Schleyer & Bakker, 1993). In the Michaelis–Menten equation, the calculated K_m and V_{max} for Cs^+ are 5 mM and 17 μmoles min^{-1} g^{-1} (dry wt) of cells and for K^+ are 0.37 mM and 27 μmoles min^{-1} g^{-1} (dry wt) of cells (Tomioka, Uchiyama, Yagi, & Fujii, 1995; Dirk Bossemeyer, 1989) where K_m is substrate concentration to achieve half V_{max} and V_{max} is the maximum rate of reaction when the enzyme is saturated with substrate (Vivian & E. Polli, 2014).

To construct a proteoliposome for Cs removal, Kup protein transporters from *E. coli* can be extracted and purified, and artificial membrane vesicles (liposomes) are

synthesized and then the extracted protein is inserted into these vesicles; proteoliposomes can be subjected to K^+ and Cs^+ uptake studies afterwards. Liposomes are microscopic vesicles, self-assembled colloidal particles, comprised of a phospholipid double layer. These phospholipids have a spherical hydrophilic head and lipophilic tail, similar to bacterial membranes, and as a result are capable of transporting hydrophobic and hydrophilic materials (Sharma & Sharma, 1997; Bangham & Horne, 1964). The following outlines the general experimental approach.

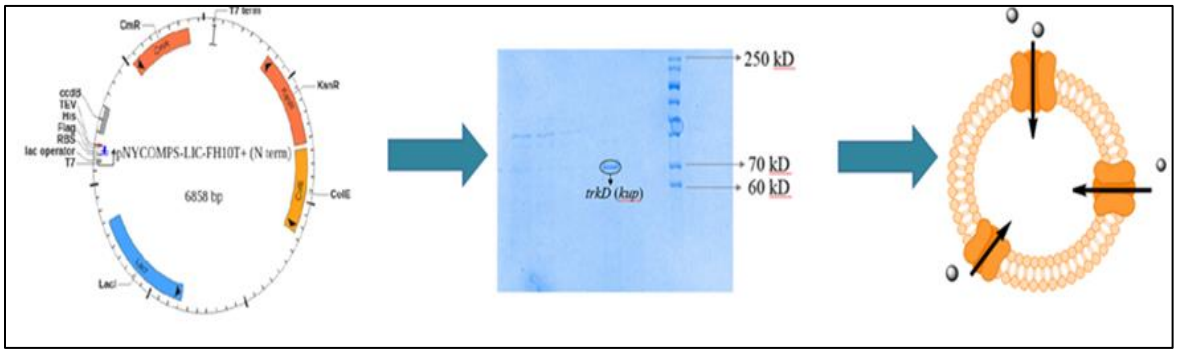

Figure 1. Schematic of the Proteoliposome to be Synthesized.

1.1 Study Objectives

The overall objective of this study is to develop an innovative and efficient method for the removal of ^{137}Cs from water using an environmentally friendly methodology with applicability in pharmaceutical and food industry. This objective will be achieved through the following tasks:

- Optimization of the method to obtain Kup protein
    - Protein extraction
    - Protein purification
- Reconstitution of purified proteins into the liposomes to make proteoliposome

- Liposome preparation
- Proteoliposome preparation

- Verification of Reconstitution of Kup protein into the liposome bilayer by two different methods
  - Cryo- Transmission electron microscopy
  - Sucrose density gradient centrifugation (SDGC)

- Evaluation of applicability and the efficiency of proteoliposome for the removal of Cs^+ in water
  - Inductively coupled plasma mass spectrometry (ICP-MS)

CHAPTER 2

LITERATURE REVIEW

2.1 What is a Liposome?

Liposomes (Weissmann, Standish, & Bangham, 1965) are microscopic vesicles, self-assembled colloidal particles comprised of one or more double layers of molecules called phospholipids that occur spontaneously when lipid components are hydrated in aqueous media (Bangham & Horne, 1964; Sharma & Sharma, 1997) and can be also prepared artificially. They were first discovered by the British researcher A. Bangham in 1964, when he noticed formation of multilamellar phospholipids (Weissmann, Standish, & Bangham, 1965; Bangham & Horne, 1964; Sharma & Sharma, 1997) and later G. Weissmann came up with the name "liposomes" for these formations (Deamer D.).

These phospholipids consist of a spherical hydrophilic or water soluble- aqueous component entrapped by and a tail-shaped lipophilic (fat soluble) bilayer components that can be either natural or synthetic (Bangham & Horne, 1964; Sharma & Sharma, 1997) and as a result they can carry both hydrophobic and hydrophilic materials (3).

The major types of liposomes (Figure 2) are the multilamellar vesicles (MLVs) sized from 500 to 5,000 nm and consist of several concentric bilayers, the small unilamellar vesicle (SUV) around 100 nm in size and formed by a single bilayer and the large unilamellar vesicle (LUV) range in size from 100 to 800 nm (Vinita, Kumar Shiv, Mahavir, & Archana, 2013; Sharma & Sharma, 1997).

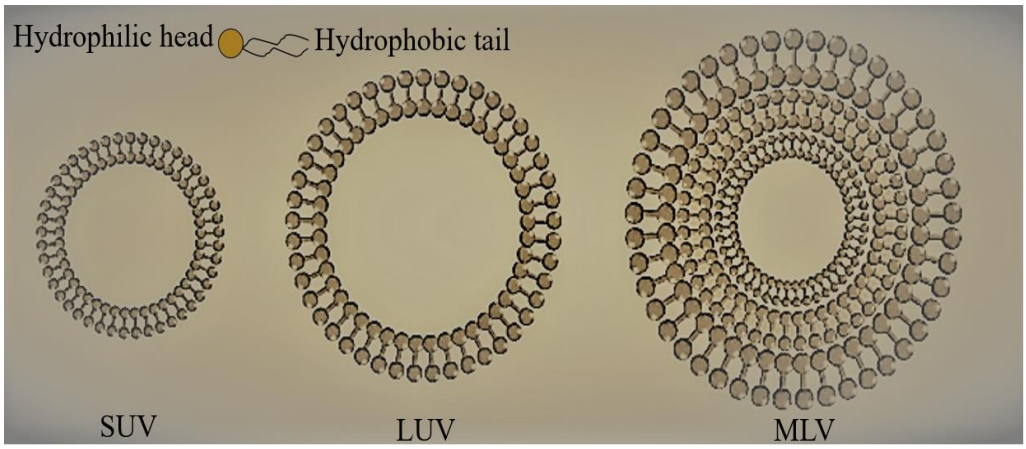

Figure 2. Three Types of Liposome. SUV: small unilamellar vesicle, LUV: Large unilamellar vesicle, MLV: Multilamellar vesicle.

2.2. Liposome Applications

Common applications of liposomes are summarized below.

2.2.1 Cosmetic

Liposomes are reported to be of great value to cosmetics industry by delivering skin care products though empty or moisture loaded liposomes that can decrease the transdermal water loss and skin dryness which is a major cause of aging (Patravale & Mandawgade, 2008). Commercially available products since 1987 include simple liposome pastes as a substitute for creams, gels, ointments and formulations of various extracts, antibiotics, moisturizers and such (Laouini, et al., 2012).

2.2.2 Food

Liposomes have also been utilized in food industry to deliver flavors, nutrients as well as for encapsulation of bioactive ingredients like antimicrobials to protect food products from microbial contamination (Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008; Reineccius, 1995).

Use of antimicrobial peptides as potential biopreservatives in hurdle technology, a method of food preservation from pathogens, has faced stability problems such as proteolytic degradation and the potential interaction of the antimicrobial peptide with food ingredients that might cause reduced antimicrobial activities (da Silva Malheiros, Daroit, & Brandelli, 2010). Hence, liposomes have been used to enhance the performance of encapsulated components by increasing physicochemical stability against a range of environmental and chemical changes and enhancing bioavailability and shelf-life of sensitive ingredients (Laouini, et al., 2012; Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008; Singh, Thompson, Liu, & Corredig, 2012).

2.2.3 Textile Industry

Liposomes can be used to improve textile preparation and dyeing because of their amphiphilic properties. Use of liposomes in the textile process can improve the mechanical properties of the product and subsequently better leveling effect and wash fastness and handling properties. Liposomes allow for a reduction in applied temperature during textile preparation reducing energy costs (Barani & Montazer, 2008). Multilamellar vesicles (MLVs) made from soya lecithin have been used to transfer dye molecules into wool fiber. The study of liposomes at various temperatures and concentrations on the wool fabric indicated that presence of liposomes in the dye-bath increases the dye absorption on the wool fabric before 80 ^{0}C; however, higher temperature might form a hydrophobic barrier against absorption of dye, due to resulting deposition of phospholipids unimers on the fabric surface (Montazer, Validi, & Toliyat, 2006). Dyeing uniformity of polyamide knitted

goods is also enhanced by encapsulation of anionic dyes and ammonium sulfate into the liposome vesicles (Prichystal, Burgert, Hrdina, Purev, & Cerny, 2013).

Recently, liposomes have been utilized to produce biofunctional textiles. These biofunctional textiles contain liposome vesicles carrying therapeutic or cosmetic ingredients that would be released when the fabric rubs the skin (Martí, et al., 2010).

2.2.4 Drug Delivery

Liposome vesicles were first utilized as drug-delivery vehicles for intended organs and tissues in the 1970s (Lasic, 1998); however, due to their colloidal and biological instability some problems including unstable and ineffective drug encapsulation into the vesicles as well as possible fast elimination from the blood might occur (Lasic, 1998; Torchilin, 2005; Sharma & Sharma, 1997). Thus, in order to improve efficiency of drug delivery, different methods such as attaching specific ligands, capable of recognizing and binding to the desired cells to liposomes (called immunoliposomes), liposome surface modification by coating with inert, biocompatible polymer such as polyethylene glycol (PEG) (long-circulating liposome) and combining the two methods of immunoliposomes and long-circulating liposomes are suggested (Torchilin, 2005) (Sharma & Sharma, 1997).

2.2.5 Bacterial Efflux Pump Encapsulation into the Liposome

Study of various organisms' genomes has revealed that about 25% of sequenced genes encode hydrophobic proteins that are integrated into cell membranes (Rigaud, Pitard, & Levy, 1995; Wang & Tonggu, 2014). Membrane proteins are responsible for different

critical biological processes, such as ion and solute transport (ion channels and protein transporters) and energy conversion (proteins for respiration and ATP synthesis) (Rigaud, Pitard, & Levy, 1995; Wang & Tonggu, 2014). They represent 70% of all known pharmacological targets and 50% of potential new drug targets that are involved in diseases like cancer, diabetes and neurological disorders (Weinglass, Whitelegge, & Kaback, 2004).

Although it is ideal to study membrane proteins in their native environment due to their complicated biological structures, reconstitution of purified membrane proteins into liposomes has been developed and has utility (Long, Tao, Campbell, & MacKinnon, 2007; Gonen, et al., 2005; Schmidt, Jiang, & MacKinnon, 2006; Werner, Simeonov, Haupt, Tanabe, & Bacia, 2013).

Membrane proteins show their whole activity only when oriented and inserted in a lipid bilayer properly (Rigaud, Pitard, & Levy, 1995). Functionality of the target protein may be easily affected by changing factors like lipid composition (Wang & Tonggu, 2014). Racker and colleagues began the studies on function of membrane proteins inserted into liposomes (Kagawa & Racker, 1971; Hinkle, Kim, & Racker, 1972 ; Racker, 1972) and in the past four decades, many reconstitution methods have been developed and modified (Wang & Tonggu, 2014).

Utilizing proteoliposomes have enabled us to study different ion channels like sulfate transporters (CyzZ), the inner membrane protein of *Escherichia coli*, that facilities the sulfate uptake (Zhang, Jiang, Nan, Almqvist, & Huang, 2014), potassium channels (Brohawn, del Mármol, & MacKinnon, 2012; Wang & Sigworth, 2009; Dirk Bossemeyer, 1989), calcium channels (Nakao, Ebata, T., Kagawa, & Hirata, 1988), and sodium channels

(Tanaka JC, 1983), among others, by measuring the changes in ion concentration or membrane potentials changes.

CyzZ, an inner membrane protein of *E. coli*, is a sulfate transporter that facilities sulfate uptake, which could perhaps be engineered to deal with elevated sulfate in sewer systems (Zhang, Jiang, Nan, Almqvist, & Huang, 2014). MexB is another transporter that utilizes the proton-motive force to transport antibiotics (Picard, Verchère, & Broutin, 2013).

MexAB efflux pump from the Gram-negative bacteria *Pseudomonas aeruginosa* was reconstituted into liposomes accompanied with bacteriorhodopsin, a light-activated proton pump that causes a proton gradient that energizes transport of antibiotics (Verchère A. , Dezi, Broutin, & Picard, 2014). (Kapoor & Wendell, 2013) developed a light-dependent antibiotic removal system by engineering the AcrA-AcrB-TolC efflux complex into a proteovesicle system. RND (Resistance-Nodulation-Division) efflux pump AcrB (a proton powered motor protein) of *E. coli* forms a complex with AcrA and TolC to remove intracellular toxins such as antibiotics, bile salts, and detergents (Kapoor & Wendell, 2013; Nikaido & Takatsuka, 2009). AcrA and TolC are outer membrane channel and a periplasmic adaptor protein joining TolC to AcrB, respectively which both provide a passive exit funnel (Kapoor & Wendell, 2013; Nagano & Nikaido, 2009) whereas AcrB converts the proton-motive force (pmf) to mechanical work expelling the toxins. In the paper, AcrB was powered with the bacteriorhodopsin (BR) proton pump, which was driven by light (Kapoor & Wendell, 2013).

Inspired by these bacteria efflux pumps, we suggest a new method using an uptake protein reconstituted into liposome in order to capture ^{137}Cs. Using this new method, which is easy and economical compared to current energy intensive engineering solutions, we learn more about the behavior and activity of bacteria in the presence of contaminants such as ^{137}Cs and as a result can solve the existing issue regarding Cs^{+} removal when present at low concentrations.

2.3 Methods of Liposome Preparation

Though liposomes can form spontaneously due to the hydrophobicity of lipid in an aqueous environment, often there's a need to have some mechanical agitation. Different liposome preparation methods have been developed to control the size and structure of the liposome vesicles as well as to enhance the encapsulation efficiencies and consequently inhibit the leakage of the entrapped molecules from the vesicles (Çağdaş, Demir Sezer, & Bucak, 2014). Factors that should be considered for method selection include: 1) physicochemical properties, concentration and potential toxicity of the materials that are going to be encapsulated into the liposomes, 2) characteristics of the compounds and buffers used to prepare liposome, 3) additional features applied during delivery of the liposomes, 4) optimum size, polydispersity and shelf-life of the liposomes, and 5) batch-to-batch reproducibility and possibility of a safe and efficient large-scale liposome production (Gomez-Hens & Fernandez-Romero, 2006; Mozafari M.R, 2008; Dua J.S, 2012).

Liposome size and number of lipid bilayers can be considered the most important factor affecting circulation half-life of liposomes in drug delivery and the amount of materials that can be encapsulated into the vesicles (Çağdaş, Demir Sezer, & Bucak, 2014). According to the desired formulation, different liposome preparation methods can be employed. These methods can be classified as mechanical agitation, solvent dispersion and detergent removal (Çağdaş, Demir Sezer, & Bucak, 2014; Akbarzadeh, Rezaei-Sadabady, Davaran, Woo Joo, & Zarghami, 2013). The main difference in these methods is their approach to overcome the low solubility of lipids in water (Çağdaş, Demir Sezer, & Bucak, 2014).

2.3.1 Mechanical Agitation

Agitation methods include: Sonication (2.3.1.1), French pressure cell (2.3.1.2), Membrane extrusion (2.3.1.3), Freeze-thaw (2.3.1.4), Hydration of a thin lipid film (2.3.1.5), Micro-emulsification (2.3.1.6) (Riaz, 1996; Himanshu, Sitasharan, & Singhai, 2011).

2.3.1.1 Sonication

Possibly, the easiest and most common method utilized for disrupting the MLVs to form SUVs is sonication. However, there are some drawbacks such as possible degradation of the lipids or compounds to be reconstituted into the liposome due to heat release during sonication, titanium contamination in case of using the probe sonicator as well as presence of some MLVs along with SUVs, and very low internal volume/encapsulation efficacy since SUVs would be formed through sonication (Çağdaş, Demir Sezer, & Bucak, 2014; Riaz, 1996). Therefore, when it comes to drug delivery applications, mechanical agitation

methods are not proper enough owing to their possible size instability and high leakage of encapsulated drugs (Çağdaş, Demir Sezer, & Bucak, 2014).

Two sonication techniques that can be used to convert MLVs into SUVs are probe and bath sonication (Arcadio & Cullis, 1995 ; Awada, et al., 2004). Probe sonication is used for disruption of highly concentrated lipid solutions that require high energy. The process involves the insertion of a probe into the liposome solution, however, because of localized heat released from the probe tip, the sample should be kept on ice during sonication (Akbarzadeh, Rezaei-Sadabady, Davaran, Woo Joo, & Zarghami, 2013). In bath sonication, the liposome solution is put in a sonication bath. This method has less disadvantages compared to the probe sonication since sample can be protected in a sterile vessel or under an inert atmosphere to protect it against heat generation (Kataria, et al., 2011). Also, unlike the probe sonication large volumes of sample can be sonicated. As a result, bath sonication is the most commonly used technique for SUVs preparation (Çağdaş, Demir Sezer, & Bucak, 2014).

2.3.1.2 French Pressure Cell

In this method, MLVs are forced through a small orifice under high pressure (Sharma & Sharma, 1997; Riaz, 1996). One advantages of this technique over sonication (and detergent removal methods discussed later) is that the protein integrity is retained. Also, due to larger vesicle formation than the SUVs produced by sonication, vesicles can retain encapsulated solutes longer (Song, et al., 2011; Mozafari M. R., 2005). Although, there are also drawbacks, including production of relatively small working volumes (maximum of ~ 50 mL) (Riaz, 1996; Himanshu, Sitasharan, & Singhai, 2011).

2.3.1.3 Membrane Extrusion

The most common preparation method is extrusion through polycarbonate filters of defined pore sizes which creates a uniform size distribution of liposome vesicles (Wang & Tonggu, 2014; Hope, Bally, Webb, & Cullis, 1985). Extrusion can be done using a device called extruder (manufactured by Avanti and LipexTM), which are composed of two syringes, rings, polycarbonate. Either extruder can be used to generated unilamellar vesicles of different sizes (Wang & Tonggu, 2014; Olson, Hunt, Szoka, Vail, & Papahadjopoulos, 1979). Repetitive extrusion of MLVs through 100-nm or less pore sizes leads to homogenous size distribution of unilamellar vesicle while membranes with larger pore sizes (e.g., 200 and 400 nm) produce larger and mostly multilamellar vesicles (Wang & Tonggu, 2014).

2.3.1.4 Freeze-thaw

In this method, rapid freezing (-80 ^{0}C or liquid nitrogen) and slow thawing of SUVs form unilamellar vesicles (Alpes, et al., 1986 ; Gabizon, 2001; Geertsma, Nik Mahmood, Schuurman-Wolters, & Poolman, 2008). Aggregated materials are dispersed into LUVs via short-lived sonication (Wang & Tonggu, 2014; Pick, 1981; Ohsawa, Miura, & Harada, 1985; Liu & Yonetani, 1994). However, the effectiveness of this method can be significantly reduced by lipid concentration and high ionic strength of the medium. The reported encapsulation efficacies achieved using this method are from 20% to 30% (Pick, 1981).

2.3.1.5 Hydration of a Thin Lipid Film

This method involves the hydration of a thin film of lipid in a proper buffer (aqueous medium or organic solvent) at the temperature above the lipid transition temperature (T_c); T_c is a temperature required to change the lipid physical state from the gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented and fluid (Avanti Polar Lipids, 2018; Dumaual, Jenski, & Stillwell, 2000). The solution is thoroughly mixed manually or mechanically with bath sonication or vortexing. Although, this method is very simple and commonly used, it forms a milky suspension of a heterogonous population of MLVs (1–5 μm diameter) (Laouini, et al., 2012; Sharma & Sharma, 1997; Çağdaş, Demir Sezer, & Bucak, 2014). To control the size and homogeneity of the resulting vesicle solutions during hydration, it has been suggested to follow up with additional methods. One is to convert MLVs to LUVs by introducing an aqueous sucrose solution down the side of the flask by tilting the flask to one side and returning the flask to the upright orientation gently, allowing the fluid to run slowly over the lipid layer on the bottom of the flask. The swelling is done typically without any shaking or agitation. The suspension is then centrifuged and the layer of MLVs floating on the surface is removed, resulting in only LUVs in solution (Çağdaş, Demir Sezer, & Bucak, 2014).

2.3.1.6 Microemulsification

Another method to produce SUVs is microemulsification. Here, using a microfluidizer, liposome vesicles in the size range of 50-200 nm with an encapsulation efficiency of up to 75% can be generated (Çağdaş, Demir Sezer, & Bucak, 2014).

2.3.2. Solvent Dispersion

The solvent injection method involves the dissolution of the lipid into an organic phase (ether or ethanol), followed by the injection of the lipid solution into aqueous media to form liposome vesicles (Szebeni, et al., 1984; Sharma & Sharma, 1997).

2.3.2.1 Ether Injection

In this method lipids are dissolved in an immiscible solvent like diethyl ether or ether-methanol and then the mixture is slowly added into an aqueous solution of the materials to be encapsulated into the vesicles. Temperatures above the boiling point of the ether (about 55°C to 65°C) or reduced pressure would be applied to the aqueous solution so that ether would be vaporized and removed once it comes to the contact with the aqueous phase that results in the formation of mainly unilamellar liposomes vesicles (Akbarzadeh, Rezaei-Sadabady, Davaran, Woo Joo, & Zarghami, 2013; Laouini, et al., 2012; Sharma & Sharma, 1997; Çağdaş, Demir Sezer, & Bucak, 2014).

An advantage to the ether injection method compared to the ethanol injection method is that ether causes lower risk of oxidative degradation of the lipid membranes. Also, since the removal of the solvent (ether) from the product is at the same rate that it's being introduced, it allows the process to carry on for a longer time forming a concentrated liposomal product of high encapsulation efficiencies (Çağdaş, Demir Sezer, & Bucak, 2014; Laouini, et al., 2012).

The main disadvantages are that the vesicle population is heterogeneous (70 to 200 nm in size) and since the temperature of the aqueous solution is elevated, the materials to be encapsulated may be affected (Deamer & Bangham, 1976 ; Schieren, Rudolph,

Finkelstein, Coleman, & Weissmann, 1978). Additionally, it takes a long time to produce a batch of liposomes and the introduction of lipid solution must be done with delicacy (Çağdaş, Demir Sezer, & Bucak, 2014).

2.3.2.2 Ethanol Injection

The ethanol injection method was first described in 1973 (Batzri & Korn, 1973). In this method, using a fine needle, an ethanolic lipid solution is injected swiftly into excess saline or other aqueous medium (Laouini, et al., 2012; Akbarzadeh, Rezaei-Sadabady, Davaran, Woo Joo, & Zarghami, 2013; Batzri & Korn, 1973). The injection force is usually enough to achieve complete mixing, subsequently ethanol is diluted in water and lipids are distributed evenly throughout the medium. This method results in a high proportion of heterogenous small vesicles (under 100 nm) (Laouini, et al., 2012; Batzri & Korn, 1973; Akbarzadeh, Rezaei-Sadabady, Davaran, Woo Joo, & Zarghami, 2013).

This technique is simple with a very low risk of degradation for sensitive lipids. However, there are some downsides such as the limitation of solubility of lipids in ethanol and the volume of ethanol that can be added into the medium. This restricts the amount of dispersed lipid which results in a diluted liposome solution that reduces the fraction of encapsulation of the hydrophilic materials. Another disadvantage is the difficulty of the removal of ethanol from the lipid solution since it forms an azeotrope with water (Çağdaş, Demir Sezer, & Bucak, 2014; Batzri & Korn, 1973).

2.3.2.3 Reverse Phase Evaporation (REV)

In this method, by short sonication of a two-phase system of phospholipids (can be numerous lipid formulations) in organic solvent such as isopropyl ether, diethyl ether, or a

mixture of isopropyl ether and chloroform with aqueous buffer, an emulsion of water and oil is formed (Laouini, et al., 2012; Akbarzadeh, Rezaei-Sadabady, Davaran, Woo Joo, & Zarghami, 2013); Organic solvent is removed under reduced pressure and continuous purging with nitrogen to remove any residual solvent. Aqueous buffer is then added to form large unilamellar and oligolamellar vesicles.

Vesicles prepared by this method have an aqueous to lipid ratio of about thirty times more than the vesicles formed by sonication and four times more than MLVs. As a result, a high percentage of the aqueous compounds including small, large and macromolecules (up to 65% in a medium of low ionic strength) can be encapsulated into the vesicles. However, there can be issues like adverse effects of either organic solvent or brief sonication on the encapsulated compounds that may cause protein denaturation or breakage of DNA strands (Szoka & D Papahadjopoulos, 1978).

2.3.3 Detergent Removal

In this method, detergents of critical micelle concentrations (CMC) are used to solubilize lipids and form mixed micelle; CMC of a surfactant or a detergent is a concentration that above that, micelles which are self-assembled structures composed of hydrophilic heads and hydrophobic tails, are formed (Tadros, 2013). By removing detergents through controlled dialysis, the micelles convert to LUVs (Laouini, et al., 2012; Sharma & Sharma, 1997; Çağdaş, Demir Sezer, & Bucak, 2014; Daemen, Hofstede, Ten Kate, Bakker-Woudenberg, & Scherphof, 1995). Detergent removal includes dialysis, dilution, polystyrene beads application and gel filtration.

2.3.3.1 Dialysis

Dialysis can be performed using a commercial dialysis system called LipoPrep (Diachema AG, Switzerland) or in dialysis bags immersed in large detergent-free buffers (equilibrium dialysis) (Shaheen, et al., 2006). Mixture of lipid-detergent- materials to be encapsulated (e.g. protein), is dialyzed against detergent-free buffer (in ~200-1000-fold excess) in a dialysis bag (cellulose membrane of high permeability) with a cut-off of ~10 kD under gentle stirring (Wang & Tonggu, 2014). During dialysis, only the detergent monomers diffuse through the dialysis bag and the removal rate is associated with the monomer detergent concentration throughout the bag (Rigaud, Pitard, & Levy, 1995). This method is mostly suitable and applicable for detergents with high CMC. Ionic detergents (sodium cholate, sodium deoxycholate), octylglucoside, or 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) can be removed by dialysis in 1–2 days (Rigaud, Pitard, & Levy, 1995). In order to control the removal rate and reduce the dialysis time, flow-through dialyzers can be used (Rigaud, Pitard, & Levy, 1995). Additionally, polystyrene beads can be added outside the bag to reduce the number of changes of buffer during dialysis and consequently decrease the time of dialysis (Philippot, Mutaftschiev, & Liautard, 1983).

Advantages of this method are low cost, simplicity and applicability for many membrane proteins. Disadvantages include unmanageable rate of dialysis, poor reproducibility, possible retention of molecules on the dialysis membrane and long duration of test run due to the number of changes of buffer that can damage the materials to be encapsulated into the vesicles (Rigaud, Pitard, & Levy, 1995). Also, this method can

be applied to many membrane proteins, it is not a proper method for detergents of low CMCs, such as polyoxyethylene glycols, that require much longer dialysis time (1–2 weeks) that can denature membrane proteins (Allen, Romans, Kercret, & Segrest, 1980).

2.3.3.2 Dilution

A simple method for detergent removal is dilution of the mixture of lipid, materials to be encapsulated (e.g. protein), and detergent by addition of detergent-free buffer into it. Doing this reduces the total detergent concentration below its CMC which leads to spontaneous transition from poly-dispersed micelles to vesicles formation (Rigaud, Pitard, & Levy, 1995; Wang & Tonggu, 2014; Akbarzadeh, Rezaei-Sadabady, Davaran, Woo Joo, & Zarghami, 2013).

Advantages of this technique includes controlled detergent removal rate, monitored vesicle reconstitution (Seras-Cansell, Ollivon, & Lesieur, 1996), and shortened time to reduce detergent concertation.

Disadvantages include an additional centrifugation step to concentrate the diluted vesicles, and incomplete detergent removal (Rigaud, Pitard, & Levy, 1995; Wang & Tonggu, 2014).

2.3.2.3 Polystyrene Beads Resins

Efficient removal of detergents with low CMCs can be accomplished by adding hydrophobic polystyrene beads such as Amberlite XAD and Bio-Beads SM-2 into the lipids-detergent-compounds mixture (Ollivon, Lesieur, Grabielle-Madelmont, & Paternostre, 2000; Levy, Bluzat, Seigneuret, & Rigaud, 1990; Verchère A. , Dezi, Broutin, & Picard, 2014; Rigaud, Pitard, & Levy, 1995). Since detergents are amphiphilic, the

hydrophobic tail binds to the insoluble hydrophobic bead surface (Holloway P. , 1973). After binding, the detergent-coated beads are removed by centrifugation or filtration (Wang & Tonggu, 2014).

Advantages of this method over conventional methods like dialysis or dilution include the ease of detergent removal by controlling only the amount of beads added to the mixture. Moreover, the resins are efficient at detergent removal from the solution, enabling the production of proteoliposomes (liposomes encapsulating proteins) of low ionic permeability, a critical element in the study of protein membrane transporters (Rigaud, Pitard, & Levy, 1995).

2.3.2.4. Gel Filtration

Gel filtration, also known as gel exclusion chromatography, separates based on the size differences of mixed micelles and liposomes. Passage of a micellar mixture through the gel allows for faster elution of the smaller aggregates (monomers, mixed micelles) and formation of liposome vesicles that are excluded from the pores (Rigaud, Pitard, & Levy, 1995; Wang & Tonggu, 2014).

This method is simple, efficient, highly reproducible and quick to perform (5-20 min) using a Sephadex (G-50) column. Disadvantages are the resulting diluted sample that would need an additional concentration step, limited contact time between detergent and materials to be encapsulated (e.g. protein) that would lead to inhomogeneous and/or incomplete materials' incorporation and heterogeneous size distribution and possible lipid retention on the gel (loss of lipid) (Wang & Tonggu, 2014; Abeywardena, Allen, & Charnock, 1983; Akbarzadeh, Rezaei-Sadabady, Davaran, Woo Joo, & Zarghami, 2013).

Hence, use of gel chromatography in detergent-mediated reconstitution of membrane proteins is not recommended (Rigaud, Pitard, & Levy, 1995).

CHAPTER 3

STEPWISE OPTIMIZATION OF PROTEIN EXTRACTION AND PURIFICATION

3.1 Introduction

Preparation of purified Kup, the target membrane transporter, is the initial major step to construct proteoliposome vesicle. Published methods as well as commercially available kits for protein extraction are not the most economical and easy to use. Furthermore, there is no specific protocol published for extraction and purification of Kup proteins. There remains a need to develop a simple, fast, cost-effective approach to achieve efficient protein extraction.

In this work, two new extraction methods were developed for the preparation of purified Kup transporter. However, since samples obtained from extraction include all cytoplasmic, periplasmic, and membrane-bound proteins, protein purification is also required to yield target protein from *E. coli* cell. Affinity purification, known as immobilized metal affinity chromatography (IMAC), is a common method to purify protein and is explored here.

In this chapter, the development of an efficient membrane protein extraction and purification step-wise procedure is presented, and it is based on the IMAC method.

3.2 Materials and Methods

3.2.1 Bacterial Strain and Plasmid

Strain K-12 (MG1655) was acquired from Arizona State University Plasmid repository (DNASU 14142). The host cell was *E. coli*, which had been transformed with our genetically engineered plasmid of interest, vector pNYCOMPS-LIC-FH10T+(N term)

containing histidine-tagged Kup (DNASU Plasmid Repository) under the direction of a T7 promoter, with a kanamycin resistance cassette (Figure 3).

The Kup system, a low affinity potassium transporter encoded by the TrkD gene in *E. coli*, is capable of high cesium accumulation (Tomioka, Uchiyama, Yagi, & Fujii, 1995) and encodes a protein of 622 amino acids with a calculated molecular weight of 69.293 kDa (Schleyer & Bakker, 1993)

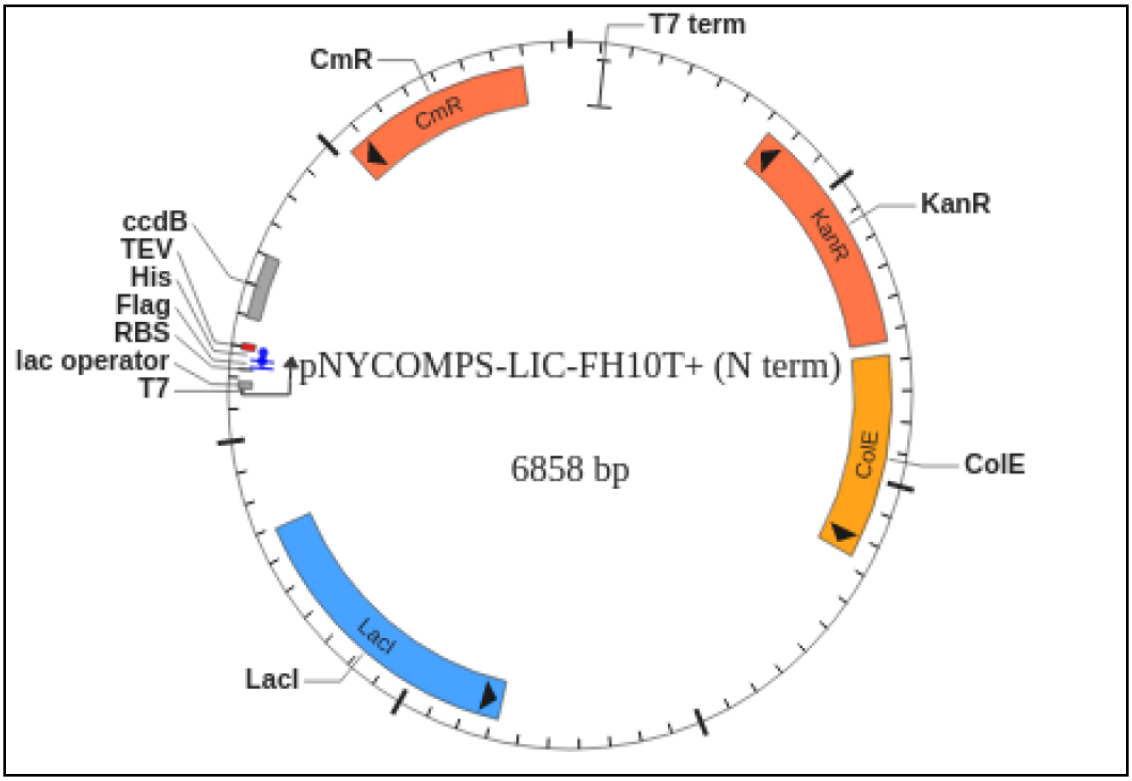

Figure 3. Vector Map of the Plasmid (DNASU Plasmid Repository)

3.2.2 Bacterial Growth and Culture Preparation

Kup cells arrived as frozen glycerol stocks in small cryogenic tubes and were subsequently streaked on kanamycin plates (refer to Appendix for kanamycin stock

solution and LB plate preparation) were prepared. Plates were incubated overnight at 37 ºC and frozen glycerol stocks (in 20% glycerol) were stored in -80 ºC freezer for future use.

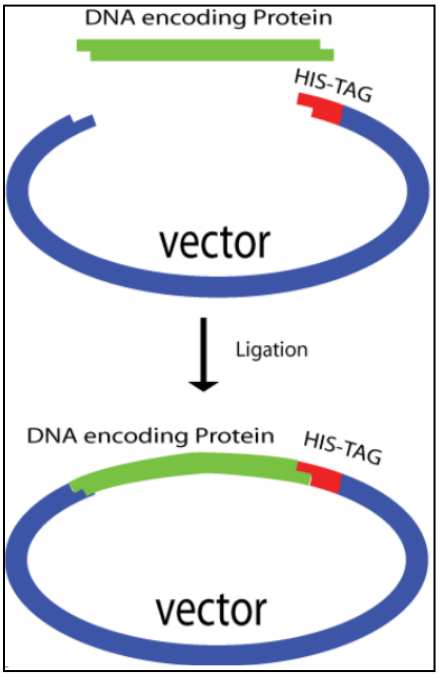

Figure 4. Illustration of Histidine tagged to a Vector

In order to perform extraction and purification, cells harboring the plasmid were grown in LB broth containing kanamycin. Kup was precultured by inoculation of 10-ml LB broth and 1000X dilution of kanamycin stock (50 mg/ml) with a single colony, followed by incubation at 37 ºC and 200 rpm on an incubated orbital shaker. A larger batch (1L) was seeded with the overnight culture containing LB-broth and kanamycin and grown to mid-log phase (OD600 0.6-0.7).

Protein expression was induced by adding 4-ml of 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution to a final concentration of 0.4 mM; IPTG

reagent is a molecular mimic of allolactose (a lactose metabolite) that prompts transcription of the lac operon (functioning unit of DNA essential for the transport and metabolism of lactose), and consequently employed to induce *E. coli* protein expression (IPTG Induction Theory). After IPTG addition, the culture was incubated until OD600 of 1.6-1.8 was reached. The prepared bacterial culture was stored at 4 ^{o}C overnight for protein extraction and purification.

3.2.3 Protein Extraction

Different methods of protein extraction are described including commercially available extraction kits and published methods. In this work, two methods, new processing method (NPM) for protein extraction (3.2.3.1) and optimizing the new protein extraction technique (3.2.3.2) were tested to develop an easy, fast, cost-effective approach to achieve successful protein extraction.

3.2.3.1 New Processing Method (NPM) for Protein Extraction

Bacteria cultures (1-L) were split into four 200-ml centrifuge conical tubes and spun at 4100 rpm for 16 minutes at 19 °C. The supernatant was removed and pellets were combined and resuspended in 200-ml phosphate-buffered saline (PBS) followed by centrifugation at 4100 rpm for 16 minutes. Supernatants were discarded. This wash was repeated twice to reduce the amount of contaminants and salts that impact the purity and efficiency of the protein extraction process.

The resulting pellet was resuspended in 15-ml Tris-buffer (pH 8.0), phenylmethylsulfonyl fluoride (100X of 100 mM PMSF), lysozyme (10X of 10 mg/ml), Deoxyribonuclease I (DNase I) storage (10X), and DNase I reaction buffer were added to

the solution and stirred at 4 °C for 40 minutes. Lysozyme was used to lyse/digest the cell walls, DNase to cleave the DNA strands to reduce the viscosity of the lysate, and PMSF served as the protease inhibitor to preserve the protein. To solubilize membranes, the entire solution was sonicated on ice for 6-8 minutes in 3-4 cycles with 30 seconds rest between each cycle. SDS (100X of 10%) was added to samples and the tube was inverted to mix. Sample was centrifuged at 4100 rpm for 1 hour 30 minutes to pellet the lipid membrane. The supernatant was transferred into a new tube and stored -20 ^{o}C overnight. The next day, the sample was thawed and centrifuged again at 4100 rpm. The final supernatant was the extracted proteins and ready to be purified on nickel-nitrilotriacetic acid (Ni-NTA) resin column (refer to Appendix). Summary of the new processing method is presented in Figure 5.

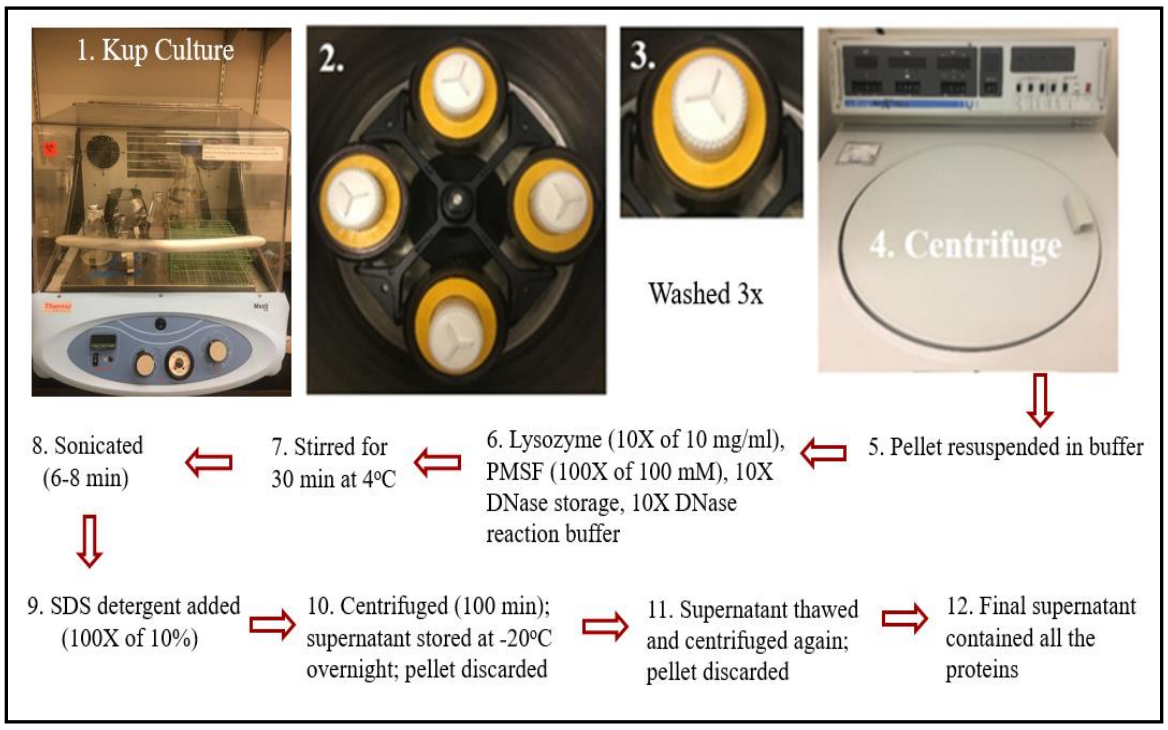

Figure 5. Illustration of New Processing Method (NPM) for Protein Extraction

3.2.3.2 Optimizing the New Protein Extraction Technique

In this method, PMSF, DNase and lysozyme were eliminated, and all the pellets were kept and purified to see where (for example supernatant or pellets) the membrane proteins are. Step by step procedure is presented below (Figure 6).

1. Each 1-L sample was split into four 200-ml centrifuge conical tubes and spun at 4100 rpm for 16 min.; supernatants were removed and pellets were combined and resuspended in about 200-ml phosphate-buffered saline (PBS) followed by centrifugation at 4100 rpm for about 16 min. Supernatant was discarded. This step was repeated two more times.
2. Resultant pellet was resuspended in 15-ml Tris-buffer (pH 8.0).
3. The whole solution was sonicated on ice for about 12-15 minutes (1 min each time with 30 seconds rest in between).
4. Sample was centrifuged at 4100 rpm for 1 hr and 40 min; 500 µl of this supernatant was kept (I) to be run on the gel and the rest of the solution was transferred into a new tube and stored overnight at -20 °C freezer.
5. Pellets from stage 4 was suspended in 2-ml PBS and 100X SDS; 500 µl was taken from this solution (II) to be run on the gel and the rest was centrifuged at 4100 rpm for 1 hr and 20 min at 17 °C and the resultant supernatant was kept in -20 °C so it could be purified next day by Ni-column and run on the gel (III).
6. Next day, the frozen supernatant from step 4, was thawed and centrifuged again at 4100 rpm for 1 hr and 30 min, so the final supernatant could be purified by Ni Column and prepared to be run on the gel (IV).

7. Pellets from step 6 were resuspended in 8-ml of PBS and 100X of SDS; 500 µl of this supernatant was kept (V) to be run on the gel and the rest of the solution was transferred into a new tube and centrifuged for 1 hr and 20 min.; the resultant supernatant was purified by Ni-Column to be run on the gel as well (VI).

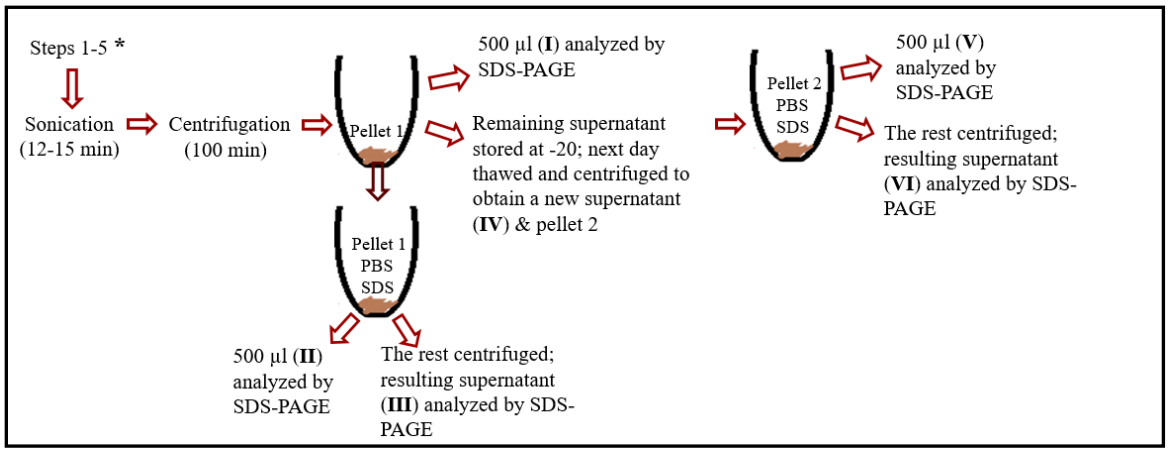

Figure 6. Illustration of Optimizing the New Protein Extraction Technique.
Note: *Steps 1-5 are as NPM in Figure 5.

3.2.4 Protein Purification

Histidine-tagged proteins can be purified because the string of histidine residues (10x) binds to several types of immobilized metal ions, such as nickel, copper and cobalt. This is the base for affinity purification known as immobilized metal affinity chromatography (IMAC) (Thermo Fisher Scientific).

In this process, purified protein fractions were obtained according to (Thermo Fisher Scientific) with some modification (refer to appendix B). The gravity-flow column (Figure 7) used in this experiment for Kup purification was packed with Ni – NTA (nitrilotriacetic acid) resin. The NTA resins function as chelators to immobilize the desired

metal ions. Elution of captured his–tagged Kup protein from the column was accomplished by adding imidazole which has an affinity to histidine due to structure similar to histidine.

In order to prepare sample to perform purification, the final volume (about 15 mL) of the protein extract was mixed with the same volume of equilibration buffer (20 mM sodium phosphate, 300 mM sodium chloride (PBS), 10 mM imidazole, pH 7.4).

The gravity-flow column with Ni- NTA resin was first equilibrated by adding equilibration buffer, twice the resin-bed volume (BV = 3 ml) and then drained slowly by gravity. The prepared extracted protein was added to the column and the flow-through was collected as several fractions in 1.5-ml centrifuge tubes. The column was washed with 2 resin-bed volume of wash buffer (PBS with 25 mM-250 mM imidazole, pH 7.4) and flow though fractions were collected ($W_{1\text{-}1}$-$W_{1\text{-}4}$). This step was repeated 2-3 more times. His-tagged Kup was eluted from the resin by adding 2 resin-bed volume of elution buffer (PBS with 250 mM-1000 mM imidazole, pH 7.4) and the flow through samples ($E_{1\text{-}1}$-$E_{1\text{-}4}$) were collected. This step was repeated 2 more times. All the fractions were then stored at -20 ºC to be run on the gel for protein bands visualization. Table 1 summarizes three steps of purification.

A range of imidazole concentrations in wash and elution buffers were tested, as there's no specific, predetermined concentration that would lead to a purified protein band. To compare the different concentrations and visualize yield, SDS-PAGE and Coomassie Blue staining were performed.

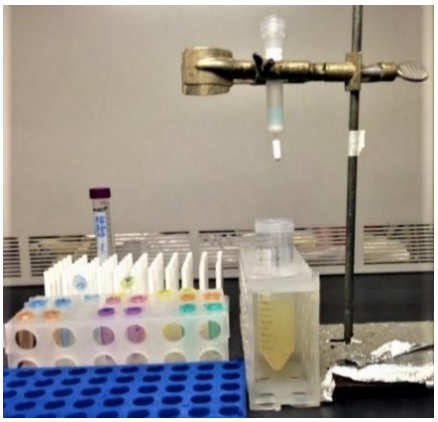

Figure 7. Gravity-flow Column Packed with Nickel Resin

Table 1. Summary of the Steps in Optimizing the Purification Process

<table>
<tr><th>Equilibration (Eq.)</th><th colspan="2">Wash (W)
25-250 mM *</th><th colspan="2">Elution (E)
250-1000 mM *</th></tr>
<tr><td rowspan="16">Twenty fractions were collected and run on SDS-PAGE; all the fractions had band intensity similar to the extracted sample before the purification (Figure 10); thus, there was no need to run the fractions on the gel anymore and focus was mostly on wash and elution steps.</td><td rowspan="4">1st Wash (W_1)</td><td>W_{1-1}</td><td rowspan="4">1st Elution (E_1)</td><td>E_{1-1}</td></tr>
<tr><td>W_{1-2}</td><td>E_{1-2}</td></tr>
<tr><td>W_{1-3}</td><td>E_{1-3}</td></tr>
<tr><td>W_{1-4}</td><td>E_{1-4}</td></tr>
<tr><td rowspan="4">2nd Wash (W_2)</td><td>W_{2-1}</td><td rowspan="4">2nd Elution (E_2) **</td><td>E_{2-1}</td></tr>
<tr><td>W_{2-2}</td><td>E_{2-2}</td></tr>
<tr><td>W_{2-3}</td><td>E_{2-3}</td></tr>
<tr><td>W_{2-4}</td><td>E_{2-4}</td></tr>
<tr><td rowspan="4">3rd Wash (W_3)</td><td>W_{3-1}</td><td rowspan="8">3rd Elution (E_3) **</td><td>E_{3-1}</td></tr>
<tr><td>W_{3-2}</td><td>E_{3-2}</td></tr>
<tr><td>W_{3-3}</td><td rowspan="5">E_{3-3}</td></tr>
<tr><td>W_{3-4}</td></tr>
<tr><td rowspan="4">4th Wash (W_4)</td><td>W_{4-1}</td></tr>
<tr><td>W_{4-2}</td></tr>
<tr><td>W_{4-3}</td></tr>
<tr><td>W_{4-4}</td></tr>
</table>

Note: *After performing many purification experiments, testing a range of 25-250 mM and 250-1000 mM for imidazole concentration in wash and elution buffer respectively, and their associated SDS-PSGE analyses (presented in results & discussion section), it was realized that: ** the target protein band (Kup of 70 kDa) was obtained from the first dilution (E_1) step and as a result no further elution steps (E_2 and E_3) are required.

3.2.5 SDS- PAGE Gel for Detection of Kup Protein

According to (Sambrook, J.; Russell, D. W., 2006) protein fractions were separated by a hand cast polyacrylamide gel electrophoresis (PAGE). Ten mL of 10% dissolving gel prepared by mixing 6 components as shown in Table 2 and added to the area between two glasses of vertical gel cast device using Pasteur pipette. Isopropanol was poured on top layer of the gel and left to polymerize. After ~ 1 hour, isopropanol was removed by pouring it off and 8-ml of 5% stacking gel was prepared by quick mixing and gentle swirling of the components presented in Table 3.

Table 2. 10-ml of 10% Resolving Gel

Components	Volume (ml)
H_2O	4.6
30% acrylamide mix	2.7
1.5 M Tris-Base (pH=8.8)	2.5
10% SDS	0.1
10% ammonium persulfate (APS)	0.1
TEMED	0.006

Table 3. 8-ml of 5% Stacking Gel

Components	Volume (ml)
H_2O	5.5
30% acrylamide mix	1.3
1.0 M Tris-HCl (pH=6.8)	1.0
10% SDS	0.08
10% ammonium persulfate (APS)	0.08
TEMED	0.008

The stacking gel was poured over the dissolving gel, and the well comb was inserted. Complete polymerization occurred in two hours. Sample buffer (25 □L, 2X) including 0.125 M of Tris-HCl or preferably Tris-base (pH 6.8), 4% SDS, 5% BME (2-mercaptoethanol), 20% glycerol, and 0.004% bromophenol blue, as a tracking dye to visualize protein bands during gel electrophoresis, was mixed with 25 μL of each purified protein fractions and were heated for 3-4 minutes at 85-100 ºC in a water bath. The chambers of the mold were filled with 1X running buffer (25 mM Tris base, 250 mM glycine, pH 8.3, 10% SDS, dH_2O). Protein ladder (5-10 μL; 60 – 250 kDa) was used (Figure 8, first well), then 15 μL of each sample was loaded into each well. The samples were run through the stacking gel for approximately 1 hour (50V) until the dye hit the resolving gel border, wherein, the voltage increased to 110 Volt, for about 2 hours to force the migration through the resolving gel (Figure 9). Once the run was complete, the gel attached to the glass plates was immersed in water until the gel was easily removed from the glass, and then the gel was placed in staining solution (Appendix A) for 12-24 hours on a shaker with gentle agitation at room temperature. The gel was destained with destaining solution (Appendix A) for another 12-24 hours until almost all of the blue dye was removed and protein bands could be visualized. Hence, the approximate molecular weight of the visualized protein bands could be roughly determined by comparing them with the ladder. Here, SDS-PAGE indicated that over-expressed, purified Kup (TrkD) was present at the expected molecular weight of about 69 kDa.

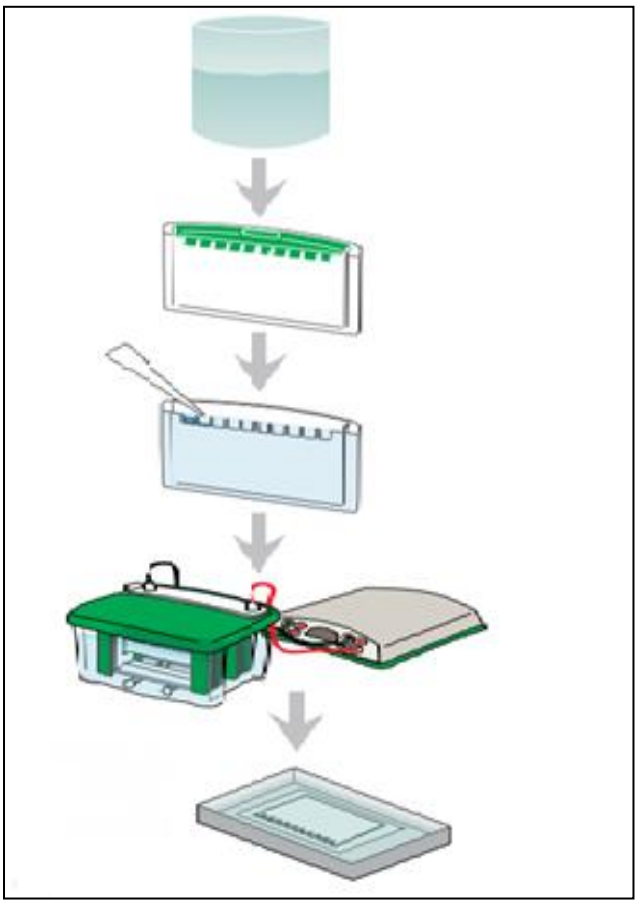

Figure 8. General Steps for Protein Detection in SDS-PAGE (OpenWetWare, 2013)

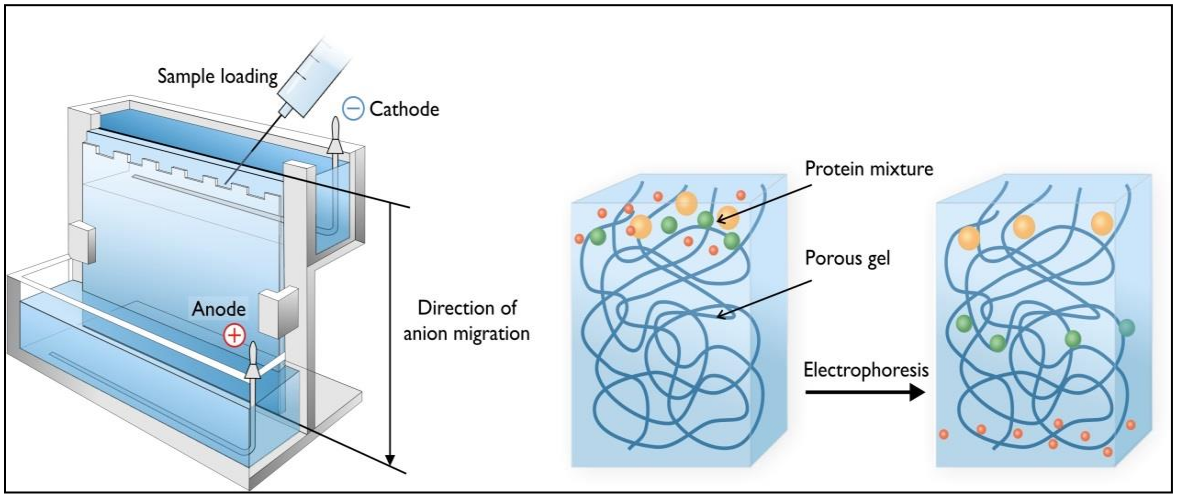

Figure 9. The Porosity of SDS-PA Gel and Migration of Different Size of Macromolecules (Hegyi, et al., 2013)

3.2.6 Protein Quantification using BCA Assay

One of the methods used to measure total protein concentration is bicinchoninic acid (BCA) assay (Smith, et al., 1987; Brady & Macnaughtan, 2015). This assay is a colorimetric detection technique similar to the Lowry assay, based on the reduction of cupper (II) cation (Cu^{+2}) to cupper (I) cation (Cu^{+}), caused by proteins under alkaline conditions (the biuret reaction), followed by the chelation of BCA with Cu^{+}. Bicinchoninic acid is a stable, water-soluble compound that can form an intense purple complex with Cu^{+}. The color produced from this reaction is stable and changes from green to purple in proportion to protein concentration.

Lowry and BCA have similar sensitivity, however, stability of BCA reagent under alkaline conditions, provides a simple, one-step process of higher tolerance to the nonionic detergents and simple buffer salts that restrict the efficiency of two-step Lowry assay (Walker, 1996; Smith, et al., 1987).

In this study, total protein concentration was estimated using Biovision's BCA protein assay kit. The reaction leading to formation of intense purple color caused by Cu^{+1} BCA complex shows a strong absorbance at 562 nm that is linear over a range of protein concentrations between 25-2000 μg/ml. Since in this assay, the production of Cu^{+1} is a function of protein concentration, the protein content of unknown samples can be estimated spectrophotometrically by comparison with protein standards of known concentration. The standard used in this kit was Bovine Serum Albumin (BSA) (Smith, et al., 1987; BioVision).

The BCA assay can be performed in a microtiter plate or test tube format; in this work, microplate procedure was employed (details on samples and reagents preparation are presented in the Appendix B).

3.2.6.1 Microplate Procedure for BCA assay.

Four purified protein samples obtained from four separate sets of extraction and purification were prepared in triplicate using dilution factors of 0, 2 and 4 as presented in Table 4.

1. BSA standard and protein samples (25 µl of each) was added into 96-well microplate. Here, three rows of the microplate were used for standards (triplicates) to obtain standard deviation.
2. BCA working reagent (200 µl) was added to the standard and sample wells and mixed thoroughly for 30 seconds.
3. The Microplate was covered with adhesive film and incubated at 37 °C for 30 minutes; after incubation, the plate cooled to room temperature.
4. Absorption wavelength of the microplate reader was set to 562 nm (OD_{562}) and all standards and samples were read.

Table 4. Preparation of Protein Samples for BCA assay

Tube	Volume of Kup protein sample (µl)	Volume of PBS (µl)
1	25	0
2	25	25
3	25 of tube 2	25

3.2.6.2 Calculation of Protein Concentration

A plot of OD_{562} (Y-axis) versus BSA concentration (X-axis) yields the slope (a) and intercept (b), which can be determined and used to calculate the Kup concentration in the purified protein samples by the following equation.

$$C = DX = D\,\frac{Y-b}{a} \qquad \text{Equation 1}$$

Where:

C= Protein concentration in sample (µg/ml)

D = Dilution factor of protein sample

X= concentration of protein sample

Y = OD_{562} of protein sample

a = Slope of the BSA Standard curve

b = Y-intercept of the Standard Curve

3.3 Results and Discussion

The results of the two methods of protein extraction, and varying imidazole concentration are presented here.

3.3.1 Method Selection and Optimization Using SDS-PAGE Analysis

SDS-PAGE images provide the details and documentation of extraction and purification of Kup protein transporter.

3.3.1.1 SDS-PAGE Images for new processing method (NPM) for protein extraction

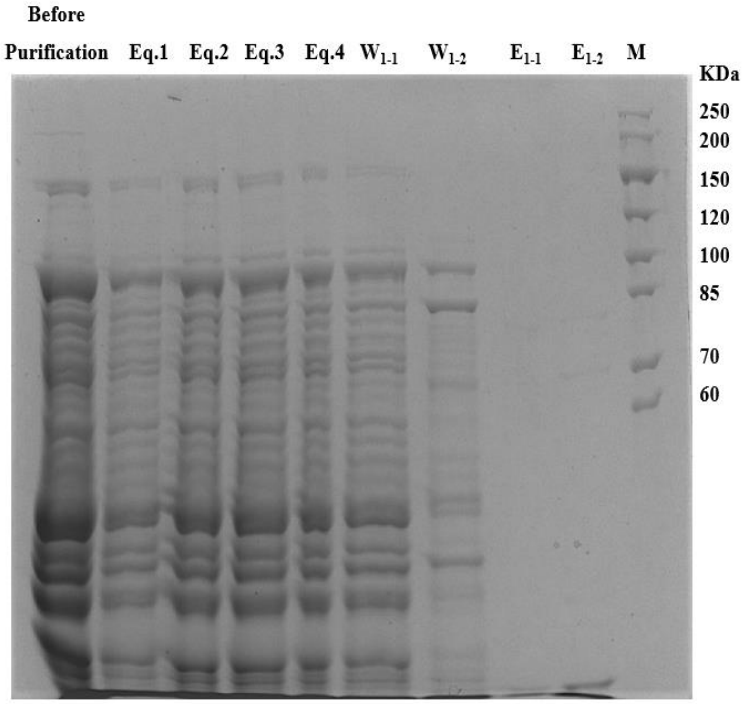

Figure 10. Protein Bands obtained from testing 10, 25 & 250 mM Imidazole Conc. in Eq., W & E. Buffers, respectively. Abbreviations are found in Table 1. M: Marker/ladder. As can be seen, Kup protein band of expected M.W. of 70 KDa was not found in the elution fractions (E); that means 20 and 250 mM imidazole concentrations are not high enough and need to be optimized.

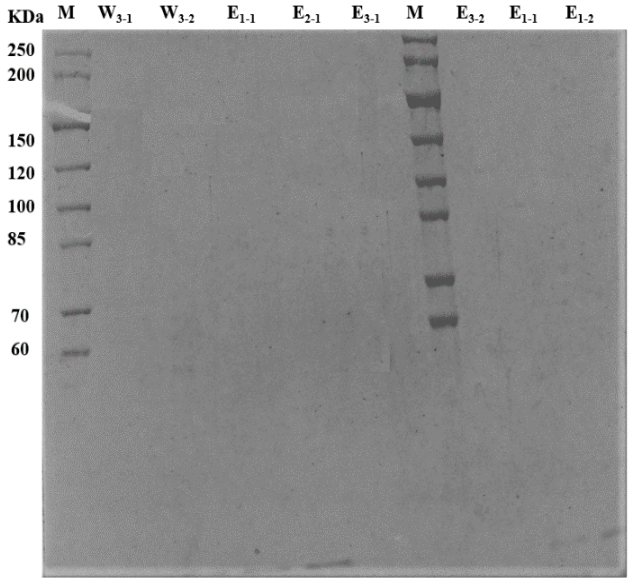

Figure 11. Protein Bands for 20, 40, 300 mM Imidazole Conc. in Eq., W & E Buffers, respectively. Kup protein band of expected M.W. of 70 KDa was not found in the elution fractions (E), meaning 40 and 300 mM imidazole were not high enough and need to be optimized.

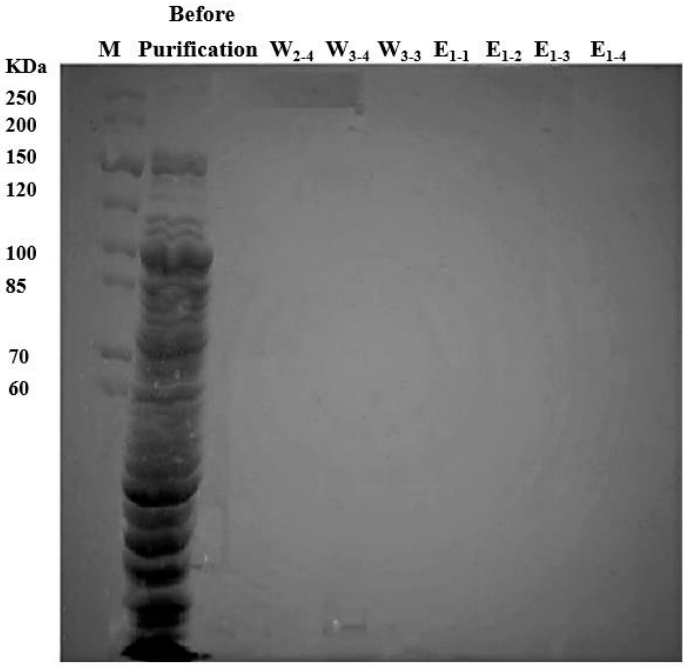

Figure 12. Protein Bands using 10, 250, 1000 mM Imidazole Conc. in Eq., W & E Buffers, respectively. Kup protein band of expected M.W. of 70 KDa was not found in the elution fractions (E); 250 mM imidazole in wash buffer was extremely high.

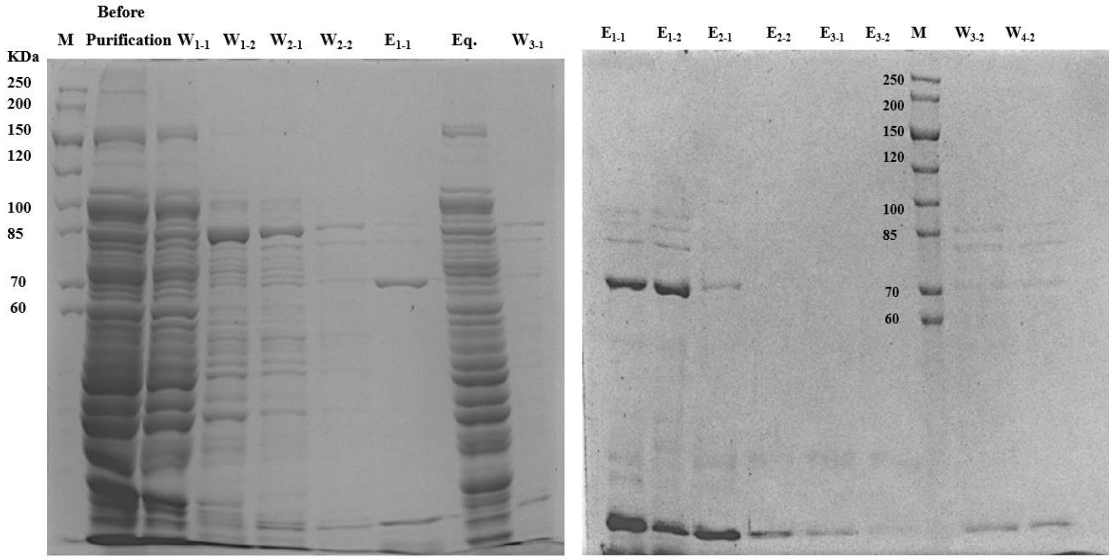

Figure 13. Protein Bands for 10, 25, 1,000 mM Imidazole Conc. in Eq., W & E Buffers, respectively. Kup protein band of expected M.W. of 70 KDa was found in the elution fractions (E); however, there were also some other unspecific bands in the elution fractions, therefore, imidazole greater than 25 mM in wash buffer needed further analysis.

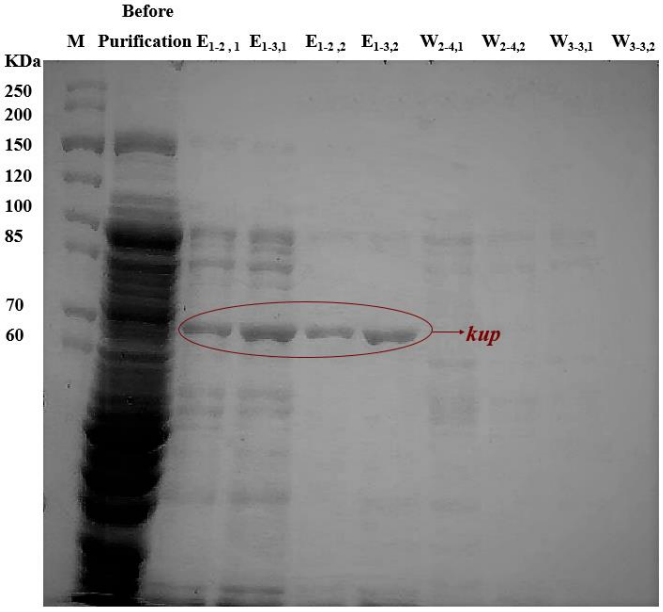

Figure 14. Comparing the Effect of 25 & 50 mM imidazole in W Buffer on Eluted Fractions (E_{1-2} and E_{1-3}) while using 1000 mM Imidazole in E Buffer. $E_{x-y,\,z}$ where z=1 means using 25 mM and z=2 using 50 mM. As can be seen, compared to 25 mM, 50 mM imidazole conc. resulted in more purified proteins.

As can be seen in these images, increasing imidazole concentration from 250 mM to 1,000 mM in elution buffer and from 25 mM to 50 mM in wash buffer (Figure 14), noticeably increased the band intensity and the purification efficiency and the target protein bands (Kup) could be observed. However, in the eluted fractions, there were also some nonspecific bands along with Kup protein; thus, some changes were made to the suggested new processing method (illustrated in Figure 6) to potentially increase the purification efficiency.

3.3.1.2 SDS-PAGE Images for new protein extraction technique

All the pellets and the supernatants collected using the new technique (Figure 6) were analyzed on SDS-PAGE gel as presented below (Figures 15-16).

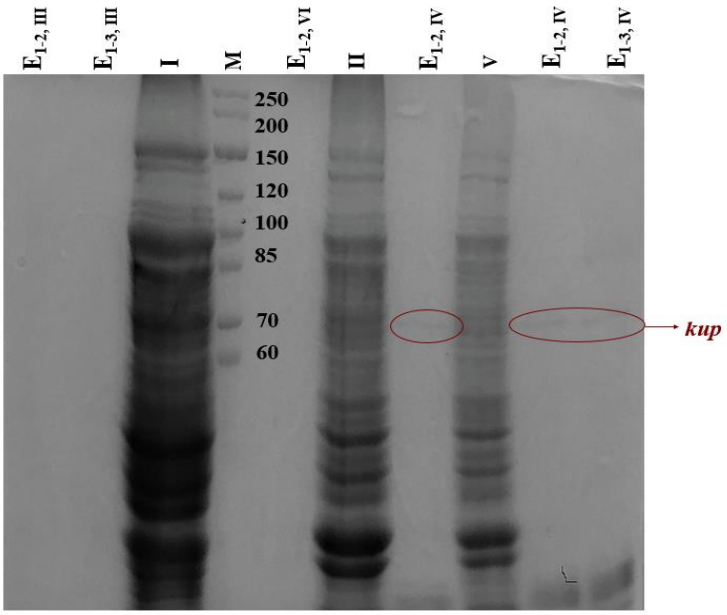

Figure 15. Protein Bands using 10, 75, 1,000 mM Imidazole in E, W & E Buffers, respectively. Kup protein bands were found only in the elution fractions obtained from purification of sample IV and are of low intensity. Imidazole (75 mM) is a high conc. in wash buffer and 50 mM can be chosen as the optimum conc.

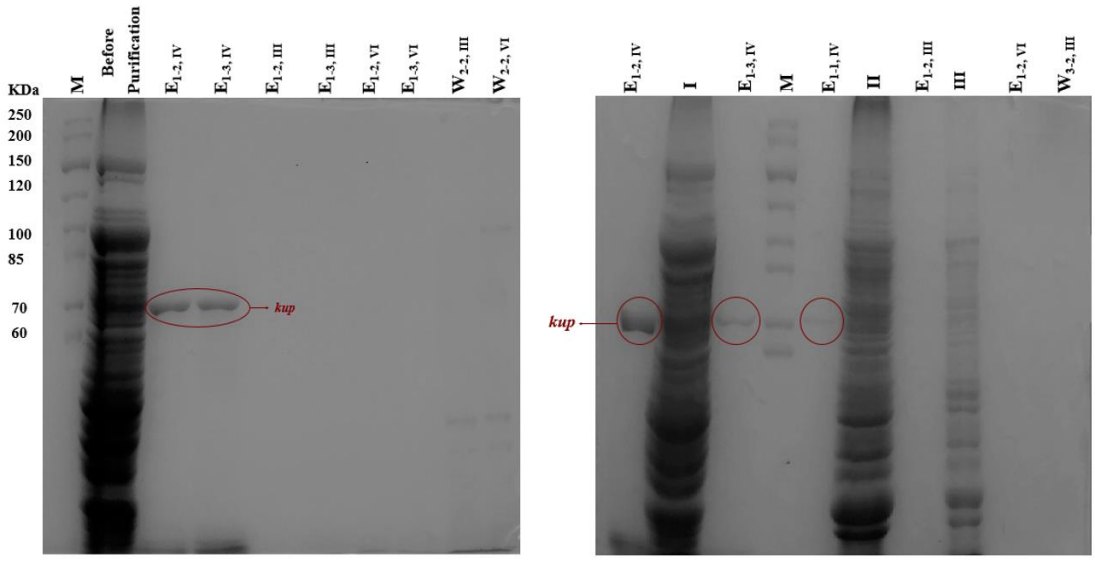

Figure 16. Protein Bands when using 10, 50, 1000 mM Imidazole Conc. in Eq., W & E Buffers, respectively. Kup protein bands were found only in the elution fractions obtained from purification of sample IV.

As can be seen in the SDS-PAGE images, out of all the samples analyzed on the gel, sample IV which is the first resultant supernatant after freeze-thaw step (Figure 6) includes the target Kup protein with no associated unspecific bands; therefore, the new technique described here got more simplified as presented in Figure 17. The optimized imidazole concentrations led to a purified protein band determined to be 50 mM and 1000 mM in wash and elution buffers, respectively (Table 4).

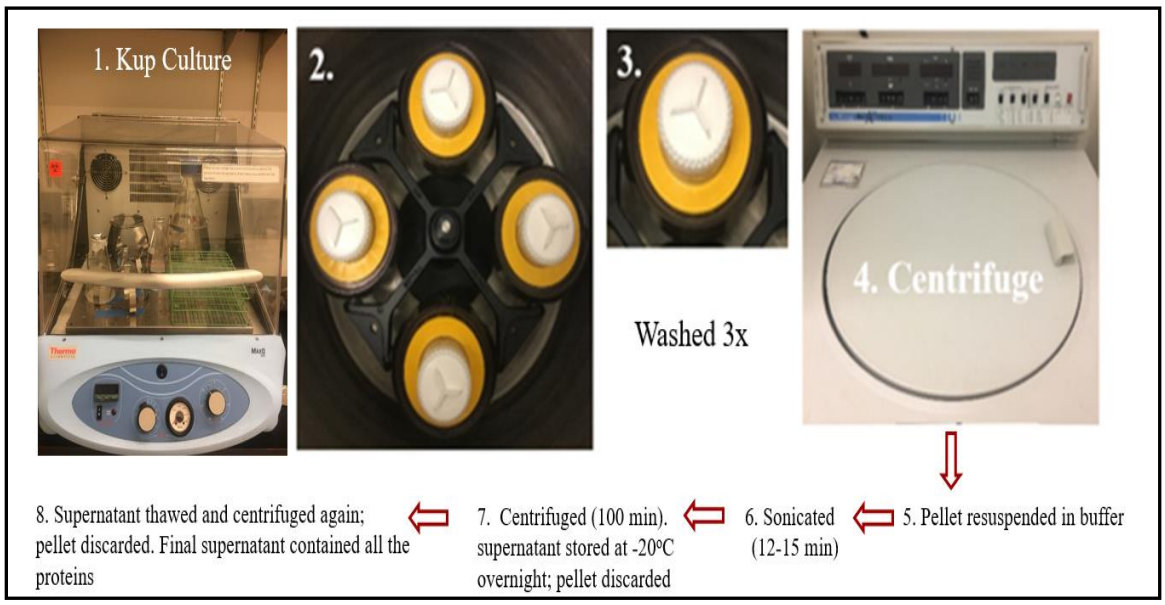

Figure 17. Simplified New Protein Extraction Technique

Table 5. Optimized Concentrations of Buffers used in Protein Purification

Equilibration (Eq.) Buffer	20 mM Na_2HPO_4, 300 mM NaCl (PBS) with 10 mM imidazole; pH 7.4
Wash (W) Buffer	PBS with 50 mM imidazole; pH 7.4
Elution (E) Buffer	PBS with 1000mM imidazole; pH 7.4

3.3.2 Protein Quantification Data

To estimate the Kup protein concentration, the BCA assay was performed as described and optical density (OD_{562}) of all the standards and purified Kup protein samples were measured using a microplate reader (Appendix B). Using the calibration curve for BSA standards (Figure 18), values of a and b were determined to be 0.0012 and 0.0431 and using Equation 1, total protein concentration of each triplicate (dilution) for four different

purified protein samples were calculated (Table 6). According to Figure 19, the average total protein concertation of Kup protein determined to be about 1 mg/ml.

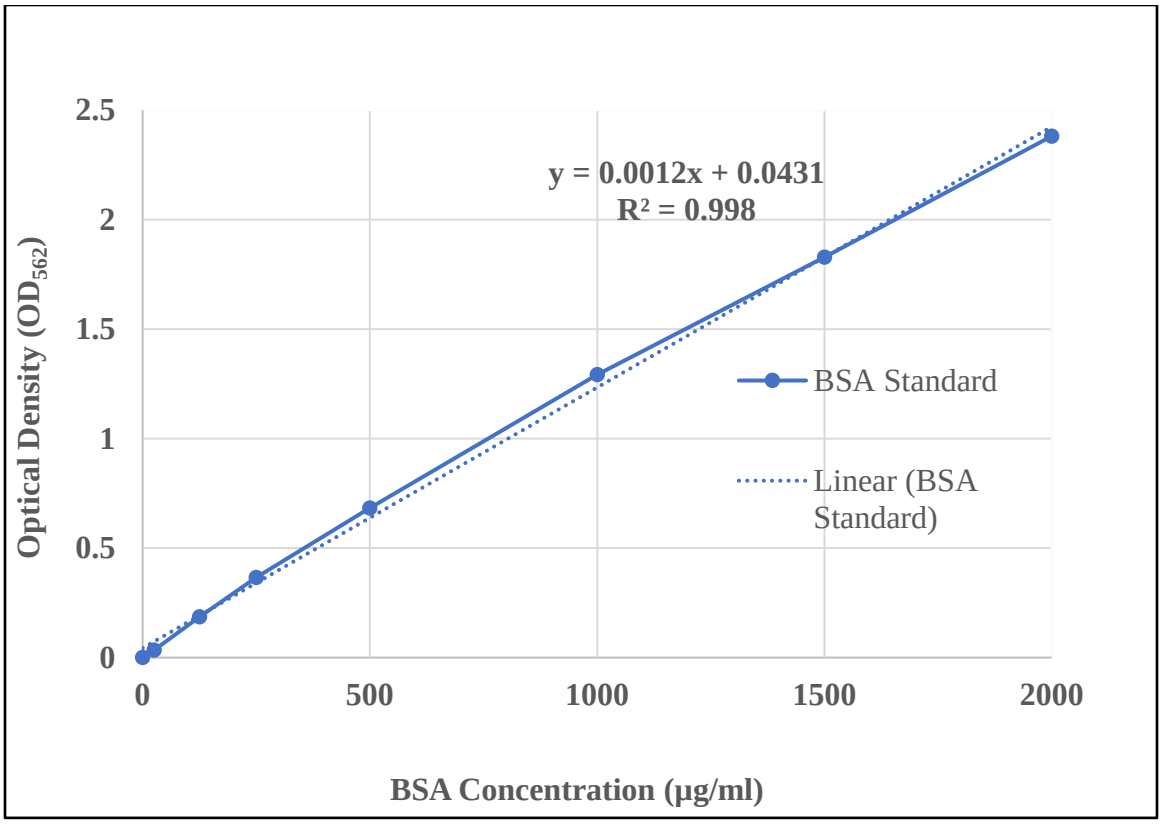

Figure 18. Calibration Curve for BSA Standards

Table 6. Calculated Total Protein Concentration (µg/ml) of Four Purified Protein Samples

Total Protein Concentration (µg/ml)				
Dilution Factor	Sample #1	Sample #2	Sample #3	Sample #4
0X	629.08	681.58	749.08	713.25
2X	1059.83	1179.83	1098.17	1231.50
4X	1173.00	1349.67	1266.33	1536.33
Average (µg/ml)				
953.97		1070.36	1037.86	1160.36

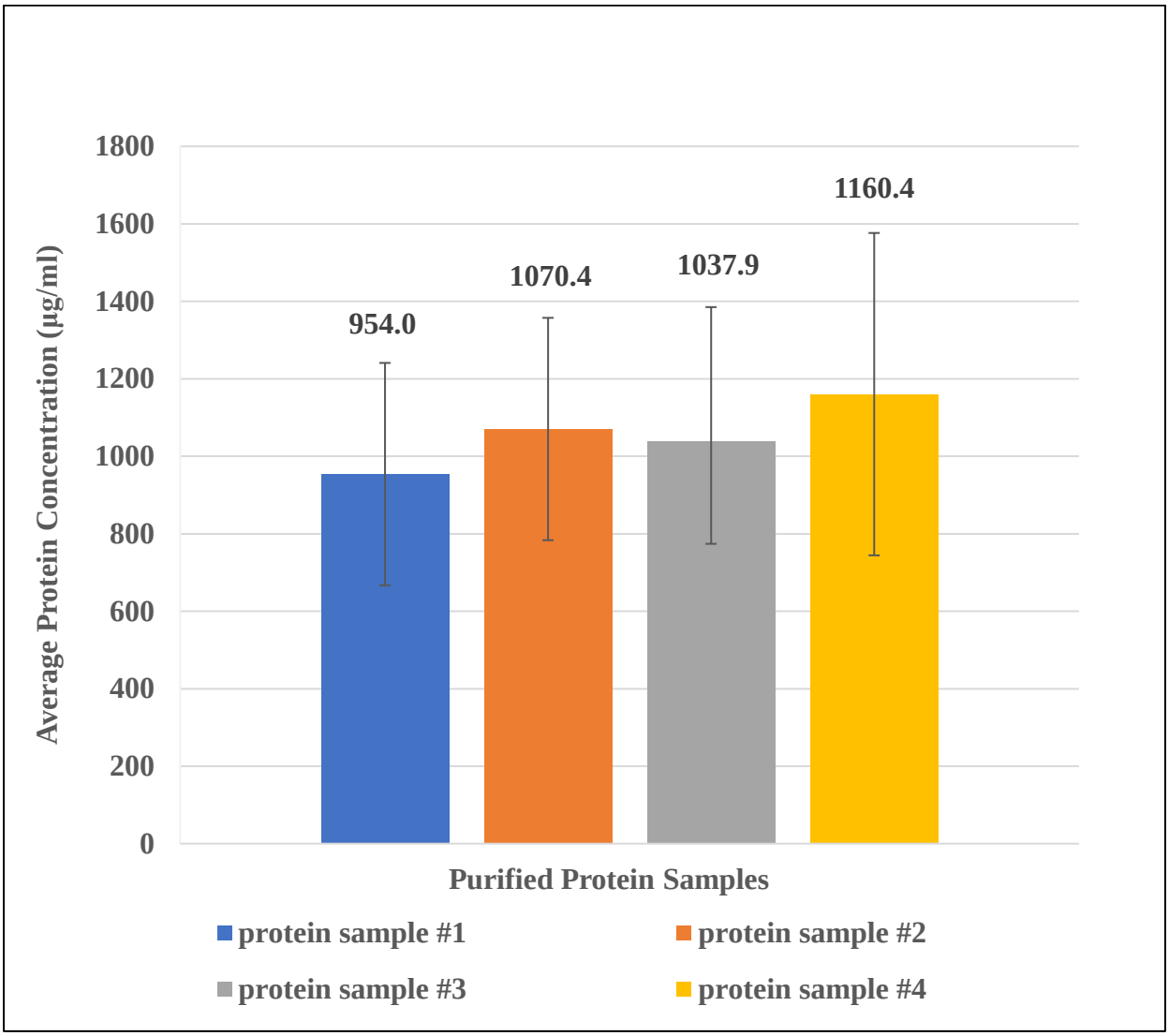

Figure 19. Average Total Protein Concentration Calculated for Four Different Purified Protein Samples.

3.4 Conclusion

- SDS-PAGE indicated that over-expressed, purified Kup was present at the expected molecular weight of ~69 kDa. Calculated average total protein concentration found to be approximately 1 mg/ml.

- 50 mM and 1000 mM imidazole in wash and elution buffers, respectively, proved to be the optimized concentrations to be used for the purification of Kup protein.
- Eluted fractions obtained in new processing method for protein extraction, showed target Kup protein as well as some unspecific bands.
- Simplified new extraction technique led to a protein band with higher intensity without any unspecific bands in the purified sample.
- SDS-PAGE analyses simply showed that the new protein extraction technique developed in this study is highly superior to other available conventional methods by means of higher efficiency, less test run time, less chemical usage which leads to less processing and easier and more cost-effective extraction and purification processes. This is significant since extraction and purification of membrane proteins are the initial major parts of proteoliposomes preparation process.

CHAPTER 4

DESIGN AND CONSTRUCTION OF PROTEOLIPOSOME TRANSPORTER

4.1 Introduction

After nuclear plant accidents in Chernobyl (1986) and Fukushima (2011), ^{137}Cs contamination became an emerging issue (Buesseler, et al., 2012; Ellen Tanudjaja, 2017). Exposure to ^{137}Cs can cause serious health problems such as body cell damage, medullar and reproductive function disorders, cardiovascular disease and etc. (ATSDR, 2004; Namiki, et al., 2012; Rosoff, Cohn, & Spencer, 1963). Hence, there is a crucial need to design a new method for Cs removal from contaminated water.

Proteoliposomes have been suggested as a tool for the evaluation of nanoscale membrane proteins, in particular membrane transporters (Simeonova, Werner, Haupta, Tanabe, & Bacia, 2013). The Kup system, the low affinity potassium transport system encoded by the TrkD gene in *E. coli,* is capable of high cesium accumulation (Tomioka, Uchiyama, Yagi, & Fujii, 1995).

The objective of this chapter is to develop a fast, easy, and environmentally friendly method to capture ^{137}Cs from contaminated water by constructing a proteoliposome transporter. To achieve this, the target protein, Kup, is inserted into the liposome vesicle to create a biological container suitable for capturing radioactive Cs.

4.2 Materials and Methods

In this study, we developed a preparation method by combination of mechanical agitation, solvent dispersion and detergent removal that includes lipid hydration with an organic solvent, solvent evaporation using N_2 gas, rehydration, brief sonication, free-thaw,

extrusion using Avanti mini extruder, lipid solubilization using Triton X-100 and detergent removal using Bio-Beads SM-2. The verification of our suggested reconstitution method was accomplished by sucrose density gradient centrifugation and electron transmission microscopy.

The materials and methods used in the development of proteoliposomes are described below.

4.2.1 Liposome Preparation

Liposomes were prepared by mixing 1.5 mL phosphatidyl choline (PC): 1.5 mL phosphatidic acid (PA), and 1 mL of diethyl ether solvent to a final concentration (20 mg/mL), followed by addition of 0.0048 mg of cholesterol to enhance the stability of liposome. Once all lipids were dissolved, the solvent was evaporated under a steady stream of N_2, and dissolved in 1- ml of appropriate buffer. Here, two different batches of liposomes using different buffers including PBS buffer and 50 mM KPi buffer (KH_2PO_4 and K_2HPO_4, pH 7.0) were tested. A glass pipette was used for mixing to generate a population of multilamellar liposomes. The lipid suspension was sonicated for 90 seconds (15 sec ON, 45 sec OFF) on ice that led to formation of small unilamellar vesicles (SUVs) with average size of 20-100 nm. The resulting SUV suspension was placed in -80 ºC for 10 minutes and then thawed at room temperature. This step was repeated twice more to convert the SUVs to large multilamellar vesicles. A mini extruder with a polycarbonate membrane size of 200 nm was used (Figure 20) to fuse the SUVs to LUVs. Figure 21 shows the lipid suspension loaded into the extrusion syringe attached to a mini extruder (Figure 22). The suspension was extruded 10-15 times until the solution became clear to slightly blue

(Figure 23). Prepared liposome solutions can be stored in 4 ºC for 3-4 days to conduct reconstitution experiment later (Geertsma, Nik Mahmood, Schuurman-Wolters, & Poolman, 2008). Images of prepared liposomes are presented in results & discussion section.

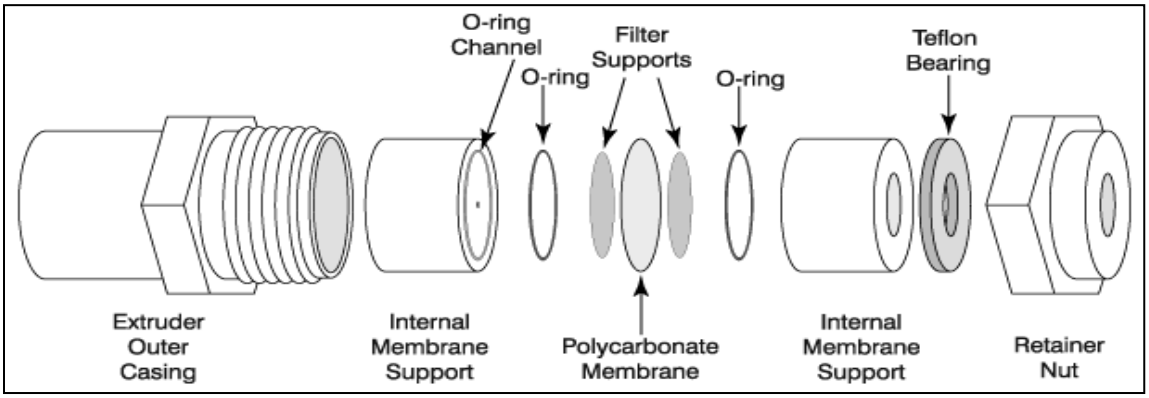

Figure 20. Avanti Mini Extruder (Avanti polar lipids, 1969)

Figure 21. Loading the Suspension into the Mini Extruder Syringes (Avanti polar lipids, 1969)

Figure 22. Avanti Extruder for Converting SUVs to LUVs

Figure 23. Lipid Suspension Before and After Extrusion

4.2.2 Kup Protein Insertion into the Liposome (Proteoliposome Preparation)

In this work, two different methods were used to evaluate reconstitution efficiency.

4.2.2.1 Proteoliposome Preparation

In this method, the published work of Geertsma et al, 2008 was modified. Prepared LUVs were diluted to 4 mg/ml using a mixture of 50 mM Kpi (KH_2PO_4 and K_2HPO_4 solution, pH 7.0) and 20% glycerol. The proper choice of detergent is an important factor in achieving a successful reconstitution as it serves to make the membrane likely to accept the protein. A variety of detergents such as sodium dodecyl sulfate (SDS), n-dodecyl-β-D-maltoside (DM) and Triton X-100 can be used for lipid solubilization (Knol, Sjollema, & Poolman, 1998; Surma, Szczepaniak, & Króliczewski, 2014) and studies have been

conducted on the effect of detergents on lipid solubilization. Those studies have revealed that SDS has strong surfactant properties and subsequently wouldn't be mild enough as other non-ionic detergents like DM and Trion X-100 that can also lyse liposomes at concentration higher than five times than SDS (Helenius & Simons, 1975).

Therefore, Triton X-100 was used here, as it is effective in isolation and reconstitution of different membrane proteins (Levy, Gulik, Bluzat, & Rigaud, 1992; Freisleben, et al., 1995), and creates an environment suitable for stabilization of membrane protein structure (Arnold & Linke, 2016; Tikhonova, Devroy, Lau, & Zgurskaya, 2007).

Prepared LUV liposomes were destabilized with 10% stock solution of Triton X-100 (~ 10 µl for each cycle of addition, 15 times in total). Purified protein was added to detergent-destabilized liposomes (100 µg protein per 2.5-ml liposome solution) in a glass vial and placed on a stirrer (15 minutes, room temperature). Next, detergent was removed from the solubilized protein-liposome solution, the reverse of the solubilization process. This causes the formation of bilayers into which proteins will insert (Ollivon, Lesieur, Grabielle-Madelmont, & Paternostre, 2000; Knol, Sjollema, & Poolman, 1998). Due to the low critical micelle concentration (CMC) of Triton X-100, removal by dialysis would be difficult. Polystyrene beads are a quick, efficient way to initiate proteoliposome formation (Verchère A. , Dezi, Broutin, & Picard, 2014; Holloway, 1973).

Here, a batch of Bio-beads SM-2 Resin (Polystyrene beads from Bio-Rad, 300–1180 µm in size), washed previously with methanol, ethanol, and water (Appendix A) was added to the liposome-protein mixture (40 mg beads per 1-ml of mixture) and incubated for 30 minutes at room temperature with gentle agitation. Afterwards, another batch of Bio-

beads was added to the suspension and incubated in a shaker overnight at 4 ºC and was repeated the next day for two hours.

The sample was filtered using a Poly-Prep chromatography column from BIO-RAD (Figure 24) to remove Bio-beads leaving only proteoliposome in solution. Proteoliposomes were eluted in 50 mM Kpi buffer to reduce the glycerol concertation but resulted in a 10-fold dilution. To collect the proteoliposomes, the diluted solution was centrifuged at 267,000 g (Beckman SW 41-Ti rotor) for 20 minutes at 4 ºC, and the resulting pellet was resuspended in 1 mL of 50 mM Kpi buffer, aliquoted and flash-frozen in liquid nitrogen and stored at -80 ºC. Pellets obtained from this method were not large enough to visualize, however, the structure can be seen using Cryo-TEM.

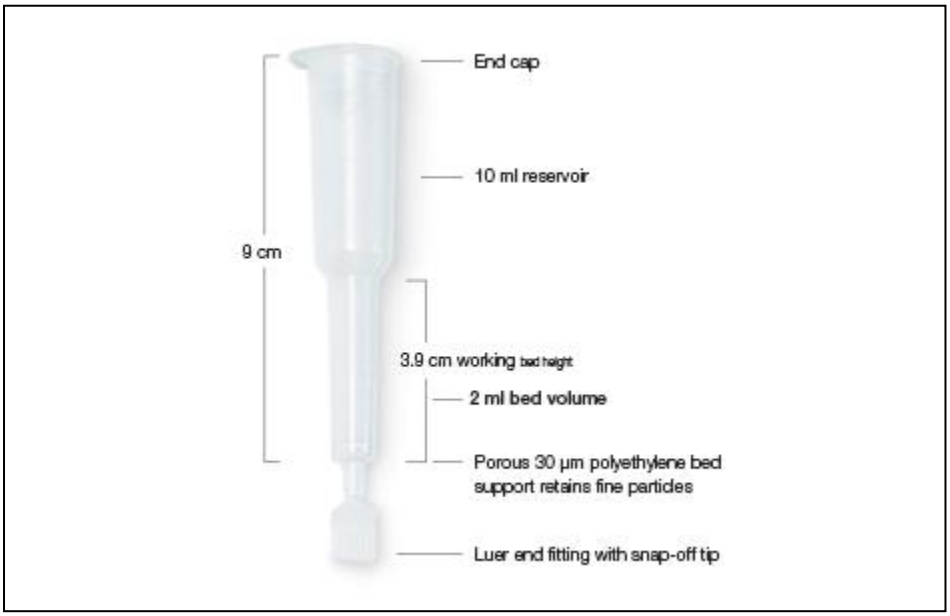

Figure 24. Poly-Prep chromatography column (BIO-RAD)

4.2.2.2 New Method of Proteoliposome Preparation

A new method to insert Kup, the target protein into the liposome vesicles was developed and is described here. A volume of 2 mL of prepared LUV liposomes was destabilized with 10% stock solution of Triton X-100. To see the effect of Triton on proteoliposome formation, three 2-ml batches of liposome were destabilized using 3.01 mM, 4.64 mM and 10.05 mM of Triton that would lead to final concentrations of 0.15%, 0.3% and 0.65%, respectively. Purified protein was added to detergent-destabilized liposomes (~300 μg protein per 2-ml liposome solution) in a glass vial and was put on the stirrer and incubated for 45 minutes at room temperature. A batch of previously washed Bio-beads SM-2 resin was added to the liposome- protein mixture (80 mg beads per 1-ml) and stirred for 1 hour at room temperature to remove Triton X-100. This was repeated two more times with two new batches of fresh Bio-beads, one hour at room temperature and one hour at 4 ºC, respectively. Filtration using a Poly-Prep chromatography column from BIO-RAD was performed to remove the Bio-beads and isolate the proteoliposome in solution. Proteoliposomes that ended up in the filtration eluate were diluted two-three times with iced cold PBS buffer and collected by centrifugation at 260,000 x g for 1 hour at 4 ºC. The final pellet was resuspended in 2-3 ml of PBS buffer and sonicated for 3-4 minutes in a sonication bath; the pellet obtained by this method was larger in quantity than pellets from the published method of Geertsma et al, 2008. Final samples were aliquoted in small glass vials and flash-frozen in liquid nitrogen and stored in -80 ºC. Summary of both methods of protein reconstitution into the liposome (published and new) is shown in Table 7.

Table 7. Summary of the Two Suggested Methods of Proteoliposome Preparation

Published Method (Geertsma, Nik Mahmood, Schuurman-Wolters, & Poolman, 2008)	1. Dilute the LUVs to 4 mg/ml with 50 mM Kpi+ 20% glycerol. 2. Titrate the LUVs with Triton X-100. 3. Add 100 µl protein per 2.5-ml liposome solution; incubate for 15 min at RT with gentle agitation. 4. Add 40 mg Bio-Beads SM-2 per 1-ml of mixture; incubate for 30 min at RT with gentle agitation. 5. Repeat step 4 but incubate for 60 min at 4 ºC with gentle agitation. 6. Repeat step 5; incubate overnight at 4 ºC with gentle agitation. 7. Repeat step 6; incubate for 2 hrs at 4 ºC with gentle agitation. 8. Remove Bio-Beads SM-2 by filtration using polyprep column. 9. Dilute the pellets tenfold in 50 mM Kpi to lower the glycerol concentration from 20% to 2%. 10. Collect the new pellet by centrifugation at 267,000g (20 min at 4 ºC). 11. Suspend the pellet in 50 mM Kpi to make final conc. of 15 mg/ml. flash-freeze in aliquots of 0.2 ml and keep in -80 ºC.
New Method	1. Destabilize volume of 2-mL of prepared LUV liposomes with 10% stock solution Triton X-100. 2. Add 300 µl protein per 2-ml liposome solution; incubate for 45 min at RT with gentle agitation. 3. Add 80 mg Bio-Beads SM-2 per 1-ml of mixture incubate for 1 hr at RT with gentle agitation. 4. Repeat step 3 with fresh Biobeads at 4 ºC. 5. Repeat step 4 with fresh Biobeads; incubate for 2 hrs at 4 ºC. 6. Remove Bio-Beads SM-2 by filtration using polyprep column. 7. Dilute the proteoliposomes two-three times with cold PBS buffer. 8. Collect the pellet by centrifugation at 260,000 g (1 hour at 4 ºC). 9. Resuspend pellet in about 2-3 ml of PBS buffer. 10. Briefly sonicate for 3-4 minutes in a sonication bath. Flash-freeze in aliquots of 0.2 ml and keep in -80 ºC.

As can be seen from Table 7, the new method can be completed in approximately 6 hrs unlike the published method that takes much longer.

4.3 Verification of Reconstituted Kup into Liposome

Assessment of the efficiency of the protein reconstitution into the liposome can be visualized using electron microscopy (EM) techniques (Mouro-Chanteloup, et al., 2010) and sucrose density gradient centrifugation (SDGC) followed by SDS-PAGE visualization (Verchère A. , Dezi, Broutin, & Picard, 2014; Haginoya, et al., 2005) with EM technique. First, EM was used to see which method of proteoliposome preparation led to a successful reconstitution and was further validated with SDGS/SDS-PAGE/EM.

4.3.1 Cryo- Transmission Electron Microscopy

Cryo-Transmission electron microscopy has become a useful imaging tool for biological specimens due to its high resolution as a result of advances in image processing software and sample preparation developments (Thompson, Walker, Siebert, Muench, & Ranson, 2016). This imaging requires samples to be frozen in vitreous ice for preservation in their native hydrated state (Liu & Sigworth, 2014). Cryo-electron microscopy examines biological or soft-organic materials in thin aqueous films on a carbon support layer that is perforated with holes.

Here, Titan Krios (Field Electron and Ion Company, FEI) cryogenic electron microscope (Figure 25) (John M. Cowley Center for High Resolution Electron Microscopy, ASU) was used to visualize the samples obtained from both proteoliposome preparations (Figures 27-28). The specimen was rapidly frozen by plunge freezing into liquefied ethane

at -179 ºC so that freezing occurs faster than the reorganization of water molecules into crystalline ice forms. Liposome samples were prepared by applying 3 µl of sample onto a 400-mesh carbon copper mesh grid coated with a regular array of 1.2 micron holes spaced 1.3 microns apart. The liquid was blotted to form a thin vicinal film containing liposomes and plunged rapidly into the nitrogen cooled ethane. Cryogenically prepared samples were examined at 300 KeV at the indicated magnifications on the FEI Titan Krios G2 at the John Cowley Center High Resolution TEM. Images were collected on either a 4K x 4K Hybrid C MOS camera at a dose rate of 25-50 electron per angstrom squared or on a direct electron detector in super-resolution movie mode at a dose of 2 electron per angstrom squared second. Each DED movie frame was 0.2 seconds and a total of 8 seconds were used per exposure. Movie files were motion corrected and sum images obtained.

Figure 25. Cryogenic Electron Microscope, Titan Krios (FEI)

4.3.2 Sucrose Density Gradient Centrifugation

To see if Kup proteins were reconstituted in liposomes, proteoliposome suspensions were purified on a discontinuous sucrose gradient, by piling layers of 40%, 37%, 29.5%, 25.1%, 21%, 17.2%, 13.4%, 9.0% and 5% (w/v) sucrose solutions in a 5-ml Beckman ultracentrifuge tube such that the densest sucrose solution is at the bottom.

In order to achieve distinct sucrose layers, 5-ml tube was placed between the clamps of a metal stand and a Pasteur pipette inserted into an electric pipette controller, set on the lowest speed, was used to pour the 40% sucrose solution gently in the tube. This procedure was continued in the same way by layering other gradients gently on top of previous layers as demonstrated in Figure 26

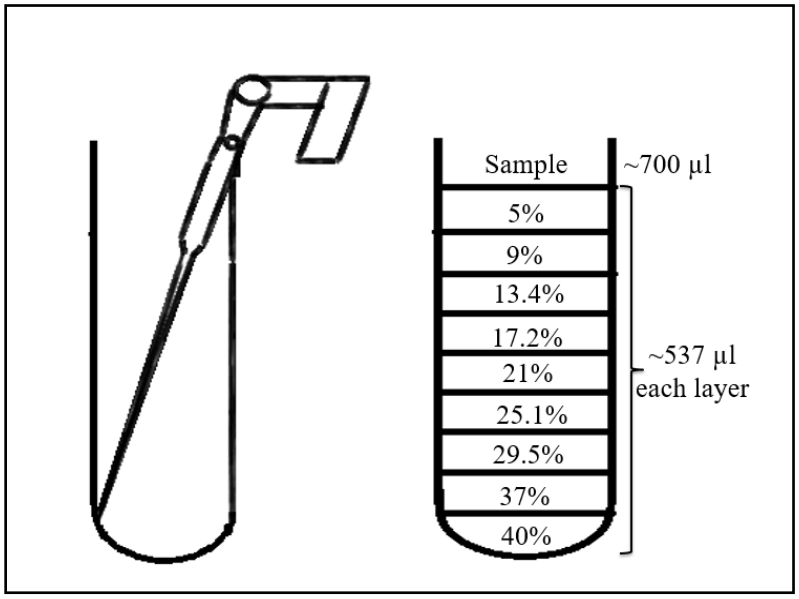

Figure 26. Sucrose Gradient Preparation

After preparing discrete layers of sucrose solution that can be visualized (Figure 27), approximately 700 µl of proteoliposome sample was loaded on top layer of the sucrose

(5%) and the tube was transferred into the ultracentrifuge machine very carefully to not disturb the sucrose layers. Samples were centrifuged (160,000 x g) for 3 hours at 4 ºC and samples from each gradient fraction/layer were collected using a needle (Figure 28). The tube was placed in a sturdy position and a tiny hole on the side of the tube was created next to each layer were the sample was located in. The summary of sample fractions collected are presented in Table 8.

Here, SDGC was done for three batches of proteoliposomes (#1-3) that were prepared using 3.01 mM, 4.64 mM and 10.05 mM of 10% Triton X-100, respectively, to see which one gives us a better formation of proteoliposome.

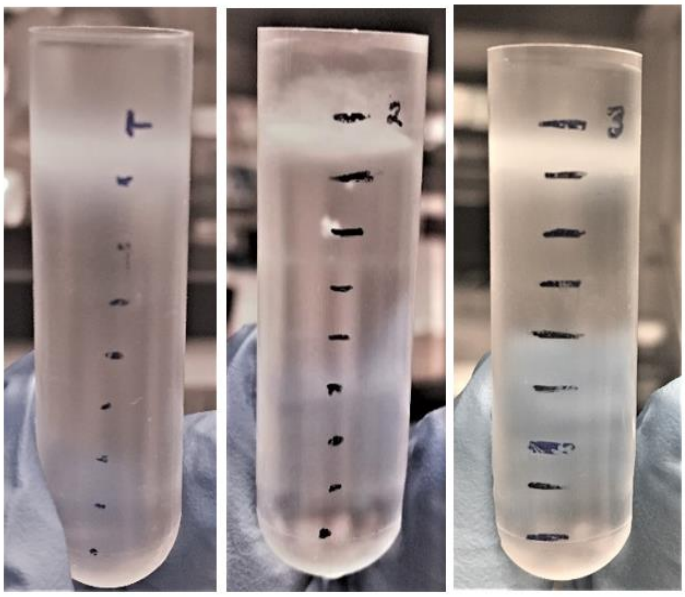

Figure 27. Nine Layers of Sucrose Gradient (5-40%) Overlayered by Proteoliposome Samples (#1-3)

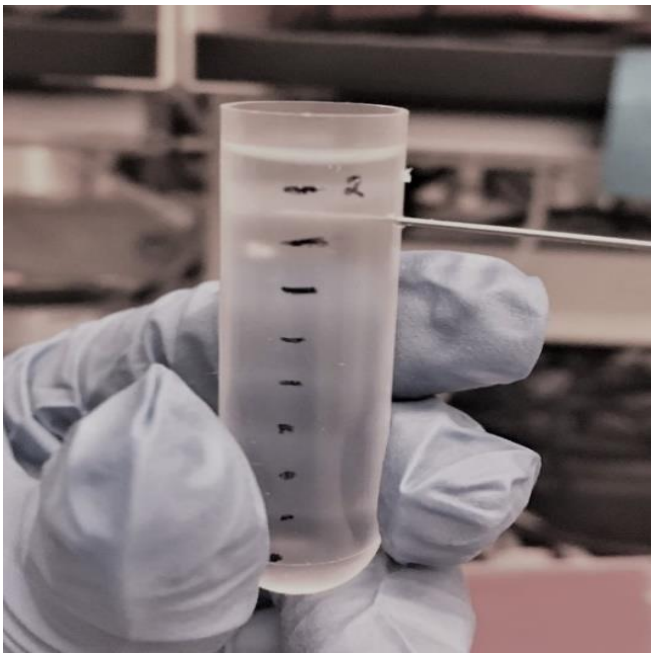

Figure 28. Fraction Collections in SDGC Method Using a Needle

Table 8. Sample Fractions Collected During SDGC

Sample*	Top Layer	5%	9%	13.4%	17.2%	21%	25.1%	29.5%	37%	40%
#1	S_{1-0}	S_{1-1}	S_{1-2}	S_{1-3}	S_{1-4}	S_{1-5}	S_{1-6}	S_{1-7}	S_{1-8}	S_{1-9}
#2	S_{2-0}	S_{2-1}	S_{2-2}	S_{2-3}	S_{2-4}	S_{2-5}	S_{2-6}	S_{2-7}	S_{2-8}	S_{2-9}
#3	S_{3-0}	S_{3-1}	S_{3-2}	S_{3-3}	S_{3-4}	S_{3-5}	S_{3-6}	S_{3-7}	S_{3-8}	S_{3-9}

Note: Samples #1, #2 and #3 are made using 0.15%, 0.3% and 0.65% of 10% Triton X-100, respectively.

Each gradient fraction was collected (25 µl) and run on SDS-PAGE to visualize protein bands and possibly proteoliposomes, and the rest of samples were kept at -80 ºC for future use. Results showed that none of the fractions contained protein which was due to being highly diluted with sucrose. Therefore, the remaining fractions were concentrated to get rid of sucrose and buffers and make it more suitable for SDS-PAGE. The fractions were spun down at 14,000 rpm for about 8 minutes and the collected pellets were resuspended in 50 µl of PBS buffer. Samples were again collected (25 µl) for analyzing on SDS-PAGE as shown in Figure 34.

4.4 Evaluation of Applicability and the Efficiency of the Constructed Proteoliposome

To evaluate the efficiency of the constructed proteoliposome transporter in capturing cesium, inductively coupled plasma mass spectrometry (ICP-MS) technique was employed to measure the cesium concentration in water before and after treatment.

ICP-MS is an analytical technique that uses a high temperature ionization source (ICP) coupled to a mass spectrometer. This technique is the advanced version of Gray and Houk's work in the early 1980s (Houk, et al., 1980) and is a powerful technique for the quantification and analysis of trace elements. This is mostly because of low detection limits, speed of analysis and relative freedom from interference. Inductively coupled plasmas, developed in the early 1960s (T.B., 2004), are flame-like discharges that can reach up to 10,000 K in temperature and are generated in a stream of argon flowing through an electromagnetic field (Fadi, 2003). Here, ICP-MS, XSERIES 2 (Figure 29) from Thermoscientific in Keck lab, ASU was used to measure the concentration of Cs in samples before and after treatment with proteoliposomes.

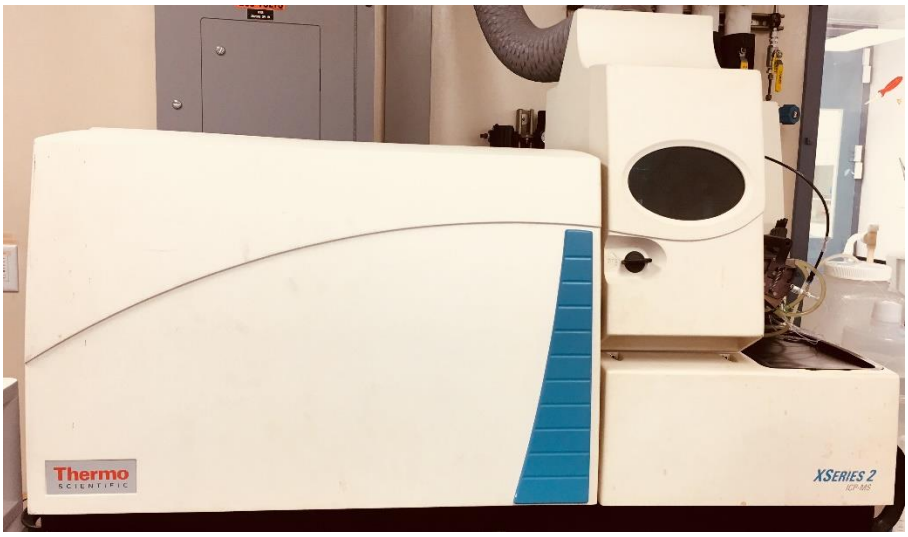

Figure 29. ICP-MS XSERIES 2

4.4.1 Treatment of Cesium-Contaminated Water using Proteoliposome

Ultrapure water spiked with about 10 ppb and 100 ppb of cesium chloride (CsCl) was used to test the effect of proteoliposomes on cesium removal. To do this, 0.1 mL, 0.5 mL, 1 mL, 2 mL, 4 mL and 8 mL of proteoliposome solution were added to water samples having 100 ppb CsCl, followed by 1-hour incubation at room temperature. To remove proteoliposomes from water sample, centrifugation at 260,000 g for 1 hour at 4 °C was done and supernatant was saved. Stock solution of about 100 ppm CsCl (the untreated sample) and resulting supernatants from centrifugation (treated samples) were run on ICP-MS to compare the Cs values before and after treatment and determine the percentage of Cs removal. All the tests were performed in duplicate.

To evaluate the effect of proteoliposome on water contaminated with ~ 10 ppb CsCl, 0.1, 0.5 mL, 1 mL and 2 mL of proteoliposome solution were used; each volume of proteoliposome samples was added to two samples (duplicates), incubated for 1 hour

followed by centrifugation for 1 hour at 4 ^{0}C. The resulting supernatant was then tested on ICP-MS along with stock solution of 10 ppb of CsCl to compare the Cs values in both samples, before and after treatment with proteoliposome. Moreover, as a control test, CsCl solution and empty liposome vesicles solution were tested separately, in the same way as the proteoliposome solution, to determine the effect of centrifugation and liposome vesicles on Cs removal. All results are presented in the results and discussion section

4.4.2 Sample Preparation and Calibration Curve for ICP-MS

Since the proteoliposome samples are mixed in PBS buffer, in order to prevent interference of salts, the sample should be diluted prior to ICP-MS. Here, a factor of 350X was used to dilute 0.15 mL of each proteoliposome sample using nitric acid. Five cesium chloride (CsCl) standards of expected concentration of 0.0186 ppb, 0.185 ppb, 0.981 ppb, 9.73 ppb and 48.7 ppb were run on ICP-MS and their concentrations were measured to develop a calibration curve. Linearity was determined by r^2. All the results and detailed procedures are presented in the results and discussion as well as Appendix C.

4.5 Results and Discussion

Cryo-EM images of the constructed proteoliposomes as well as measured cesium percentage removal for water treated using proteoliposomes have been presented herein.

4.5.1 Cryo-TEM Images of Liposomes

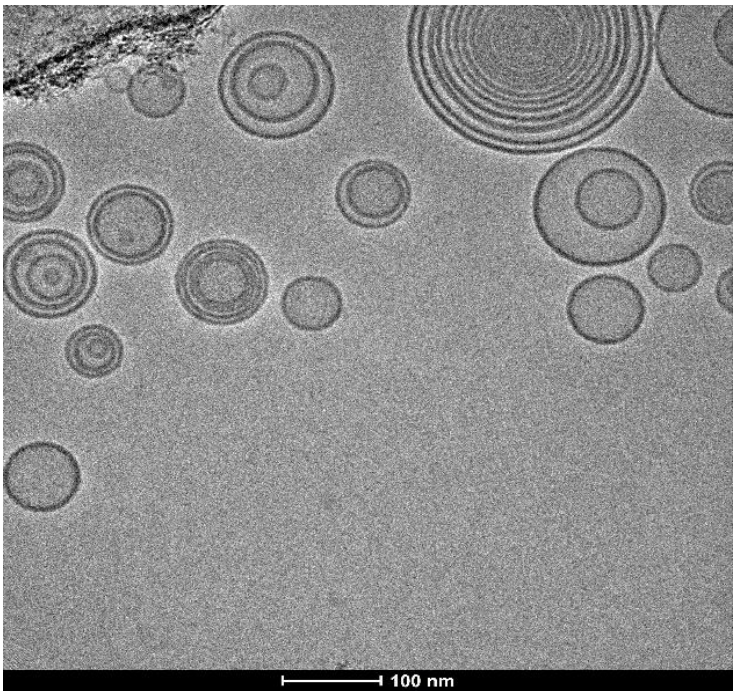

Figure 30. Cryo-TEM Image of Large Multilamellar Vesicles (MLVs) Formed During Freeze-Thaw Step of Liposome Preparation

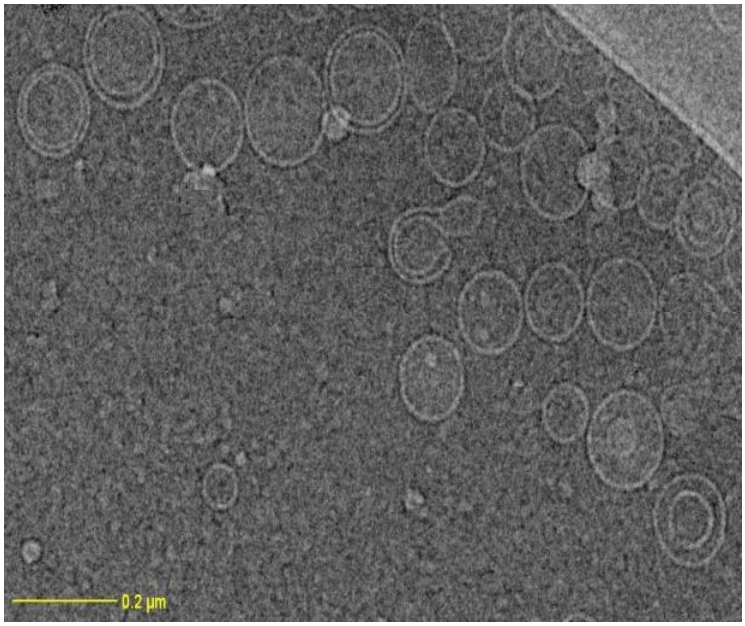

Figure 31. Cryo-TEM Image of Large Unilamellar Vesicles (LUVs)

An interesting feature was observed during preparation. Here, during the liposome preparation experiments, lipid suspension after extrusion did not turn blueish as expected, thus, freeze-thaw was done (three cycles of 10 min) followed by extrusion. Once more, no change in color was noticed. The sample was sonicated again followed by two freeze-thaw-extrusion cycles, which resulted in a color change, but not bluish as expected. Cryo-TEM images of this liposome suspension is presented in Figure 32 and as can be seen, surprisingly, multivesicular liposomes (MVLs) can be formed by this method. MVLs are a different liposomal morphology than multilamellar vesicles (MLVs), they compose of smaller, non-concentric liposomes entrapped in larger vesicles while MLVs are typically structures with more than five lamellae (Vergara-Irigaray, Riesen, Piazza, & Nosonovsky, 2012; Daneshamouz, M. Danyali, Mohammadi-Samani, & Jaafari, 2012).

MVLs are mostly used in drug delivery and it's also reported that the loading efficiency of the MVLs are 3 to 6 times higher than conventional multilamellar vesicles (Jain, et al., 2005).

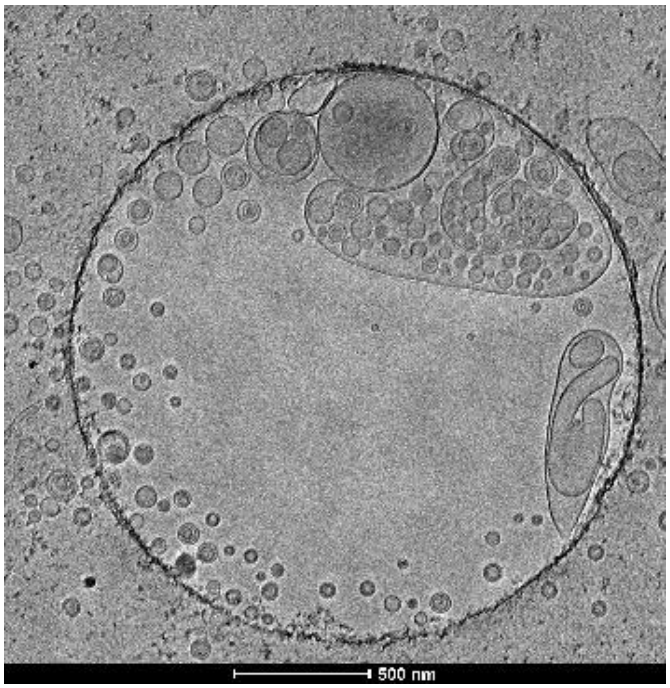

Figure 32. Cryo-TEM Images of Multivesicular Liposomes (MVLs)

4.5.2 Cryo-TEM Images of Proteoliposome

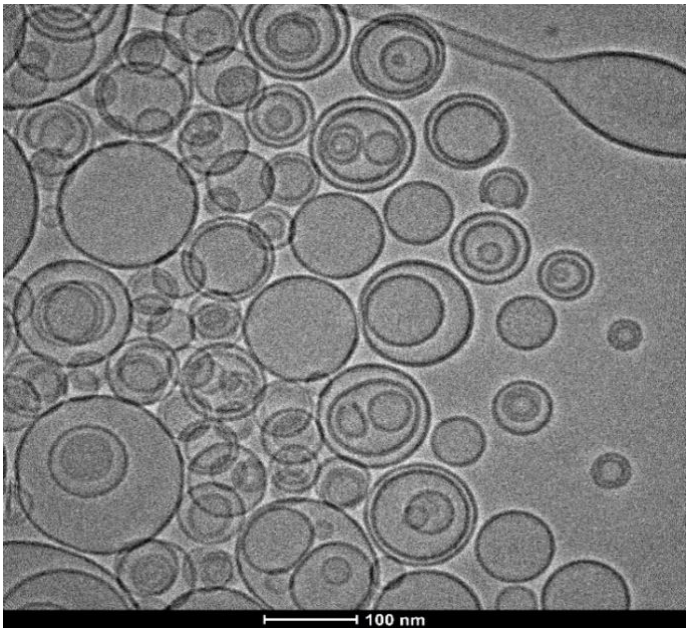

Figure 33. Cryo-TEM Images of Samples from the Published Method of Proteoliposome Preparation.

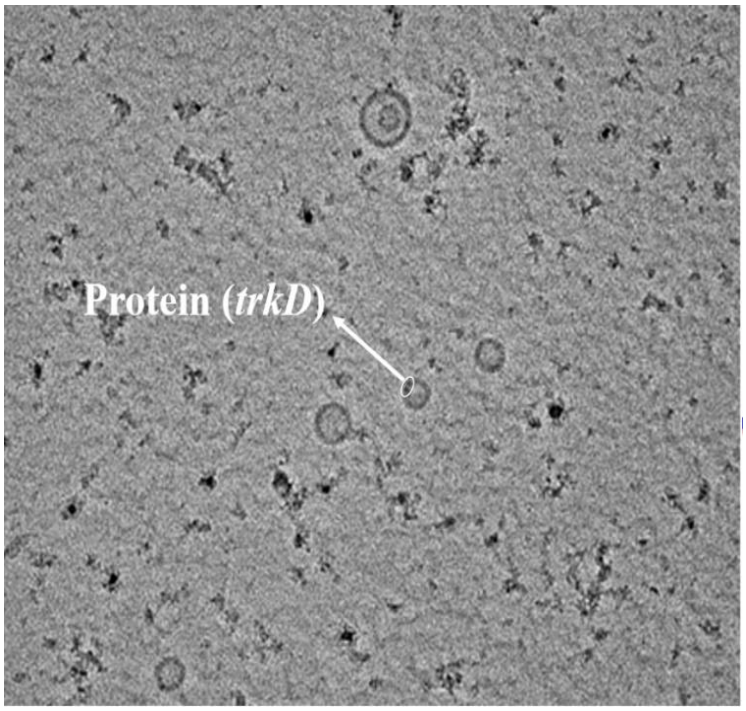

Figure 34. Cryo-TEM Images of Samples from the New Method of Proteoliposome Preparation.

Using published method of proteoliposome preparation (Figure 33), mostly empty liposome vesicles, with no protein resides in the lipid bilayers could be obtained. However, in Figure 34 protein residues (small dark dots) can be seen in the bilayer of vesicles which reveals that the second method should be chosen to accomplish protein reconstitution.

4.5.3 SDS-PAGE and EM Analysis of Samples Obtained from SDGC TEST

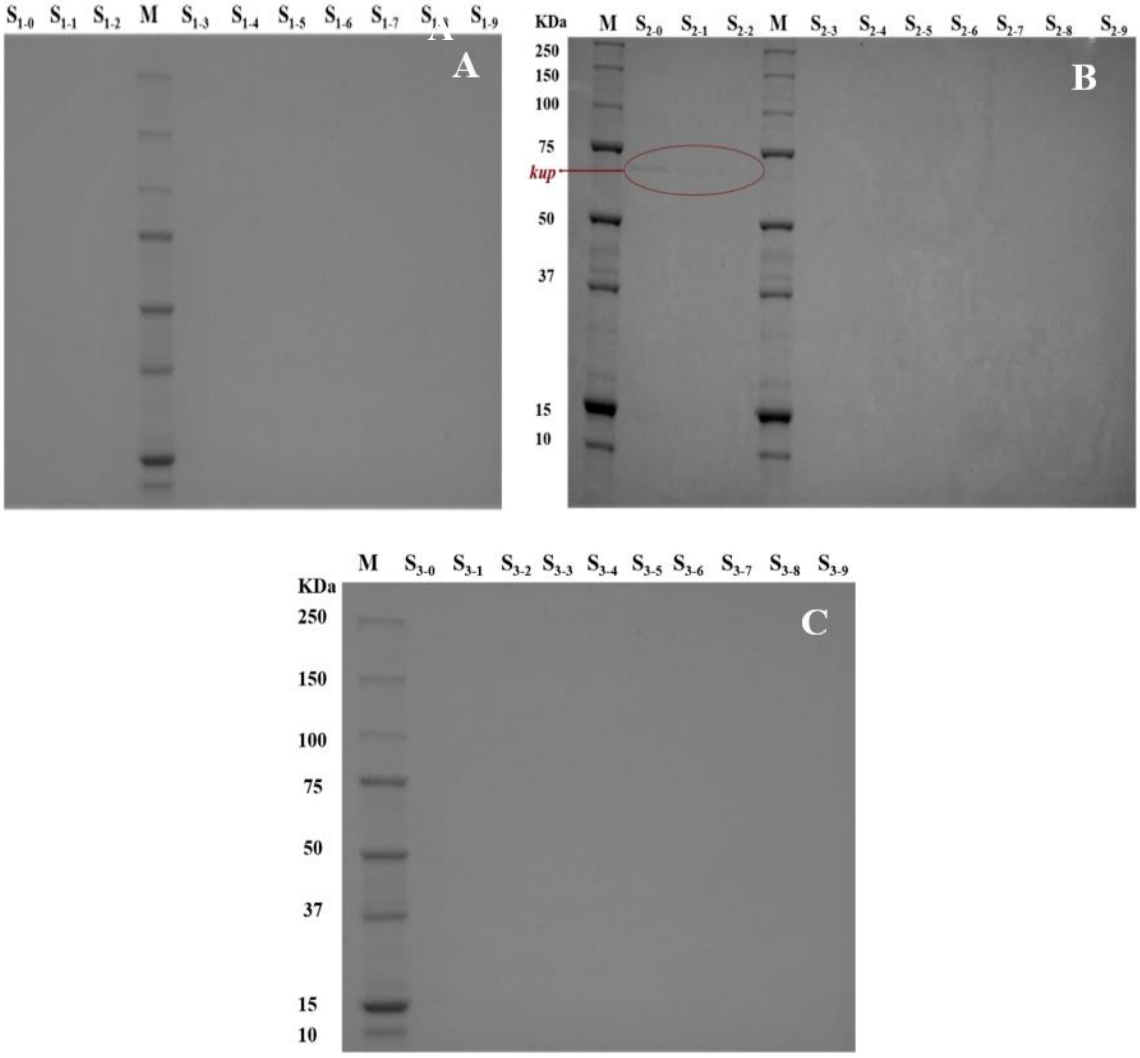

Figure 35. SDS-PAGE Images of Concentrated Samples Collected in SDGC Test.
A. fractions collected testing sample #1 prepared using 0.15% Triton X-100; B. fractions of sample #2 prepared using 0.3% Triton X-100; C. fractions of sample #3 prepared using 0.65% Triton X-100. For more details refer to Table 8.

As can be seen in Figure 35, only concentrated fractions associated with sample 2 (prepared using final concentration of 0.3% Triton X-100), collected from the three top layers of SDGC tube (5%- 13.2%) had the target protein bands. Furthermore, since the protein band intensity of the first two concentrated fractions were higher, they were saved

for cryo-transmission electron microscopy to look for proteoliposomes. EM images are presented below (Figure 36).

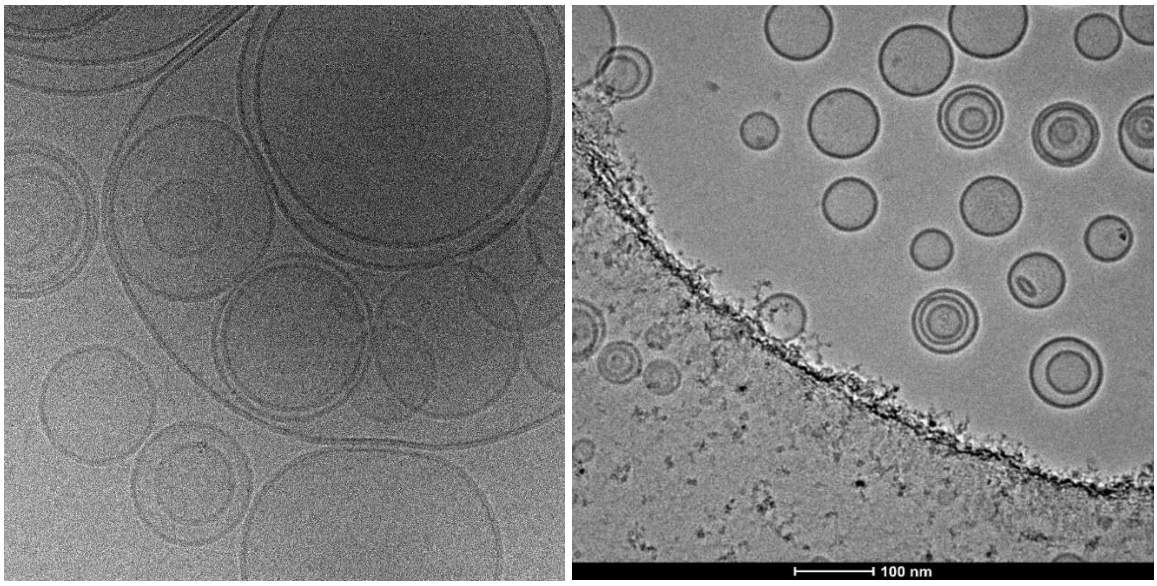

Figure 36. Cryo-TEM Images of Proteoliposomes Purified using SDGC

4.5.4 Cesium Concentration Data Obtained from ICP-MS

Using ICP-MS, the calibration curve (Appendix C) produced $r^2 = 1$, which showed cesium (Cs) concentration would be close to the expected and actual concentration. Cs in samples before and after treatment was measured (Figures 37 and 38). Using ultrapure water, stock solutions of 10 ppb and 100 ppb were prepared and used as untreated samples; however, as can be seen, the measured concentration in ICP-MS is slightly below or above 10 and 100 ppb. This was likely due to errors associated with sample preparation as well as accuracy of ICP-MS in measurement.

Percentage removal of cesium was also calculated (Tables 9 and 10) and the average values of removal as well as their associated standard deviation numbers were

plotted (Figures 39 and 40). Cs percentage removal due to centrifugation and empty liposome vesicles were calculated and presented in Figure 43.

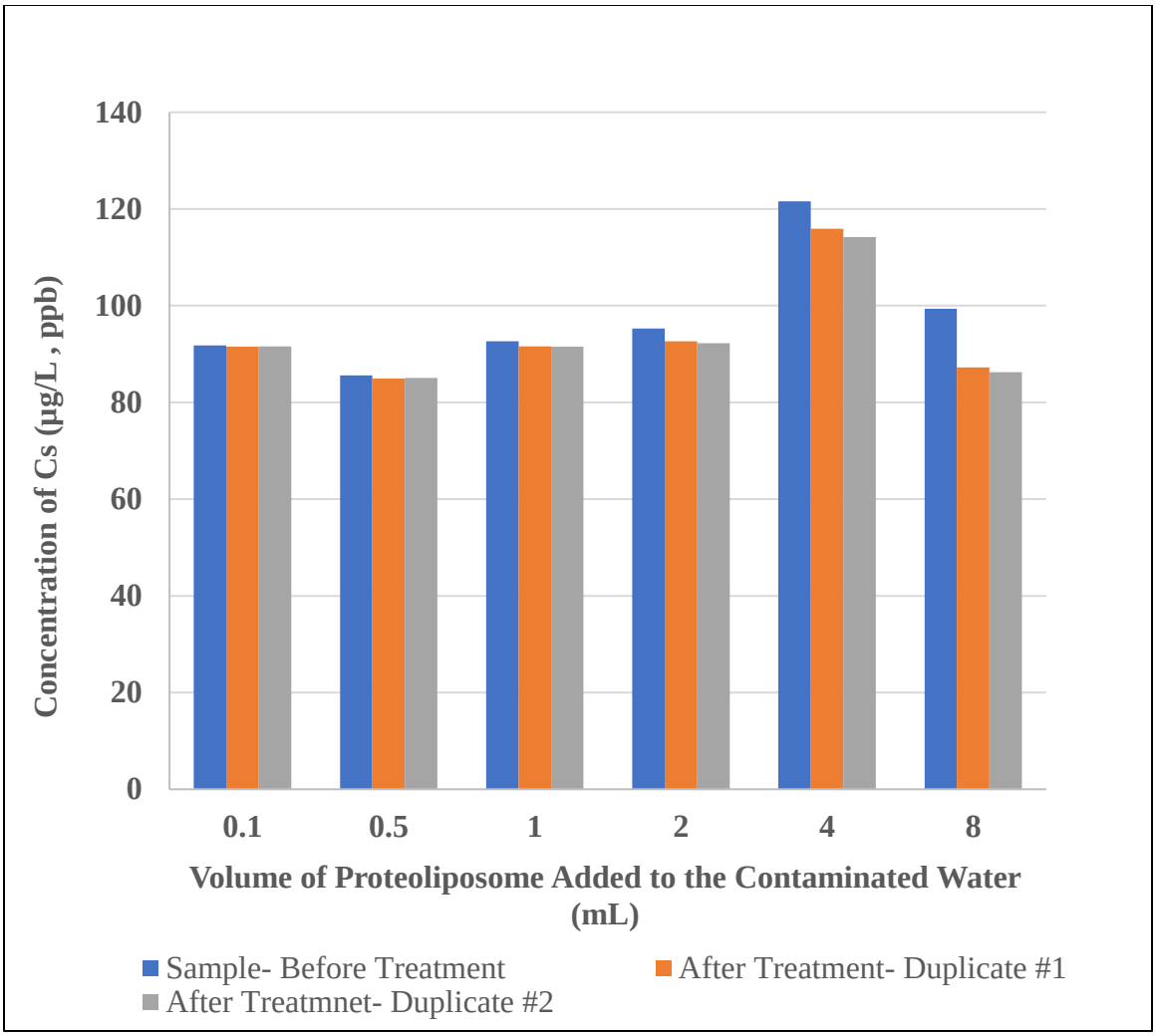

Figure 37. Effect of Proteoliposome on Cs Removal from Water Spiked with ~100 ppb CsCl.

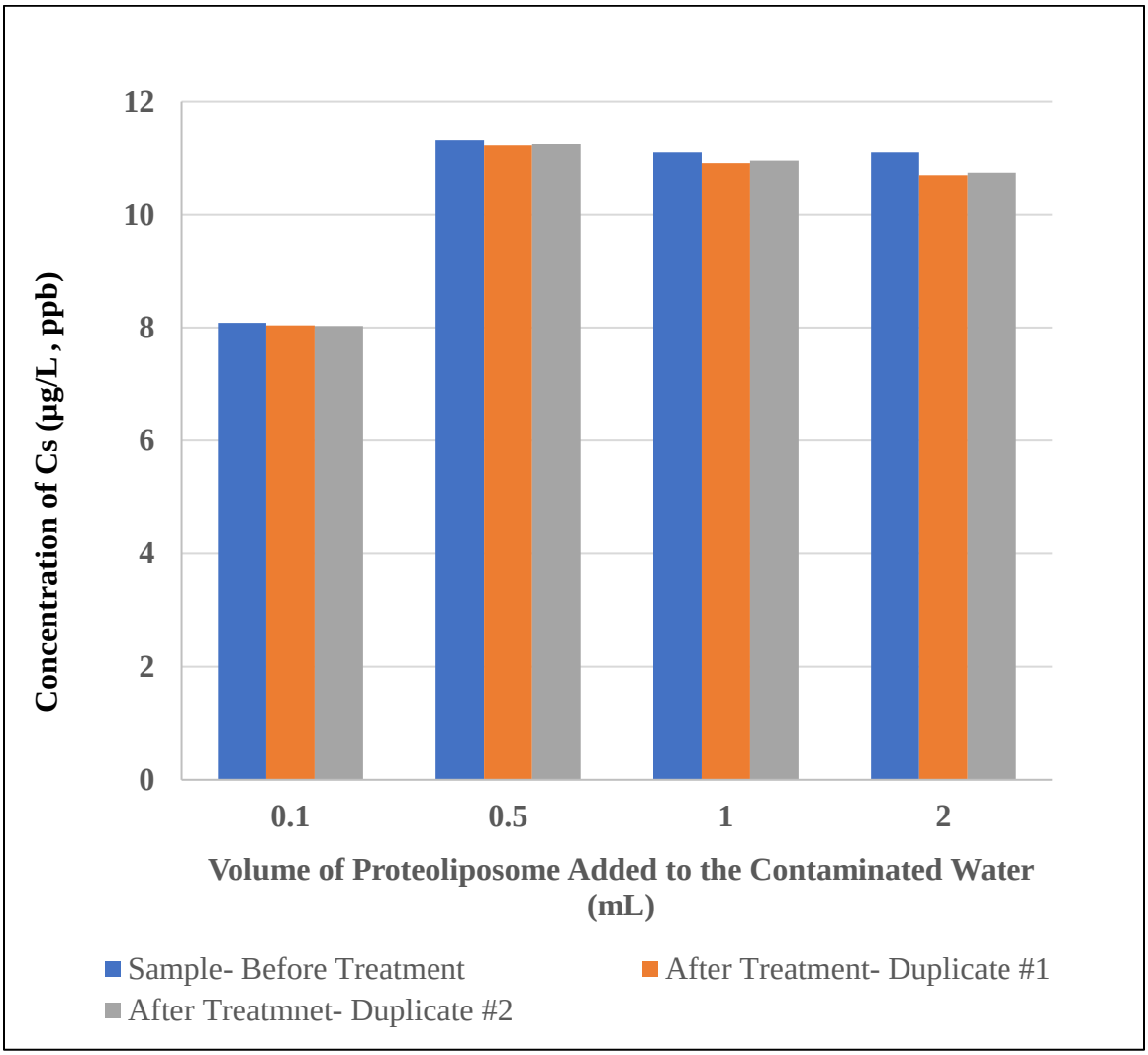

Figure 38. Effect of Proteoliposome on Cs Removal from Water Spiked with ~10 ppb CsCl.

Removal of cesium for samples treated with proteoliposome was calculated (Tables 9 and 10).

Table 9. Percentage Removal of Cesium (Cs) using Proteoliposome in Water Samples Spiked with about 100 ppb of Cs

Percentage Removal of Cesium						
Sample	Volume of Proteoliposome added to Contaminated Water					
	0.1 mL	0.5 mL	1 mL	2 mL	4 mL	8 mL
Duplicate #1- AT	0.33%	0.72%	1.12%	2.77%	6.11%	12.24%
Duplicate #2-AT	0.26%	0.62%	1.19%	3.17%	4.69%	13.23%

AT: After Treatment

Table 10. Percentage Removal of Cesium (Cs) using Proteoliposome in Water Samples Spiked with about 10 ppb of Cs

Percentage Removal of Cesium				
Sample	Volume of Proteoliposome added to Contaminated Water			
	0.1 mL	0.5 mL	1 mL	2 mL
Duplicate #1- AT	0.59%	0.93%	1.70%	3.61%
Duplicate #2-AT	0.70%	0.76%	1.29%	3.25%

AT: After treatment.

Average values of percentage removal and associated error bars for samples treated with proteoliposome presented in Figures 39 and 40.

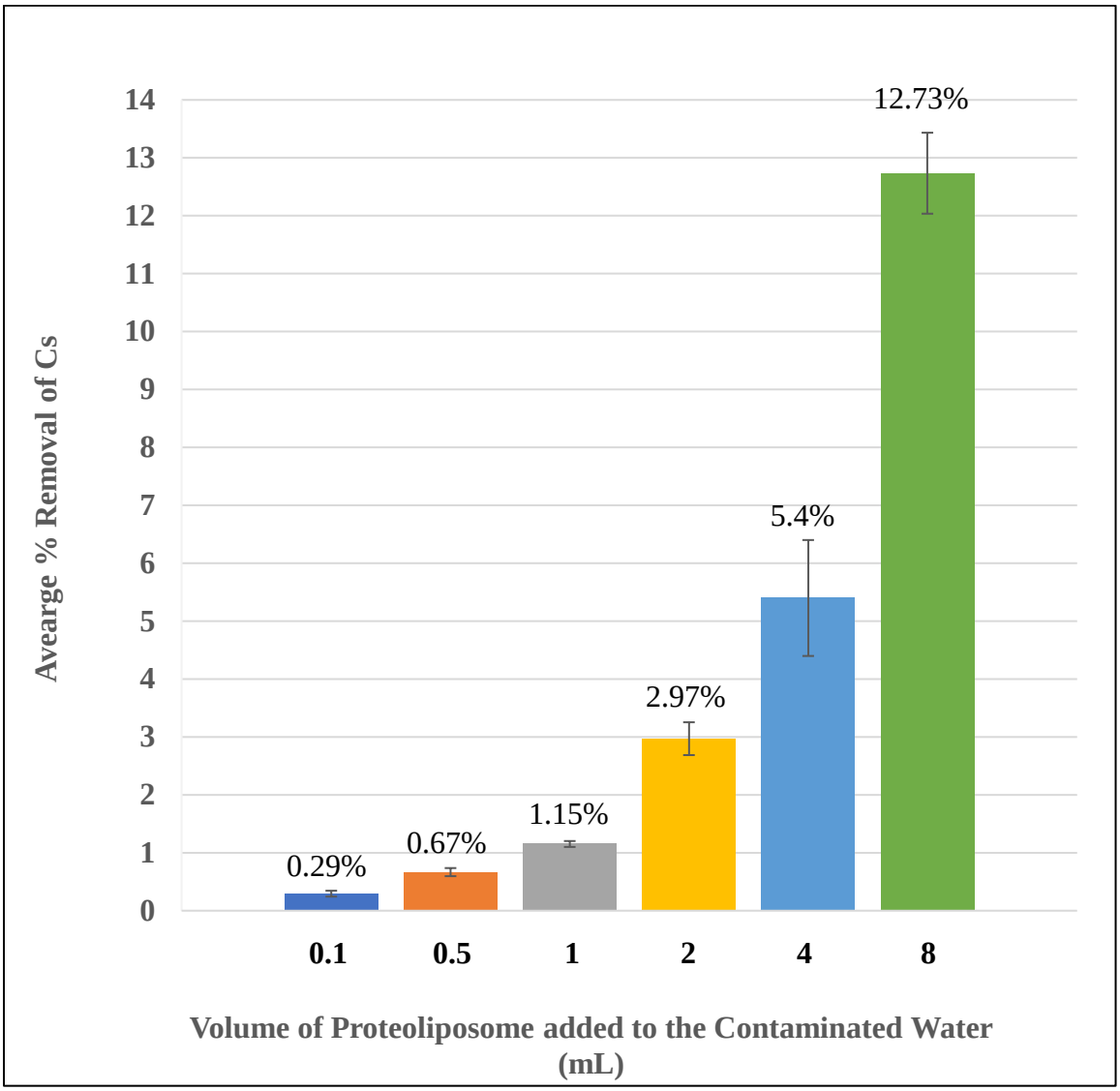

Figure 39. Average Percentage Removal of Cesium (Cs) for Water Samples Spiked with about 100 ppb of Cs.

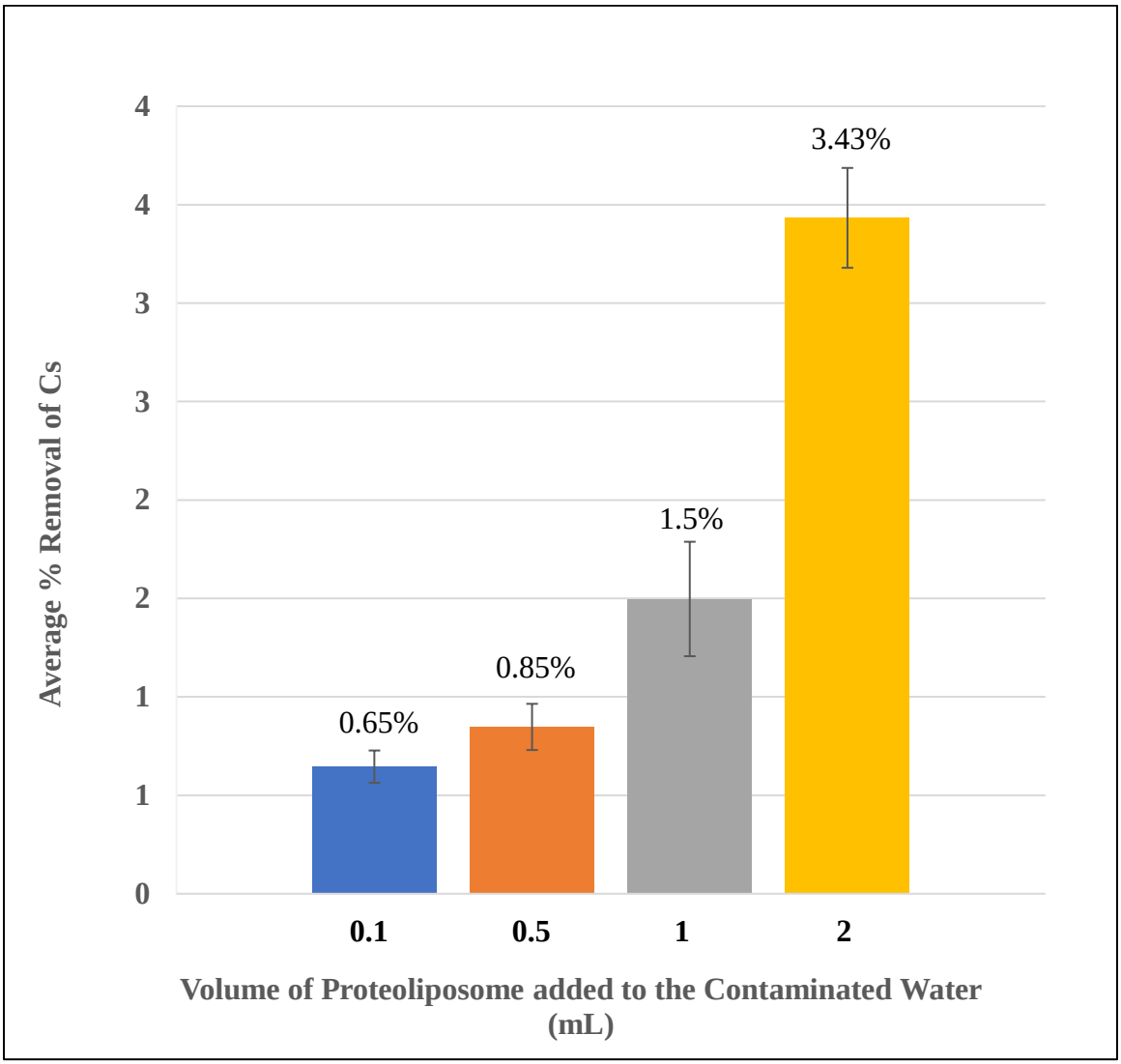

Figure 40. Average Percentage Removal of Cesium (Cs) for Water Samples Spiked with about 10 ppb of Cs.

According to Figures 39 and 40, increase in removal is proportional to increasing the amount of proteoliposome added to the contaminated water. It can be concluded that by doubling the volume of proteoliposome, removal would be increased two-fold. Figure 41 illustrates the linear relathipnship between the volume of proteoliposome and avearge percentage removal.

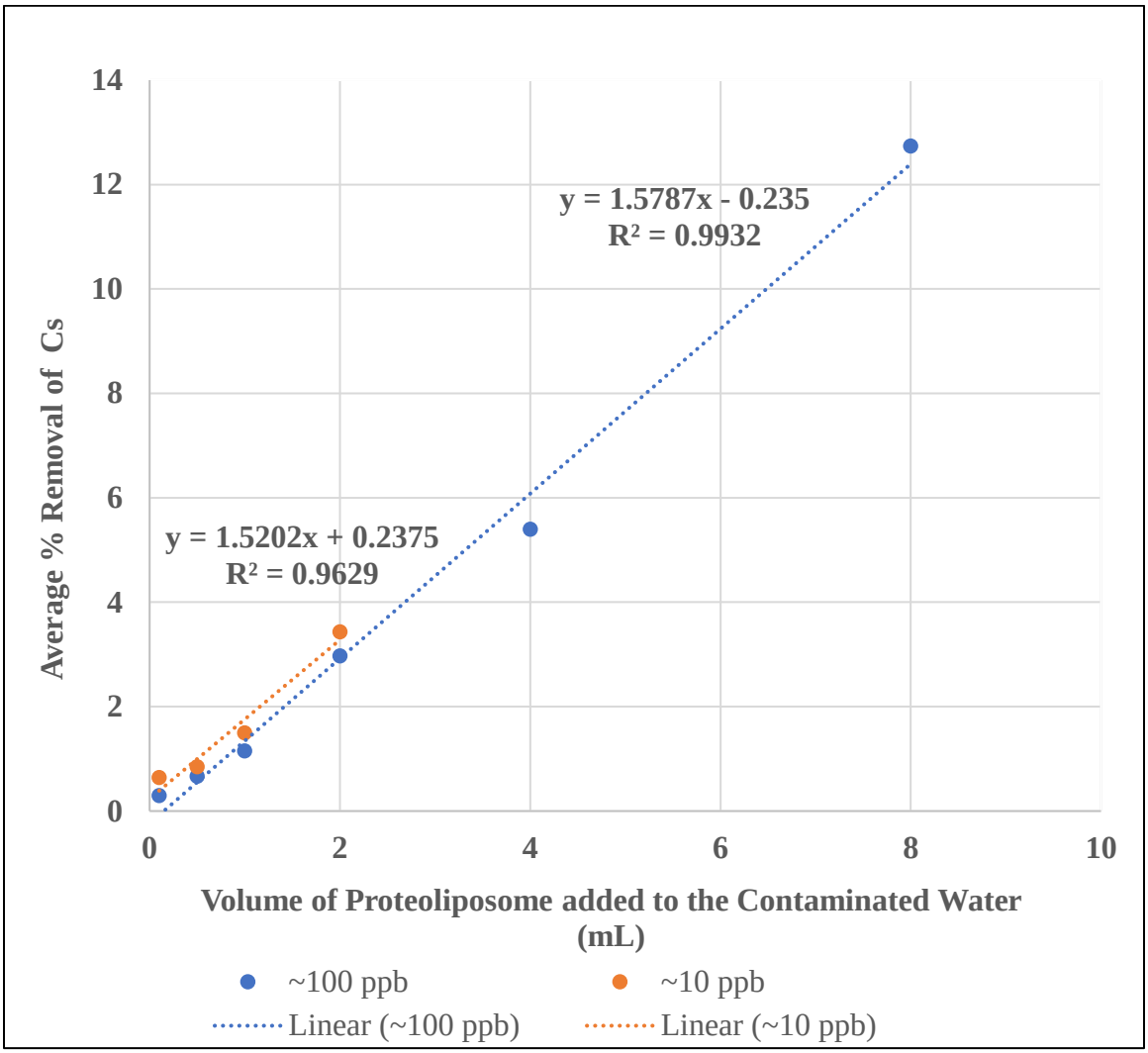

Figure 41. Linear Relationship between the Volume of Proteoliposome and Average Percentage Removal of Cs.

The equilibrium adsorption capacity (q_e) per gram dry mass of the protein transporter (adsorbent) was calculated (Appendix C) as presented in Figure 42.

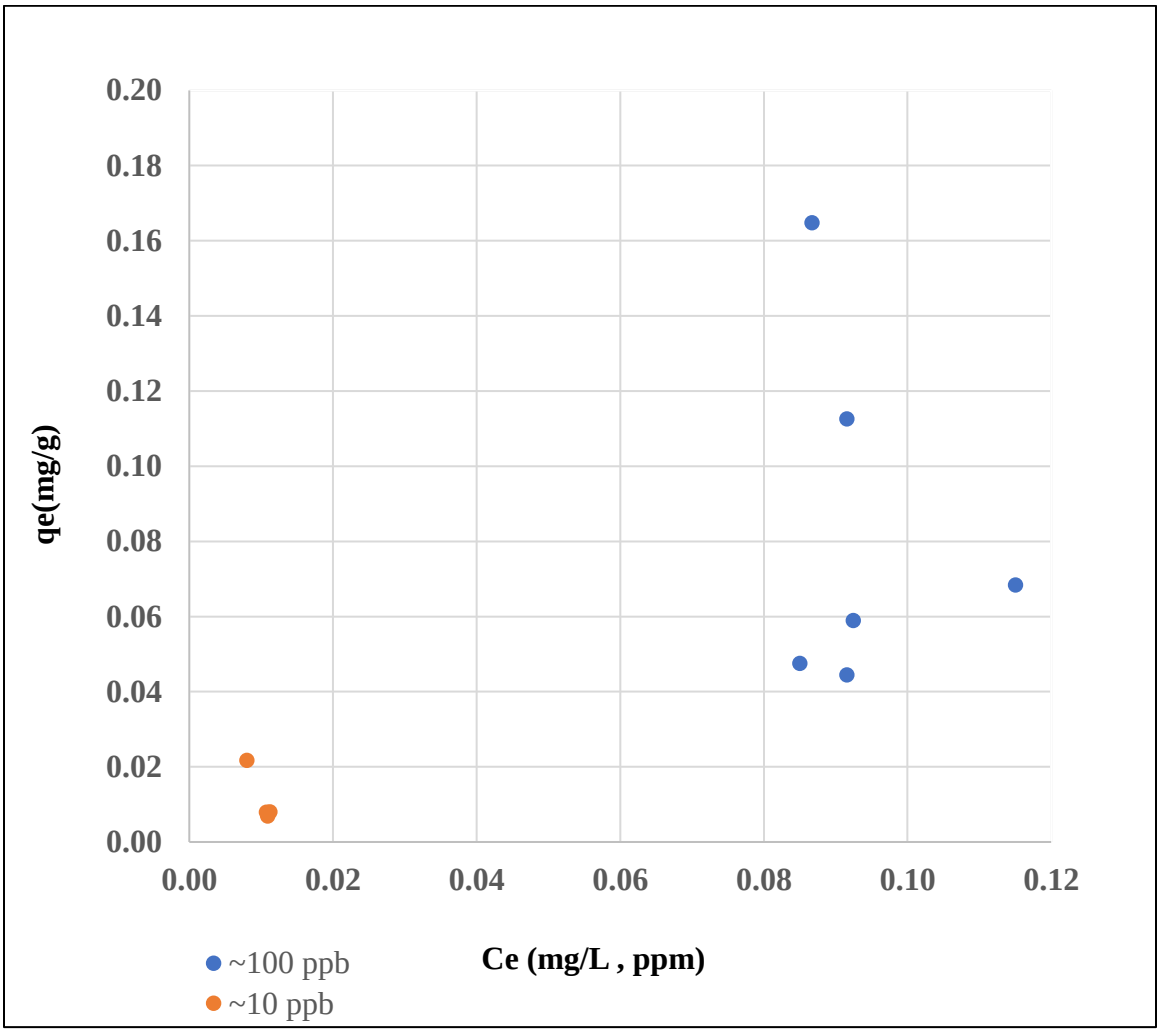

Figure 42. Estimated Uptake Capacity of Proteoliposome. q_e is mass of Cs adsorbed on adsorbent per mass of adsorbent (protein) (mg/g), $C_{e:}$ is the concentration of Cs after uptake/treatment (mg/L).

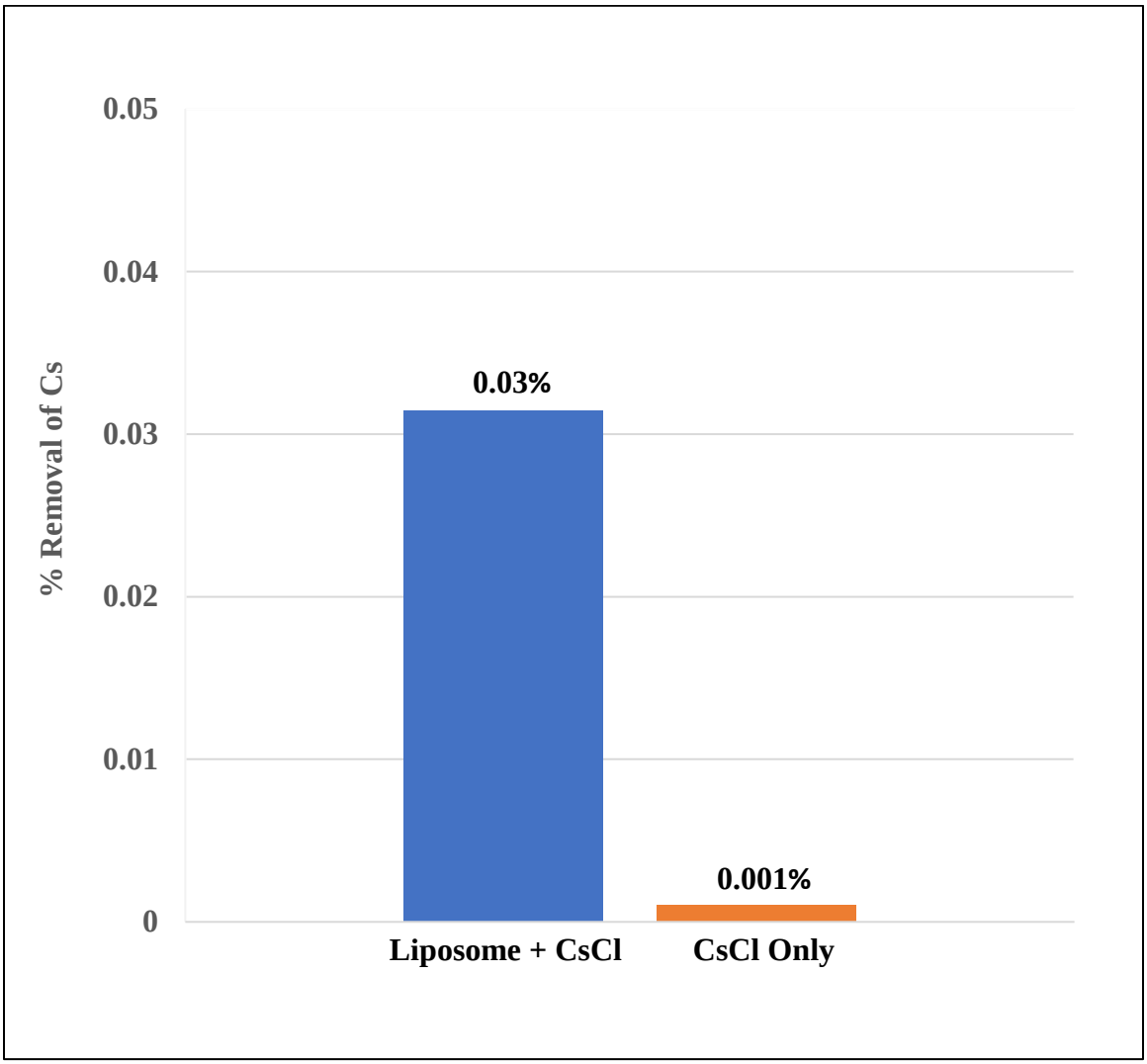

Figure 43. Effect of Centrifugation (260,000 x g) and Empty Liposomes on Cs Removal

Removal of Cs with proteoliposome is greater than the controls. As can be seen in Figure 43, centrifugation and empty liposome vesicles only contributes to 0.001% and 0.03% removal which is significantly low. Therefore, according to this control test, Kup transporter inserted into the liposome is responsible for the highest amount of removal.

4.6 Conclusions

A simple, efficient, eco-friendly method using a proteoliposome transporter to remove radioactive Cs, present in low concentrations of about 10 ppb and 100 ppb from water was developed. This method is a combination of mechanical agitation, solvent dispersion and detergent removal. The results provide new insight on the effectiveness and applicability of proteoliposome transporter as well as contributing to science by resolving the existing issue as for challenging removal of cesium when present in low concentrations (~10 ppb) which is the typical concentrations found in the radioactive contaminated areas. In summary, the following conclusions can be drawn from this study:

- Adding 0.1 - 8 mL of proteoliposome to contaminated water with about 100 ppb CsCl resulted in 0.29 - 12.73% Cs removal, respectively.
- Adding 0.1 - 2 mL of proteoliposome to contaminated water with about 10 ppb CsCl resulted in 0.65 - 3.43% Cs removal, respectively.
- Comparing the results obtained from both 10 ppb and 100 ppb tests, showed high similarity in percentage removal indicating that this method would be also efficient in removal of Cs in range of 10- 100 ppb.
- There is a linear relationship between the amount of proteoliposome added to the contaminated water and removal percentage. Increasing the amount of proteoliposome, removal can be simply enhanced significantly. That means using about 60-70 mL proteoliposome that can be made easily using our suggested technique, removal of about 90% can be expected.

CHAPTER 5

SUMMARY

In this study, a new protein extraction method as well as an optimized purification process for Kup membrane transporters were developed. The method was superior to other existing traditional protocols by means of higher efficiency, reduced experimental time, less chemical usage leading to less processing and easier and more cost-effective extraction and purification processes. This is of importance since extraction and purification of membrane proteins are the major steps required for proteoliposome construction.

An environmentally friendly method to remove cesium using the Kup proteoliposome transporter was developed. This method proved to be efficient in removing cesium when present in low concentrations of about 10 ppb and 100 ppb.

Future research on this topic includes testing a more concentrated proteoliposome solution to demonstrate greater percentage removal of Cs as well as spiking the tap water (instead of ultrapure water), with Cs, to study the impact of other background and interfering ions on removal efficiency. Making a larger volume of proteoliposome would be cost-effective since preparation of about 16 mL of proteoliposome solution cost about $170.

Exploration of other metal transporters may prove beneficial in remediation and treatment efforts of heavy metals. The transporter classification database (www.tcdb.org) lists proteins that are involved in the uptake of lead, cobalt, cadmium, and zinc in bacteria, while uptake transporters for arsenic, mercury, or chromium do not exist. However, the chemistry of arsenic (V) and chromium (VI) ions is similar to phosphate and sulfate,

respectively, which do have uptake proteins in bacteria. Based on this work, molecules of similar chemistry may have an affinity for the same protein. In theory, proteoliposomes can be constructed to contain multiple metal transporters for efficient and broad removal, and the internal environment can be designed to precipitate metals upon uptake.

REFERENCES

Abeywardena, M. Y., Allen, T. M., & Charnock, J. S. (1983). Lipid-protein interactions of reconstituted membrane-associated adenosinetriphosphatases. Use of a gel-filtration procedure to examine phospholipid-activity relationships. *Biochim. Biophys. Acta.*, 729(1):62-74.

Agency for Toxic Substances and Disease Registry. (n.d.). Retrieved from https://www.atsdr.cdc.gov/PHS/PHS.asp?id=575&tid=107

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Woo Joo, S., & Zarghami, N. (2013). Liposome: classification, preparation, and applications. *Nanoscale Res Lett*, 8:102.

Allen, T. M., Romans, A. Y., Kercret, H., & Segrest, J. P. (1980). Detergent removal during membrane reconstitution. *Biochim Biophys Acta.*, 601(2):328.

Alpes, H., Allmann, K., Plattner, H., Reichert, J., Rick, R., & Schulz, S. (1986). Formation of large unilamellar vesicles using alkyl maltoside detergents. *Biochim Biophys Acta (BBA)*, 862:294.

Arcadio, C., & Cullis, P. R. (1995). Recent advances in liposomal drug-delivery systems. *Curr Opin Biotechnol* , 6:698–708.

Arnold, T., & Linke, D. (2016). The Use of Detergents to Purify Membrane Proteins. *Curr Protoc Protein Sci.*, 84:4.8.1-4.8.35.

ATSDR. (2004). *Agency for Toxic Substances and Disease Registry*. Retrieved from https://www.atsdr.cdc.gov/PHS/PHS.asp?id=575&tid=107

Avanti polar lipids, I. (1969). Retrieved from http://www.linkedin.com/redirect?url=http%3A%2F%2Favantilipids%2Ecom&urlhash=0MN8&trk=Avanti+Polar+Lipids%2C+Inc%2E_website

Avanti Polar Lipids, I. (2018). Retrieved from https://avantilipids.com/tech-support/faqs/transition-temperature

Awada, A., Gil, T., Sales, F., Dubuisson, M., Vereecken, P., Klastersky, J., . . . Piccart, M. J. (2004). Prolonged schedule of temozolomide (Temodal) plus liposomal doxorubicin (Caelyx) in advanced solid cancers. *Anticancer Drugs* , 15, 499–502.

Bangham, A. D., Standish, M. M., & Watkins, J. C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *J Mol Biol.*, 13(1):238-52.

Bangham, A., & Horne, R. (1964). Negative staining ofphospholipids and their structural modification by surfaceactive agents as observed in the electron microscope. *J.Mol. Biol.*, 8, 660-668.

Bangham, A., Standish, M., & Weissmann, G. (1965). The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *J. Mol. Biol.*, 13, 253-259.

Barani, H., & Montazer, M. (2008). A review on applications of liposomes in textile processing. *J Liposome Res.* , 18(3):249-62.

Batzri, S., & Korn, E. D. (1973). Single bilayer liposomes prepared without sonication. *Biochim Biophys Acta.*, 298(4):1015-9.

Bee, A., Massart, R., & Neveu, S. (1995). Synthesis of very fine maghemite particles. *J. Magn. Magn. Mater*, 149, 6–9.

BioVision. (n.d.). *BCA Protein Assay Kit II.* Retrieved from https://www.biovision.com/documentation/datasheets/K813.pdf

Brady, P. N., & Macnaughtan, M. A. (2015). Evaluation of Colorimetric Assays for Analyzing Reductively Methylated Proteins: Biases and Mechanistic Insights. *Anal. Biochem.* , 491: 43–51.

Brohawn, S., del Mármol, J., & MacKinnon, R. (2012). Crystal structure of the human K2P TRAAK, a lipid- and mechano-sensitive K+ ion channel. *Science*, 335: 436–441.

Buesseler, K., Jayne, S., Fisher, N., Rypina, I., Baumann, H., Baumann, Z., Breier, C., Douglass, E., George, J., Macdonald, A. (2012). Fukushima-derived radionuclides in the ocean and biota off Japan. *Proc. Natl. Acad. Sci.*, 109, 5984–5988.

Bystrzejewska-Piotrowska, G., & Urban, P. (2004,). Accumulation and translocation of cesium-137 in onion plants (Allium cepa). *Environ. Exp. Bot.*, 51, 3–7.

Çağdaş, M., Demir Sezer, A. D., & Bucak, S. (2014). Liposomes as Potential Drug Carrier Systems for Drug Delivery. In *Application of Nanotechnology in Drug Delivery* (p. Chapter1).

da Silva Malheiros, P., Daroit, D. J., & Brandelli, A. (2010). Food applications of liposome-encapsulated antimicrobial peptides. *Trends in Food Sci Technol*, 21, 284-292.

Daemen, T., Hofstede, G., Ten Kate, M. T., Bakker-Woudenberg, I. A., & Scherphof, G. L. (1995). Liposomal doxorubicin-induced toxicity: depletion and impairment of phagocytic activity of liver macrophages. *Int J Cancer.*, 61(5):716-21.

Daneshamouz, S., M. Danyali, M., Mohammadi-Samani, S., & Jaafari, M. (2012). Preparation and characterization of multivesicular vesicles (MVVs) containing gentamicin as a depot drug delivery system. *RPS*, 7(5).

Deamer, D. (n.d.). From "Banghasomes" to liposomes: A memoir of Alec Bangham, 1921–2010.

Deamer, D., & Bangham, A. D. (1976). Large volume liposomes by an ether vaporization method. *Biochim Biophys Acta.*, 443(3):629-34.

Dirk Bossemeyer, A. S. (1989). Specific Cesium Transport via the Escherichia coli Kup (TrkD) K+ Uptake System. *J Bacteriol.*, 2219-2221.

DNASU Plasmid Repository. (n.d.). Retrieved from Detailed Vector Information: pNYCOMPS-LIC-FH10T+ (N term): http://dnasu.org/DNASU/GetCloneDetail.do?cloneid=91443#

Dua J.S, R. A. (2012). Liposome: Methods of Preparation and Applications. *Int J Pharm Stu Res*, 3(2), 14-20.

Dumaual, A. C., Jenski, L. J., & Stillwell, W. (2000). Liquid crystalline/gel state phase separation in docosahexaenoic acid-containing bilayers and monolayers. *Biochimica et Biophysica Acta*, 1463, 395-406.

Ella Zakharyan, A. T. (2001). K‡ in£ux by Kup in Escherichia coli is accompanied by a decrease in H+ efflux. *FEMS Microbiol Lett*, 61-64.

Ellen Tanudjaja, N. H.-H. (2017). Kup-mediated Cs+ uptake and Kdp-driven K+ uptake coordinate to promote cell growth during excess Cs+ conditions in Escherichia coli. *Sci Rep.* , 7: 2122.

Entry, J. V. (1996). Phytoremediation of soil contaminated with low concentrations of radionuclides. *Water Air Soil Pollut* , 88: 167.

EPA. (2002). *EPA Facts about Cesium-137*. Retrieved from https://semspub.epa.gov/work/HQ/176309.pdf

Fadi, R.-S. (2003). Biomedical applications of inductively coupled plasma mass spectrometry (ICP–MS) as an element specific detector for chromatographic separations. In *Handbook of Analytical Separations* (pp. 351-371). Elsevier BV.

Freisleben, H. J., Zwicker, K., Jezek, P., John, G., Bettin-Bogutzki, A., Ring, K., & Nawroth, T. (1995). Reconstitution of bacteriorhodopsin and ATP synthase from Micrococcus luteus into liposomes of the purified main tetraether lipid from Thermoplasmaacidophilum: proton conductance and light-driven ATP synthesis. *Chem Phys Lipids* , 78: 137–147.

Gabizon, A. A. (2001). Stealth liposomes and tumor targeting: one step further in the quest for the magic bullet. *Clin Cancer Res.*, (2):223-5.

Geertsma, E. R., Nik Mahmood, N. A., Schuurman-Wolters, G. K., & Poolman, B. (2008). Membrane reconstitution of ABC transporters and assays of translocator function. *Nat Protoc*, 3(2):256-66.

Gomez-Hens, A., & Fernandez-Romero, J. M. (2006). Analytical Methods for the Control of Liposomal Delivery Systems. *Trends in Analytical Chemistry* , 25, 167-178.

Gonen, T., Cheng, Y., Sliz, P., Hiroaki, Y., Fujiyoshi, Y., & Harrison, S. (2005). Lipid-protein interactions in double-layered two-dimensional AQP0 crystals. *Nature*, 438: 633–638.

Haginoya, K., Kato, T., Higuchi, M., Shitomi, Y., Asakura, T., Hayakawa, T., Hori, H. (2005). Preparation of stable liposomes using sucrose density gradient centrifugation and their interaction with insecticidal CrylA toxins of Bacillus thuringiensis . *AGRIS*, 57, 115-120.

Hamasaki, T., Nakamichi, N., Teruya, K., & Shirahata, S. (2014). Removal Efficiency of Radioactive Cesium and Iodine Ions by a Flow-Type Apparatus Designed for Electrochemically Reduced Water Production. *PLOS ONE*, 9, p. e102218.

Hegyi, G., Kardos, J., Kovács, M., Málnási-Csizmadia, A., Nyitray, L., Pál, G., Venekei, I. (2013). Eötvös Loránd University.

Helenius, A., & Simons, K. (1975). Solubilization of membranes by detergents. *Biochim Biophys Acta* , 415: 29–79.

Himanshu, A., Sitasharan, P., & Singhai, A. K. (2011). Liposomes as drug carriers. *Int J Pharm life Sci. (IJPLS)*, 2(7), 945–95.

Hinkle, P. C., Kim, J. J., & Racker, E. (1972). Ion Transport and respiratory control in vesicles formed from cytochrome oxidase and phospholipids. *J Biol Chem*, , 247: 1338–1339.

Holloway. (1973). A simple procedure for removal of triton X-100 from protein samples. *Anal Biochem*, 53, 304-308.

Hope, M., Bally, M. B., Webb, G., & Cullis, P. R. (1985). Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta*, 812(1):55-65.

Houk, R., Fassel, V., Flesch, G., Svec, H., Gray, A., & Taylor, C. (1980). Inductively coupled argon plasma as an ion source for mass spectrometric determination of trace elements. *Anal. Chem.*, 52 (14), pp 2283–2289.

IPTG Induction Theory. (n.d.). Retrieved from biologics international corp: https://www.biologicscorp.com/blog/iptg-induction-protein-expression/

Jain, S. K., Jain, R. K., Chourasia, M. K., Jain, A. K., Chalasani, K. B., Soni, V., & Jain, A. (2005). Design and development of multivesicular liposomal depot delivery system for controlled systemic delivery of acyclovir sodium. *AAPS PharmSciTech* , 6 (1).

Kagawa, Y., & Racker, E. (1971). Partial resolution of the enzymes catalyzing oxidative phosphorylation: XXV. Reconstitution of vesiclescatalyzing 32Pi—adenosine triphosphate exchange. *J Biol Chem*, 246: 5477–5487.

Kapoor, V., & Wendell, D. (2013). Engineering Bacterial Efflux Pumps for Solar-Powered Bioremediation of Surface Waters. *Nano Lett.*, 13 (5), 2189–2193.

Kataria, S., Sandhu, P., Bilandi, A., Akanksha, M., Kapoor, B., Seth, G. L., & Bihani, S. D. (2011). Stealth liposomes: a review. *IJRAP* , 2(5), 1534–1538.

Knol, J., Sjollema, K., & Poolman, B. (1998). Detergent-Mediated Reconstitution of Membrane Proteins. *Biochemistry*, 37,16410-16415.

Koji Kosaka, K., Asami, M., Kobashigawa, N., Ohkubo, K., Terada, H., Kishida, N., & Akiba, M. (2012). Removal of radioactive iodine and cesium in water purification

processes after an explosion at a nuclear power plant due to the Great East Japan Earthquake. *Water Research*, 46, 4397-4404.

Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., Sfar, S., Charcosset, C., & Fessi, H. (2012). Preparation, Characterization and Applications of Liposomes: State of the Art. *J. Colloid Sci. Biotechnol*, (1), 147–168.

Lasic, D. D. (1998). Novel applications of liposomes. *Trends Biotechnol.* , 16(7):307-21.

Laurent, S., Forge, D., Port, M., Roch, A., Robic, C., Vander Elst, L., & Muller, R. (2008). Magnetic iron oxide nanoparticles: Synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications. . *Chem. Rev.* , 108, 2064–2110.

Levy, D., Bluzat, A., Seigneuret, M., & Rigaud, J. (1990). A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal. *Biochimica et Biophysica Acta*,, 1025, 179-190.

Levy, D., Gulik, A., Bluzat, A., & Rigaud, J. L. (1992). Reconstitution of the sarcoplasmic reticulum Ca(2+)-ATPase: mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. *Biochim Biophys Acta* , 1107: 283–298.

Wang, L., & Tonggu, L. (2014). Membrane protein reconstitution for functional and structural studies. *Sci China life Scis*, 58, 66–74.

Little, I., Alorkpa, E., Khan, V., Povazhnyi, V., & Vasiliev, A. (2018). Efficient porous adsorbent for removal of cesium from contaminated water. *J Porous Mater*, 1–9.

Liu, L., & Yonetani, T. (1994). Preparation and characterization of liposome-encapsulated haemoglobin by a freeze-thaw method. *J Microencapsul.* , 11(4):409-21.

Liu, Y., & Sigworth, F. J. (2014). Automatic cryo-EM particle selection for membrane proteins in spherical liposomes. *J Struct Biol*, 185, 295–302.

Long, S., Tao, X., Campbell, E., & MacKinnon, R. (2007). Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment. *Nature*, 450: 376–383.

Martí, M., Rodriguez, R., Carreras, N., Lis, M., Valldeperas, J., Coderch, L., & Parra, J. (2010). Monitoring of the microcapsule/liposome application on textile fabrics. *J. of the Textile Institute*, 19-27.

Meroufel, B., Benali, O., Benyahia, M., Benmoussa, Y., & Zenasni, M. A. (2013). Adsorptive removal of anionic dye from aqueous solutions by Algerian kaolin: Characteristics, isotherm, kinetic and thermodynamic studies. *J. Mater. Environ. Sci.* , 4 (3), 482-491

Molecular cell Physiology. (2013, November 4). Retrieved from Amsterdam University: http://www.falw.vu/~microb/Protocols/index.html

Montazer, M., Validi, M., & Toliyat, T. (2006). Influence of temperature on stability of multilamellar liposomes in wool dyeing. *J Liposome Res.* , 16(1), 81-9.

Mosquera, B., Carvalho, C., Veiga, R., Mangia, L., & Anjos, R. (2006). 137Cs distribution in tropical trees after soil contamination. . *Environ. Exp. Bot.* , 55, 273–281.

Mouro-Chanteloup, I., Cochet, S., Chami, M., Genetet, S., Zidi-Yahiaoui, N., & Engel, A. (2010). Functional reconstitution into liposomes of purified human RhCG ammonia channel. *PLoS One.* 5(1): e8921.

Mozafari M.R, J. C. (2008). Nanoliposomes and Their Applications in Food Nanotechnology . *Journal of Liposome Research* , 18, 309-327.

Mozafari, M. R. (2005). Liposomes: an overview of manufacturing techniques. *Cell Mol Biol Lett* , 10(4):711–719.

Mozafari, M., Johnson, C., Hatziantoniou, S., & Demetzos, C. (2008). Nanoliposomes and their applications in food nanotechnology. *J Liposome Res.*, 18(4):309-27.

Nagano, K., & Nikaido, H. (2009). Kinetic behavior of the major multidrug efflux pump AcrB of Escherichia coli. *PNAS*, 5854 –5858 .

Nagy, L., Ming, H., Masataka, I., Masanobu, N., & Yusuke, Y. L. (2012). Cs adsorption capability of nanostructured Prussian Blue particles with high accessible surface areas. *J. Mater. Chem.* , 22, 18261–18267.

Nakao, S., Ebata, H., T., H., Kagawa, Y., & Hirata, H. (1988). Solubilization and reconstitution of voltage-dependent calcium-channel from bovine cardiac-muscle.

Ca2+ influx assay using the fluorescent dye Quin2. *Biochim Biophys Acta*, 944: 337–343.

Namiki, Y., Namiki, T., Ishii, Y., Koido, S., Nagase, Y., Tsubota, A., Kitamoto, Y. (2012). Inorganic-organic magnetic nanocomposites for use in preventive medicine: A rapid and reliable elimination system for cesium. *Pharm. Res.* , 29, 1404–1418.

Neil Willey, S. H. (2006). Assessing the Potential of Phytoremediation at a Site in the U.K. Contaminated With 137Cs. *Int J Phytoremediation*, 321-333.

Nikaido, H., & Takatsuka, Y. (2009). Mechanisms of RND multidrug efflux pumps. *Biochim Biophys Acta*, (1794), 769-781.

Ohsawa, T., Miura, H., & Harada, K. (1985). Improvement of Encapsulation Efficiency of Water-Soluble Drugs in Liposomes Formed by the Freeze-Thawing Method. *Chem Pharm Bull* , 33(9):3945–3952.

Ollivon, M., Lesieur, S., Grabielle-Madelmont, C., & Paternostre, M. (2000). Vesicle reconstitution from lipid–detergent mixed micelles. *Biochimica et Biophysica Acta* , 1508, 34-50.

Olson, F., Hunt, C. A., Szoka, F. C., Vail, W. J., & Papahadjopoulos, D. (1979). Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim Biophys Acta.* , 557(1):9-23.

OpenWetWare. (2013). Retrieved from SDS-PAGE Protein Gels: https://openwetware.org/wiki/SDS-PAGE_Protein_Gels

Parajuli, D., Tanaka, H., Hakuta, Y., Minami, K., Fukuda, S., Umeoka, K., Kamimura, R., Hayashi, Y., Ouchi, M., Kawamoto, T. (2013). Dealing with the aftermath of Fukushima Daiichi nuclear accident: Decontamination of radioactive cesium enriched ash. *Environ. Sci. Technol.* , 47, 3800–3806.

Patravale, V. B., & Mandawgade, S. D. (2008). Novel cosmetic delivery systems: an application update. International Journal of Cosmetic Science, 30,19–33.

Philippot, J., Mutaftschiev, S., & Liautard, J. P. (1983). A very mild method allowing the encapsulation of very high amounts of macromolecules into very large (1000 nm) unilamellar liposomes. Biochim. Biophys. Acta , 734, 137-143.

Picard, P., Verchère, A., & Broutin, I. (2013). Functional Investigation of the MexA-MexB-OprM Efflux Pump of Pseudomonas Aeruginosa. *Biophysical Journal*, 104(2):286.

Pick, U. (1981). Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. *Arch Biochem Biophys.* , 212(1):186-94.

Prichystal, P., Burgert, L., Hrdina, R., Purev, N., & Cerny, M. (2013). Encapsulation of textile dyes and textile auxiliary agents into liposome systems and their use for polyamide dyeing. *coloration technology*, 64-68.

Racker, E. (1972). Reconstitution of a calcium pump with phospholipids and a purified Ca++-adenosine triphosphatase from sarcoplasmic reticulum. *J Biol Chem*, 247: 8198–8200.

Reineccius, G. (1995). *Liposomes for Controlled Release in the Food Industry.* Chapter 11, pp 113–131: ACS Publications.

Riaz, M. (1996). Liposome preparation method. . *Pak J Pharm Sci* , 9(1):65–77.

Rigaud, J., Pitard, B., & Levy, D. (1995). Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochim Biophys Acta (BBA) Bioenergetics*, 223-246.

Rosoff, B., Cohn, S., & Spencer, H. (1963). Cesium-137 metabolism in man. . *Radiat. Res.* , 19, 643–654.

Sambrook, J.; Russell, D. W. (2006). The Condensed Protocols from Molecular Cloning: A Laboratory Manual. In D. W. Joseph Sambrook. New york, USA: Cold Spring Harbor Laboratory Press.

Sangvanich, T., Sukwarotwat, V., Wiacek, R., Grudzien, R., Fryxell, G., Addleman, R., . . . Yantasee, W. (2010). Selective capture of cesium and thallium from natural waters and simulated wastes with copper ferrocyanide functionalized mesoporous silica. *J. Hazard. Mater*, 182, 225–231.

Schieren, H., Rudolph, S., Finkelstein, M., Coleman, P., & Weissmann, G. (1978). Comparison of large unilamellar vesicles prepared by a petroleum ether vaporization method with multilamellar vesicles: ESR, diffusion and entrapment analyses. *Biochim Biophys Acta.* , 542(1):137-53.

Schleyer, M., & Bakker, E. P. (1993). Nucleotide Sequence and 3'-End Deletion Studies Indicate that Indicate that the K+-Uptake Protein Kup from Escherichia coli Is Composed of a Hydrophobic Core Linked to a Large and Partially Essential Hydrophilic C Terminus. . *J Bacteriol.* , 6925-6933.

Schmidt, D., Jiang, Q. X., & MacKinnon, R. (2006). Phospholipids and the origin of cationic gating charges in voltage sensors. *Nature*, 444: 775–779.

Seras-Cansell, M., Ollivon, M., & Lesieur, S. (1996). Generation of non-ionicmonoalkyl amphiphile-cholesterol vesicles: evidence of membrane impermeability to octyl glucoside. *STP Pharma Sci*, 6: 12–20.

Shaheen, S. M., Shakil Ahmed, F. R., Hossen, M. N., Ahmed, M., Amran, M. S., & Ul-Islam, M. A. (2006). Liposome as a carrier for advanced drug delivery. *Pak J Biol Sci* , 9 (6): 1181-1191.

Sharma, A., & Sharma, U. S. (1997). Liposomes in drug delivery: progress and limitations. *Int J Pharm*, 154, 123 140.

Shokouhimehr, M., Soehnlen, E., Khitrin, A., Basu, S., & Huang, S. (2010). Biocompatible Prussian blue nanoparticles: Preparation, stability, cytotoxicity, and potential use as an MRI contrast agent. *Inorg. Chem. Commun*, 13, 58–61.

Shubair, T., Eljamal, O., Khalil, A. M., Tahara, A., & Matsunaga, N. (2018). Novel application of nanoscale zero valent iron and bimetallic nano-Fe/Cu particles for the treatment of cesium contaminated water. *J Env Chem Eng*, 6, 4253–4264.

Simeonova, P., Werner, S., Haupta, C., Tanabe, M., & Bacia, K. (2013). Membrane protein reconstitution into liposomes guided by dual-color fluorescence cross-correlation spectroscopy. *Biophysical Chemistry* , 184, 37–43.

Singh, H., Thompson, A., Liu, W., & Corredig, M. (2012). Liposomes as food ingredients and nutraceutical delivery systems. In *Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals* (pp. 287-318). Woodhead Publishing.

Smith, P., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., . . . Klenk, D. C. (1987). Measurement of protein using bicinchoninic acid. *Anal Biochem*, 163(1):279.

Song, H., Geng, H. Q., Ruan, J., K., W., Bao, C. C., Wang, J., . . . Cui, D. X. (2011). Development of polysorbate 80/phospholipid mixed micellar formation for

docetaxel and assessment of its in vivo distribution in animal models. *Nanoscale Res Lett*, 6:354.

Sung-Chan Jang, S.-B. H.-M.-W.-K.-K. (2014). Removal of Radioactive Cesium Using Prussian Blue Magnetic Nanoparticles. *Nanomaterials*, 4, 894-901.

Surma, M. A., Szczepaniak, A., & Króliczewski, J. (2014). Comparative Studies on Detergent-Assisted Apocytochrome b6 Reconstitution into Liposomal Bilayers Monitored by Zetasizer Instruments. *PLOS One*, 9, 1-10.

Szebeni, J., Breuer, J. H., Szelenyi, J. G., G., B., Lelkes, G., & Hollan, S. R. (1984). Oxidation and denaturation of hemoglobin encapsulated in liposomes. *Biochim Biophys Acta.* , 798(1):60-7.

Szoka, F., & D Papahadjopoulos, D. (1978). Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc Natl Acad Sci*, 75(9): 4194–4198.

T.B., R. (2004). Induction-Coupled Plasma Torch. *J. Appl. Phys.*, 32, 821.

Tadros, T.). (2013). *Critical Micelle Concentration.* Berlin, Heidelberg: Encyclopedia of Colloid and Interface Science. Springer.

Tanaka JC, E. J. (1983). Cation selectivity characteristics of the reconstituted voltage-dependent sodium channel purified from rat skeletal muscle sarcolemma. *J Biol Chem*, 258: 7519–7526.

TED PELLA, I. (n.d.). *PELCO easiGlow™ Glow Discharge Cleaning System.* Retrieved from https://www.tedpella.com/easiGlow_html/Glow-Discharge-Cleaning-System.htm

Thammawong, C., Opaprakasit, P., Tangboriboonrat, P., & Sreearunothai, P. (2013). Prussian blue-coated magnetic nanoparticles for removal of cesium from contaminated environment. *J. Nanopart. Res.* , 15, 1689–1698.

Thermo Fisher Scientific. (n.d.). Retrieved from https://tools.thermofisher.com/content/sfs/manuals/MAN0011700_HisPur_NiNTA_Resin_UG.pdf

Thompson, R. F., Walker, M., Siebert, A., Muench, S. P., & Ranson, N. A. (2016). An introduction to sample preparation and imaging by cryo-electron microscopy for structural biology. *Methods* , 100, 3–15.

Tikhonova, E. B., Devroy, V. K., Lau, S. Y., & Zgurskaya, H. I. (2007, January 4). Reconstitution of the Escherichia coli macrolide transporter: the periplasmic membrane fusion protein MacA stimulates the ATPase activity of MacB. *Molecular Microbiology*, 63(3), 895–910.

Tomioka, N., Uchiyama, H., Yagi, O., & Fujii, T. (1995). Kinetic Studies on Cesium Transport in Rhodococcus erythropolis CS98 and Rhodococcus sp. Strain CS402. *Biosci. Biotech. Biochem.*, 59 (12), 2219~2222.

Tomomi Koshiyama, M. T. (2018). Direct Synthesis of Prussian Blue Nanoparticles in Liposomes Incorporating Natural Ion Channels for Cs+ Adsorption and Particle Size Control. *Langmuir* , 34, 1666−1672.

Torchilin, V. P. (2005). Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov.* , 4(2):145-60.

Verchère, A., Dezi, M., Broutin, I., & Picard, M. (2014). In vitro Investigation of the MexAB Efflux Pump From Pseudomonas. *J Vis Exp* , 1-6.

Vergara-Irigaray, N., Riesen, M., Piazza, G., & Nosonovsky, M. (2012). Liposomes. In E. Jabbari, *Nanoengineered Hydrogels for Cell Engineering* (pp. 1218-1223). New York, NY: Springer.

Vikram, K., & Wendell, D. (n.d.). Engineering Bacterial Efflux Pumps for Solar-Powered .

Vinita, P., Kumar Shiv, M., Mahavir, Y., & Archana, T. (2013). Liposome: A Powerful Approach Festinates Drug Delivery System. *Int J Drug Deliv Technol* , 3(1), 4-7.

Vivian, D., & E. Polli, J. (2014). Mechanistic interpretation of conventional Michaelis–Menten parameters in a transporter system. *Eur J Pharm Sci*, 64, 44–52.

Walker, J. (1996). The Bicinchoninic Acid (BCA) Assay for Protein Quantitation. In J. Walker, *The Protein Protocols Handbook* (pp. 11-14). Humana Press.

Wang, C.-B., & Zhang, W.-X. (1997). Synthesizing nanoscale iron particles for rapid andcomplete dechlorination of TCE and PCBs. *Environ. Sci. Technol.* , 31, 2154–2156.

Wang, L., & Sigworth, F. J. (2009). Structure of the BK potassium channel in a lipid membrane from electron cryomicroscopy. *Nature*, 461:292–295.

Weinglass, A., Whitelegge, J., & Kaback, H. (2004). Integrating mass spectrometry into membrane protein drug discovery. *curr Opin Drug Dis Dev.*, 7: 589–599.

Weissmann, G., Standish, M. M., & Bangham, A. D. (1965). The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *J. Mol. Biol.*, 13, 253-259.

Werner, S., Simeonov, P., Haupt, C., Tanabe, M., & Bacia, K. (2013). Membrane protein reconstitution into liposomes guided by dual-color fluorescence cross-correlation spectroscopy. *Biophysical Chemistry*, 184, 37–43.

Zhang, L., Jiang, W., Nan, J., Almqvist, J., & Huang, Y. (2014). The Escherichia coli CysZ is a pH dependent sulfate transporter that can be inhibited by sulfite. *Biochimica et Biophysica Acta* , 1838,1809–1816.

APPENDIX A

SOLUTIONS PREPARATION

A. Ultra-Pure Water

Autoclave Ultra-pure water for about 30 min. at 120℃. then keep it at 4℃ in the refrigerator.

B. Glycerol 10% preparation

1. Prepare 18 mL of APW add into a beaker.
2. Add 2mL of glycerol and mix very well until it turns to homogenous solution.
3. Sterilize by using syringe filter 0.22 -0.45 µm.
4. Collect the filtrate into a sterilize vial, store at room temperature.

C. Kanamycin preparation

1. Add 0.5 g of Kanamycin powder into 10 mL of Milli-Q water.
2. Mix well, then sterilize by using syringe filter 0.22- 0.45 µm.
3. Aliquot filtrate into small sterilized tubes.
4. Store at -20℃ (Table A2-1 from (Sambrook, J.; Russell, D. W., 2006)).

D. LB-Broth preparation

1. Add 20 g of LB broth powder into 1000 mL of Milli-Q water.
2. Mix well, autoclave for about 30 min at 121℃.
3. Keep the medium at room temperature.

E. LB-Agar Plate preparation

1. Add 35 g of LB agar powder to one liter of Milli-Q water.
2. Autoclave for 2 hours at 121℃, once the autoclaving is done, let the medium temperature cool down about 70- 65℃
3. Add 1000 µl of Kanamycin and swirl gently to confirm mixing.
4. Immediately start to pour petri dish plates. The medium makes about 40 plates.

5. Wait for the agar to solidify about 10-15 mins. Keep the plates in refrigerator.

F. DNase I solution preparation:

1. DNase storage buffer: 10 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$ in 50% glycerol

(filter sterilized).

2. DNase reaction buffer: 100 mM Tris-HCl, pH 7.6, 5 mM $CaCl_2$, 25 mM $MgCl_2$ (filter sterilized).

G. 100 mM PMSF stock solution:

1. Mix 0.0261 gr of PMSF with about 1.5-mL of ethanol to make 1-mL stock solution.
2. Aliquot into 150 µl volumes and store in -20 ^{o}C.

H. Buffers for protein purification:

1. Equilibrium buffer: 20mM Sodium phosphate, 300mM Sodium chloride (PBS) with 10 mM imidazole; PH 7.4
2. Wash buffer: PBS with 50 mM imidazole; PH 7.4
3. Elution buffer: PBS with 1000 mM imidazole; PH 7.4
4. MES buffer: 20 mM 2-(*N* – morphine) – ethane sulfonic acid, 0.1 M Sodium Chloride, PH 5.0.

I. Staining solution for SDS-PAGE:

Add 0.25 g of Coomassie Brilliant Blue per 1-lit of 50% methanol, 10% glacial acetic acid and 40% H_2O solution

J. Detaining solution for SDS-PAGE:

50% methanol, 10% glacial acetic acid and 40% H_2O solution

K. Preparation of SM2-biobeads

Wash 2.5 gr of SM-2 Bio-Beads (Bio-Rad):

1. Once with about 100 mL of methanol
2. Once with 100 mL of ethanol,
3. Ten times with 100 volumes of ultrapure water

The clean beads stock can then be stored in ultrapure water at 4℃.

To weigh out the required amount of beads to be added to each batch of solution, some of beads mixed with water from the prepared beads stock was poured into a weighing dish. The dish was tilted, and a Pasteur pipette was used to remove extra water from the beads to be able to measure and pick the required grams of wet beads.

APPENDIX B

PROTOCOLS

A. Protein purification:

1. Pack column with an appropriate amount of Ni-NTA resin. Allow storage buffer to drain from resin by gravity flow.
2. Prepare sample by mixing protein extract with an equal volume of equilibrium buffer.
3. Equilibrate column with two resin- bed volumes of equilibrium buffer. Allow buffer to drain from the column.
4. Add the prepared protein extract to the resin. Collect the flow- through in a tube. If desired, re-apply the flow through once to maximize binding.
5. Wash resin with two resin- bed volumes of wash buffer and collect the flow-through. Repeat this step 2-3 more times.
6. Elute His- tagged proteins from the two resin-bed volumes of elution buffer. Repeat this step, twice and collecting each fraction in a separate tube.
7. Monitor protein elution by measuring the absorbance of the fractions at 280 nm.
8. Keep the fractions with appropriate labels in -20 C for future use.

   Protocol from: (Thermo Fisher Scientific)

B. Resin regeneration

   1. Wash resin with 10 resin bed volumes of MES buffer.
   2. Wash resin with 10 resin bed volumes of ultrapure water.
   3. Store resin in 20% ethanol.

C. Preparation of SDS- PAGE Gel :

   1. Prepare the apparatus for molding the gel and make sure it is not leaking.

2. For 10 mL of dissolving 10% gel prepared, by mixing: 4mL H2O, 3.3 mL 30% acrylamide mix, 2.5 mL 1.5 Tris (PH 8.8),0.1 mL10%SDS, 0.1 mL ammonium persulfate, 0.004 mL (TEMED – This should be added when it is the time to pour the gel because it helps to polymerize the gel quickly).
3. Pour the gel with specific pipette tip, should be fast and leave 1 cm below the comb.
4. Pour isopropanol immediately on dissolving gel. Wait for one hour.
5. Remove the isopropanol by pouring off all of it and using Kim wipe to remove the final drop.
6. 8 mL of stacking gel prepared by mixing 5.5 mL H2O, 1.3 mL 30% acrylamide mix, 1.0 mL 1.0 M Tris buffer (PH 6.8), 0.08 mL 10% SDS, 0.08 mL 10% ammonium persulfate, 0.008 mL TEMED.
7. Mix and pour the solution on the dissolving gel, immerse the comb into the stacking gel and pour the remaining gel to get rid of any bubbles. Wait for two hours until the gel is set. The Protocol from: (Sambrook & Russell, 2006)

D. Preparation of protein sample

1. Sample buffer was prepared by mixing 0.125 M of Tris HCl (PH 6.8), 4% SDS, 5% 2-ME, 20% glycerol, and 0.004% bromophenol blue.
2. In a micro tube mix 25μL protein fractions (from protein purification), 25 μL of sample buffer heat each for 3min at 85-100 ºC (Sambrook, J.; Russell, D. W., 2006).

E. Pouring protein samples and running the gel

1. Pour HCl buffer (PH 8.3) into the prepared mold until the comb becomes lubricant and easy to take it off (should be slowly do not break the wells).

2. Add 10 µl of protein ladder in the first well, and then add 15 µL of each of samples into each well. Connect to the power for stacking gel run on 50 Volt, 40 Amp, for 1 hour and 22 minutes, run the dissolving gel for 110 volt, 40 Amp, for 2 hours.
3. After running is done, take off the mold and soak it in water for removing the gel easily.
4. Immerse the gel in a deep dish with staining solution and leave it on a very slow shaker overnight.
5. Pour off the staining solution and put the gel in a de-staining solution for overnight with slow shaking.
6. Take the gel from the de-staining solution (ready for taking images) and store it in 20% glycerol then keep it in the fridge (Sambrook & Russell, 2006).

F. 2-mL Liposome preparation

1. 0.0048 mg of cholesterol was mixed with 1.5 mL PA and 1.5 mL PC, add them together (PA, PC were prepared previously dissolved in diethyl ether). The calculation for PA and PC volumes should be done in a way that the final concentration is 20 mg/mL.
2. Mix above solution by shaking only until everything is dissolved, then expose it to N_2 gas (pressure 0.5 bar) for about 5 min until all the liquids evaporate.
3. Add 2 mL of HEPES buffer and mix with (use only glass pipette) to eliminate any lumps.
4. Sonicate on iced water for 10 cycles each 10 sec and rest in between each cycle for 10 sec. (for total time cycles 1 min and 10 sec.)

during sonication small unilamellar vesicles (SUVs) are generated.

5. Using Avanti Polar lipids extruder for converting SUVs to LUVs. Load the solution from previous step into the extruder and run it for about 10- 20 times until the solution color becomes clear and sometimes turns to light blue at this point the (LUV)s are generated.

Pour the solutions into two vials 1mL in each, keep them in the fridge. (Geertsma, Nik Mahmood, Schuurman-Wolters, & Poolman, 2008)

G. BCA Protein Assay (BioVision)

1. Preparation of Bovine Serum Albumin (BSA) Standards:

As can be seen in the table below, BSA standards were prepared by diluting BSA standard stock using de-ionized water or same buffer added to the protein samples during purification (here PBS buffer was used). The diluted standard solutions can be stored at 4 °C for about one week.

Table B.1. Preparation of Bovine Serum Albumin (BSA) Standards

Tube	Volume of BSA (µl)	Volume of diluent (µl)	Final BSA Concentration (µg/mL)
1 (Stock)	300 of 2 mg/mL Stock	0	2000
2	300 of 2 mg/mL Stock	100	1500
3	300 of 2 mg/mL Stock	300	1000
4	300 of vial 3	300	500
5	300 of vial 4	300	250
6	300 of vial 5	300	125
7	100 of vial 6	400	25
8 (Blank)	0	400	0

2. Preparation of Protein Samples:

Three different concentrations of samples were prepared to perform the assay in triplicates to make sure that they are within the assay range (25-2000 µg/mL); in this study, samples were diluted using PBS by 0, 2X and 4X dilution factors as presented in the following table.

Table B.2. Preparation of Protein Samples

Tube	Volume of protein sample (µL)	Volume of PBS (µL)
1	25	0
2	25	25
3	25 of tube 2	25

3. Preparation of BCA working reagent:

Since each sample replicate needs 200 µl of BCA working reagent for microplate assay, sufficient amount of BCA working reagent solution should be prepared by mixing BCA reagent A with BCA reagent B in the ratio of 50:1; after mixing the reagent solution appears to be clear and green.

4. Assay Format:

BCA Assay can be performed in a microtiter plate format or test tube format; in this work, microplate procedure was employed.

5. Microplate Procedure:

i. Add 25 µl of each BSA standard and protein samples into 96-well microplate. Here, three rows of the microplate were used for standards (triplicates) to get a nice calibration curve.

ii. Using microchannel pipette, add 200 µl of BCA working reagent to the standard & sample wells, mix thoroughly for 30 seconds.

iii. Cover the plate with adhesive film and incubate at 37 °C for 30 minutes. After incubation, let the plate cool down at room temperature.

iv. Set the absorption wavelength of a microplate reader to 562 nm (OD_{562}) and read all standards and samples.

6. Calculations:

i. Subtract $OD_{562\ of}$ blank (#8) from all readings. For BSA standard curve, plot OD_{562} (Y-axis) vs standard BSA concentration (X-axis). Obtain the equation from the plot Y = aX + b to get the value of slope (a) and intercept (b).

ii. Use the following equation to calculate the protein concentration in sample.

Protein concentration in sample (µg/mL): C: DX: $Dilution\ factor \times \frac{Y-b}{a}$

Where

Y = OD_{562} of protein sample

X= concentration of protein sample

a = Slope of the BSA Standard curve

b = Y-intercept of the Standard Curve

D = Dilution factor of protein sample

Raw data/OD reading Obtained from Microplate Reading

Table B.3. Optical Density (OD_{562}) Data for Triplicates of BSA Standards

1	2	3	4	5	6	7	8 (Blank)
2.444	1.896	1.41	0.78	0.469	0.296	0.145	0.111
2.537	1.962	1.475	0.782	0.469	0.29	0.146	0.114
2.502	1.968	1.333	0.829	0.501	0.314	0.153	0.115

Table B.4. Average Optical Density (OD_{562}) and Concentration Data for BSA Standards

BSA Standards	1	2	3	4	5	6	7	8 (Blank)
Concentration μg/mL	2000	1500	1000	500	250	125	25	0
Average OD_{562}	2.49	1.94	1.41	0.80	0.48	0.30	0.15	0.11

Table B.5. Optical Density (OD_{562}) Data of Four Purified Protein Samples

Dilution Factor	Optical Density (OD_{562})			
	Sample #1	Sample #2	Sample #3	Sample #4
0X	0.911	0.974	1.055	1.012
2X	0.792	0.864	0.815	0.895
4X	0.508	0.561	0.536	0.617

Table B.6. Calculated Total Protein Concentration (μg/mL) of Four Purified Protein Samples

Total Protein Concentration (μg/mL)				
Dilution Factor	Sample #1	Sample #2	Sample #3	Sample #4
0X	629.08	681.58	749.08	713.25
2X	1059.83	1179.83	1098.17	1231.50
4X	1173.00	1349.67	1266.33	1536.33
Average (μg/mL)				
953.97		1070.36	1037.86	1160.36

APPENDIX C

SAMPLE PREPARATION FOR CRYO-EM AND RAW DATA OBTAINED FROM ICP-MS

A. Cleaning of the carbon grid with plasma

Prior to sample vitrification, carbon grids and glass slides must be cleaned with plasma, using a plasma cleaner. Here, PELCO easiGlow™ Cryo-EM glow discharge set was used (Figure C.1); this cleaning system is a glow discharge treatment with air that will clean carbon grids while making the film surface negatively charged or hydrophilic that lets aqueous solution to spread easily (TED PELLA).

Figure C.1. PELCO easiGlow™ Cryo-EM glow discharge set

B. Vitrification of the sample using the Vitrobot (FEI)

The specimen needs to be rapidly frozen by plunge freezing into liquefied ethane at -179 ºC so that freezing occurs faster than the reorganization of water molecules (crystallization). To do this, samples were prepared by applying 3 µl of sample onto a 400-mesh carbon copper mesh grid coated with a regular array of 1.2-micron holes spaced 1.3 microns apart. The liquid was blotted with a filter paper quickly to form a thin vicinal film containing liposomes and plunged rapidly into the nitrogen cooled ethane (Figure C.2). The grid with the vitrified sample was transferred into a tube filled with liquid nitrogen and the tube was stored in a Dewar with liquid nitrogen until TEM observation.

Figure C.2. Vitrification of the sample by plunge freezing into liquefied ethane using the Vitrobot (FEI)

C. Taking images using Cryo-TEM microscope

Cryogenically prepared samples were examined at 300 KeV at the indicated magnifications on the FEI Titan Krios G2 (Figure C.3). Images were collected on either a 4K x 4K Hybrid CMOS camera at a dose rate of 25-50 electron per angstrom squared or on a direct electron detector in super-resolution movie mode at a dose of 2 electron per angstrom squared second. Each DED movie frame was 0.2 seconds and a total of 8 seconds were used per exposure. Movie files were motion corrected and sum images obtained.

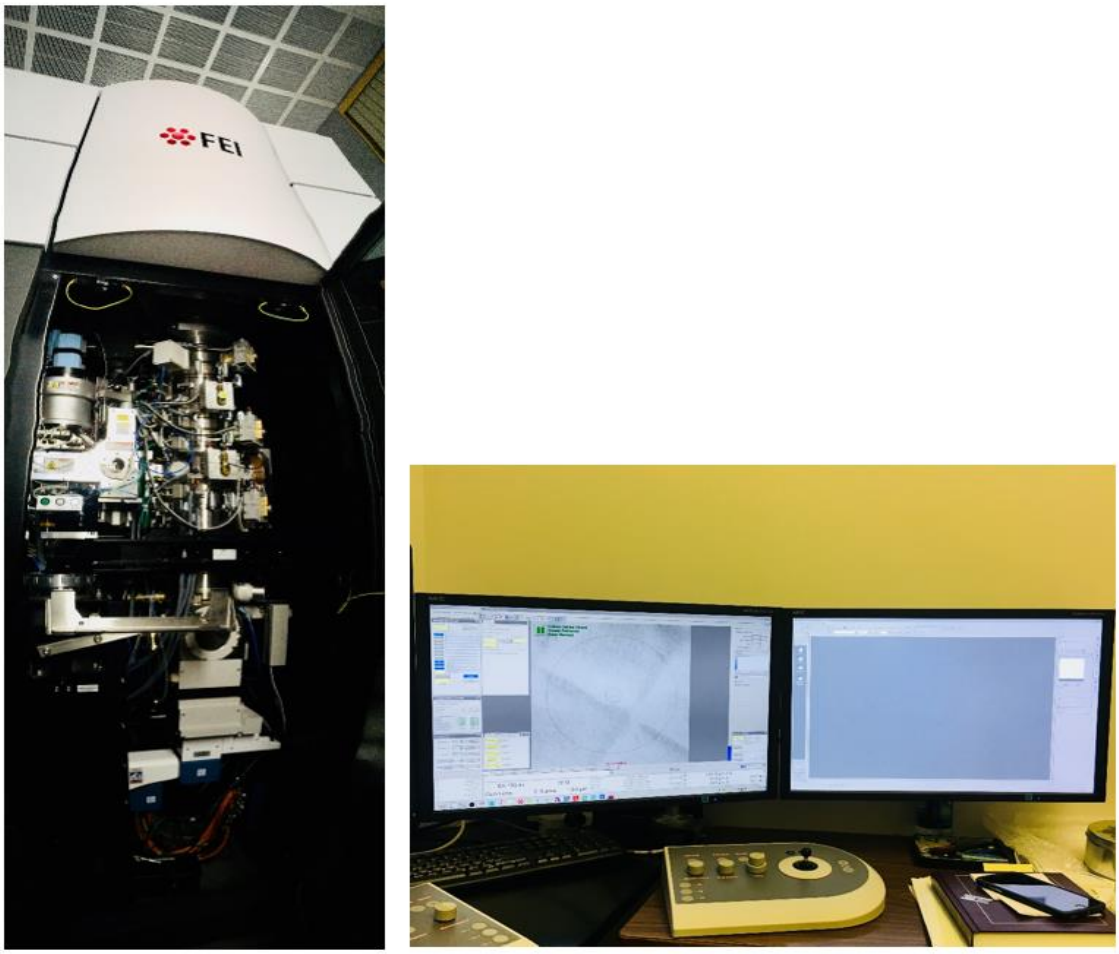

Figure C.3. Imaging and Processing using Titan Krios (Field Electron and Ion Company, FEI) cryogenic electron microscope

D. Calibration curve and raw data obtained from ICP-MS

Table C.4. Expected and Measured Cs Concentration Values of CsCl Standards

Standards	Expected Cs Concentration (ppb)	Cs Concentration (ppb) Measured by ICP-MS
Standard 1	0.0186	0.022
Standard 2	0.185	0.2
Standard 3	0.981	0.966
Standard 4	9.73	9.61
Standard 5	48.7	48.72

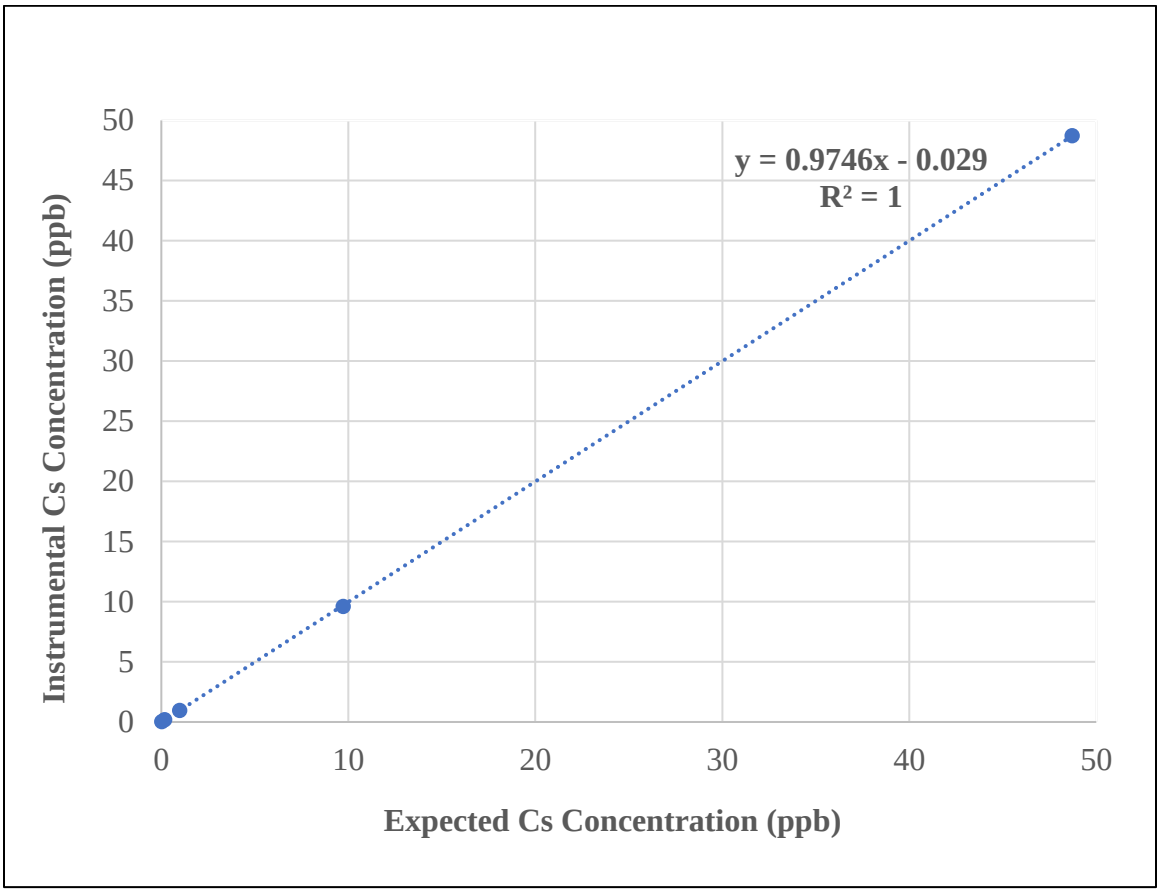

Figure C.4. Calibration Curve for CsCl Standards

Orig Conc.	45 Sc	+/-	77Ar Cl	+/-	115In-H2	+/-	133 Cs	+/-	209 Bi	+/-
	ppb		ppb		ppb		ppb		ppb	
Rinse	1.000	0.004	0.003	0.002	1.000	0.006	0.000	0.000	1.000	0.010
Blank	1.000	0.010	0.000	0.002	1.000	0.010	0.000	0.000	1.000	0.011
0.0186 ppb Cs	1.038	0.029	0.000	0.004	1.006	0.013	0.022	0.002	0.969	0.008
0.185 ppb Cs	1.085	0.016	0.002	0.004	1.036	0.002	0.200	0.002	0.985	0.004
0.981 ppb Cs	1.085	0.007	0.000	0.002	1.068	0.006	0.966	0.012	1.042	0.012
9.73 ppb Cs	1.091	0.019	0.001	0.004	1.049	0.007	9.610	0.134	0.996	0.008
48.7 ppb Cs	1.074	0.003	0.002	0.002	1.032	0.004	48.720	0.702	0.976	0.005

Table C. 5. Summary of Concentration View for a few Elements including Cs

Note: As can be seen, only Cs had concentration close to CsCl standards. The other elements showed different concentration. That means, Cs concentration measured by ICP-MS were definitely for Cs not any other interfering elements that might exist in the solution.

Table C.6. Concentration of Cesium (Cs) in Water Samples Contaminated with about 100 ppb of Cs Before and After Treatment

Cesium Concentration (μg/L, ppb)						
Sample	Volume of Proteoliposome added to Contaminated Water					
	0.1 mL	0.5 mL	1 mL	2 mL	4 mL	8 mL
Sample-BT	91.81	85.57	92.62	95.27	121.60	99.37
Duplicate #1- AT	91.51	84.95	91.58	92.63	115.90	87.21
Duplicate #2-AT	91.58	85.04	91.51	92.25	114.17	86.23

Note: BT: before treatment, AT: After treatment. As can be seen the Cs concentration in untreated sample is not exactly 100 ppb, that can be explained due to errors associated with analytical chemistry during sample preparation

Table C.7. Concentration of Cesium (Cs) in Water Samples Contaminated with about 10 ppb of Cs Before and After Treatment

Cesium Concentration (μg/L, ppb)				
Sample	Volume of Proteoliposome added to Contaminated Water			
	0.1 mL	0.5 mL	1 mL	2 mL
Sample-BT	8.09	11.32	11.09	11.09
Duplicate #1- AT	8.04	11.22	10.91	10.69
Duplicate #2-AT	8.03	11.24	10.95	10.73

Note: BT: before treatment, AT: After treatment. As can be seen the Cs concentration in untreated sample is not exactly 10 ppb, that can be explained due to errors associated with analytical chemistry during sample preparation.

Table C.8. Concentration of Cs in CsCl and Empty Liposome Solutions before and after Centrifugation.

Samples	Cs Concentration before Centrifugation (ppb)	Cs Concentration After Centrifugation (ppb)
CsCl Only	103.4582	103.4571
Liposome Only	112.4671	112.4317
Calculated Percentage Removal		
CsCl Only	0.0010%	
Liposome Only	0.0314%	

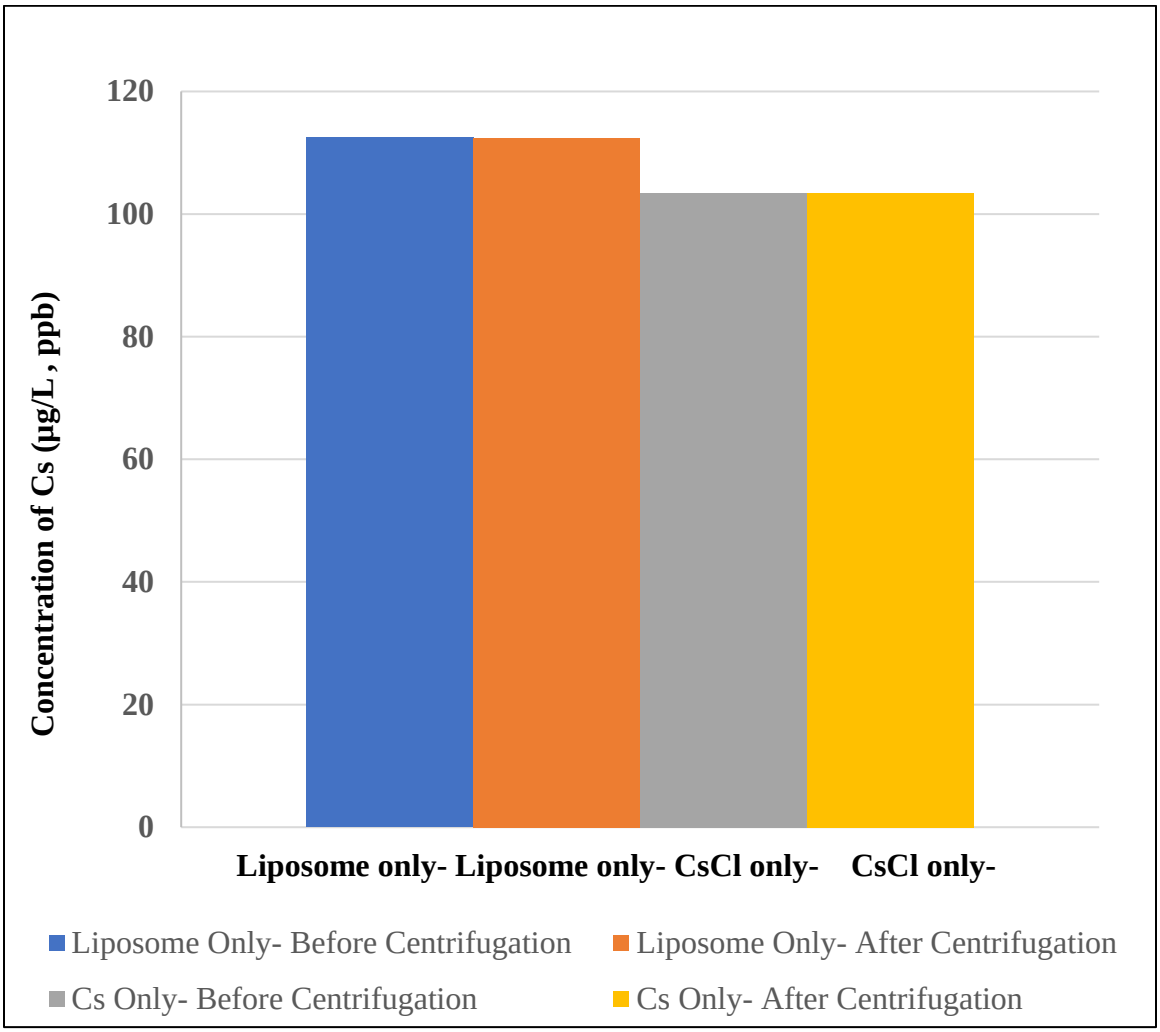

Figure C.5. Effect of Centrifugation and Liposomes on Cs Concentration

E. Langmuir isotherm model

The equilibrium adsorption capacity (q_e) per gram dry mass of the protein transporter (adsorbent) was calculated using Equation C.1 (Meroufel, Benali, Benyahia, Benmoussa, & Zenasni, 2013) and concentration data in Tables C.6 and C.7.

$q_e = ((C_0 - Ce)\ V_T)/m$ Equation C.1.

Where

q_e= mass of Cs adsorbed on adsorbent/ mass of adsorbent (protein) (mg/g)

C_0: Initial concentration of Cs in solution before treatment (mg/L)

C_e: Concentration of Cs after sorption/treatment (mg/L)

V_T: Total volume of solution (L); when testing 0.1-4 mL of proteoliposome V_T was 5 mL and when testing 8 mL of proteoliposome, V_T was 12.5 mL.

M: Mass of adsorbent /protein transporter (g)

Table C. 9. Mass of Cs Captured by Proteoliposome when Water is Spiked with ~100 ppb of Cs.

Mass of Cs Captured by Proteoliposome (μg)						
Sample	Volume of Proteoliposome added to Contaminated Water					
	0.1 mL	0.5 mL	1 mL	2 mL	4 mL	8 mL
Duplicate #1- AT	0.001516	0.003062	0.005167	0.013195	0.028525	0.152045
Duplicate #2-AT	0.001186	0.002639	0.005501	0.015105	0.037133	0.164329
Average	0.001351	0.002851	0.005334	0.014150	0.032829	0.158187

Note: AT: After treatment.

Table C. 10. Mass of Cs Captured by Proteoliposome when Water is Spiked with ~10 ppb of Cs.

Mass of Cs Captured by Proteoliposome (μg)				
Sample	Volume of Proteoliposome added to Contaminated Water			
	0.1 mL	0.5 mL	1 mL	2 mL
Duplicate #1- AT	0.000238	0.000527	0.000945	0.002004
Duplicate #2-AT	0.000284	0.000433	0.000717	0.001805
Average	0.000261	0.000480	0.000831	0.001905

Note: AT: After treatment.

Approximate mass of protein which was added to liposome solution, during reconstitution process, has been presented in Table C. 11.

Table C. 11. Estimated Mass of Protein Reconstituted into the Liposome Vesicles.

Mass of Protein (µg)	Volume of Proteoliposome Solution (mL)
12	0.1
60	0.5
120	1
240	2
480	4
960	8

Table C. 12. Calculated q_e and Ce when Water is Spiked with ~100 ppb of Cs

Ce (µg /L, ppb)	Ce (mg/L, ppm)	q_e (mg/g)
91.54	0.0915	0.112600
85.00	0.0850	0.047510
91.55	0.0915	0.044446
92.44	0.0924	0.058959
115.03	0.1150	0.068394
86.72	0.0867	0.164778

Note: Ce values are the average of Cs concentrations for both duplicates after Treatment.

Table C. 13. Calculated q_e and Ce when Water is Spiked with ~10 ppb of Cs

Ce (µg /L, ppb)	Ce (mg/L, ppm)	q_e (mg/g)
8.03	0.0080	0.021748703
11.23	0.0112	0.007995884
10.93	0.0109	0.006921329
10.71	0.0107	0.007935457

Note: Ce values are the average of Cs concentrations for both duplicates after Treatment