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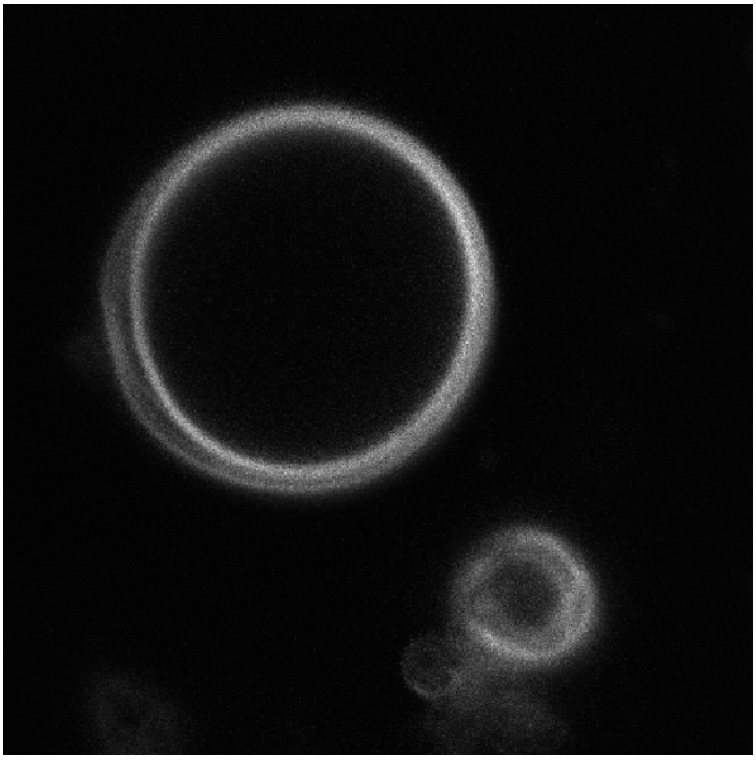

Preparation and characterization of giant niosomes

Master's thesis in Nanotechnology

Maryam Homaei

Department of Microtechnology and Nanoscience – MC2
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2016

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Maryam Homaei

Supervisor: Gavin Jeffries, Chemistry and Chemical Engineering
Co-Supervisor: Tatsiana Lobovkina, Chemistry and Chemical Engineering
Examiner: Aldo Jesorka, Chemistry and Chemical Engineering

Master's Thesis 2016
Department of Microtechnology and Nanoscience – MC2
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Cover: a confocal laser scanning micrograph of a giant niosome.

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Abstract

Niosomes, lamellar vesicles prepared from non-ionic surfactants and cholesterol, have been investigated in recent years due to their potential applications as drug delivery systems (DDSs). Niosomal drug delivery systems offer an advantage over conventional delivery systems by delivering the drugs in a controlled manner to overcome some problems associated with conventional drug delivery such as insolubility, instability and low bioavailability.
For different applications specific lamellarity, size and shape of niosomes are required. These parameters, in turn, are dependent on applied preparation method. Thus preparation method must be selected accordingly. Niosomes can encapsulate both hydrophilic and hydrophobic drug compounds.
Despite of substantial amount of studies on niosomes as drug carrier, the mechanism by which niosomes interact with biological cells and deliver drugs is not fully understood. Therefore further research is needed to expand application of niosome in drug delivery and to make it commercially available.
In order to elucidate drug delivery mechanism of niosomes, we produced giant niosomes (GNs) which are well visible by an optical microscope, and thus allow for direct observation and study of niosome-cell interaction.
In this study, we produced GNs from spin-coated amphiphile films by the thin film hydration method. GNs were formed from dry amphiphile films during hydration. We also studied effect of hydration temperature and duration on the yield of niosoms. The optimal hydration condition that maximizes noisome yield was hydration duration of 25 minutes at 70-75°C.
As a subject for future study, we also suggest an experimental method to investigate and monitor the interaction of GNs with the membrane of biological cells in order to gain insights on how niosomes deliver drugs.

Keywords: drug delivery systems, giant niosomes, spin-coated amphiphile films, thin film hydration method, yield of niosomes, lipophilic and hydrophilic drugs, biological membrane

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I also thank Celine who has developed giant vesicle preparation method and has written a good PhD thesis so that I could continue part of her research work. I am thankful to Mehrnaz who contributed to the thesis by doing the cell culture and helping me to continue the project further.
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Maryam Homaei, Gothenburg, September 2016

Contents

List of Figures

List of Tables

1
Introduction

Drug delivery systems (DDSs), such as nanoparticles, vesicles and liquid crystals provide different benefits, which include improving efficacy and biocompatibility of already established drugs, help to reduce the need for developing new drugs. In addition, recent advances in biotechnology introduce new pharmateutical agents which are macromolecules such as proteins, peptides, oligonucleotides, and plasmids. Challenges associated with these drugs such as toxicity, bioavailability and stability can also be solved with formulation of a suitable DDS [1].
Among different kind of DDSs, the success achieved with liposomal systems in drug delivery motivated further research to investigate other possibilities for developing new vesicular DDSs. Non-ionic surfactant-based vesicles (niosomes) were among the first systems which have been studied as a DDS. Niosome is a class of molecular clusters formed by self-assembly of non-ionic surfactant in an aqueous phase. For the first time they were used in the cosmetic industry in the seventies, and since then their potential as a DDS have been investigated.
Most of the studies on niosomes have considered niosomes in nano or sub-micrometer size [4]. These studies show that niosomes can encapsulate various drugs and improve their biocompability and efficacy. However the mechanism by which niosomes deliver drug compounds to the biological cells has not yet been fully understood. In order to improve our understanding of niosome interaction with biological cells, we prepared giant niosomes (GNs), which can be directly observed using standard optical interrogation schemes. In this project a novel and easily applied method for producing the GNs (in micrometer size) is introduced. This project aimed to develop a thin film hydration method to form GNs from spin-coated amphiphile films and to optimize the yield of niosomes. As potential future work, we also suggest an experimental method to investigate mechanisms by which GNs cross membranes of biological cells in order to deliver and release drug compounds.

2

Theory

Numerous drug delivery systems (DDSs) have been formulated using amphiphilic molecules to encapsulate and protect well-established traditional drugs (such as insulin, Tenoxicam [12] and Paclitaxel [15]). These DDSs improve efficacy and biocompatibility of the drugs. In this chapter, first non-ionic surfactant based vesicles, named niosomes, are introduced as a DDS and parameters affecting their self assembly and formation are discussed. In addition several basic definitions required for the study of interaction of niosomes with cell membranes (such as cell membrane and ion channels) and also the imaging technique which is used for this study are introduced.

2.1 Amphiphiles

Amphiphilic molecules have two different parts with different solubility, a hydrophilic (water-soluble) head group and a hydrophobic (organic-soluble) part. In an aqueous solution, amphiphilic molecules self-assemble in a way that the hydrophilic part of molecules has maximum contact with solvent molecules while the hydrophobic part is shielded. This property leads to self assembly of amphiphilic molecules into variety of structures (like micells, lamellae, vesicles, etc) based on the repelling and coordinating forces between hydrophilic/hydrophobic parts of the amphiphilic molecules and the solvent molecules. Some self-assembled amphiphilic structures are shown in figure 2.1.

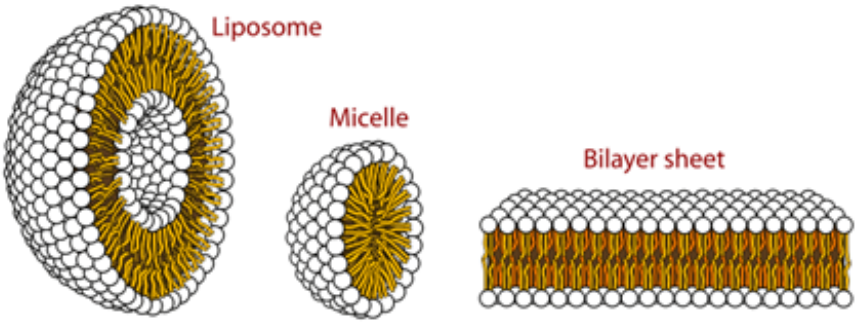


Figure 2.1: Representative images of a bilayer vesicle (liposome), a micelle and a bilayer sheet made from amphiphilic molecules in aqueous solution [5].

2.2 Niosomes

Niosomes are lamellar vesicles composed of non-ionic surfactants and cholesterol. In comparison with liposomes, they offer some advantages, such as lower cost, greater chemical stability and longer storage time. They also have high compatibility with biological systems and low toxicity due to their non-ionic nature. These advantages, together with the ability to encapsulate both hydrophobic and hydrophilic drugs, put the niosomes into the focus of attention as a potential DDS. Figure 2.2 shows a niosome prepared from sorbitanmonostearate (Span-60) in an aqueous solvent.

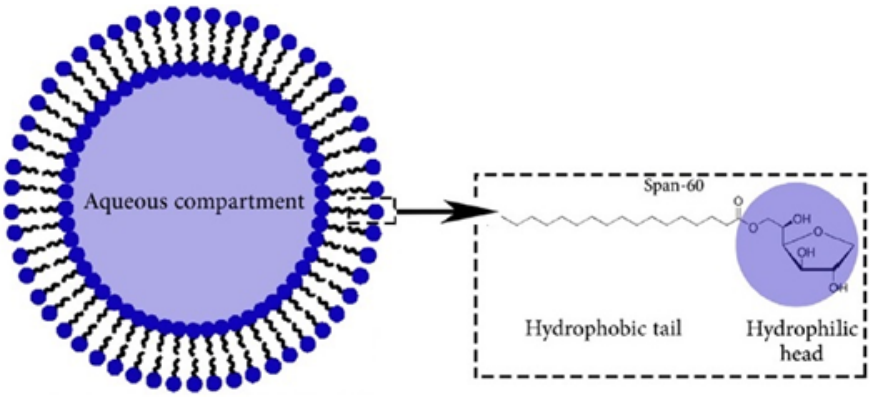


Figure 2.2: Schematic representation of niosome prepared by sorbitanmonostearate (Span-60) [4].

2.3 Classification and preparation of niosomes

Niosomes can be divided into different categories according to their size (small and large) and the number of membrane bilayers (unilamellar and multilamellar) as follows [2, 4]:

- Small unilamellar vesicles, or SUV (20-50 nm in diameter),
- Large unilamellar vesicles, or LUV (50-1000 nm in diameter),
- Multilamellar vesicles, or MLV (1-20 µm in diameter).

In this work, niosomes are classified according to their size (small, large and giant) or the number of membrane bilayers (unilamellar, oligolamellar, multishells and multilamellar). We classify niosomes in three groups based on their size:

- Small vesicles, or SV (20-50 nm in diameter),
- Large vesicles, or LV (50-1000 nm in diameter),
- Giant vesicles, or GV (1-20 µm in diameter).

Given that we are interested to produce giant niosomes, therefore we further divide giant vesicles into three groups based on lamellarity:

- Giant oligolamellar vesicles, or GOLV,
- Giant multishell vesicles, or GMSV,
- Giant multilamellar vesicles, or GMLV.

Various types of niosomes based on this classification are shown in figure 2.3. Some examples of each type of GNs are shown in figure 2.4.

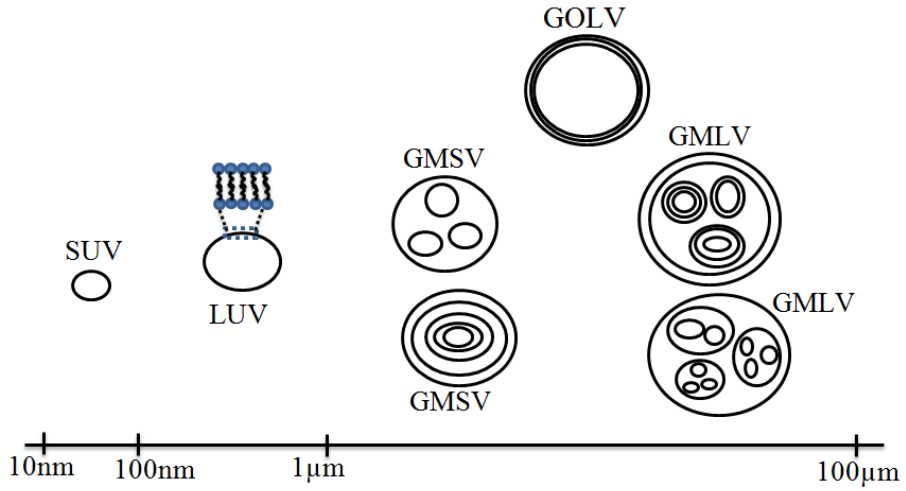
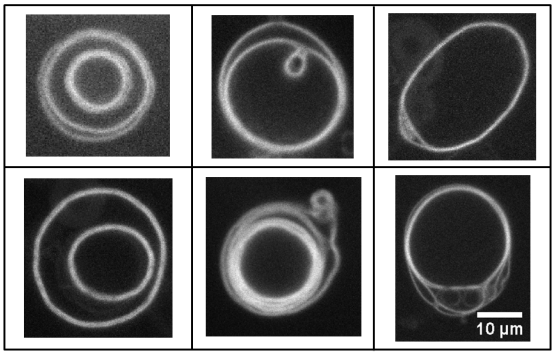
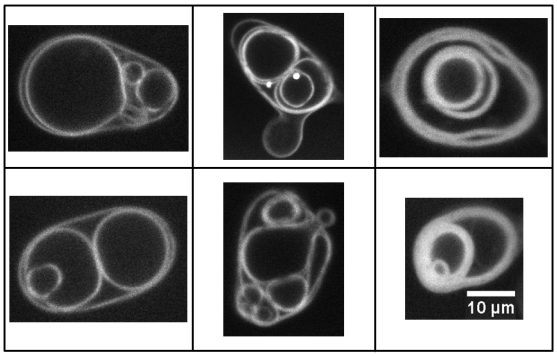


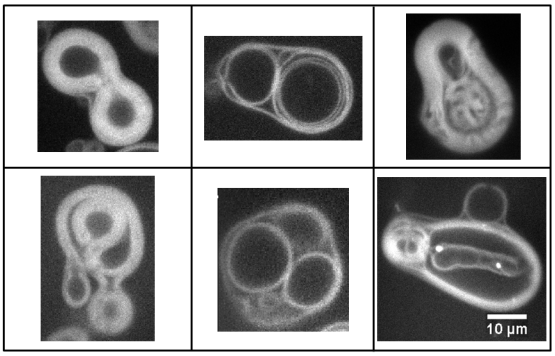
Figure 2.3: Different type of niosomes according to their size and number of membrane bilayers.



(a) Giant oligolamellar niosomes



(b) Giant multishell niosomes



(c) Giant multilamellar niosomes

Figure 2.4: Various types of GNs based on lamellarity.(a) Oligolamellar niosomes: circles (b) Multishell niosomes: like onion, (c) Multilamellar niosomes: packed onion.

There are many protocols for the preparation of niosomes as controlled DDSs and gene therapy vectors [12, 4]. Given that the preparation method affects the size

and lamellarity of niosomes, a proper method should be chosen according to their application. For example, MLVs are highly suitable as drug carrier for encapsulation of lipophilic compounds. They are also suitable for ocular drug delivery because of their micron size. In contrast, LUVs having a high aqueous/lipid compartment ratio, are mainly used for encapsulation of hydrophilic compounds and their sub-micron size makes them suitable for transdermal delivery and intravenous administration [2]. Examples of MLV and LUV preparation methods are described below.

The thin film hydration method is a simple and widely used method to produce MLVs [4]. In this method, surfactants and other additives (such as cholesterol) are dissolved in a volatile organic solvent (such as chloroform) in a round bottomed flask. Then, by evaporation of the solvent at room temperature using a rotary evaporator, a thin layer of surfactant mixture is deposited on the inside wall of the flask. The dried thin film of surfactant is hydrated above the phase transition temperature of the surfactant by adding an aqueous buffer. The MLVs are produced during hydration process. Gentle mechanical shaking (for about one hour) can increase the dispersion of MLVs. Figure 2.5 shows the schematic of the protocol for MLV preparation through thin film hydration method. This method has been used in many different applications such as preparation of niosomes as carriers for delivery of insulin [17], glucocorticoid [18] and doxorubicin [19].

Temperature of hydration medium has an effect on the self-assembly of surfactant into niosomes and leads to different shape, size and affect the yield of niosomes. Usually the hydration temperature should be above the gel-liquid phase transition temperature of system [1, 12, 22]. The volume of the hydration medium and duration of the hydration of the surfactant film has also an effect on vesicle structure and yield [22].

The reverse evaporation method is commonly used for preparation of LUVs. In this method, surfactant and other additives are dissolved in an organic solvent. Then, an aqueous phase containing drug is added to the organic phase and the mixture is sonicated in order to form an emulsion. This is followed by slow removal of the organic phase using a rotary vacuum evaporator which leads to production of LUVs. This method has been used for the preparation of niosomes as carriers for delivery of diclofenac sodium [20] and naltrexone [21].

The size of niosomes has a major impact on their in-vitro and in-vivo performance. All the surveyed experimental methods consisting of a hydration of surfactant mixture produces niosomes in size range of micrometers. Since size reduction of the niosomes to the nanoscale increases the dissolution of the drug and improve bioavailability, these methods should be combined with an optional size reduction method. Sonication is the most common size reduction method. For example, SUVs can be obtained by sonication of the MLVs prepared by the thin film hydration method [2, 7, 4].

One of the aims of this work is to study the mechanism by which niosomes interact with biological cells and to investigate its uptake and retention properties. This inspired us to generate giant niosomes (micrometer size) to directly observe their interaction with biological cells, and to study this mechanism.

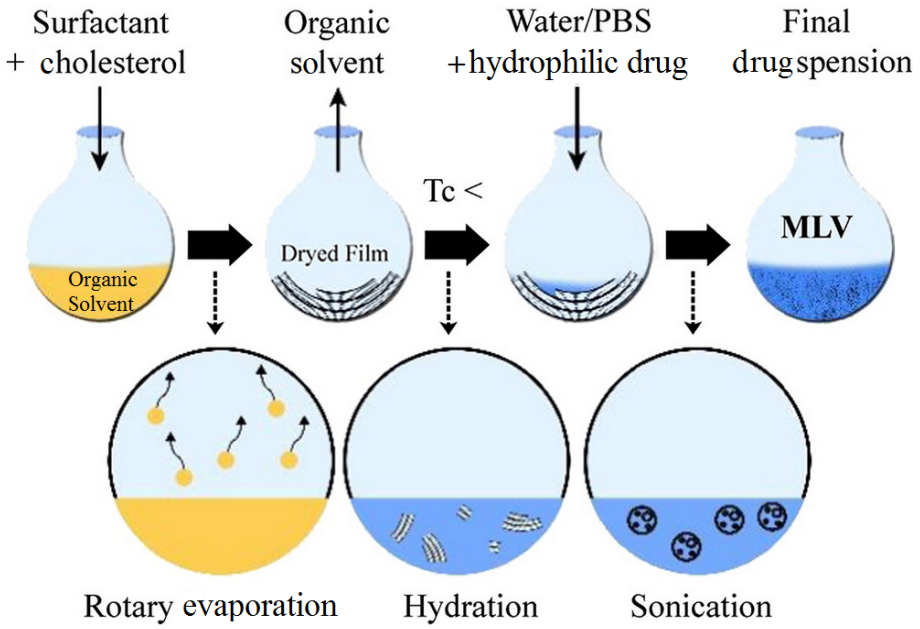


Figure 2.5: Protocol for MLV preparation via the thin film hydration method [4].

2.4 Niosomes versus liposomes

The main role of vesicular DDSs is to overcome delivery issues such as bioavalibability and solubility associated with well-established drugs. Among different types of vesicular systems, liposomes and noisomes have received great attention in drug delivery. Despite of some similarities, niosomes offer several advantages over liposomes. Both niosomes and liposomes have the ability to entrap drugs (lipophilic, hydrophilic and amphiphilc drugs) into their vesicular membrane, or the aqueous compartment. They can increase the bioavailability and efficacy of the drug by protecting the drug from the biological environment and release the drug in a more controlled manner, delaying clearance from the circulation. Optional functional groups on their hydrophilic heads lead to great ease of surface formation and modification. Thus, they can be used as targeting drug delivery carrier. The characteristics of niosomes and liposomes, such as lamellarity, size and fluidity and their performance, can be controlled by changing their formulation and preparation methods.

In comparison with liposomes, niosomes have higher chemical stability and longer storage time. Because of the non-ionic nature of niosomes, they are more biocompatible, show low-toxicity, and low-immunogenicity. Access to raw materials is convenient and cheap, and they do not require any special conditions, such as low temperature or inert atmosphere, and precautions during preparation or storage stages.

Like liposomes, niosomes are at the risk of aggregation, fusion, drug leakage, or hydrolysis of encapsulated drugs during storage [4].

2.5 Parameters affecting formation of the Niosomes

2.5.1 Non-ionic surfactant structure

Niosomes are formed by self-assembly of non-ionic surfactants in an aqueous solvent. Non-ionic surfactants are belonging to the class of surfactants which have no charge on the hydrophilic head groups. The most common non-ionic surfactants for preparing niosomes are shown in figure 2.6. The vesicle forming ability of any surfactant depends on the hydrophilic–lipophilic balance (HLB), critical packing parameter (CPP) values, and gel-liquid transition temperature (T_C), which are explained below.

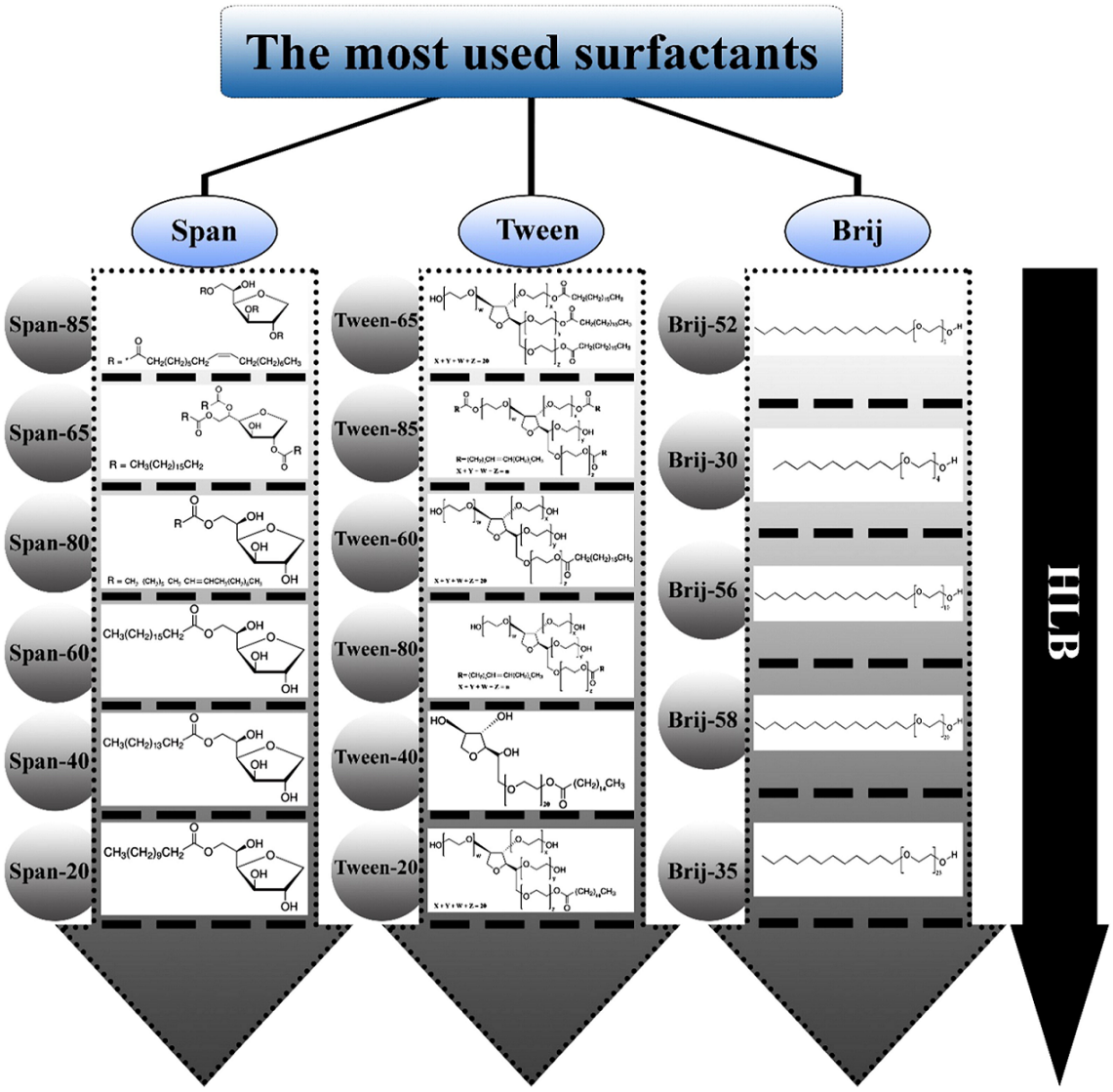


Figure 2.6: The most common surfactants for niosome preparation. The surfactants are are listed with their hydrophilic-lipophilic balance (HLB) in descending order [4].

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2.5.1.1 Hydrophilic-lipophilic balance (HLB)

The hydrophilic-lipophilic balance of a surfactant is a dimensionless parameter which varies between 0 to 20 (for non-ionic surfactants) and is applied as a guide for selecting the appropriate surfactant. The formula below is suggested by Griffin in 1949 for non-ionic surfactants but latter it is extended for ionic-surfactant as well.

$$HLB = \frac{20M_h}{M} \tag{2.1}$$

where M_h is the molecular mass of the hydrophilic portion of the molecule, and M is the molecular mass of the whole molecule, giving a result on a scale of 0 to 20. An HLB value of 0 corresponds to a completely lipophilic/hydrophobic molecule, and a value of 20 corresponds to a completely hydrophilic/lipophobic molecule. The HLB scale for predicting the non-ionic surfactants is shown in figure 2.7. The HLB range of different types of Tweens and Spans is shown as an example. It shows that Spans are more lipophilic while Tweens are more hydrophilic [8, 9].

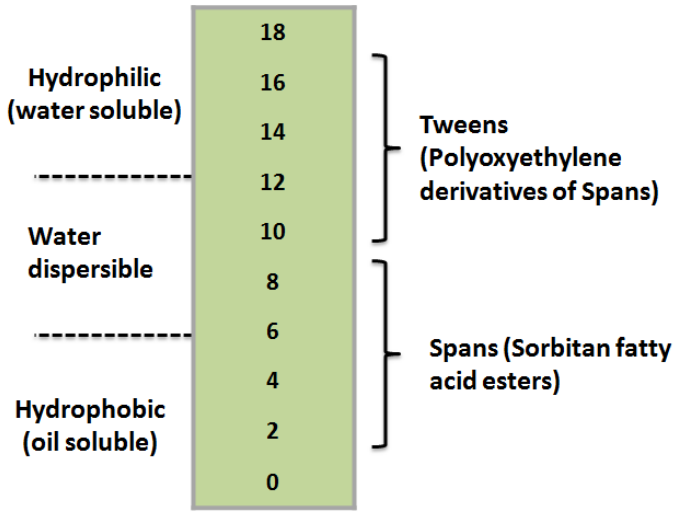


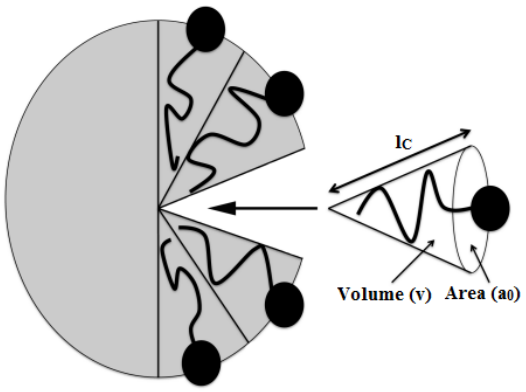
Figure 2.7: HLB scale for Spans and Tweens.

2.5.1.2 Critical packing parameter (CPP)

The molecular geometry of the amphiphiles affect the self-assembly of them and leads to different structures. For predicting the shape of the amphiphilic aggregates, CPP, a dimentionless scale of surfactant is used and defined as below:

$$CPP = \frac{v}{a_0 l_C} \tag{2.2}$$

Where v is the volume of the hydrocarbon portion, a_0 is the effective area of the head group and l_C is the length of the hydrophobic tail. The type of vesicle can be predicted by estimating the CPP value of a certain amphiphile. Different types of amphiphilic aggregates related to different ranges of CPP are shown in figure 2.8.



(a)

Critical Packing Parameter $v/a_0 l_c$	Critical Packing Shape	Liquid Crystal Structure
1/3	Cone	Spherical Micelles
1/3 - 1/2	Truncated Cone	Cylindrical Micelles
1/2 - 1	Truncated Cone	Flexible Bilayers, Vesicles
1	Cylinder	Planar Bilayers
>1	Wedge	Inverted Micelles

(b)

Figure 2.8: Critical packing parameter for amphiphilic molecules. (a) Critical packing parameters for amphiphilic molecules and the resulting critical packing shapes and (b) Resulting liquid crystal structures [11].

2.5.1.3 Gel-liquid phase transition temperature (T_C)

The bilayers of the vesicles are either in so-called liquid phase or gel phase, depending on temperature, type of surfactants or other agent additives. In the liquid phase, the alkyl chains of bilayers are more disordered while in gel phase, the bilayers have well ordered structures [12]. The surfactant and lipids are characterized by the gel-liquid phase transition temperature. This parameter has direct effect on the entrapment efficiency of the vesicles. As an example, Span 60 with high T_C (53°C) exhibits good entrapment efficiency [4]. In this work, Span 60 has been chosen for preparing niosomes, because it has a high phase transition temperature (53°C), so it is solid at room temperature. In addition, the proper HLB (4.7) and CPP values make it a good choice to form bilayer vesicles of desired size [10, 13].

2.5.2 The encapsulated drug

An amphiphilic drug can influence the formation of the vesicles by affecting charge and rigidity of the niosome bilayer. As an example, the interaction of a drug with surfactant head groups might enhance the charge and creates mutual repulsion between surfactant bilayers, which leads to vesicle size increase [1]. Also, sorbitan monostearate (Span 60) niosomes, containing dicetyl phosphate groups, forms homogenous dispersions encapsulating 5(6)-carboxyfluorescein. This system forms an aggregated dispersion when the amphipathic drug doxorubicin (DOX) is used [12]. Table 2.1 shows other observed effects of drugs.

Table 2.1: Effect of the nature of drug on the formation of niosomes [1].

Nature of the drug	Leakage from the vesicle	Stability	Other propeties
hydrophobic drug	decreased	increased	improved trans-dermal delivery
hydrophobic drug	increased	decreased	-
amphiphilic drug	decreased	-	increased encapsulation, altered electrophoretic mobility
macromolecule	decreased	increased	-

2.5.3 Stability-enhancing additives

Often, in order to prepare stable niosomal DDSs, an additive agent is required. Among different additives, which are typically used in drug delivery studies, cholesterol is the most common and important agent. The position of cholesterol in the bilayer of a vesicle, and its hydrogen bond with the hydrophilic head group of a surfactant, Span-60, is shown in figure 2.9 [4].

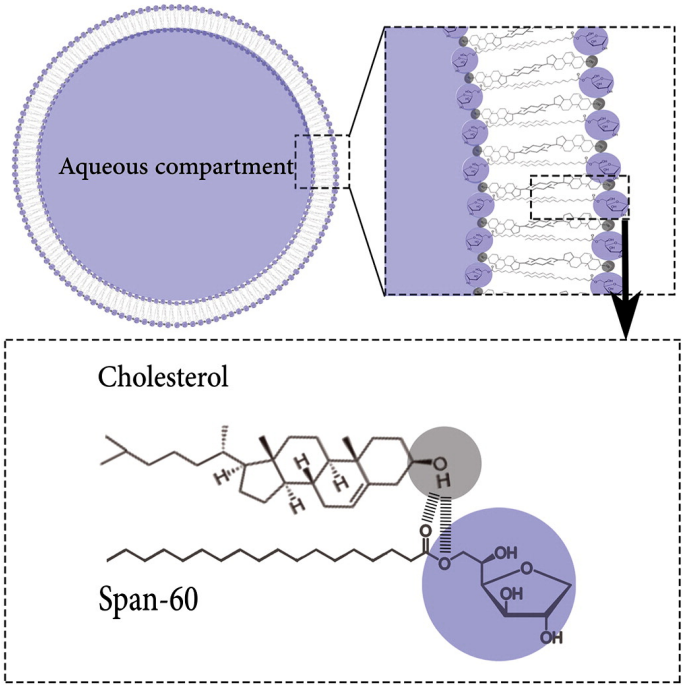


Figure 2.9: Cholesterol insertion in a niosome bilayer [4].

The cholesterol content tends to affect the important vesicular properties such as entrapment efficiency, storage time, permeability and stability. Therefore in order to get desire properties, the content of cholesterol should be optimized [4].
In cases where a mixture of surfactants and additive agents are used in the for-

mulation of niosomes, the whole components should been taken into account for calculating the CPP value. Considering this fact, adding additive agents to the membrane will change the value of CPP which can lead to the self assembly of different structures [12].
For surfactants with a HLB lower than 6, cholesterol increases the stability of the vesicles while, for a higher HLB, cholesterol is required to form a bilayer vesicle [4]. Cholesterol provides greater stability and rigidity to the surfactant bilayer by increasing the gel-liquid transition temperature (T_C) of the vesicle, and it also reduces the leakage of drugs from niosomes [4, 14]. Studies show that Span 60 can form vesicles in the absence or presence of cholesterol, because of its proper HLB and CPP values. Increasing the cholesterol content in this case can increase the rigidity of the niosomes, which leads to an increased encapsulation efficiency [13, 10].

2.6 Niosome-cell membrane interaction

The transport of drug delivery systems (DDSs) across the cell membrane is a complex biological process. Lots of studies have been performed to investigate the mechanism by which the internalisation of the drug to a cell occurs. In many of these studies giant liposomes have been used as model biomembrane systems to mimic some behavior of the cell membrane and to study their interaction with drugs or DDSs [23, 24]. However the mechanism of interaction of niosomes with cell membranes is not completely understood, and further research has to be done. Here we suggest a method by which giant niosomes can be used to address the mechanism by which niosomes interact with cell membranes in order to deliver drug compounds.

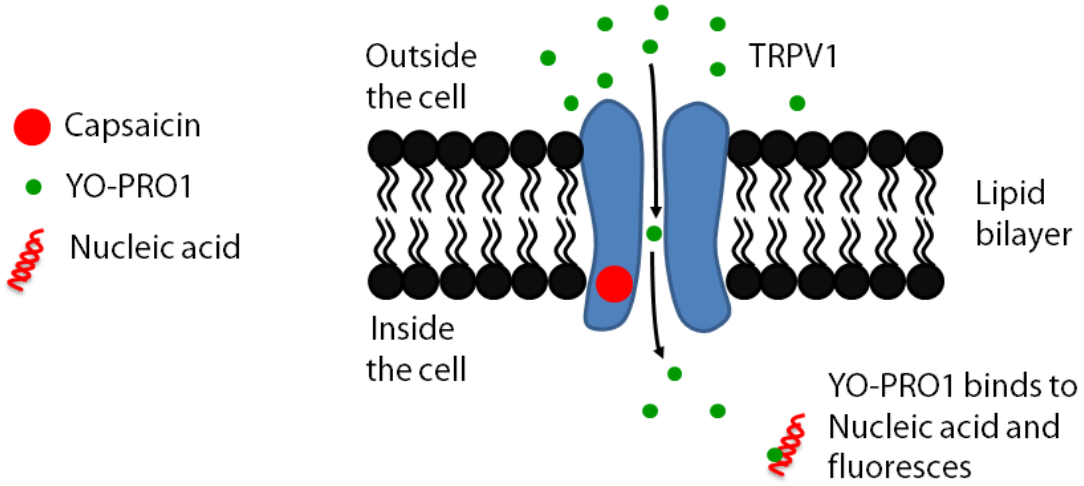


Figure 2.10: The TRPV1 ion channel activated by capsaicin allows passage of YO-PRO1 molecules. The YO-PRO1 passes through the channel and binds to nucleic acid molecules inside the cell, leading to significant increase in the quantum yield of the dye.

One way to study this interaction is to prepare fluorescently labeled capsaicin-loaded giant niosomes, and monitor their interactions with Chinese hamster ovary (CHO)

cells. The CHO cells express TRPV1 ion channels, and are surrounded by the nucleotide binding dye, YO-PRO1. Capsaicin (a small hydrophobic drug-like molecule) is entrapped in hydrophobic part of giant niosomes. Upon interaction of niosomes with the cell membrane, the capsaicin can diffuse through the cell membrane, and reach its intracellular ligand-binding site on TRPV1, leading to TRPV1 activation and cation permeation [29] (see figures 2.10 and 2.11). YO-PRO1 molecules can then pass through the ion channels and bind to the nucleic acid molecules inside the cells leading to an increase in the quantum yield of YO-PRO1 molecules, which can be followed by fluorescence microscopy. Niosomes are loaded with Nile Red (a lipophilic stain) to visualize the niosome-cell membrane interactions, also using fluorescence microscopy.

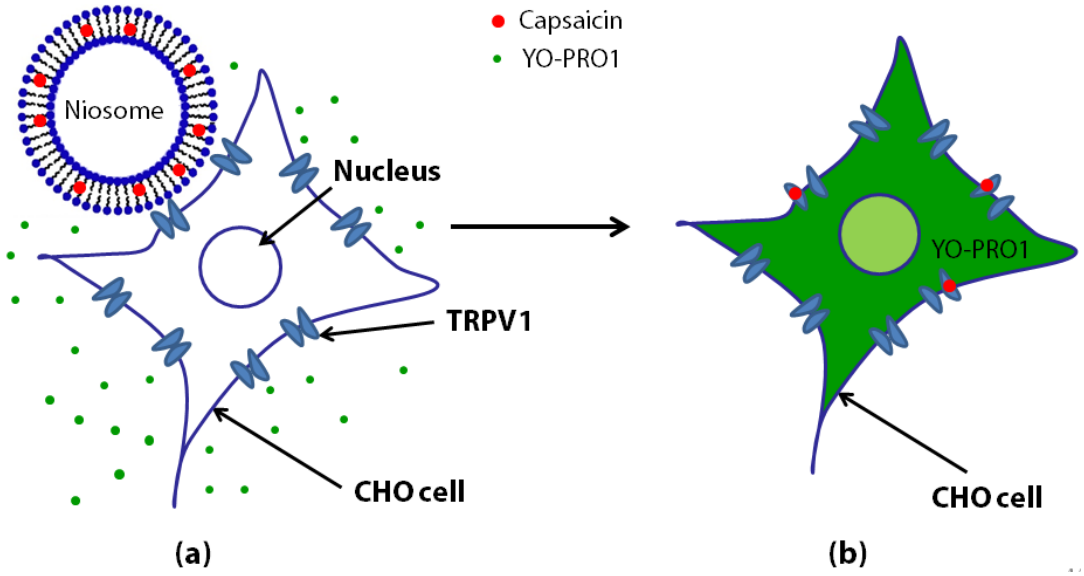


Figure 2.11: Niosome- cell interaction (a) Capsaicin-loaded giant niosomes are introduced to the cells surrounded with YO-PRO molecules. (b) After niosome-cell interaction, the capsaicin diffuse through the cell membrane and activate ion channels and make it permeable to YO-PRO. Then YO-PRO1 molecules can pass through the TRPV1 and bind to nucleic acid molecules inside the cell and floureces.

2.7 Microscopy

There are different optical microscopy techniques for visualisation of samples which are too small to be seen by the naked eye. In this work, laser scanning confocal microscopy have been used to visualize GNs.
One of the main characteristics of an optical microscope is its lateral resolution. The lateral resolution, i.e., the limit of how small objects can be and still be distinguishable, is determine by the Reyleigh criterion.

$$R = 0.61\frac{\lambda}{NA} \tag{2.3}$$

where λ is the wavelength of the radiation used to visualize the sample and NA is

the numerical aperture of the objective lens, which is a measurement of the capacity of the objective to collect light. It is given by:

$$NA = nsin\theta \tag{2.4}$$

where n is the refractive index of the medium between the objective and sample and θ is the half-angle of the maximum cone of light that can enter or exit the objective lens. The refractive index n is 1.0, 1.33 and 1.56 for air, water and oil, respectively. Using an oil immersion objective in combination with visible light (e.g. 400 nm), a resolution of ($\sim$ 200nm) can be obtained [2].

2.7.1 Fluorescence

Fluorescence is re-emission of absorbed light by certain molecules. These so-called fluorophores or fluorescent molecules, absorb light of a shorter wavelength, and emit light of a longer wavelength. The energy of a photon that is absorbed by a fluorophore is inversely proportional to its wavelength, and can be described by

$$E = \frac{hc}{\lambda} \tag{2.5}$$

where h is Planck's constant, c is the speed of light and λ is the wavelength of light. The processes of absorption and emission of light are conveniently illustrated by a Jablonski diagram. A simplified Jablonski diagram is shown in figure 2.12. By absorption of a photon, a fluorescent molecule at its singlet ground state (S_0)

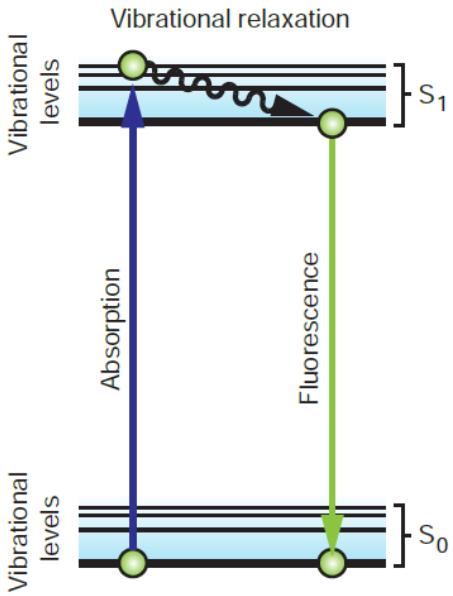


Figure 2.12: A simplified Jablonski diagram depicting absorption of light by a molecule in the singlet ground state, S_0, followed by vibrational relaxation and fluorescence [27].

can be excited to a higher vibrational level of the first excited electronic state (S_1).

The excited molecule can return to its ground state, S_0 by both radiative and non-radiative energy transfers. One of the non-radiative processes is vibrational relaxation, in which the fluorophore in higher vibrationl level rapidly relaxes to the lowest vibrational level of S_1, losing energy as heat. Fluorescence emission occurs from the lowest vibrational level of S_1 to the any vibrational level of singlet ground state, S_0. Because of energy loss during vibrational relaxation, the emitted photon has a longer wavelength than the absorbed photon.
Fluorescence is extensively used in life science, mainly to track and analyse the biological structures which are labeled by different fluorophores. Fluorophores that have been used in this project are NBD-cholesterol, which is an environment-sensitive probe that localizes in the niosome membrane's interior and helps to track and investigate niosome transport processes as well as niosome interactions with the biomembrane; and YO-PRO1, a dye that can pass through the activated TRPV1 ion channels and fluoresces after binding to nucleic acid molecules inside the cells.

2.7.2 Laser scanning confocal microscopy

A laser scanning confocal microscope uses the principle of fluorescence excitation to obtain high resolution images with depth selectivity from fluorescent specimens.
The beam path way in a confocal microscope is shown in figure 2.13. First, the laser

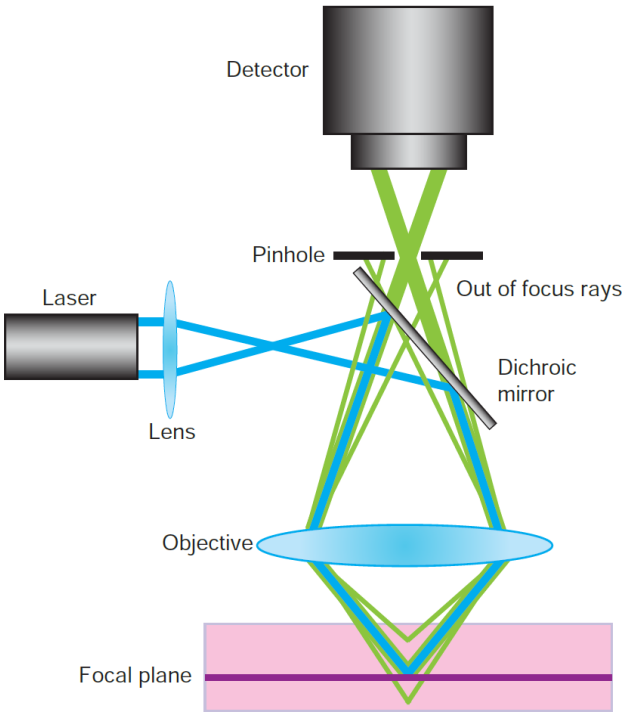


Figure 2.13: Schematic overview of the beam pathway in a confocal microscope. Only light emitted from the focal plane can pass through the pinhole and reach the detector, whereas out-of-focus light (thin green lines) is blocked by the aperture wall [27].

light is reflected by a dichroic mirror and then focused by an objective lens onto

a small focal volume of specimen. The emitted light is collected by the objective and passed through the dichroic mirror. Out-of-focus light from the area above and below of the focal plane is eliminated. Finally the intensity of light measured by a photodetection device, transforming the photons into an intensity value is recorded by a computer. For creating 2D images, the area of interest in specimen is illuminated and laterally scanned in a rectangular pattern by a laser beam. The emitted light at each point is measured and an image is built up in a computer.

3

Methods

Niosomes can be prepared by various methods such as ether injection, revers phase evaporation, sonication and thin film hydration [4]. The preparation method can affect the size, shape, entrapment efficiency and drug release. In this study, a thin film hydration method for formation of giant oligolamellar niosomes is introduced. These cell-size vesicles allow us to directly observe and monitor the interaction of niosomes with biological membranes, their uptake into cells, and retention. Here we also suggest an experimental method to study this interaction in the future.

3.1 Thin Film Hydration Method

In this study, we produce GNs from spin-coated amphiphile films by the thin film hydration method. Figure 3.1 shows sequential steps of preparation of GNs. First, a glass coverslip is covered with SU-8 photoresist by spin-coating. The SU-8 surface is used as a substrate for adhesion and formation of an amphiphile film. In the next step, amphiphile solution (containing Span 60, cholesterol and NBD-cholesterol in chloroform) is deposited on the SU-8 surface and is distributed by spin-coating. Then, a rectangular frame made of polydimethylsiloxane (PDMS) is positioned onto the amphiphile spin-coated surface via PDMS bonding, defining a chamber. This step is followed by heating the film in a convection oven for one hour removing remaining traces of the chloroform. Subsequently, the chamber is filled with an aqueous solution (HEPES buffer) and hydrated above the gel-liquid transition temperature. Finally giant niosomes are formed by hydration of amphiphile film. Each step is described in details in the following sections.

3.1.1 SU-8 Surface fabrication

The SU-8 is a novolac-epoxy resin, originally developed for microfabrication and micropaterning in microelectronic industry. In this study, SU-8 is used to modify the surface chemistry of the glass coverslip to form spin-coated amphiphile film.
First, a 10 volume (%) SU-8 solution in cyclopentanone is prepared. Then SU-8 solution is spin-coated on borosilicate cover slips at 2000 rpm for one minute (figure 3.1.(b)). The cover slips are dried for three minutes at 150°C to remove traces of solvent, and then they are illuminated with 254 nm light from a UV-source for four minutes at 12-15 mW/cm^2. Finally the cover slips are baked at 150°C for 10 minutes.

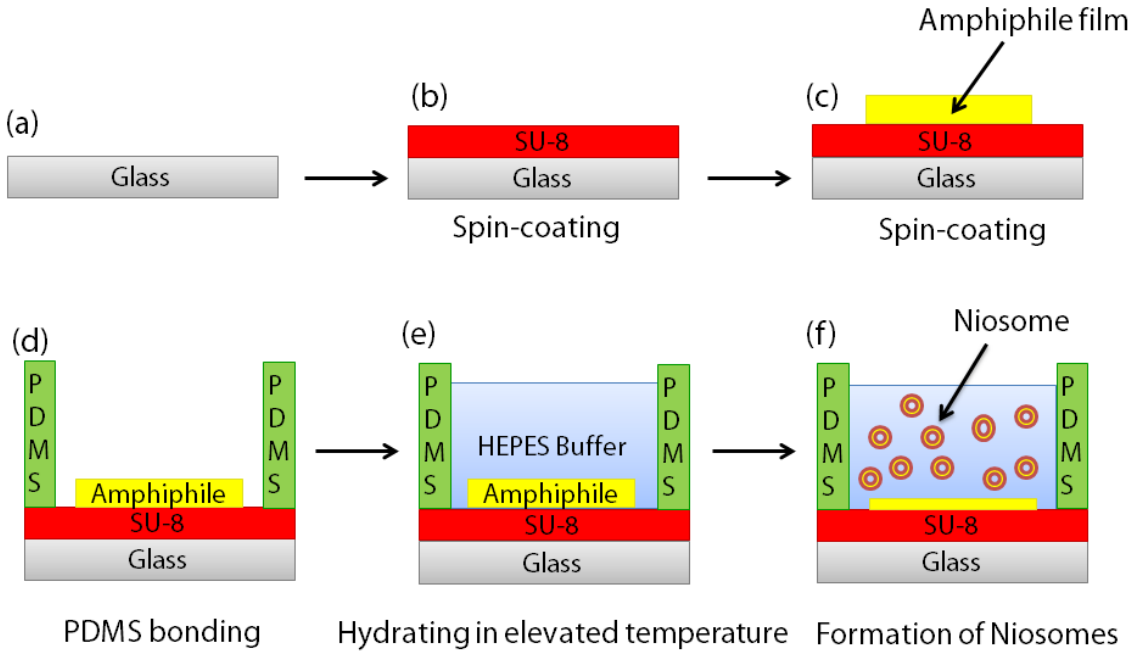


Figure 3.1: Sequential steps of formation of GNs, applying the thin film hydration method (a) Glass cover slip (b) Spin-coating of SU-8 solution onto the glass coverslip (c) Spin-coating of amphiphile solution onto SU-8 surface (d) Positioning of a PDMS frame onto the SU-8 and amphiphile spin-coated surfaces (e) Filling the chamber with HEPES buffer (f) Hydrating the amphiphile film to form giant niosomes.

3.1.2 Amphiphile film preparation

An amphiphile solution containing 80% Span 60, 19% Cholesterol and 1% NBD-cholesterol (used to visualize the membrane of niosomes) in chloroform is prepared. Then 10μl of the amphiphile solution is deposited onto a SU-8 surface (figure 3.1.(c)). Subsequently, the amphiphile film is spun using a spin-coater at 1000 rpm for 5 minutes. After formation of this film, a rectangular frame made of polydimethylsiloxane (PDMS) is positioned onto the amphiphile spin-coated surface via PDMS bonding, defining a chamber (figure 3.1.(d)). Finally the film is dried in a convection oven for 1 hour to remove remaining traces of the solvent.

3.1.3 Formation of giant niosomes

In order to form giant niosomes, the chamber was filled with 1 ml of HEPES buffer solution with pH of 7.4 (figure 3.1.(e)) and the amphiphile film is hydrated in a convection oven above the phase transition temperature of Span 60. Niosomes are formed during the hydration (figure 3.1.(f)). To investigate the effect of hydration duration and hydration temperature on niosome formation, the amphiphile film is hydrated for various periods of time (10, 15, 25 and 35 minutes) and various temperatures (55, 60, 65, 70, 75, 80, 85 and 90°C).

3.2 Niosome-Cell interaction

In order to prepare capsaicin-loaded GNs, an amphiphile solution is prepared as described in section 3.1.2 (containing Span 60, Cholesterol, and Nile Red in chloroform) and also contains additional amount of capsaicin with the mass ratios 99:1. The same experimental steps for preparation of giant niosomes are repeated with new amphiphile solution (containing capsaicin) and the capsaicin-loaded GNs are formed. The hydrophobic capsaicin molecules are encapsulated in the bilayer membrane of niosomes. The capsaicin-loaded niosomes are aspirated into a glass pipette (figure 3.2.(a)) and injected close to the CHO cells (figure 3.2.(b)). The niosome-cell interactions are studied using laser scanning confocal microscopy.

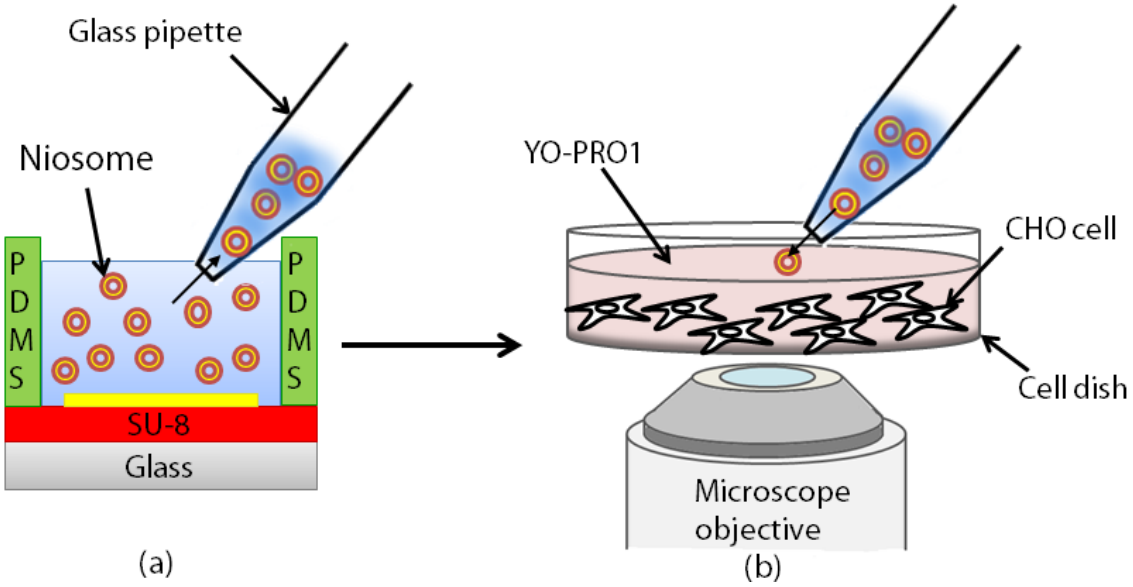


Figure 3.2: Transfer of capsaicin-loaded niosomes to the cell dish (containing CHO cells surrounded by YO-PRO1 molecules) using a glass pipette.

3.2.1 Imaging

A confocal laser scanning microscopy system (Thorlabs CLS system), equipped with a Galvo:Resonant scanner and high-sensitivity GaAsP PMTs is used for acquisition of micrographs. The micrographs are recorded into ThorImageLS software (Thorlabs Inc, New Jersey, U.S.A.). The scanner unit is mounted onto a Leica DMIRB microscope equipped with an oil immersion 63× NA 1.47 Leica HCX PL APO objective.
NBD-cholesterol is excited at 488 nm using a Coherent Sapphire 488 LP laser (Coherent Inc., CA, U.S.A.) and emission was between 500-550 nm.

4

Results and discussion

The aim of this study is to develop a novel method for generating GNs from spin-coated amphiphile films and to optimise the yield of niosomes. The important parameters that affect the formation and the yield of niosomes are the type of surfactant, additives, the drugs (see section 2.5), hydration temperature and hydration duration (for definitions see section 3.1.3). The previous work by Billerit *et al.* has shown that amphiphile solution containing 80% Span 60, 19% Cholesterol and 1% NBD-cholesterol in chloroform forms GNs, using the thin film hydration method [2]. However, the effect of hydration temperature and duration on the formation and yield of niosomes is not clear. In this work we provide details on these parameters. In particular, the formation of niosomes was tested for various hydration times at different temperatures, as shown in table 4.1.

Table 4.1: Parameters affecting formation of niosomes.

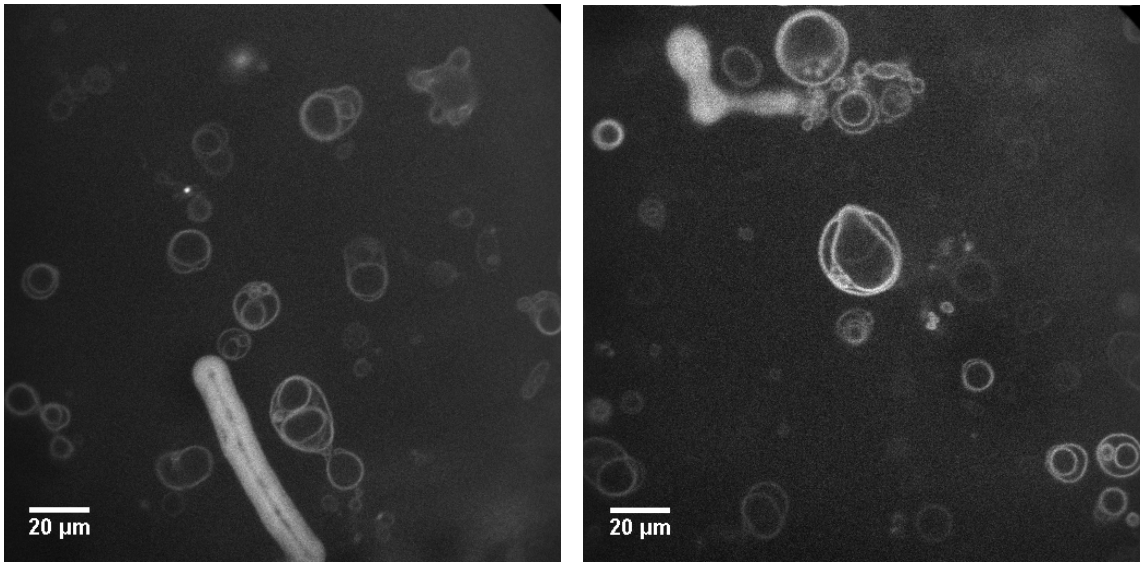
Hydration time (min)	10	15	25	-	-
Hydration temperature (°C)	55	65	70	75	80

The experimental conditions shown in table 4.1 were investigated for amphiphile solution containing Span 60, Cholesterol, and NBD-cholesterol. For each experimental condition, the experiment was performed in triplicate, to remove effects of sample variances. Laser scanning confocal microscopy was used to capture high resolution images of the niosomes. For each sample, tens of images have been taken. Figure 4.1 shows two confocal laser scanning micrographs with the same microscope acquisition setting, for 25 minutes hydration duration at 80°C as an example. Figure 4.2 also shows two confocal laser scanning micrographs for 25 minutes hydration duration at 90°C as another typical example.
Note that GOLVs, GMLVs and GMSVs were present in each sample. In comparison with GMLVs and GMSVs, GOLVs have simpler shapes which make them suitable for niosomal application to biological cells. Therefore we were interested in maximizing the percentage of GOLVs in each sample. The percentage of GOLVs, η, can be calculated as

$$\eta = \frac{n_1}{n_1 + n_2} \times 100\% \tag{4.1}$$

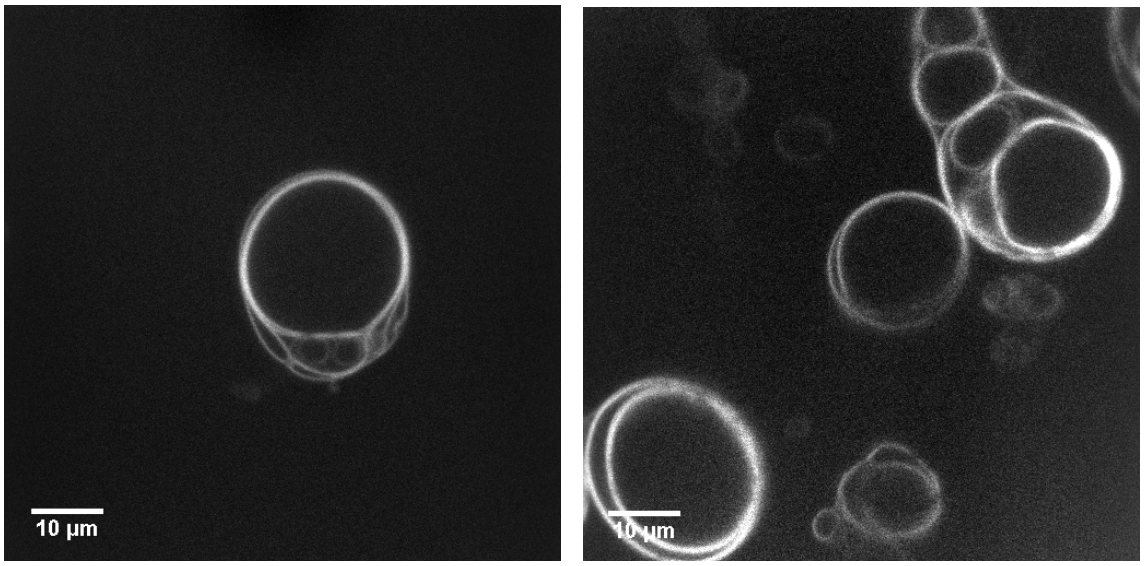
where n_1 is the number of GOLVs and n_2 is the number of both GMSVs and GMLVs in each sample. We estimate n_1 and n_2 as their average over three recordings.

The following results are obtained for elevated temperatures of 55°C to 80°C and different hydration duration of 10, 15 and 25 minutes. In order to reveal a relationship between number of niosomes and the hydration conditions, we plotted n_1 and n_2 versus different hydration temperatures for each hydration experiment. Figure 4.3 shows micrographs of niosomes formed under defined conditions.



(a) (b)

Figure 4.1: Confocal laser scanning micrographs of giant niosomes for 25 minutes hydration duration at 80°C.



(a) (b)

Figure 4.2: Confocal laser scanning micrographs of giant niosomes for 25 minutes hydration duration at 90°C.

The charts in figure 4.3.(a) shows the general trend in GOLV formation, indicating

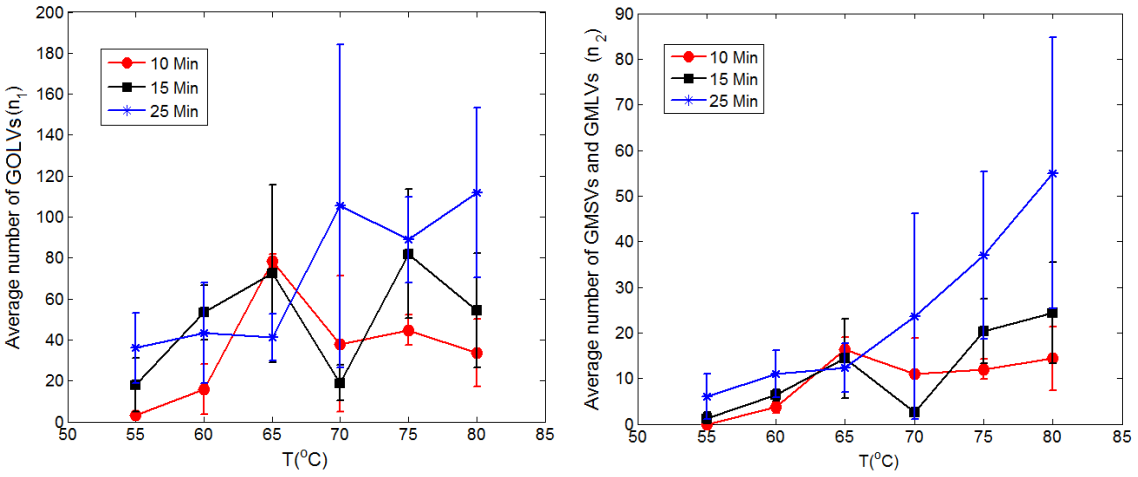


Figure 4.3: Formation of GNs at different conditions. Average number of (a) giant oligolamellar niosomes (GOLVs) and (b) multishell and multilamellar niosomes (GMSVs and GMLVs) per sample as a function of temperature for different hydration duration (The bars represent the standard deviation, 3 experiments per data point).

that increasing hydration temperature and duration leads to an increased number of GOLVs.

For studying the effect of hydration temperature and duration on GMSVs and GMLVs, n_2 is plotted as a function of temperature for different hydration durations (figure 4.3.(b)). It shows n_2 is clearly proportional to hydration duration and temperature, *i.e.* the higher hydration temperature and the longer hydration duration, the larger n_2.

According to figure 4.3.(a), the largest n_1 is obtained for 25 minutes hydration duration at 70°C and 80°C (105.33 and 111.66, respectively). Considering figure 4.3.(b), corresponding n_2 values are 23.66 and 55.00. From equation 4.1, for 25 min hydration duration at 70°C and 80°C, η is 81.6% and 67%, respectively. Overall the optimal conditions are about 25 minutes hydration duration and 70-75°C hydration temperature.

We have also tested experimental conditions by extending hydration time and temperature to 35 minutes and 90°C, respectively (not presented in figure 4.3). We found that by increasing these parameters, η decreases. At a hydration time above 35 minutes, and hydration temperature above 80°C, the sample evaporated.

In summary, we conclude that the optimal experimental condition which can be used for cell studies is hydration temperature of 70-75°C and hydration duration of about 25 minutes.

5

Conclusion

In this project, we have used an easily applied method for generation of giant oligolamellar niosomes (GOLVs) from spin-coated amphiphile films. By this method, we produced fluorescently labeled GNs from amphiphile solution (containing Span-60, Cholesterol and NBD-cholesterol in chloroform), which can be monitored using fluorescence microscopy. We studied the effects of hydration temperature and hydration duration on the yield and lamellarity of niosomes. We found that the optimal experimental condition is hydration duration about 25 minutes and hydration temperature of 70-75°C.
Elucidating the drug delivery mechanism of niosomes will greatly extend the application of niosomes as DDS. As a subject for future study, we suggested a scheme by which one can investigate the interaction of GNs with the membrane of biological cells, in order to gain insights on how niosomes deliver drugs (capsaicin).

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