Titre: Title:	Studying the Characteristics of Liposomes Composed of DMPC by Lipid Film Hydration Technique to be Manipulated as Carriers for Magnetotactic Bacteria
Auteur: Author:	Hussein Salama
Date:	2015
Type:	Mémoire ou thèse / Dissertation or Thesis
Référence: Citation:	Salama, H. (2015). Studying the Characteristics of Liposomes Composed of DMPC by Lipid Film Hydration Technique to be Manipulated as Carriers for Magnetotactic Bacteria [Master's thesis, École Polytechnique de Montréal]. PolyPublie. https://publications.polymtl.ca/1711/

UNIVERSITÉ DE MONTRÉAL

STUDYING THE CHARACTERISTICS OF LIPOSOMES COMPOSED OF DMPC BY LIPID FILM HYDRATION TECHNIQUE TO BE MANIPULATED AS CARRIERS FOR MAGNETOTACTIC BACTERIA

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MÉMOIRE PRÉSENTÉ EN VUE DE L'OBTENTION
DU DIPLÔME DE MAÎTRISE ÈS SCIENCES APPLIQUÉES
(GÉNIE BIOMÉDICAL)
MARS 2015

UNIVERSITÉ DE MONTRÉAL

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

Ce mémoire intitulé :

STUDYING THE CHARACTERISTICS OF LIPOSOMES COMPOSED OF DMPC BY LIPID FILM HYDRATION TECHNIQUE TO BE MANIPULATED AS CARRIERS FOR MAGNETOTACTIC BACTERIA

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DEDICATION

To my family especially my mother and my new baby, Ahmed.

ACKNOWLEDGEMENTS

I would like to express my great appreciation to my supervisor Dr. Sylvain Martel and my co-supervisor Dr. Mahmood Mohammadi who gave me the opportunity to pursue my master's degree in Canada under their supervision. Their support, advice, motivation, and guidance were very valuable to me. Without them I would not have been able to successfully publish this work.

I am grateful for the technical assistance given to me by Charles C. Tremblay and the scientific assistance provided to me by Samira Taherkhani during the research period. I would like to offer special thanks to my colleagues Nina Olamaei, Ouajdi Felfoul, Nasr Tabatabaei, Alexandre Bigot, Benjamin Conan, Dominic de Lanauze, Azadeh Sharafi, Guillermo Vidal, and Viviane Lalande who joined me for the best moments in the laboratory.

I am most grateful for my parents, brothers, and sisters who supported me in completing to get this scientific degree. I wish to acknowledge my wife for her support and patience throughout this time. Also thanks are sent to my friends and relatives in Canada and Libya who were in contact with me, providing me with support and motivation.

RÉSUMÉ

Les bactéries magnétotactiques (BMT) peuvent être utilisées pour des applications biomédicales telles que la délivrance de médicaments. Les BMT sont encapsulées dans des transporteurs comme les liposomes, afin d'être capable de contrôler la navigation des BMT à travers les vaisseaux sanguins et de protéger les cellules normales des effets nuisibles des médicaments anticancéreux attachés aux BMT.

Dans notre étude, nous avons étudiés les caractéristiques des liposomes composés de DMPC par la technique d'hydratation de couche lipidique afin d'évaluer leurs disponibilité à être manipulé en tant que transporteur de BMT. Les résultats ont montré que la technique d'hydratation des couches de lipides présente une importante reproductibilité des liposomes. Environ 1,500,000 liposomes ayant un diamètre de 8 à 23 μm pour chaque ml de solution de liposome ont été préparés par cette technique. Les liposomes préparés ont encapsulés 14,1% de la quantité ciblée de BMT.

Les filtres de polycarbonate ont séparé 90,63% de BMT non encapsulées qui sont restées dans l'échantillon à la fin du processus d'encapsulation. En outre, les filtres de polycarbonate n'ont pas montré d'effets négatifs reconnaissables sur l'intégrité des liposomes, du fait que 79.6% des liposomes qui ont subi le procédé de séparation par des filtres de polycarbonate pour l'isolement de BMT non encapsulées, ont été maintenus intacts après que la séparation des BMT non encapsulées a eu lieu.

La faible fréquence des ultrasons, $3W/cm^3$, pendant 3 minutes a libéré 95% des liposomes composés de DMPC. Selon nos résultats, la température corporel et le pH du corps n'ont pas pu causer la libération de liposomes composés de DMPC pendant 30 minutes d'exposition.

Nous avons conclu le fait que la technique d'hydratation des couches de lipides est fortement reproductible. Cette technique est capable de produire des liposomes ayant un grand diamètre pouvant piéger une quantité suffisante de particules ayant un diamètre de l'ordre des micromètres. De plus, les filtres de polycarbonate sont capables de séparer les BMT non encapsulés des échantillons de liposomes sans affecter l'intégrité des liposomes. La faible fréquence de l'ultrason a permis d'obtenir un fort pourcentage de liposomes alors que la

température corporel et le pH n'ont pas été capable de causer la libération de liposomes composés de DMPC pendant les 30 premières minutes d'injection dans le corps.

ABSTRACT

Magnetotactic bacteria (MTB) could be used in biomedical applications such as drug delivery. To be able to control the navigation of MTB through blood vessels and protect the normal cells from the harmful effects of anticancer medications attached to MTB, we have to encapsulate MTB inside carriers such as liposomes.

In our study, we studied the characteristics of liposomes composed of DMPC by lipid film hydration technique to evaluate their availability to be manipulated as MTB carriers. The results showed that the lipid film hydration technique manifests high liposomes reproducibility. Around 1,500,000 liposomes with diameters between 8-23 μm in every 1ml of liposomes solution are prepared using this technique. The prepared liposomes encapsulated 14.1% of the targeted quantity of MTB to be entrapped inside liposomes.

The polycarbonate filters segregated 90.63% of non-encapsulated MTB that remained in the sample after the encapsulation process is achieved. In addition, the polycarbonate filters did not show recognizable negative effects on the liposomes' integrity since 79.6% of the liposomes underwent the separation process by polycarbonate filters for the isolation of non-encapsulated MTB are kept intact after the separation of non-encapsulated MTB had taken place.

The low frequency ultrasound $3W/cm^3$ for 3 minutes released 95% of the liposomes composed of DMPC. According to our results, the both body temperature and body pH could not cause the release of liposomes composed of DMPC during 30 minutes of exposure.

We concluded that the lipid film hydration technique is high reproducible technique. This technique is able to produce liposomes with big diameters that could entrap sufficient amount of particles in micrometers in their diameters. In addition, the polycarbonate filters are able to separate the non-encapsulated MTB from liposomes sample without affecting the integrity of liposomes. The low frequency ultrasound releases a high percentage of liposomes while both body temperature and body pH are not able to release liposomes during the first 30 minutes of their injection into the human body.

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AC	Alternating current
DLPC	1,2-dilauroyl-*sn*-glycero-3-phosphocholine
DMPC	1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine
DMPE	1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine
DNA	Deoxyribonucleic acid
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPE	1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
GUV	Giant unilamellar vesicles
ITO	Indium tin oxide
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
MRI	Magnetic resonance imaging
MTB	Magnetotactic bacteria
PBS	Phosphate buffer saline
PE	Phosphatidylethanolamine
Rpm	Round per minute
SUV	Small unilamellar vesicles
Tc	Transition temperature

INTRODUCTION

According to Canadian statistics, cancer is the first cause of death in Canada. Cancer is responsible for 29% of the total number of deaths in Canada [1]. In 2013 approximately 39400 men and 36100 women died in Canada from different types of cancer [2].

Conventional ways for administrating chemotherapies lack specificity because chemotherapeutic agents kill both normal and cancerous cells that frequently lead to toxicity and complications that could be lethal [3].

Professor Sylvain Martel invented a new technique for the targeted administration of therapeutic agents based on the exploitation of a specific kind of bacteria called magnetotactic bacteria (MTB) strain MC-1 to be used as a carrier for chemotherapeutic agents [4].

MTB MC-1 is a specific strain of MTB that are characterized by their spherical shape. Each MTB MC-1 bacterium measures 2 μm in diameter and has two flagella bundles providing a thrust force exceeding 4 picoNewtons (pN). These flagella allow the MTB to swim in water at room temperature at speeds exceeding 200 μm/s [5].

Each MTB has a chain of nanoparticles called magnetosomes. These magnetosomes can be manipulated for controlling swimming speeds and direction of MTB by applying magnetic fields to them. In addition, magnetosomes could also be exploited to track the MTB inside human blood vessels because magnetosomes cause a local distortion of the magnetic field inside the bore of a clinical MRI system [4].

Anticancer medications can be attached to the MTB by antibodies. Therefore, we can use the MTB as a carrier to transport anticancer medications to tumor lesions [4]. To be able to control the navigation of the MTB through the blood vessels and protect normal cells from the harmful effects of anticancer medications attached to the MTB, we have to encapsulate the MTB inside carriers like liposomes.

To be able to manipulate liposomes to encapsulate MTB attached to anticancer medications, we have to study the characteristics of liposomes aimed to be manipulated for the targeted delivery.

In the first chapter, we explain the cancer and medications available for it, the MTB and how can be used as nanorobots inside the human body, and liposomes and their role as medications carriers. We explain the methods that we manipulate to encapsulate the MTB in liposomes, to separate non-encapsulated MTB from liposomes sample, and to release liposomes composed of DMPC in the second chapter. We present our results in the third chapter and discuss them in the fourth chapter.

The general objective of this work:

The general objective of this research is to study the characteristics of liposomes composed of DMPC by lipid film hydration technique. We will evaluate the reproducibility of the lipid film hydration technique and assess the size of produced liposomes. We aim to evaluate the encapsulation efficacy for liposomes composed of DMPC produced by the lipid film hydration method for bacteria in micrometer in their sizes. In addition, the release efficacy of release techniques on liposomes composed of DMPC will be studied as well.

CHAPTER 1 LITERATURE REVIEW

1.1 Cancer

Cancer is a disease in which abnormal cells grow uncontrollably due to the loss of control of cell division by normal mechanisms of cell division. In this disease, normal cells convert to cancerous cells that divide without control resulting in solid masses called tumours [6]. There are many types of cancer influences on the most organs in the human body [7].

Tumours are classified according to their degree of aggressive growth to benign and malignant tumours. Benign tumours usually exist in certain tissues without invading the adjacent tissues while malignant tumours have the ability to invade other tissues in the body through a process called metastasis [8].

Metastasis is a multistep process during which cancerous cells spread from the initial tumour to distant tissues. This complex process starts by the separation of cancerous cells from the initial tumour and the invasion of nearby tissues until the cancerous cells reach the blood or lymphatic circulations. By immigration through the blood and lymphatic circulations, cancerous cells reach distant tissues in the body where they stop and colonize these tissues. When cancerous cells invade new tissues, they proliferate and induce a process called angiogenesis [9].

Angiogenesis is the process that involves the formation new blood vessels from the normal existing blood vessels by the activation of the migration of endothelial cells of pre-existing blood vessels to form new blood vessels. The angiogenesis process is essential for the growth of tumours because it enhances the growth and spreading of tumours by providing cancerous tissues with oxygen and nutrients and removing waste products [10, 11].

According to the International Agency for Research on Cancer (IARC), the estimated number of deaths related to cancer in the world in 2012 was around 8.2 million accompanied with 14.1 million new cancer cases. These numbers are higher than the number estimated in 2008 with 12.7 million new cases and 7.6 million death cases. Lung cancer, breast cancer, and colorectum are the most commonly diagnosed cancers worldwide making up 13.0%, 11.9%, and 9.7% respectively of the total percentage of cancer cases while lung, liver, and stomach cancer are the most common causes of death from cancer making up 19.4%, 9.1%, and 8.8% of the total

number of deaths respectively worldwide. Estimations predict that there will be 19.3 million new cancer cases per year by 2025 because of increasing number of people and ageing populations in the world. According to the IARC, in 2012 around 64.9% of the total cancer cases and 56.8% of the total number of cancer related deaths took place in less developed countries. Incidences of cancer increased in all countries with more cases estimated in the more developed countries, but death cases are higher in less developed countries because they lack the ability to detect cancer early and they also have less treatments available to them [7].

There are many risk factors that increase the probability of incidences of cancer. In general, any type of cancer is associated with risk factors that promote the development of the specific type of cancer. For instance, smoking and inhalation of tobacco smoke, the consumption of saturated fats, red meat, dairy products and alcohol enhance the development of lung cancer [12]. The risk of devolving breast cancers decreases in women with histories of breast-feeding while it increases in overweight women, women who consume alcohol and women who smoke [13]. Oral contraceptives, elevated iron storage in the body, obesity, tobacco smoking, and alcohol consumption are the most common risk factors of liver cancer [14].

There are a lot of symptoms that are associated with cancer diagnosed people, such as fatigue, pain, lack of energy, weakness, loss of appetite, weight loss, dry mouth, anxiety, early satiety, sore mouth, insomnia, depressed mood, taste changes, confusion, dysphagia, nausea and vomiting, constipation, bleeding, irritability, and diarrhea [15].

1.1.1 Radiation therapy

Around 50% of cancer patients are treated with radiotherapy with the aim of preventing cancerous cells from multiplying by inhibiting the cell division of cancerous cells. This type of treatment is either used alone or with other cancer medications such as chemotherapy or surgery depending on the medical purpose it is used for. For instance, radiotherapy is utilized before surgery to shrink the tumour size and it is used after surgery to destroy microscopic cancerous cells that are not removed by surgery [16, 17]. Radiation is a physical treatment that depends on using x-rays and gamma rays to kill cancerous cells. The radiation used in radiotherapy is called ionizing radiation because it composes ions. When radiation passes through cancerous tissues, it deposits energy in the cancerous cells of the tissues and the deposited energy kills cancerous

cells or causes genetic modifications in the cancerous cells leading to their death [17]. Generally, radiation is directed towards cancerous cells using two approaches. One of the procedures of delivery is called external beam radiation, which is based on directing high-energy rays such as photons, protons or particle radiation from outside the body to the place of cancerous cells inside the body. Another procedure called internal radiation is when radiation is delivered from inside the body by radioactive sources sealed in catheters [17]. The main drawback of using radiotherapy to treat cancers is the exposure of the normal cells to radiation during the exposing of cancerous tissues to radiation therapy. The symptoms of destroying normal cells by radiotherapy will appear during the period of treatment or later. For instance, symptoms such as fibrosis appear six weeks after irradiation of a lung. These symptoms appear as a result of cell death in irradiated tissue [17, 18]. Acute damage due to radiation also occurs in tissues that are characterized by rapid proliferating cells such as in the epithelial surfaces of the skin or the alimentary tract [18]. In addition, radiotherapy does not kill cancer cells right away because radiation takes time to kill cancerous cells. It takes hours, days or weeks of treatment before cancerous cells start to die [17]. People treated with radiotherapy are suspected to develop erythema in the skin and experience an elevation in intracranial pressure in the central nervous system [18]. Determining the dose of radiation needed is also another problem related with utilizing radiation because there are only a few studies that have been developed to detect the maximum tolerated dose of radiation at any specific place in the body. Some damage in some tissues might be acceptable especially when the benefits earned are greater than the damage caused, while the damage is not allowed to happen especially in vital organs such as the central nervous system [18]. Using this type of treatment for cancer patients achieves different degrees of efficacy. For example, the probability of staying alive after radiotherapy for a patient with some cancerous cells such as an early stage of larynx cancer and a non-small-cell lung cancer is high whereas for some cancerous cells such as sarcomas and advanced non-small-cell lung cancer the likelihood of liveability is low. In addition, many patients will experience recurring disease after radiotherapy [16]. Some latest effects due to exposure to radiation appear many years after treatment, such as fibrosis, necrosis, atrophy, and vascular damage [18].

1.1.2 Surgery

Surgery is one of the most commonly used techniques to eradicate solid tumours, but many complications are associated with using this technique for cancer treatment, such as pain, tissue damage, and inflammation. Moreover, recurrence of cancer and metastasis happens sometimes after the removal the initial cancer by surgery [19]. In addition to the general complications related with cancer surgery, many complications are related to surgical eradication of specific types of cancers. For example, there are many drawbacks associated with breast cancer surgery, such as wound infections, seromas, and hematomas that happen in around 30% of patients. Theses complications require prolonged hospitalization of patients. Mondor's disease, thrombosis of the thoracoepigastric vein, pneumothorax, and brachial plexopathy occur in some patients who undergo surgical breast procedures [20]. Many medical problems arise as a result of the surgical treatment of pancreatic cancer, such as cardiac problems, cerebrovascular accidents, respiratory distress, renal dysfunction, pneumonia, pulmonary embolism, hepatic and metabolic dysfunction [21].

1.1.3 Chemotherapy

A lot of medications are utilized as chemotherapies, such as antimetabolites, alkylating agents, antibiotics, plant alkaloids, hormones, and biologic response modifiers. This type of cancer treatment is based on using therapeutic agents that target the killing of rapidly dividing cells through different mechanisms of action. For instance, antimetabolites overlap with the production of the nucleic acids of rapidly dividing cancerous cells by different mechanisms. One of these mechanisms is the prevention production of the deoxyribonucleoside triphosphates, which are precursors for DNA synthesis [22]. Since chemotherapies kill rapidly dividing cells, these medications do not differentiate between normal and cancerous rapidly dividing cells. Therefore, using these medications for killing cancerous tissues is usually associated with toxicity [3]. Nausea and vomiting are the most common side effects of anticancer medications that could lead to other complications, such as dehydration and electrolytes imbalance that require hospitalization for a period of time. Many patients refuse to continue their treatment courses because of these complications [23]. Alopecia (losing hair) is a common side effect of chemotherapies that has negative effects on the psychology of patients. Many patients avoid participation in social activities and going to work because of their appearance [24]. Hepatic

toxicity is one of the dangerous drawbacks of chemotherapeutic agents. These agents could cause mild toxicity via the elevation of liver enzymes or severe toxicity via composing fibrosis or cirrhosis [25]. Cardiotoxicity happen as a result of using some chemotherapeutic agents such as doxorubicin and daunorubicin. The negative effects of using these medications include sinus tachycardia, premature ventricular and atrial contractions [25]. In addition to previously mentioned side effects of chemotherapies, there are many neurological side effects as a result of using these medications such as confusion, disorientation, cerebellar ataxia, cranial nerve palsy, and autonomic neuropathy. Moreover, chemotherapeutic agents cause genitourinary toxicity, pulmonary toxicity, chronic skin changes, mucositis, and other complications [25].

1.2 Magnetotactic bacteria (MTB)

MTB are gram-negative motile bacteria that are able to migrate along geomagnetic field lines because they have small intracellular mineral organelles bounded to their membranes called magnetosomes [26].

Magnetosomes are nanometre-sized particles called magnetite of iron oxide (Fe_3O_4) or greigite of iron sulfide (Fe_3S_4) arranged in 1, 2 or more chains. These mineral particles are surrounded by a bilayer membrane of phospholipids. The magnetosomes that are in the middle of a chain are bigger than the magnetosomes that are present at the end of a chain since the magnetosomes that are at the end of a chain are the newly synthesized ones. These magnetosomes have many shapes. For example, some of them have a bullet shape while others take on a cubooctahedral shape and some of them are rectangular [26, 27].

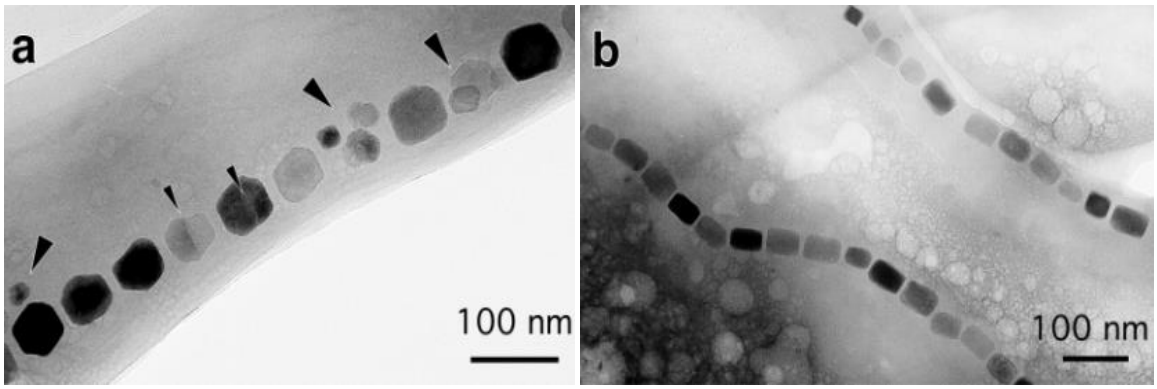


Figure 1-1: Images of shapes and arrangements of magnetosomes [28].

Magnetic interactions between the magnetosomes in a chain create their magnetic dipole moments. Therefore, the total magnetic dipole moment of any cell is the sum of the overall dipole moments of the magnetosomes. This magnetic dipole moment causes the cell to arrange itself along geomagnetic field lines while it swims in a phenomenon called magnetotaxis [28].

The direction of movement of MTB is controlled by chemotaxis, aerotaxis, and magnetotaxis, but when the MTB are exposed to a significant magnetic field the magnetotaxis overcomes the influence of chemotaxis and aerotaxis and subsequently the MTB are fully controlled by the effect of the magnetotaxis [4].

MTB exist in many morphological shapes, such as bacillus, vibrios, spirilla, and cocci. Some MTB strains have the ability to live in fresh water whereas other MTB live in marine water. Specific species of MTB produce iron oxide while others produce iron sulfide and some species produce both iron oxide and iron sulphide. Iron oxide-producing MTB exist just in the fresh water while MTB producing both iron oxide and iron sulphide are found in marine water and lakes [27].

MTB are classified according to their response to magnetic fields. Axial MTB have the ability to migrate to both magnetic poles with continuous switching in their migration direction along magnetic field lines. Contrarily, polar MTB migrate in the direction of one pole. For instance, polar MTB that migrate in direction of the North Pole exist in the Northern Hemisphere and are called north-seeking MTB while polar MTB that migrate in the direction of the South Pole are exist in the Southern Hemisphere and are called south-seeking MTB [29].

Although there are a lot of strains of MTB in marine and fresh water, there are specific strains that are isolated in pure cultures, such as Magnetospirillumgryphiswaldense MSR-1, Magnetospirillummagneticum AMB-1, Magnetospirillummagneticum MGT-1, Magnetovibrio MV-1, Magnetococcus sp. MC-1, Marine Magnetic spirillum QH-2, Magne- tospirillumsp.WM 1 and Magnetospirillummagnetotacticum MS-1 [27].

1.2.1 Choosing a strain of MTB to be manipulated as a medication carrier

As we mentioned earlier, few strains of MTB can be isolated and cultured. The fundamental criteria for selecting a species of MTB to be applied as microrobots inside the human body are

the swimming speed of the MTB, the size of the MTB, and the ability to control the movement of the MTB [4].

Strains of MTB have different swimming speeds. For instance, MTB MV-4 have a swimming speed around 30-80 μm s^{-1}. Magnetotactic spirilla have a swimming speed of less than 100 μm s^{-1} while Magnetococcus sp. MC-1 have swimming speeds around 200-300 μm s^{-1}. The MV-4 strain of MTB is the smallest in size. Each MV-4 bacterium is 0.5 μm in length while the MC-1 strain is bigger in size since MTB MC-1 are 2 μm in size. Both MV-4 and MC-1 are polar MTB because both of them only have 2 flagellum on 1 side of the MTB. This feature makes them swim in only 1 direction through the magnetic field. Therefore, their movement is easier to control than the axial MTB who have flagella on both sides of the cells such as the Magnetospirillum gryphiswaldense MTB. The swimming direction of the axial MTB is unpredictable since this strain of MTB migrates in the both directions of the magnetic field with approximately the same number of MTB migrating in each direction. From the previous data, we can conclude that MC-1 MTB is the best strain of MTB to be exploited as microrobots for transport medications inside the human body [4, 29, 30].

1.2.2 MTB MC-1

MC-1 MTB is a specific stain of MTB which has a spherical shape with a diameter of around 2 μm. Each bacterium has 2 flagella on 1 side of the cell providing it with a thrust force of around 4 pN. The thrust force provided by bundles of flagella enables every cell to swim in water at room temperature and without load at speeds around 200-300 μm s^{-1}[29].

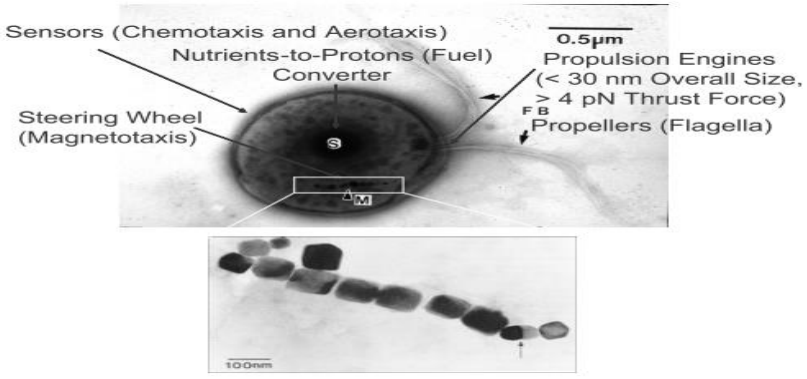


Figure 1-2: Image showing MC-1 bacterium cell morphology [4].

The MC-1 strain grows in a chemoheterolithotrophic liquid medium under microaerobic conditions. Iron-enrichment of the medium is achieved using 50 μM of ferrous sulfate heptahydrate $FeSO_4.7H_2O$ [31].

The MC-1 strain of MTB has magnetosomes that are responsible for magnetotaxis that has an effect on determining the movement direction of the MTB, but the movement direction of MC-1 is also affected by chemotaxis and aerotaxis. Magnetotaxis is the most convenient way to guide MC-1 inside the maze of blood vessels in the human body. By applying a magnetic field higher than the magnetic field of the earth (0.5 Gauss), the movement direction of the MC-1 will be fully influenced by magnetotaxis [29].

Tracking movement of MTB inside the human body is done using the Resonance Imaging System (MRI). The magnetosomes of the MTB are manipulated to track the movement of MTB using the MRI system because the magnetosomes cause disturbances in the local magnetic field that have an affect on the spin-lattice T_1 and spin-spin T_2 relaxation times during MRI [32].

In vitro studies are done to evaluate the ability of MC-1 to penetrate through solid tumours. The results collected from studying the penetration of MC-1 through 3D models composed of multicellular tumour spheroids (MCTS) simulating the structure of solid tumours show that MC-1 are able to penetrate inside the 3D multicellular tumour spheroids [31,33].

MTB MC-1 are loaded with particles with sizes of around 150 nm. The MC-1 are attached to these particles using antibodies. The size of these particles is sufficient to provide the tumour lesions with enough concentration of anticancer medication without affecting the swimming speed of the MTB [4].

1.3 Liposomes

Liposomes are spherical vesicles composed of 1 or more lipid bilayers enclosing aqueous compartments inside them [34, 35]. They have the ability to encapsulate particles inside both the aqueous solution and in the lipid bilayer. For instance, liposomes encapsulate the lipophilic drugs in the lipid bilayer and encapsulate the hydrophilic drugs in the aqueous compartment [34].

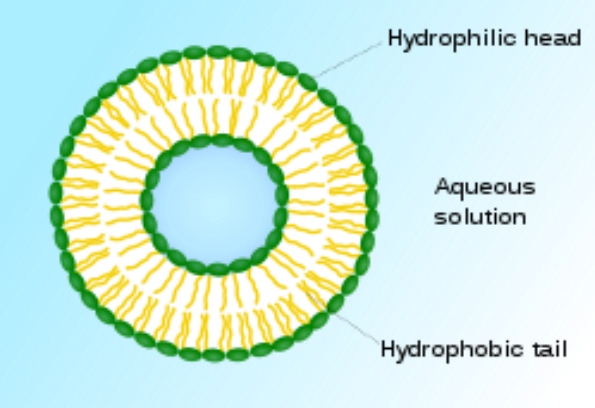


Figure 1-3: Sketch explaining the general structure of liposomes [36].

Liposomes can be classified according their sizes and the number of bilayers as in the following [37]:

1. Small unilamellar vesicles (SUV) are between 20-100 nm.
2. Large unilamellar vesicles (LUV) are more than 100 nm.
3. Giant unilamellar vesicles (GUV) are bigger than 1000 nm in size.
4. Oligolamellar vesicles (OLV) are 100-500 nm in size.
5. Multilamellar vesicles (MLV) are bigger than 500 nm in size but are composed of many layers.

1.3.1 Advantages of using liposomes as medication carriers [38]

1. Liposomes are composed of phospholipids that do not cause any toxicity inside the human body.
2. They are biodegradable and biocompatible.
3. Liposomes are usually used for the targeted delivery of medications because they allow us to deliver medications to desired places.
4. Liposomes enable us to achieve higher therapeutic efficacy.
5. Encapsulation of medications inside liposomes decreases the toxicity of medication by increasing the concentration of the drug in the targeted area and decreasing side effects of drugs on normal cells.
6. Liposomes keep encapsulated particles stable until they reach the place of release.

1.3.2 Disadvantages of using liposomes as medication carriers [39]

1. Liposomes are expensive to produce because of the high cost of artificially synthesized phospholipids.
2. Liposomes are unstable.

1.3.3 Structure of liposomes

In general, liposomes are composed of phospholipids and cholesterol [40]. Phospholipids have a general structure composed of glycerol, fatty acids, and organic alcohol. Glycerol is considered to be the backbone of phospholipids and is composed of 3 carbon atoms. 2 of these carbon atoms are connected to 2 chains of fatty acids from 1 side and the 3rd carbon atom is attached to organic alcohol that is connected to a phosphate group from another side. The fatty acid region of phospholipids is called the non-polar region or the hydrophobic region. The alcohol group that is attached to the phosphate group forms hydrogen bonds with water. This region of phospholipids is called the polar region or the hydrophilic region. Because the phospholipids contain both hydrophilic and hydrophobic regions they are called amphipathic molecules [41].

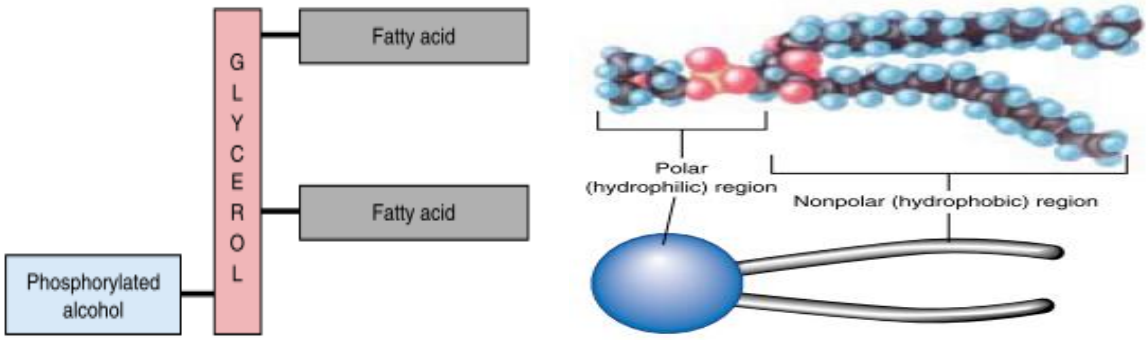


Figure 1-4: Sketch representing the general structure of phospholipids [41].

When a phospholipid is exposed to water, the polar alcohol molecules (heads) arrange against the non-polar fatty acids (tails). As a result, the polar regions face the water while the non-polar regions face each other away from the water, forming a lipid bilayer [41].

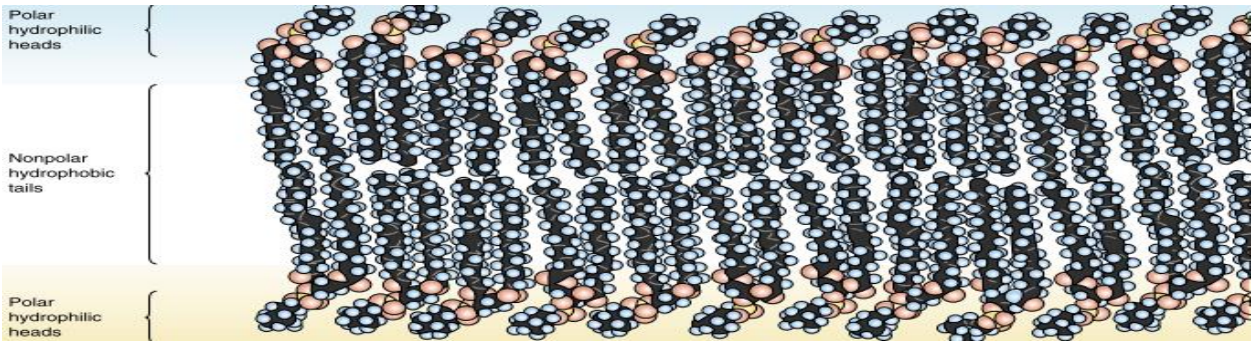


Figure 1-5: Sketch of the arrangement of the lipid bilayer [41].

Each phospholipid has its own transition temperature. The transition temperature (Tc) of the phospholipids is the temperature that is needed to change the lipid from the gel phase to the liquid phase. The main factors that determine if a phospholipid has a low or high transition temperature are the hydrocarbon length of the fatty acids, saturation, and the head group species [42].

Table 1-1: Transition temperature of the main synthetic phospholipids [42]

Name of the phospholipid	**Transition temperature**
DLPC	- 1°C
DMPC	23°C
DPPC	41°C
DSPC	55°C
DOPC	- 20°C
DMPE	50°C
DPPE	63°C
DOPE	- 16°C

Cholesterol is a steroid composed of 4 fused rings with one hydroxyl group at carbon atom number 3, and a double bond between carbon atoms 5 and 6, and an iso-octyl hydrocarbon side chain at carbon atom 17 [43].

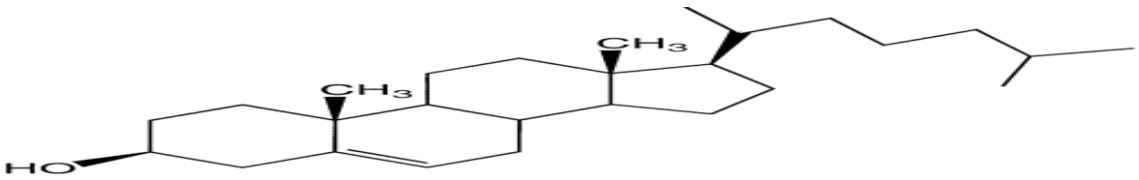


Figure 1-6: The chemical structure of cholesterol [43].

Liposomes are prepared of just phospholipids or phospholipids and cholesterol. Corporation of cholesterol in liposomes changes physic-chemical characters of liposomes [44]. Cholesterol has the ability to increase the transition temperature of liposomes and enhances the rigidity of the liposomes. In addition, it increases the stability and decreases the deformity of liposomes. As a result, it prolongs the life of liposomes inside the blood vessels. Cholesterol performs these actions because it increases the packing density of the phospholipids fatty acids chains by reducing the rate of motion of the hydrocarbon chains of phospholipids fatty acids [44].

1.3.4 Techniques used for preparation of liposomes

1.3.4.1 The electroformation technique

The electroformation technique was introduced by Angelova and Dimitrove, who prepared GUV with diameters exceeding 10 µm. In this technique, a phospholipid solution in chloroform is spread at a constant speed with a micropipette tip on the electrode substrates of indium tin oxide or silicon. The electrode substrate is separated from the ITO counter-electrode using a 1mm silicon rubber spacer. After the phospholipid film is dried under a vacuum a swelling solution is introduced between the 2 electrodes. Electroformation is performed using a sinusoidal AC field (10 Hz) for at least 90 minutes. After using the electricity, the liposomes are produced with relatively large sizes (10 -100µm) [45-47].

1.3.4.2 Microfluidic devices

Microfluidic devices are widely used for the preparation of liposomes. Microfluidic devices that are used to produce liposomes are fabricated out of many substances such as silicon and polymethyl methacrylate. Many research groups prepare liposomes by injecting lipids dissolved in an organic solvent such as ethanol or chloroform as a lipid phase from the central inlet by

injection pump and injection buffer saline or water containing particles to be entrapped inside liposomes from 2 side inlets as a water phase in microfluidic devices by injection pumps. Liposomes are composed when the oil phase intersects with the water phase and collected at the outlet of the microfluidic device [48, 49].

1.3.4.3 The lipid film hydration technique

One of the most frequently used techniques to produce liposomes is lipid film hydration. In this method, the phospholipids dissolve in an organic solvent and then the solvent evaporates in a rotary evaporator under a low vacuum until the lipid film is composed on the conical flask sides. Once the lipid film is totally dry, the phosphate buffer saline is added and rotated for 30 minutes inside the water bath above the transition temperature of the used phospholipid and 30 minutes outside the water path to get liposomes. This method produces liposomes with large diameters [50-52].

1.3.4.4 Sonication

Sonication is a resizing process to prepare SUV vesicles with diameters between 15-50 nm from the MLV. There are many instruments used to get SUV from MLV such as bath and probe tip sonicators. The SUV are prepared from MLV by placing a glass vial containing the MLV in a bath sonicator or immersing the tip of the sonicator in a glass vial and sonicating it for 10 minutes. This technique produces small liposomes that have diameters less than the diameter of MTB [53, 54].

1.3.4.5 Extrusion

Extrusion is another method for resizing the liposomes. We can get SUV from MLV using this technique. In this method, MLV are extruded through polycarbonate membranes with definite pore sizes to produce SUV. This method is better than the sonication method because it is simple and rapid and does not have negative effect on the stability of materials used for the preparation of liposomes, but the MTB is very large comparing to liposomes prepared by this procedure [55, 56].

1.3.4.6 The ether injection method

The ether injection method is a method to prepare SUV. This technique includes the preparation of liposomes by slowly injecting of phospholipids dissolved in diethyl ether by infusion pump to the aqueous solution of the particles that are targeted to be entrapped inside liposomes at 55-65°C followed by removing the solvent from the sample using a vacuum to get liposomes. This technique yields liposomes with tiny diameters that are smaller in size than the MTB [57, 58].

1.3.4.7 The ethanol injection method

The ethanol injection method is a method used to prepare liposomes by slowly injecting of a phospholipid dissolved in ethanol to buffer solutions that contain the material aimed to be entrapped inside liposomes at 55-65°C with continuous stirring by a magnetic stirrer. The solvent is removed from the sample either by heating or stirring. Disadvantage of this technique is that the sizes of the liposomes are small compared to the MTB sizes [59-61].

1.3.4.8 The reverse phase evaporation method

The reverse phase evaporation method is a method used to prepare LUV around 400 nm in diameter that have around 65% encapsulation efficacy. We can prepare liposomes by this method using the sonication of phospholipids dissolved in organic solvent and aqueous buffers for 5 minutes at 25°C to compose inverted micelles. After getting the micelles, organic solvents are removed from the sample under low pressure until achieving a viscous gel. To get liposomes, we agitate the gel in the vortex. The main drawback of this method is that liposomes prepared by this technique are smaller than MTB that are meant to encapsulate them [62, 63].

1.3.4.9 Freeze drying (lyophilization)

This method is usually used for the preparation of unilamellar vesicles with sizes of around 200 nm. In this technique, phospholipids and lyoprotectans such as sucrose or lactose are dissolved in a solvent such as chloroform to compose a monophase solution. After that, the monophase solution undergoes to sterilization and it is loaded in vials to be freeze dried. The freeze drying is performed in a freeze drier by freezing at - 40°C for 8h followed by primary

drying at - 40°C for 48h and secondary drying at 25°C for 10h. The pressure is kept constant at 20 Pascal during the drying. The liposomes are produced by adding water to the lyophilized product. The liposomes produced by this technique are very small compared to the sizes of MTB that will be entrapped inside them. Therefore, this technique is inappropriate for encapsulating the MTB [37, 64].

1.3.4.10 Detergent dialysis

This technique is used to prepare unilamellar liposomes between 40–200 nm in their sizes that have high encapsulation efficacy. This method is based on the preparation of liposomes from micelles. These micelles are composed of solubilizing phospholipids with detergent in the desired buffer solution. When the micelles are prepared, the detergent is removed by dialysis to get homogeneous unilamellar liposomes. Unfortunately, liposomes produced by detergent dialysis are smaller than MTB that are aimed to be encapsulated inside liposomes [37].

CHAPTER 2 METHODOLOGIES

This project is composed of 3 main steps. The first step is the encapsulation of MTB in large liposomes. After achieving this step successfully, the non-encapsulated MTB are removed by separation techniques to get rid of non-encapsulated MTB for 2 main reasons. The first reason is to study the encapsulation efficacy of liposomes prepared by lipid film hydration technique. The second reason is to evaluate the efficacy of the release techniques on release of liposomes composed of DMPC. When the non-encapsulated MTB are removed, the sample free of non-encapsulated MTB are released by the known techniques for the release liposomes.

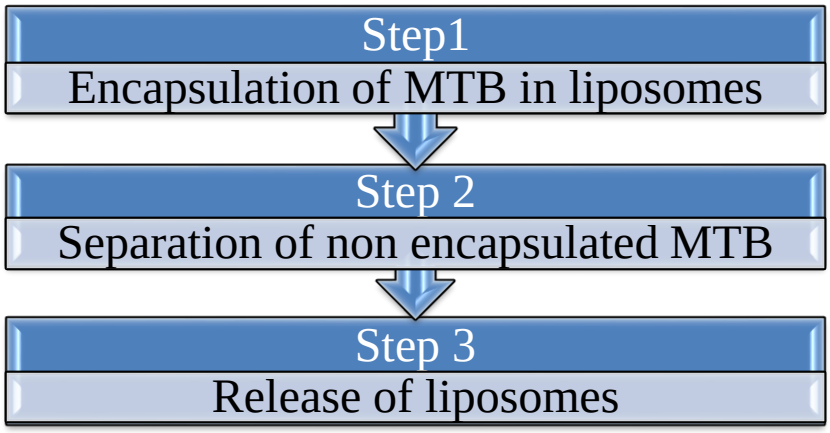


Figure 2-1: The plan of work for the studying the characteresics of liposomes composed of DMPC by lipid film hydration technique.

2.1 MTB MC-1 culture and microscope setup

MTB MC-1 are cultured in chemoheterolithotrophic liquid medium under microaerobic conditions. The iron-enrichment of the medium is completed using 50 µM of ferrous sulfate heptahydrate $FeSO_4.7H_2O$.

All observations are carried out using a Zeiss Imager Z1 microscope (Carl Zeiss Canada Ltd., Toronto, Canada) equipped with a Zeiss Axiocam camera. Observations are completed under dark illumination using 2 powers of magnification 20X and 50X.

2.2 Experiments setups

All equipments used in preparation of the liposomes by lipid film hydration are vitreous. The conical flask utilized to preparation the liposomes is washed with soap and water followed by rinsing it with deionized water many times followed by washing it with acetone (Sigma Aldrich) to remove residuals of water from the conical flask. The conical flask is kept in a vacuum hood to make sure to get rid of water and acetone from the conical flask. All glass vials used for keeping phospholipid are washed with acetone to make sure to remove any water that might be present in them. The preparation of the exact concentration of phospholipid is carried out in a vacuum hood. A glass syringe is used to withdraw the needed volumes of DMPC and chloroform to prepare the exact concentration of phospholipid solution. The phospholipid solution is preserved in a tightly closed glass vial covered with parafilm in a cold container until it is used.

2.3 Keeping materials

DMPC vials are kept in a refrigerator adjusted at − 18°C. We avoid exposing phospholipids to room temperature for long periods during the withdrawal of the required volumes of DMPC from the original DMPC volume. Both chloroform and acetone are preserved in a chemical room.

2.4 Counting the number of MTB and liposomes in 1 ml of a sample

Each drop of the sample is located on a microscope slide using a pipette. In the case of counting the number of MTB in the MTB sample, each drop of MTB is covered with a cover slide separated from a microscope slide by 1 cover slide from both sides with a thickness of 150 μm whereas in counting the number of liposomes and MTB in the liposome sample, 2 cover slides are utilized with an overall thickness equal to 300 μm for the separation. The dimensions of each area which are observed under a microscope with a lens of 50X is 180 μm (the length) × 134 μm (the width) while the dimensions of the area which is observed under the microscope with a lens of 20X is 440 μm × 330 μm. To calculate the number of liposomes and MTB in 1ml of liposomes sample, we calculate the number of liposomes and MTB in 8 different specific sizes each of which has dimensions of 180 μm (the length) ×134 μm (the width) × 300 μm (the depth of the drop). After we calculate the number of liposomes and MTB in this specific size, we calculate the number of liposomes and MTB in each 1ml of the liposomes solution. The same

steps are done to count the number of MTB in the MTB sample except by multiplying 180 μm × 134 μm or 440 μm × 330 μm in 150 μm instead of multiplying them by 300 μm. Every experiment has been repeated 3 times and in every experiment, 8 different readings have been recorded.

2.5 Encapsulation of MTB in liposomes using the lipid film hydration technique

Materials and instruments

1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC) (Avanti polar lipids, Alabaster, Alabama, USA), Chloroform (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), Acetone (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), glass pipette, glass vials, glass syringe, MTB, rotary evaporator (BUCHI Switzerland ,Flawil, Switzerland), vacuum source, parafilm (Parafilm M Barrier Film, West Chester, PA, USA).

Procedure

6.6 mM of DMPC in chloroform is evaporated under a low vacuum in a rotary evaporator equipped with a water bath adjusted at 26°C with a rotation speed of 220 rpm for 45 minutes. After we get a thin lipid film on the corners of the conical flask, we let the thin lipid film dry more by extra evaporation for an additional 45 minutes under a low vacuum to make sure to evaporate the residuals of chloroform from the lipid film. When the thin lipid film has dried completely, 1.5 ml of MTB in their media with concentration of 5.79×10^{6} per 1 ml are added to the lipid film in the conical flask and the conical flask is rotated with a speed of 220 rpm inside the water path adjusted at 26°C for 45 minutes to transfer the DMPC from the gel phase to the liquid phase. Then the conical flask rotates outside the water path for 45 minutes to transfer the DMPC to liposomes that entrap the MTB inside them.

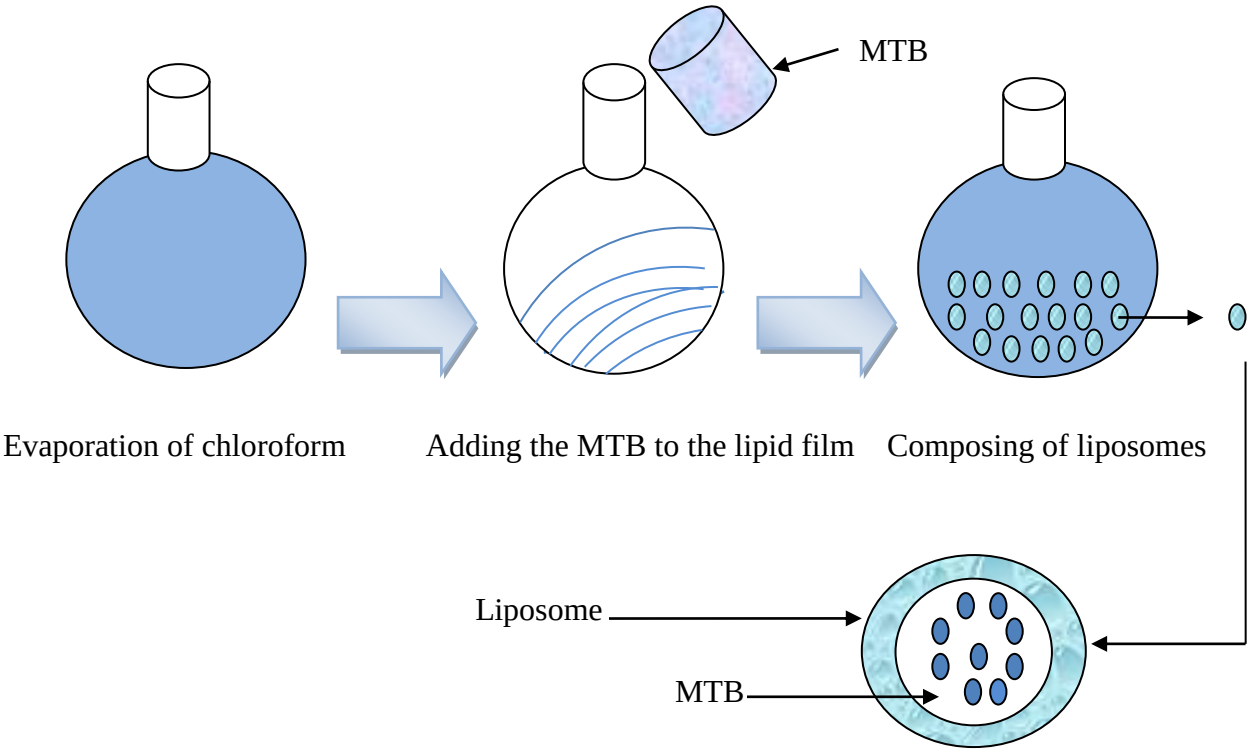


Figure 2-2: Sketch explaining the encapsulation of MTB in liposomes using the lipid film hydration technique.

2.6 Preparation 6.6 mM DMPC in solvent

Molecular weight of DMPC = 677.933

1M of DMPC solution composed of 677.933 gram in 1000 ml of solvent

1ml 1M contain 0.6779 gram of DMPC

0.6779 in 1ml of DMPC ⟶ 1000 m M

X in 1ml of DMPC ⟶ 6.6 m M

X = 0.6779 ×6.6 ÷ 1000

X = 0. 004474 gm in 1ml solvent

4.47 mg / ml of solvent

The concentration of DMPC is 25 mg in 1 ml of chloroform

25mg ⟶ 1000 µl of chloroform

4.47 ⟶ X

$X = 4.47 \times 1000 \div 25$

$X = 178.8$ µl

By adding 178.8 µl of DMPC to 821 µl of chloroform, we prepare 1 ml phospholipid solution of 6.6 m M in chloroform.

2.7 Separation of non-encapsulated MTB from liposomes by polycarbonate filters

Equipments and materials

1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC) (Avanti polar lipids, Alabaster, Alabama, USA), chloroform (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), acetone (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), glass pipette, glass vials, glass syringe, MTB, Rotary evaporator (BUCHI Switzerland, Flawil, Switzerland), vacuum source, Parafilm (Parafilm M Barrier Film, West Chester, PA, USA), deionized water, PBS (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), polycarbonate filters with pores of 5 µm (Sterlitech Corporation. Kent, WA, USA), Zeiss Imager Z1 microscope (Carl Zeiss Canada Ltd., Toronto, Canada).

Procedure

The segregation procedure is been done by passing 1.5 ml of deionized water through the polycarbonate filter of 5 µm pores followed by passing 1.5 ml of the liposomes solution through the polycarbonate filter while continually washing the liposomes solution with PBS to make sure to separate most of the non-encapsulated MTB from the liposomes solution. The liposomes that remain on the top of the filter are collected and suspended in PBS to make a volume of 1.5 ml.

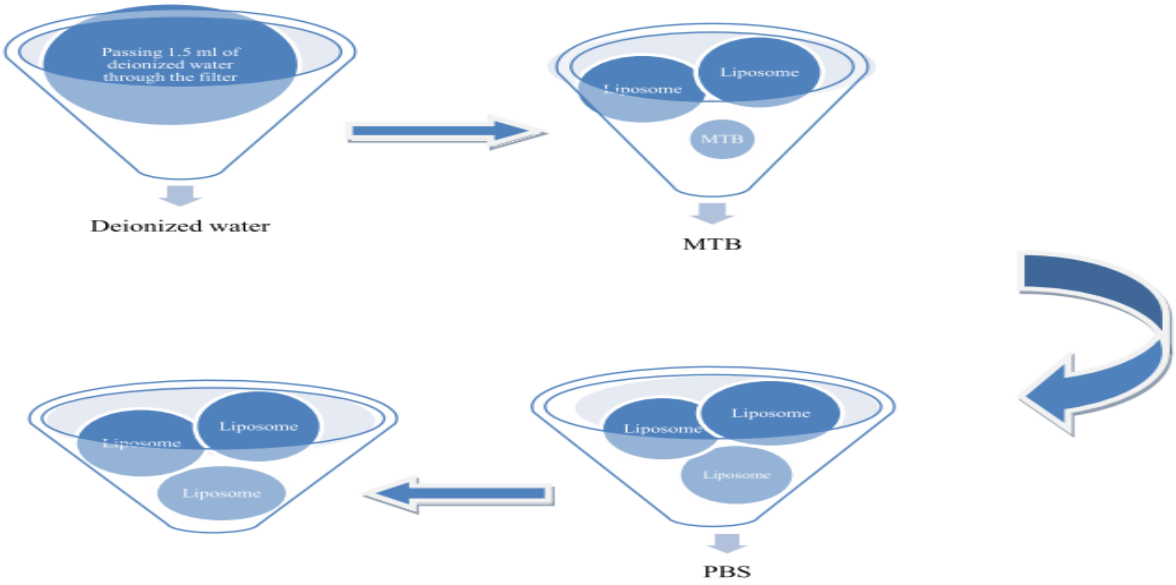


Figure 2-3: Sketch explaining the separation of non-encapsulated MTB from the sample by polycarbonate filters.

2.8 Release of liposomes

There are many techniques that could be manipulated for the releasing of liposomes, such as LFUS, temperature, and pH. The LFUS has the advantage over the other 2 techniques for the releasing of liposomes because it is recognized as a controlled way for the release of liposomes while the temperature and pH methods are uncontrolled ways for the release of liposomes.

2.8.1 Release of liposomes by low frequency ultrasound (20 kHz)

Materials and equipments

1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC) (Avanti polar lipids, Alabaster, Alabama, USA), chloroform (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), acetone (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), glass pipette, glass vials, glass syringe, MTB, rotary evaporator (BUCHI Switzerland ,Flawil, Switzerland), vacuum source, parafilm (Parafilm M Barrier Film, West Chester, PA, USA), deionized water, PBS (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), polycarbonante filter with 5µm pores (Sterlitech Corporation. Kent, WA, USA), Zeiss Imager Z1 microscope (Carl Zeiss Canada Ltd., Toronto, Canada), ice path, ultrasonic processor (QSONICA, Newtown, *CT*, USA).

Procedure

After the preparation of liposomes and the separating of the non-encapsulated MTB, 1.5 ml of the liposomes solution is added to a glass vial situated in an ice bath to prevent release of liposomes by temperature generated during the sonication process. The release of liposomes is achieved by the immersion of the sonication probe in the liposomes solution vial and applying LFUS of $3W/cm^3$ for 3 minutes. The amount of liposomes and MTB is counted before and after the sonication process and the efficacy of the low frequency ultrasound to release of liposomes is studied.

2.8.2 Release of liposomes by body temperature and body pH

Materials and equipments

1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC) (Avanti polar lipids, Alabaster, Alabama, USA), chloroform (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), acetone (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), glass pipette, glass vials, glass syringe, MTB, rotary evaporator (BUCHI Switzerland, Flawil, Switzerland), vacuum source, parafilm (Parafilm M Barrier Film, West Chester, PA, USA), deionized water, polycarbonate filter with pores of 5 μm (Sterlitech Corporation. Kent, WA, USA), Zeiss Imager Z1 microscope (Carl Zeiss Canada Ltd., Toronto, Canada), plastic syringes, PBS (Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), controlled water bath (BUCHI Switzerland, Flawil, Switzerland).

Procedure

After the preparation of liposomes and the separation of the non-encapsulated MTB by the polycarbonate filters, the liposomes are suspended in 1.5 ml PBS and the liposomes solution is poured into 6 different plastic vials. These vials incubate in water bath controlled at 37°C for different time intervals and the release of liposomes is studied by counting the number of liposomes and MTB in the samples before and after exposure to body temperature and body pH.

2.9 Studying the effect of body temperature on liveability of MTB

Materials and instruments

MTB, plastic vials, pipette, controlled water path, Zeiss Imager Z1 microscope (Carl Zeiss Canada Ltd., Toronto, Canada).

Procedure

We study the effect of the body temperature 37°C on the liveability of MTB by incubating 6 different vials each filled with 250 µl of MTB inside the water path adjusted at 37°C. The MTB withdraw for different time intervals and are investigated under the microscope to study their liveability.

CHAPTER 3 RESULTS

3.1. Encapsulation of MTB in liposomes

3.1.1 The number of produced liposomes in 1ml of liposome solution

We can produce around 1,500,000 liposomes in each 1 ml of liposome solution by using the lipid film hydration technique.

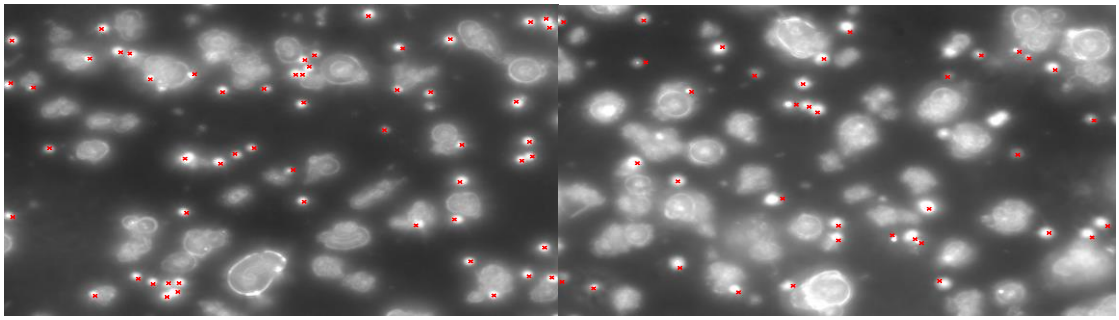


Figure 3-1: Transmission electron microscopy images of the liposomes prepared by the lipid film hydration technique.

3.1.2 Diameters of liposomes produced by lipid film hydration

The sizes of liposomes in a specific size of liposomes solution are measured by our group to figure out their size distribution. Results attained from these measurements indicate that the produced liposomes are 8-23 μm in their diameters and 56% of the liposomes' own diameters are between 11-16 μm. Liposomes with this range of sizes are eligible to capture a sufficient number of MTB inside them since they are 4-11 times bigger than the MTB aimed to be encapsulated inside them.

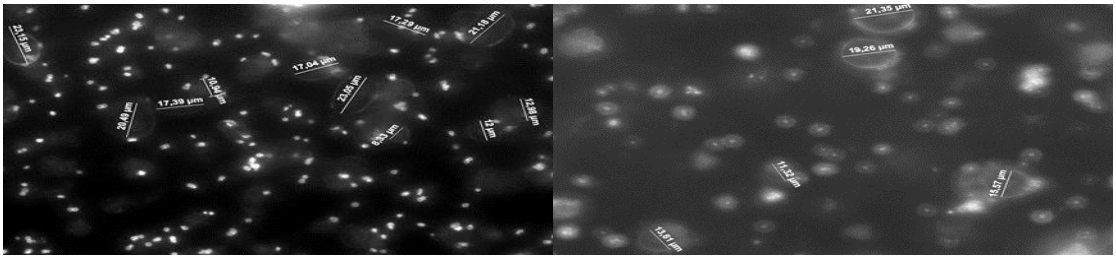


Figure 3-2: Transmission electron microscopy images showing the diameters of liposomes prepared by the lipid film hydration technique.

Table 3-1: Diameters of liposomes in 1ml of liposomes solution produced by lipid film hydration technique

Sizes of liposomes	Number of liposomes	Percentage of liposomes of total number of liposomes
8-10 µm	183977	20%
11-13 µm	275966	30%
14-16 µm	239171	26%
17-19 µm	119585	13%
20-23 µm	101187	11%

3.2 Separation of non-encapsulated MTB by polycarbonate filters

3.2.1 The separation efficacy of polycarbonate filters

Polycarbonate filters are able to separate around 3.5×10^6 non-encapsulated MTB from the total amount of non-encapsulated MTB in liposomes solution (3.9×10^6 non-encapsulated MTB). These filters separate around 90.6% of the total percentage of non-encapsulated MTB while 9.4% of non-encapsulated MTB are kept in the sample.

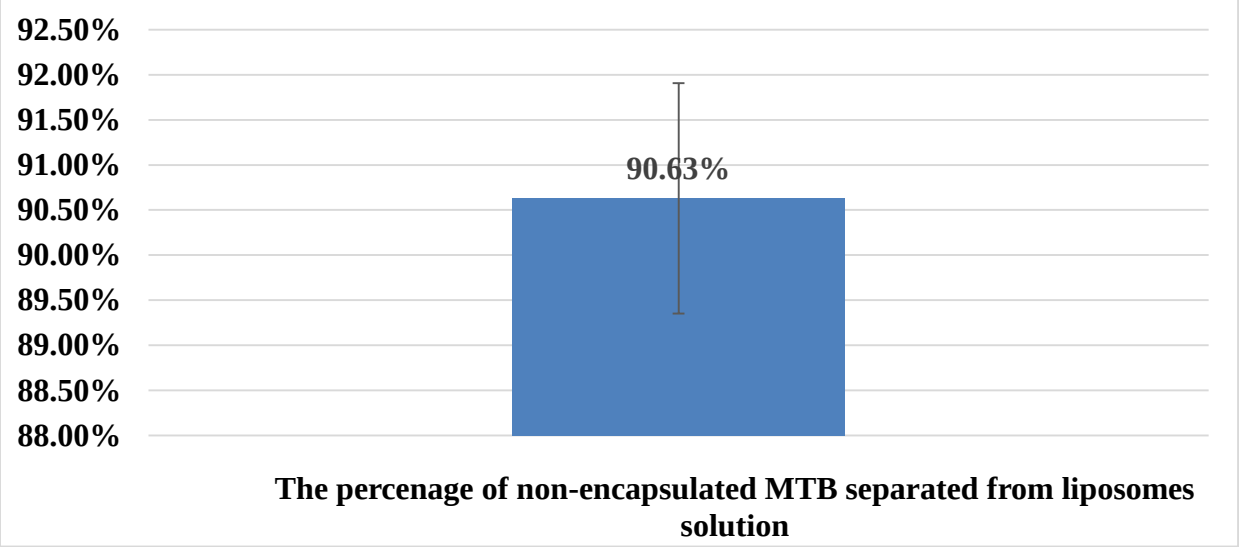
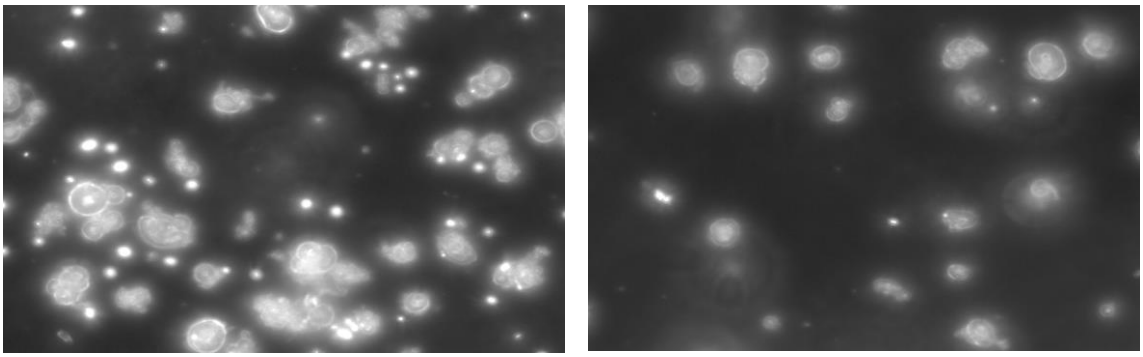


Figure 3-3: Graph representing percentage of non-encapsulated MTB that have been separated from liposomes solution by polycarbonate filters.



A) The sample before separation of non-encapsulated MTB.

B) The sample after separation of non-encapsulated MTB.

Figure 3-4: Transmission electron microscopy images for the sample before and after the separation of non-encapsulated MTB by polycarbonate filters.

3.2.2 Studying the influence of polycarbonate filters on the stability of liposomes

The polycarbonate filters achieve segregation of a high percentage of non-encapsulated MTB reaching 90.63%, but we have to know their effect on the stability of liposomes. In general, the liposomes are still intact after the separation of the non-encapsulated MTB, but we observe that there is a decrease in the number of liposomes after the separation process is complete. Therefore, we count the number of liposomes before and after the separation of MTB. We find that 79.6% of liposomes are kept intact in the sample after the separation of non-encapsulated MTB by polycarbonate filters.

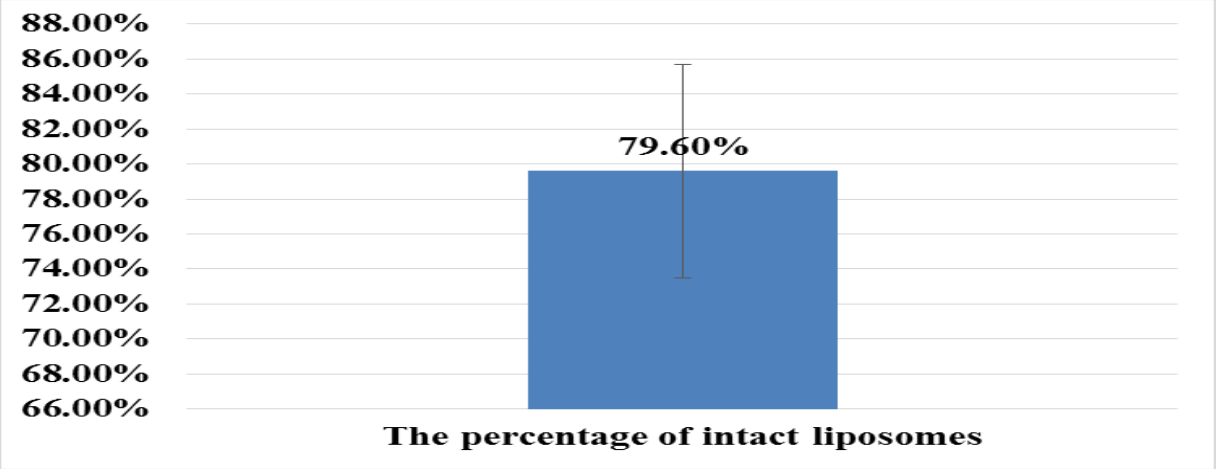


Figure 3-5: Graph showing the percentage of intact liposomes after their separation by polycarbonate filters.

3.3 Release of liposomes

3.3.1 Release of liposomes composed of DMPC by LFUS

The low frequency ultrasound shows high performance in release of liposomes composed of DMPC since 95% of liposomes have been released by using low frequency ultrasound.

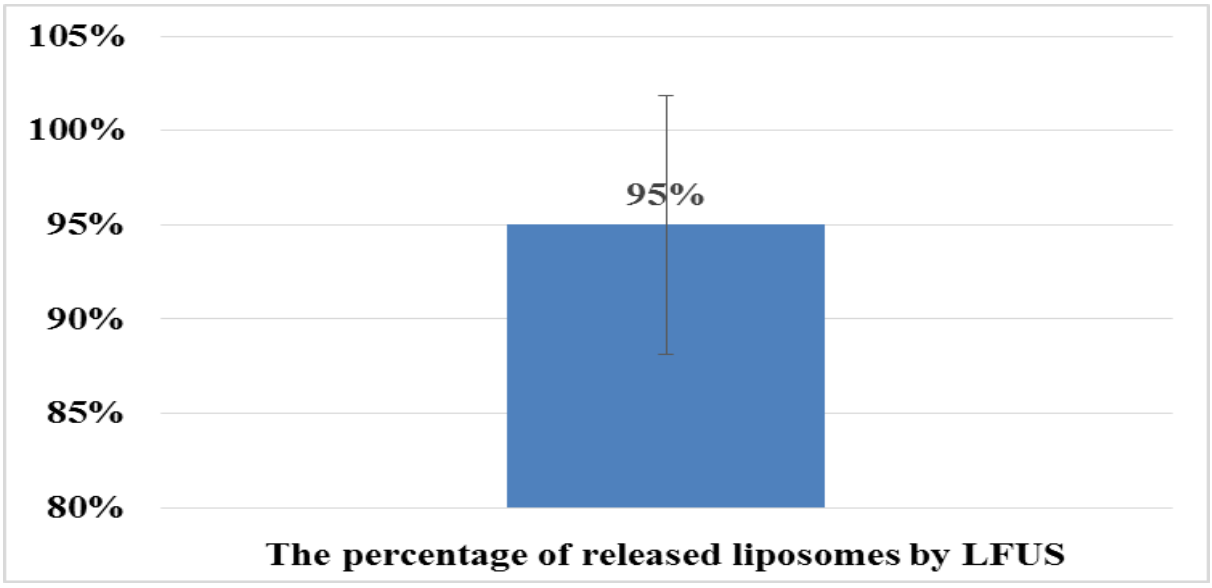
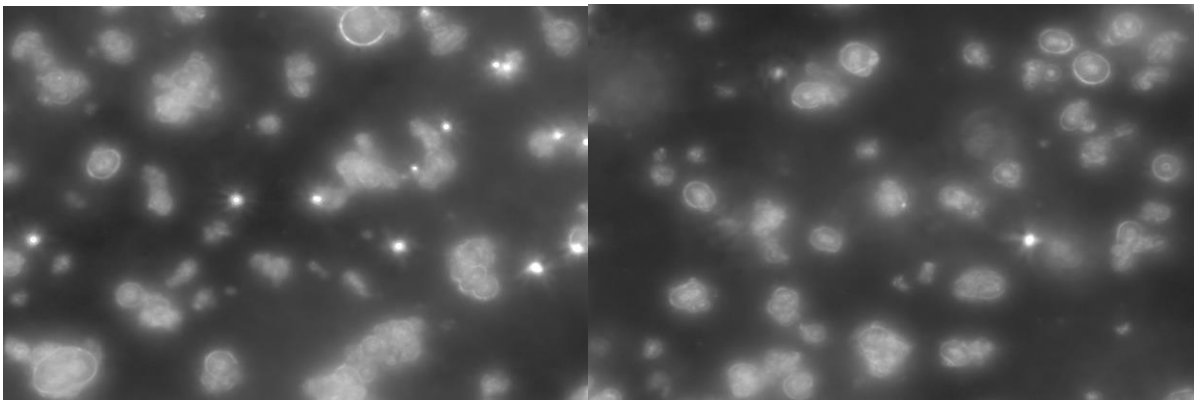
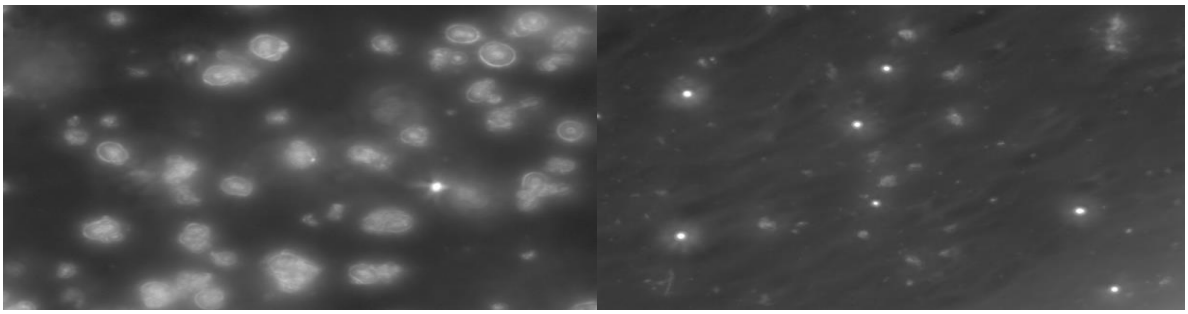


Figure 3-6: Graph explains the percentage of released liposomes by LFUS.



(A).The sample before separation of non-encapsulated MTB.

(B) The sample after separation of non-encapsulated MTB.



(C) The sample before release of liposomes by LFUS (D) The sample after release of liposomes by LFUS

Figure 3-7: Transmission electron microscopy images representing the release of liposomes by LFUS.

3.3.1.1 The encapsulation efficacy of liposomes prepared by the lipid film hydration technique.

The results obtained by our group show that we can encapsulate around 857807 MTB in every 1ml of liposome solution.

3.3.1.2 The number of non-separated non-encapsulated MTB in every 1 ml of liposome solution.

57550 non-separated non-encapsulated MTB stayed in every 1 ml of liposomes solution.

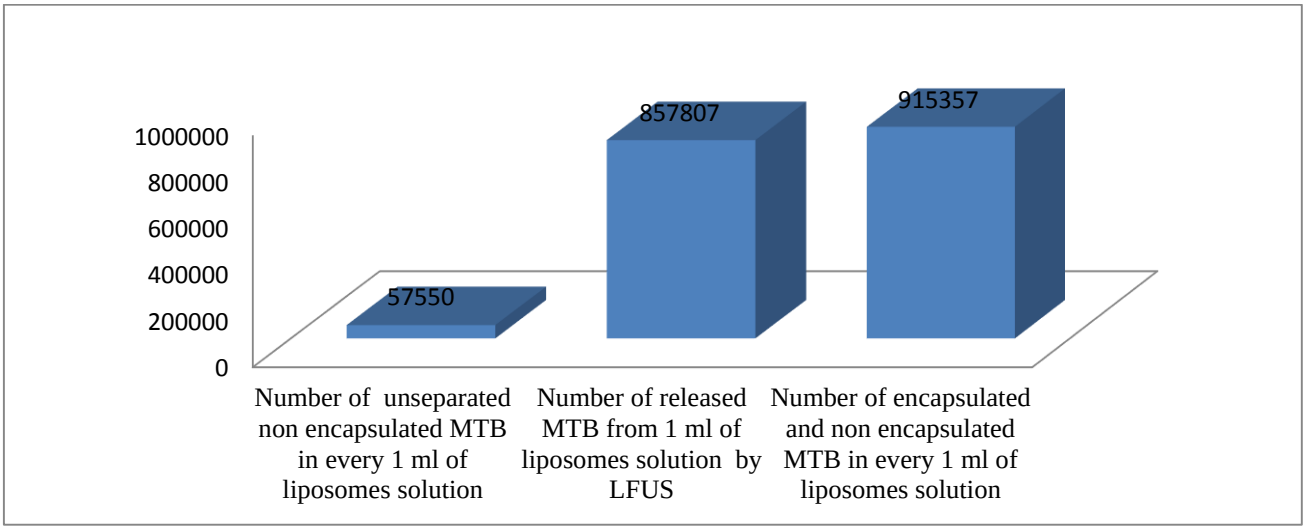


Figure 3-8: Graph showing the number of non-encapsulated and encapsulated MTB in the sample after the separation process.

3.3.1.3 The percentage of MTB entrapped inside liposomes

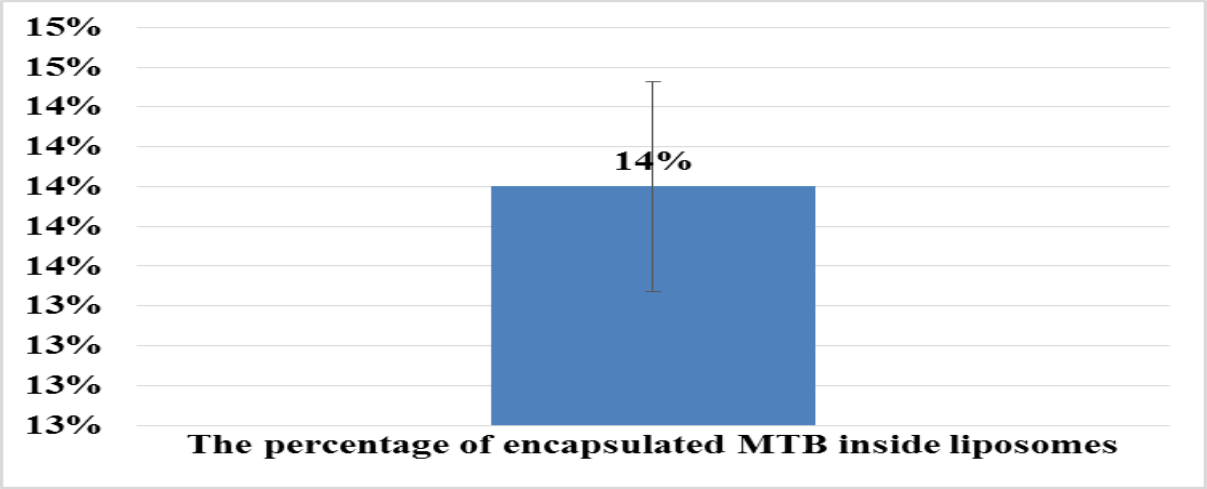


Figure 3-9: Graph manifesting the percentage of encapsulated MTB from the total number of MTB aimed to be encapsulated in the liposomes.

3.3.2 Release of liposomes by the effect of body temperature (37°C) and body pH

Both body temperature and body pH are unable to release liposomes composed of DMP by lipid film hydration technique during 30 minutes of exposure.

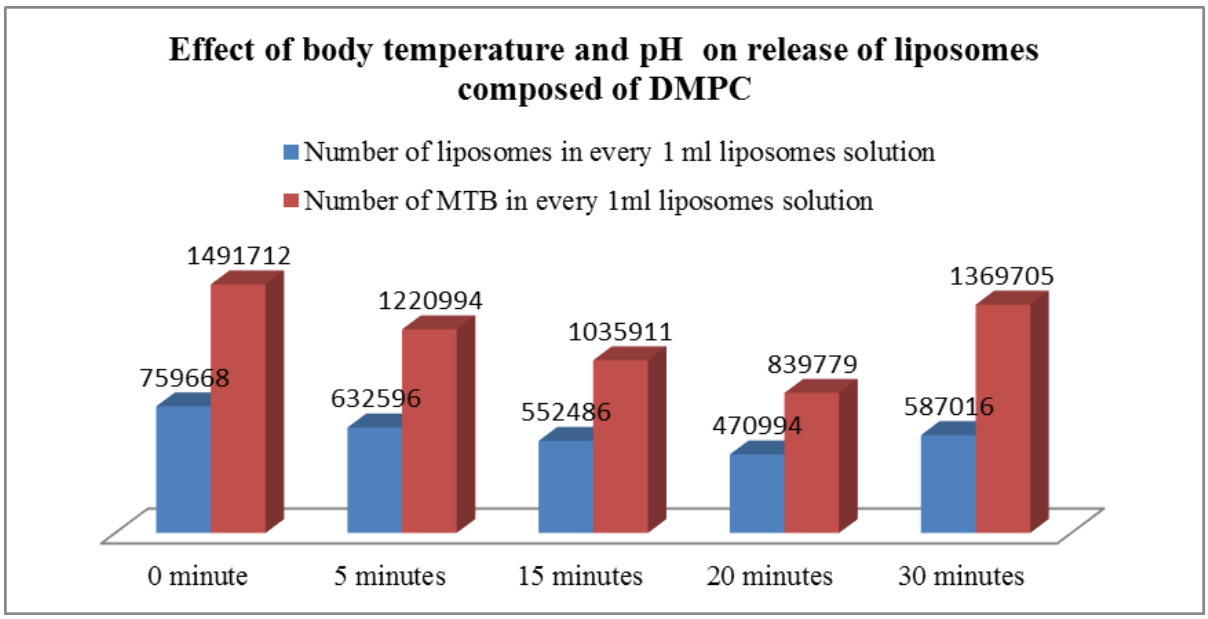
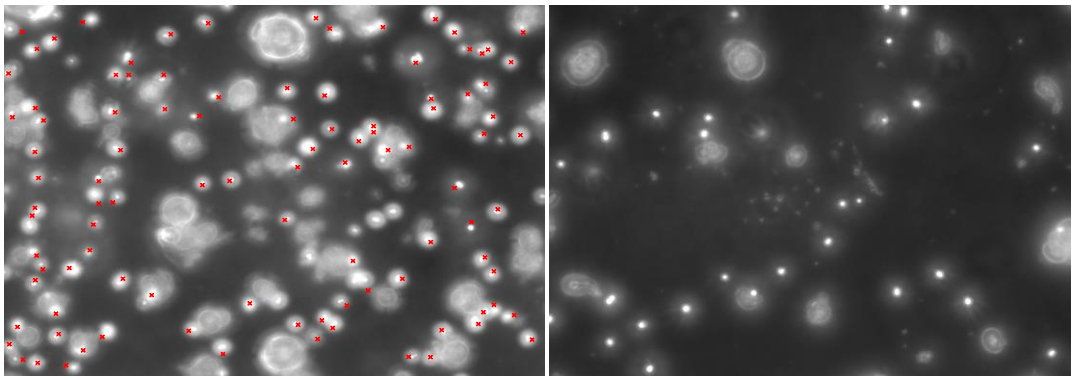
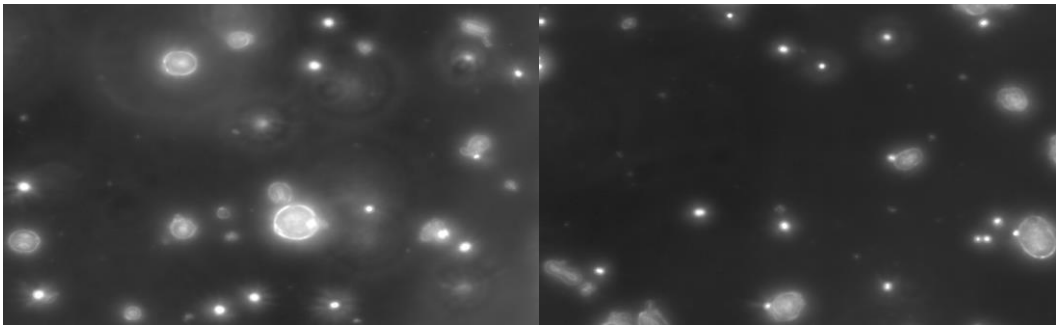


Figure 3-10: Graph representing the effect of body temperature and body pH on the release of liposomes composed of DMPC.



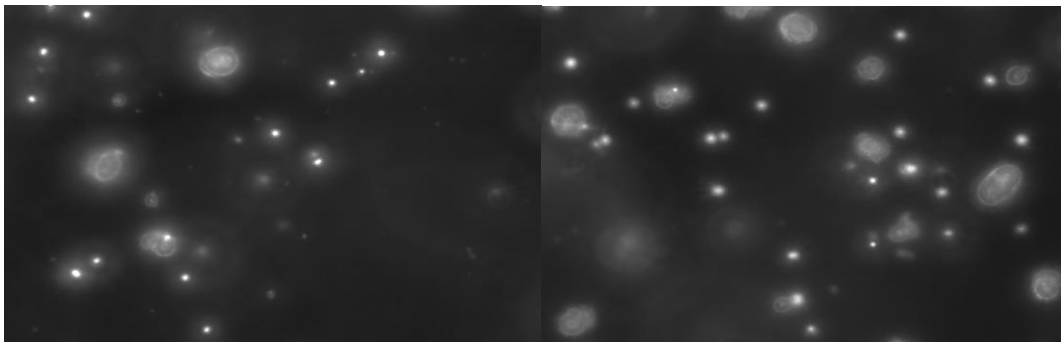
(A) The sample before the separation of non-encapsulated MTB.

(B) The sample after the separation of non-encapsulated MTB.



(C) The sample 5 minutes after exposure to 37 ºC and body pH .

(D) The sample 15 minutes after exposure to 37 ºC and body pH.



(E) The sample 20 minutes after exposure to 37 °C and body pH.

(F) The sample 30 minutes after exposure to 37 °C and body pH.

Figure 3-11: The transmission electron microscopy images showing the effect of body temperature and body pH on the release of liposomes composed of DMPC.

3.4 The effect of body temperature on the liveability rates of MTB

Around 96.53% of MTB have survived during first 15 minutes of their exposure to human body temperature whereas 92.6% of them survived after their exposure to 37ºC for 20 minutes. The sharp decrease in the percentage of alive MTB after exposure to human body temperature has appeared after 25 and 30 minutes where 55.6 % and 17.3% of MTB have survived respectively.

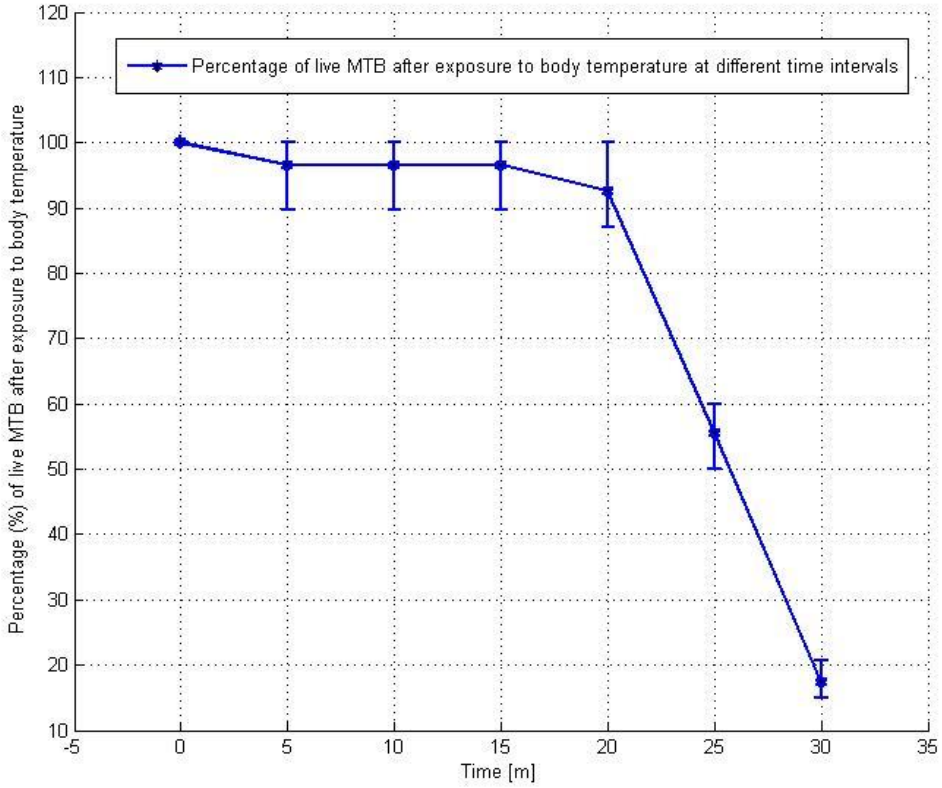


Figure 3-12: Graph representing the percentage of living MTB after exposure to body temperature for different time intervals.

CHAPTER 4 DISCUSSION

4.1 Encapsulation of MTB inside liposomes

The lipid film hydration technique shows high reproducibility of liposomes that are big enough to encapsulate big particles such as MTB inside them. This technique produces more than one million liposome in every 1ml of liposomes solution. Liposomes produced by this technique are between 8-23 μm in their diameters and 56% of them have diameters between 11-16 μm. According to our results, liposomes encapsulate 14.1% of the targeted number of MTB. Although the produced liposomes manifest low encapsulation efficacy, 857807 MTB encapsulate inside each 1ml of liposomes solution with around 1 MTB entrapped inside each liposome. This high number of encapsulated MTB is able to carry sufficient quantities of anticancer medications since each MTB is able to carry particles with sizes around 150 nm. We could encapsulate the MTB inside liposomes using MTB in their media as a water phase instead of using water or PBS as a water phase as it is used in all of the liposomes production techniques. The successes in preparation of liposomes using MTB media as a water phase enables us to encapsulate the MTB inside liposomes with their media. Because the produced liposomes are opaque, we are unable to see inside them to investigate the encapsulation efficacy of liposomes produced by lipid film hydration. The investigation of encapsulation efficacy is achieved after the separation of non-encapsulated MTB and the release of liposomes.

4.2 Separation of liposomes by polycarbonate filters

Liposomes are very fragile particles because they are composed of two layers of phospholipids. Since they are susceptible to breaking, the separation techniques used for the separation of particles could puncture or break them easily. The gel chromatography technique is employed to separate the liposomes by many research groups. Unfortunately, it is impossible to use this technique for the separation of non-encapsulated MTB because it cannot be used for segregating particles that are bigger than 300 nm. Another method utilized for separating particles is cellulose filters, but we lost our sample when we studied the isolation of the non-encapsulated MTB using cellulose filters. Liposomes precipitate and attach at the bottom of centrifuge tube when

we exploit the centrifugation technique to isolate the non-encapsulated MTB although we study the separation by centrifugation for different time intervals with different centrifugation powers. The polycarbonate filters of 5 μm pores show the best results regarding the separation of non-encapsulated MTB. These filters manifest a high separation performance since they segregate 90.63% of the non-encapsulated MTB. The 9.37% of non-encapsulated MTB remain in the sample after the separation process because some MTB adhere to the liposomes since liposomes are composed of phospholipids. Moreover, there are millions of non-encapsulated MTB in the sample and achieving 100% isolation is very hard for these numbers. Polycarbonate filters keep the integrity of the most liposomes since 79.6% of the liposomes collected are intact at the top of the polycarbonate filters. 20.4% of the liposomes are lost during the isolation process for the non-encapsulated MTB. Either they are broken during the separation process or they escape from the corners of the polycarbonate filters during the isolation process. Polycarbonate filters are hydrophobic which makes them resistant to passing water through them. As a result, any solution will slowly pass through them. By adding PBS or water, we can compensate the solution passed through the filters and collect liposomes suspended in PBS or water. In contrast, when we study the efficacy of cellulose filters to separate the non-encapsulated MTB, the solution passes quickly through the filters and the liposomes are broken because these filters are hydrophilic.

4.3 Release of liposomes composed of DMPC

The efficacy of utilizing the LFUS to release liposomes is tested before the other techniques for release such as body temperature and body pH because it is a controlled method for liposomes release. The LFUS 3W/cm^3 released 95% of liposomes during 3 minutes of exposure. This technique achieves a great releasing performance as a controlled liposomes release technique. The results collected by our group are close to the results achieved by other groups that promote exploiting the LFUS for the controlled release of liposomes. Efficiency of releasing liposomes by the LFUS seems to be caused by the cavitation (formation bubbles in a liquid and collapsing them) that happens besides liposomes membranes or inside liposomes induced by LFUS. When cavitation

occurs, pores formed in liposomes membranes followed by their collapse leads to the release of their entrapped content.

The body temperature and body pH are uncontrolled methods for the release of liposomes. We study the effects of body temperature and body pH on the release of liposomes composed of DMPC by suspending the liposomes in PBS of pH 7.4 and exposing them to 37°C for 30 minutes. The results attained from these experiments indicate that there is no significant increase in the number of MTB or significant decrease in the number of liposomes during the exposure to body temperature and body pH during the 30 minutes of their exposure. The cause of the stability of liposomes composed of DMPC at the body pH is attributed to the chemical characteristics of DMPC. DMPC is not recognized among pH-sensitive liposomes composing phospholipids such as 1, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and phosphatidylethanolamine (PE) that could release their contents as a response to changes in the pH in the environment that surrounds them. As a result, liposomes composed of DMPC resist releasing their contents under the influence of body pH. Liposomes prepared from DMPC are temperature sensitive liposomes because their transition temperature is lower than the body temperature. Since liposomes prepared from DMPC are thermosensitive, they have to release their contents when the temperature is elevated more than their transition temperature. When the temperature is elevated more than the transition temperature of phospholipids that composes liposomes, the lipid bilayer of liposomes starts to lack its ordering and becomes disorganized. With continuous exposure to body temperature, the fluidity of the lipid bilayer increases and the liposomes start to release their contents. Liposomes composed of DMPC do not manifest a significant release of their entrapped MTB during 30 minutes of exposure to the body. MTB is too large to go through the small pores formed in the membrane of the liposomes as a result of exposure to body temperature. MTB are 2 μm in size and the liposomes' own sizes are between 8-23 μm. Therefore, pores larger than 2 μm have to be composed in the lipid bilayer of liposomes or the liposomes have to collapse, so the MTB can escape from them.

4.4 Effect of body temperature on liveability of MTB

Generally, MTB prefer to live and grow at room temperature, but the human body temperature is 13-14°C higher than the temperature preferred for growth of MTB. Therefore, we study the surviving ability of MTB at body temperature to determine the availability of navigation the MTB inside the human body as nano-robotics while keeping them alive. According to our results, 92.6% of MTB stay alive during the first 20 minutes of exposure to the body temperature. This indicates that we can manipulate the MTB as nano-robotics inside the human body for 20 minutes without losing them. The percentage of alive MTB have dropped to 55.6 % after 25 minutes of exposure to 37°C. This decrease in number of alive MTB indicates that the MTB are able to survive just for 20 minutes at body temperature and after that they will start to loss their activities and die. The sharp decrease in number of alive MTB has appeared after 30 minutes of exposure to the body temperature where 17.3% of MTB survive after exposure to body temperature.

CONCLUSION AND RECOMMENDATIONS

We study the characteristics of liposomes composed of DMPC by lipid film hydration technique. To achieve our goal we design a protocol composed of 3 steps. In the first step, we use the lipid film hydration technique to prepare liposomes composed of DMPC that entrap MTB inside them. We succeeded with the preparation of liposomes with diameters between 8-23 μm. These liposomes encapsulated 14.1% of the total amount of MTB targeted to be encapsulated inside liposomes. Lipid film hydration technique produced liposomes that are able to entrap big particles with 2 μm in their diameters. In addition, this technique showed high reproducibility of liposomes. These characteristics make lipid film hydration technique superior to produce liposomes that could encapsulate big particles inside them. We have succeed in obtaining liposomes by creation some modifications in the protocol of the preparation of liposomes through the lipid film hydration technique using MTB in their media as a hydration solution instead of utilizing water or a buffer saline as a hydration solution. Polycarbonate filters are manipulated to separate the non-encapsulated MTB in the second step. According to our knowledge, this is the first time we are able to manipulate polycarbonate filters to separate non- encapsulated particle. These filters are able to separate 90.63% of the non-encapsulated MTB while keeping most of liposomes intact during the encapsulation procedure. During the third step, we assess the efficacy of LFUS as a controlled way to release liposomes composed of DMPC. 95% of liposomes are released by low frequency ultrasounds (3 W/ cm^3) during 3 minutes of exposure to LFUS. According to our results, both body pH and body temperature did not show any efficiency in the release of liposomes composed of DMPC 30 minutes after their exposure to body temperature or body pH.

After we succeed in encapsulation of MTB inside liposomes, we are able to navigate MTB through the blood vessels and we can protect the normal healthy tissues from anticancer medications that are attached to MTB by antibodies. The produced liposomes can be manipulated as vectors to deliver medications attached to MTB to tumor masses. The movement direction of these vectors inside the human body can be controlled by applying magnetic fields on magnetosomes of MTB encapsulated inside these carriers

while the location of these vectors inside the human body can be determined by MRI system. When liposomes reach tumor masses can be released by LFUS to release the entrapped medications.

Future works that could be done to achieve the encapsulation of MTB in liposomes:

1. Exploiting the lipid film hydration technique to entrap MTB in liposomes composed of phospholipids with a transition temperature lower than the DMPC transition temperature although the low stability of these liposomes will be an obstacle in achieving the goal successfully. Cholesterol could be used to enhance the stability of theses liposomes.

2. Manipulation of pH-sensitive phospholipids such as DOPE and PE instead of DMPC to prepare liposomes although their lower transition temperature could cause the very quick release of liposomes after their injection in the human body. Adding cholesterol to them could increase their stability.

Contributions

1. According to our knowledge this is the first time that a researcher manipulate polycarbonate filters to separate the non-encapsulated particles from liposomes solution. This kind of filters can be utilized to separate the non-encapsulated MTB from liposomes solution and to separate liposomes from each other depending on their sizes since these filters are available with many pores sizes.

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