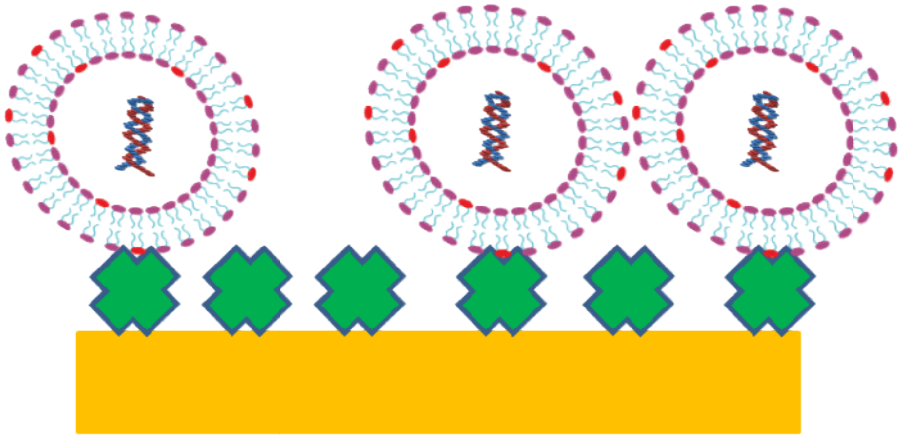
UNIVERSITY OF CRETE

SCHOOL OF SCIENCES AND ENGINEERING

BIOLOGY DEPARTEMENT

MASTER THESIS

"A novel method for the quantification of surface bound DNA-loaded liposomes"



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Finally many thanks to my family for always being there to offer support and for making me who I am. With their trust and their love they always make things look easy.

Abstract

Liposomes are used as drug delivery systems in the past two decades and they have quite a lot of applications in different fields of science. In acoustic biosensors liposomes due to their structure are used to determine different characteristics of liposomes and to find out innovative properties of the bound vesicles. Within this thesis, the potential of a novel-method for the quantification of surface bound DNA-loaded liposomes has been investigated.

In this work liposomes were produced using the widely known thin film hydration method. During this process DNA was diluted into the medium and entrapped into the liposomes. After the extrusion and the formation of DNA-loaded liposomes, the latter were purified from non-encapsulated DNA *via* several different methods, including overnight dialysis and centrifugation. These methods were compared during this project to determine the most efficient method for removing the non-entrapped DNA. The encapsulation efficiency of the liposomes has been determined by quantifying separately the encapsulated DNA and the number of liposomes produced. The quantification of the encapsulated DNA via qPCR measurement along with the lipid quantification provided the basis for the calculation of the DNA/liposome ratio. This ratio was used for the quantification of bound liposomes during the acoustic experiments.

The acoustic biosensor used was the Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). In this work, liposomes containing 1% of biotinylated lipids were captured on the sensor surface using the biotin-neutravidin capture system. Liposomes are bound on the previously Neutravidin-coated biosensor surface *via* their biotinylated lipids, changing the acoustic wave propagation characteristics. In order to quantify the liposomes bound, the liposomes were disrupted using detergents and the waste coming out of the sensor chambers was collected and analyzed. For this purpose, two different methods were used and compared. In the first one, the quantification was succeeded indirectly, *via* qPCR experiments and *via* the DNA/liposome ratio. In the second method, the number of liposomes previously bound on the surface was determined directly by Phosphatidylcholine quantification (PC hydrolysis). Finally, a correlation between the number of bound liposomes calculated from the two different methods and the signal from QCM-D was performed.

Chapter 1 – Introduction

1.1 Introduction to liposomes

In the 1960s, Alec D. Bangham discovered the existence of liposomes at the Babraham Institute, University of Cambridge (Bangham, Standish and Watkins, 1965). The word "liposome" derives from the two Greek words "Lipo" (fat) + "Soma" (Body) (Çağdaş, Sezer and Bucak, 2014). Liposomes have been extensively studied in depth until these days and they continue to constitute an interesting field of research and to be used in innovative methods. The important role of liposomes on the delivery of drugs, as well as on biophysical studies is important to be mentioned.

1.1.1 Structure of liposomes

Liposomes are closed lipid bilayers surrounding an aqueous internal space (Bangham, Standish and Watkins, 1965). Phospholipids are the basic lipids composing these lipid bilayers, separating the internal from the external environment. A hydrophilic group and one or two hydrophobic alkyl chains are the main components of phospholipids; the ones commonly used in liposomes are phosphatidylcholine (PC) or phosphatidylethanolamine (PE). The thickness of the bilayer formed by the phospholipids is about 4-7 nm (Balgavý *et al.*, 2001). The particle diameter size can range from 25 nm to several micrometers depending on the number of bilayers and the method of preparation. Size and lamellarity are the basic characteristics of liposomes. The different types of liposomes include unilamellar vesicles and multilamellar vesicles (MLV) (Figure 1). Unilamellar vesicles can be classified into three categories: small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and Giant unilamellar vesicles (GUV) (Yang *et al.*, 2011). SUV have a diameter of 20-100 nm, LUV have a diameter between 200 and 1000 nm and GUV a diameter greater than 1 μm. MLV have 5-25 lipid bilayers and diameter of 500-5000 nm and these vesicles have an onion structure (Shaheen, Sharif Mohammad, *et al., 2006*).

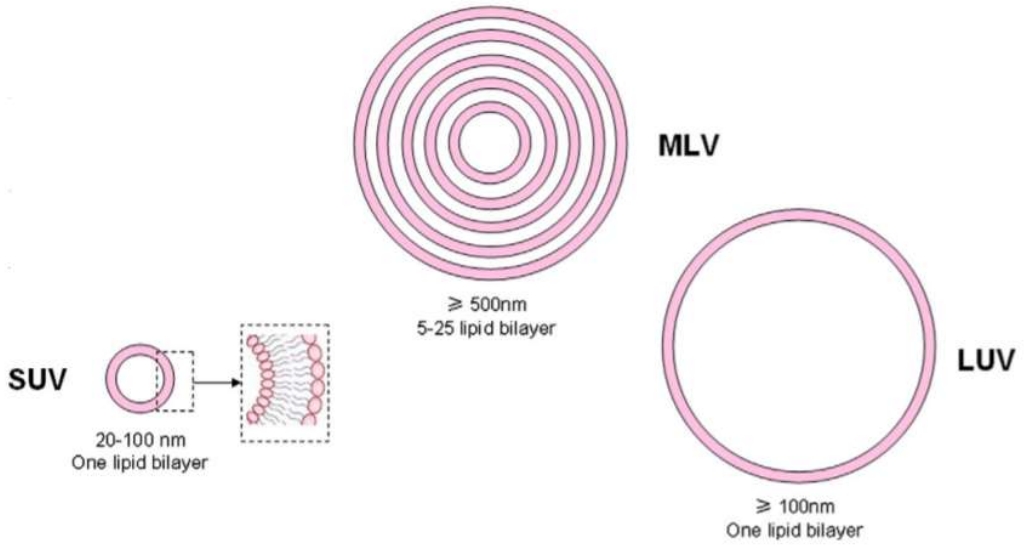


Figure 1. Classification of liposomes and their relative sizes. SUV: small unilamellar vesicles, LUV: large unilamellar vesicles, MLV: multilamellar vesicles (Yang *et al.*, 2011).

1.1.2 Use of liposomes

Due to their structure and physiological characteristics, liposomes have a wide range of applications. They are used as carriers in cosmetic and pharmaceutical industries, while food and farming industries have studied the use of liposomes for the encapsulation of various compounds. Liposomes are widely used in medicine and pharmacology; drug-loaded liposomes can be used as a tool to study cell-interactions, recognition or mechanisms of action of certain substances, but also as carriers for delivering the drug to its target (Banerjee *et al.*, 2004). Finally, liposomes can help understanding the material properties of bilayer systems and due to their quasi-spherical lipid structures they are developed as membrane models.

1.1.3 Liposome preparation

The preparation of unilamellar liposomes can be made by several methods such as detergent dialysis, ethanol injection and thin film (TF) hydration (Levine *et al.*, 2013). In detergent dialysis, a solution of mixed micelles is converted to bilayered vesicles during removal of detergent from the solution (Wacker and Schubert, 1998). This method is appropriate for encapsulation of lyophilic drugs and also for incorporation of membrane proteins into bilayers (Madani, Perálvarez-Marín and Gräslund, 2011). In ethanol injection, liposomes are formed with injection of ethanol-

dissolved lipids into an aqueous phase. This method is more efficient for encapsulation of ethanol soluble molecules (Kordges and Weddeling, 2015) (Batzri and Korn, 1973).

As DNA that was used in this study is neither lyophilic neither ethanol-soluble, none of the previous preparation methods can be used for liposome production. The method used was TF hydration (Figure 2). During this process, firstly, the phospholipids are mixed in organic solvent, chloroform being the most commonly used. Then, the solvent is evaporated and a thin lipid film is formed in a vial (Ulrich, 2002). Afterwards, liposomes are produced by hydrating the lipid film formed with an aqueous solution (Lasic, 1988). In this solution, DNA or other soluble compounds can be added in order for them to be encapsulated into the liposomes. The hydration step produces multilamellar vesicles which then can be extruded to form unilamellar vesicles (Akbarzadeh *et al.*, 2013).

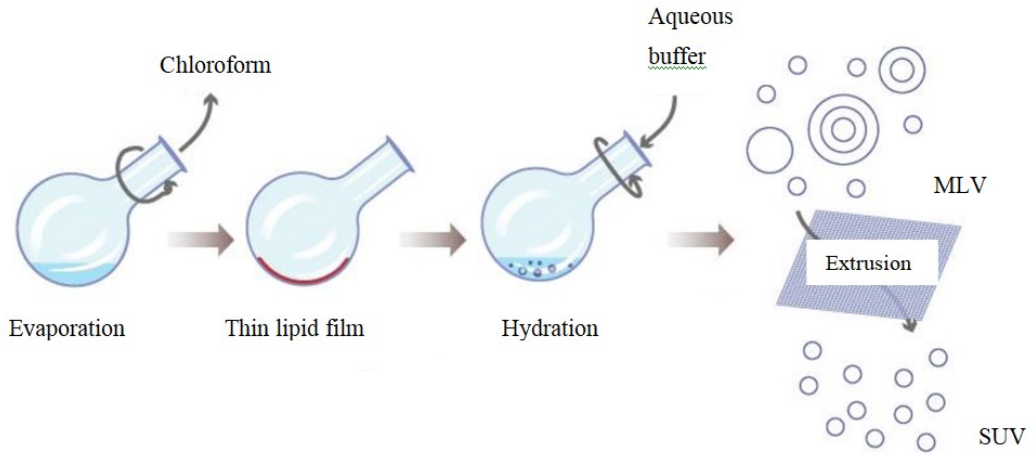


Figure 2. Liposome production by thin film (TF) hydration. Modified from (Calle *et al.*, 2015).

1.1.4 Liposome purification

After the extrusion of DNA loaded liposomes, it is necessary to separate them from any non-encapsulated material. There are several methods of liposome purification of non-encapsulated material. Some of them are based on the difference in size between the materials, such as dialysis method and others based on the difference in density of the materials, such as centrifugation. In dialysis, the liposome suspension is placed in a porous membrane and is left stirring overnight. The membrane dialysis must be chosen so that the molecular weight of the encapsulated material is lower than the membrane pores. In this way, the non entrapped material is

removed from the liposome preparation, while liposomes cannot penetrate membrane pores due to their bigger size. During centrifugation, non-encapsulated DNA is separated from liposomes due to lower density. After centrifugation, any non-encapsulated material remains in the supernatant that is discarded, while the liposomes are in pellet which is dissolved again.

1.1.5 Determination of encapsulation efficiency

The possibility of controlled encapsulation and release represents one of the main advantages of liposomes. For defining the encapsulation efficiency, the encapsulated probes as well as the liposomes formed have to be quantified. Depending on the nature of the encapsulated molecules, different techniques can be used for this purpose. Measurement of DNA molecules per liposome in this study was performed by calculating the number of liposomes formed, as well as by measuring the encapsulated DNA.

The number of liposomes produced was calculated *via* the quantification of PhosphatidylCholine (PC) phospholipids in the liposome preparation. The phospholipid quantification is succeeded through a fluorometric assay *via* the hydrolysis of PC.

The quantification of the encapsulated DNA was performed using quantitative polymerase chain reaction (qPCR) after disruption of the liposomes produced. qPCR is an evolved type of PCR but the difference between them is that in conventional PCR the accumulation of products can be measured for each cycle. Measurement of amplified DNA is realized by using fluorescent molecules which are either specific or non-specific for the targeted DNA sequence. As the reaction progresses, the increased fluorescence reflects the amplification of DNA. Two primers are needed, reverse and forward, which are specific for the targeted DNA. The components in a qPCR reaction are a polymerase, a set of primers, a fluorescent dye, deoxyribose nucleotide triphosphates, $MgCl_2$ and components for the pH optimization (Eckert and Kunkel, 1991). This method requires high technical sensitivity and precision, while one of its most important advantages is that there is a wide dynamic range of quantification of 7-8 logarithmic decades (Dieter Klein, 2002). The quantification of the encapsulated DNA along with the lipid quantification provided the basis for the calculation of DNA mass/Liposome ratio.

1.2 Quartz Crystal Microbalance

The quartz Crystal Microbalance is a technique that allows the measurement of small mass changes on the surface of a piezoelectric crystal. This material is a bulk acoustic wave (BAW) sensor comprising an AT-cut quartz crystal sandwiched between two gold electrodes. The application of an alternative current (AC) voltage to the crystal results to crystal deformation in an oscillatory manner. The fundamental resonance of a quartz oscillator is 5 MHz for a 0.33 nm crystal. The resonant frequency of the crystal decreases when there is a mass loaded on the surface and more specifically the changes of the surface mass are proportional to the shift of the crystal resonant frequency.

1.2.1 Quartz Crystal Microbalance with Dissipation monitoring

Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) is an advanced type of QCM that is based on the "ring-down" technique (Rodahl *et al.*, 1997) where the AC voltage is turned off intermittently and the oscillation decays exponentially (Reviakine, Johannsmann and Richter, 2011). During these decaying oscillations a voltage is generated and the signal is recorded. The characteristics of the signal studied are resonant frequency, (F) and dissipation, (D). Any changes on the ΔF are related to mass changes occurring on the surface, while dissipation gives information about the structure of the molecules adhered to the sensor and equals to the ratio between the energy dissipated during one period of oscillation and the energy stored in the oscillating system.

1.2.2 The acoustic ratio ΔD/ΔF

The quantification of changes in resonant frequencies and dissipation can be measured and give information about viscoelastic properties of the bound molecules. Recent studies in Biosensors lab (Tsortos *et al.*, 2008) have shown that the combination of the aforementioned characteristics, (D) and (F), can provide valuable information about the conformation of the bound analyte. This information is extracted by the acoustic ratio ΔD/ΔF, which represents the energy losses per unit mass absorbed to the surface (Milioni *et al.*, 2017).

Chapter 2 – Materials and Methods (M&M)

2.1 Materials and chemicals

The materials used for the purposes of the research were purchased by the followed companies:

- KAPA2G Fast HotStart ReadyMix PCR Kit, KAPA SYBR FAST qPCR Master Mix (2X) Kit: Kapa Biosystems, USA.
- PBS tablets: Sigma-Aldrich, USA.
- Avidine, NeutrAvidine Biotin-binding Protein: ThermoFisher Scientific, USA.
- POPC, b-DOPE lipids: Avanti polar lipids, INC, USA.
- Triton X-100: 108643 Merck KGaA, Germany.
- Biotech Cellulose Ester (CE) Membrane: Spectrum, USA
- GelRed Nucleic Acid Gel Stain: Biotium, USA.
- Forward primer (SF), Reverse primer (SR): Metabion international AG, Germany

  Sequence of SF: **5'-TCCTTTTCCAGATTACGCAACAGATACT-3'**

  Sequence of SR: **5'-TTGGGTTCTGGATTTTTGATTATCCTGC-3'**

2.2 Preparation and characterization of DNA

For this study, template Salmonella DNA was used for amplification through Polymerase Chain Reaction (PCR); the sequence was chosen because it is a fragment very often used in Biosensors Lab. The length of this double stranded DNA is 88 base pairs (bp). For every reaction of 10 μl volume, 5 μl KAPA2G Fast HotStart ReadyMix PCR Kit, 1 μl reverse primer (SR) (10 pmol/ μl), 1 μl forward primer (SF) (10 pmol/ μl), 2 μl ddH_2O and template Salmonella DNA (1 ng/ μl) were used. The PCR product was submitted to electrophoresis in the presence of GelRed in a TBE agarose gel of 1% (1 g agarose in 100 ml TBE 0.5x). For DNA purification, gel-extraction took place according to the protocol provided by the commercial kit for gel extraction (Nucleospin Gel and PCR Clean-up, MACHEREY-NAGEL). The extraction product was submitted to electrophoresis in the presence of gel-red in a TBE agarose gel of

2%, in order to check the size of the purified DNA. In addition, NanoDrop (ND-1000) spectrophotometer was used to check the DNA concentration (typically between 80 and 100 ng/μl).

2.3 Method of liposome preparation

99% of POPC and 1% of b-DPPE dissolved in chloroform were mixed in a round-bottom flask with a final lipid concentration of 1 mg/ml. Chloroform was evaporated at room temperature using dry nitrogen to generate a homogenous lipid film. The lipid film was re-hydrated with 1 ml of PBS. All buffers were filtered through Sarstedt Filtropur S 0.2 μm Syringe Filter. For DNA-loaded liposomes, the PBS contained DNA in the desired concentration (100 nM). The hydrated film was vortexed for 1 h in order for the MultiLamellar Vesicles (MLVs) to be formed. Finally, in order for the liposomes to be formed, the MLVs were extruded using the Avanti Polar Lipids (USA) mini-extruder. For the extrusion, the liposome suspension is forced 21 times through a polycarbonate filter of 100 nm pore size using 1 ml glass syringes and the mini-extruder. After the extrusion, the liposome preparation was diluted 5 times, with a final liposome concentration calculated to be 0.2 mg/ml.

2.4 Purification of liposomes, removal of non-encapsulated DNA

For the separation of non-entrapped DNA, two different methods were used, centrifugation and dialysis. During centrifugation, the liposome suspension was centrifuged at 14,100x g for 50 minutes. The supernatant was discarded and the pellet (containing the liposomes) was dissolved in PBS. During the second method, the dialysis tubing was put in ddH_2O for 10 minutes to remove any preservatives. Then, the tubing was cut into small pieces of 10 cm length and secured with clips on both ends. The liposome preparation was placed in a closed 1000 kDa MWCO membrane and was left stirring overnight at 4^{o}C. After dialysis, the DNA of the surrounding buffer was quantified. The two methods were compared *via* the signal recorded upon injection of the purified liposomes on the QCM-D sensor.

2.5 Phospholipid quantification of DNA-loaded liposomes

Phospholipid quantification was carried out using Phosphatidylcholine Assay Kit (ab83377, abcam), which is a fluorometric assay used to quantify phospholipid

concentration. This assay is based on an enzyme-coupled reaction which hydrolyses PC and releases choline. The latter oxidizes the OxiRed probe to be developed and to produce fluorescence (Ex/Em 535 nm/587 nm). This method was used for measuring PC in the extruded liposomes, as POPC were the main lipids in the liposomes (99%). The preparation of the standard curve by using 0.05 mM PC standard is described in the table below (Table 1).

Standard #	Volume of 0.05 mM PC standard (µl)	ddH_2O	Final volume standard in well (µl)	End [PC] in well (nmol/well)
1	0	150	50	0
2	6	144	50	0.1
3	12	138	50	0.2
4	18	132	50	0.3
5	24	126	50	0.4
6	30	120	50	0.5

Table 1. Preparation of standard curve dilution for fluorometric assay.

For the phospholipid quantification (fluorometric assay), PC Reaction Mix (50 µl) was prepared for each reaction with the addition of 45.8 µl Assay Buffer, 2 µl PC Hydrolysis Enzyme, 2 µl PC Development Mix and 0.2 µl OxiRe Probe. Each standard well contained 50 µl of reaction mix and 50 µl of standard and each sample well contained 50 µl of reaction mix and 50 µl of liposomes. The well plate was incubated under stirring for 30 min in the dark (RT). Then, the samples were measured on a microplate reader (Synergy-HT by BioTek, USA).

2.6 Quantification of encapsulated DNA

Quantification of the encapsulated DNA was performed by quantitative polymerase chain reaction (qPCR). The fluorescent dye that was used for the quantification of double stranded DNA was SYBR Green I. The latter, is a non-specific dye that binds to any double stranded DNA (Broeders *et al.*, 2014), (Agilent-Techologies, 2010). The qpcr instrument used is the CFX Real-Time PCR Detection

System, Biorad. The results were analyzed with CFX Maestro Analysis Software. The preparation of each experimental method was performed in a laminar flow hood.

Each time, prior to performing qPCR on unknown samples, a calibration curve of successive 10-fold dilutions in ddH_20 from the initial DNA preparation (~ 10^{-4} ng/μl) was realized. The qPCR mixture of 10 μl was prepared using 5 μl KAPA SYBR FAST qPCR Master Mix (2X), 0.2 μl reverse primer (SR) (10 pmol/ μl), 0.2 μl forward primer (SF) (10 pmol/ μl), 2.6 μl ddH_2O and 2 μl of diluted template DNA Salmonella. Water as negative control was used each time.

In order for the liposome-encapsulated DNA to be quantified, 0.1% Triton (in ddH_2O) was added in the purified liposomes preparation. Then, the DNA was purified and concentrated according to the protocol of DNA purification kit (Nucleospin Gel and PCR Clean-up, MACHEREY-NAGEL). After the purification step, 10-fold dilutions in ddH_2O from the purified DNA were carried out. For the determination of the DNA concentration, every reaction (10 μl volume) consisted of 5 μl KAPA SYBR FAST qPCR Master Mix (2X), 0.2 μl reverse primer (SR) (10 pmol/ μl), 0.2 μl forward primer (SF) (10 pmol/ μl), 2.6 μl ddH_2O and 2 μl of the purified DNA.

2.7 Acoustic measurements using QCM-D

A four-sensor quartz crystal microbalance with dissipation monitoring (QCM-D, Q-Sense E4) acoustic device was used for the measurement of frequency (ΔF) and energy dissipation (ΔD) changes. The Au-coated sensor chips (AWS SNS 000042 A) were cleaned with ddH_2O and 70% ethanol and were placed into a UV Ozone Cleaner for 30 minutes (L2002A2-UK, Ossila). The buffer used was PBS and the flow rate was 50 μl / min. The liposomes were captured on the sensor surface using the biotin-neutravidin capture system. It is noted that the interaction between biotin and neutravidin is characterized by one of the highest affinities in nature ($K \approx 10^{13}\ M^{-1}$) (Williams and Sebastine, 2005). 200μl of neutravidin (0.2 μg/μl) were injected in the sensor chamber. After PBS rinsing and re-equilibration of the system, the liposomes were injected into the chamber in various concentrations [500, 400, 300, 200 μl of liposomes preparation (0.2 mg/ml) in a total volume of 500 μl PBS]. The next step included the rinsing with PBS for 30 minutes. The last step consisted of the lysis of the liposomes *via* detergent addition (0.1% Triton in ddH_2O). After the addition of the detergent, the waste of each sensor was collected during 20 minutes in order for the DNA previously encapsulated in the bound liposomes to be quantified. The QCM-D

wastes were purified and concentrated (≈13 times) according to the protocol of DNA purification (Nucleospin Gel and PCR Clean-up, MACHEREY-NAGEL). Except of the DNA that was quantified from the waste of each sensor, the liposomes bound were quantified directly from the waste by the Phosphatidylcholine Assay Kit.

3.1 Confirmation of DNA size

Gel electrophoresis was performed to verify the length of PCR products and the purified DNA after gel extraction. Figure 1 shows the PCR products on a 1% gel agarose gel and the purified DNA after the gel extraction on a 2% agarose gel. DNA size appears to be 88 bp as expected.

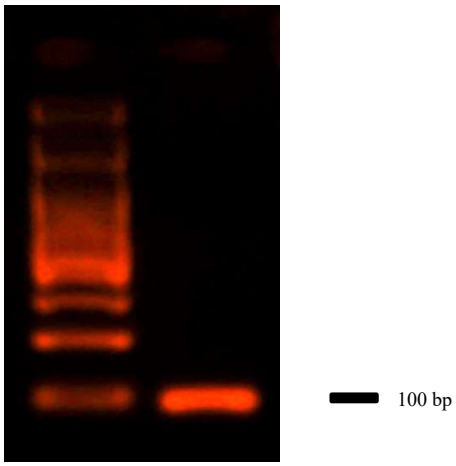


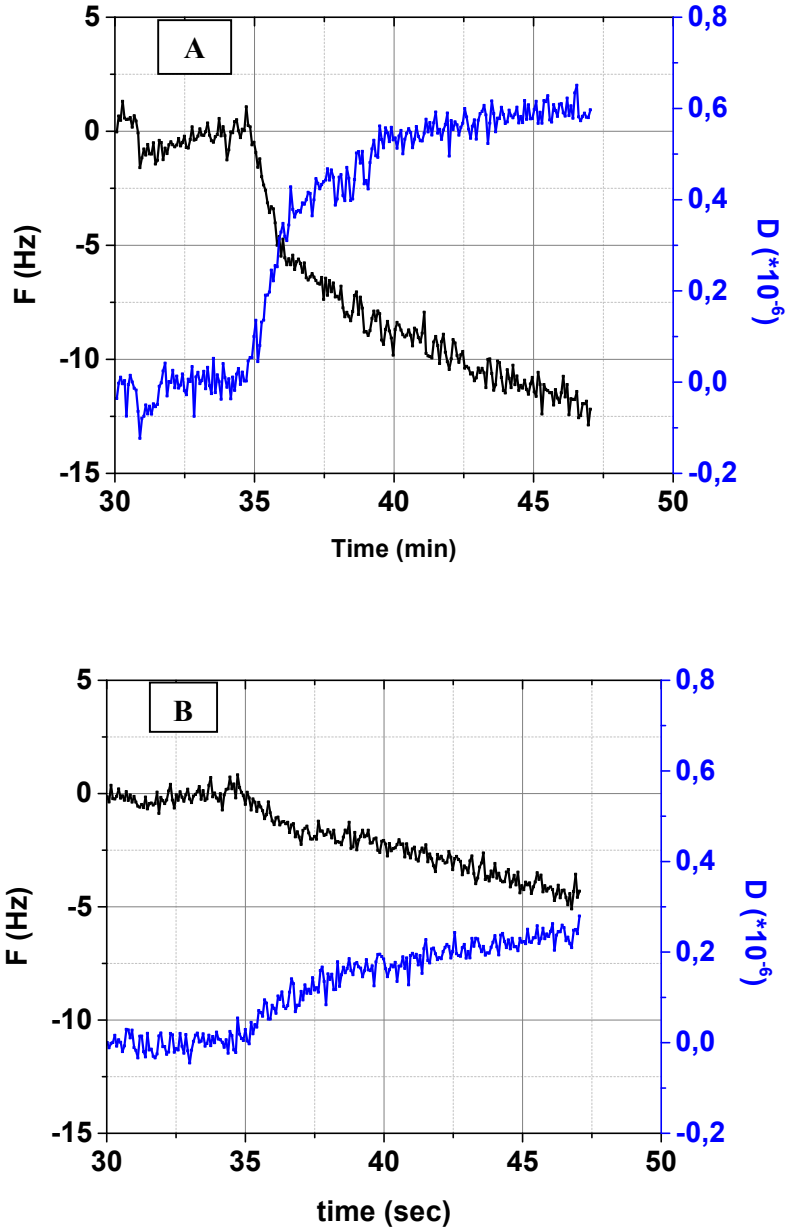
Figure 3. Agarose gel electrophoresis of PCR products and purified DNA after gel extraction.

3.2 Liposome purification

Between the two purification methods of DNA loaded liposomes described in M&M (§2.4), dialysis and centrifugation, the dialysis method appeared to produce better results. More precisely, after purification of liposomes from the same liposome preparation, using QCM-D measurements the two techniques were performed. During the experiment, any energy dissipation (ΔD) and frequency changes (ΔF) after injecting the same volume of liposomes were recorded (Graph 2).

According to the results shown (Graph 2), we concluded that there is a lower loss of liposomes using the dialysis technique, thus this is the method used during this work. In order to be sure that all the non-encapsulated has been removed, the buffer

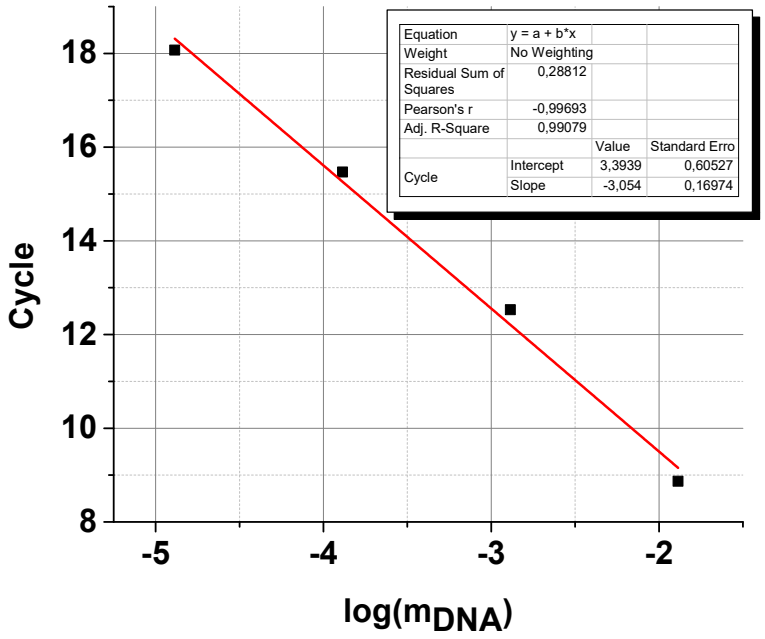
was refreshed after 2-3 hours and the surrounding buffer of the membrane was tested until no DNA was detected by qPCR.



Graph 2. Measurement of energy dissipation (ΔD) and frequency changes (ΔF) after liposome bound. Graph (A) shows liposomes bound after dialysis and graph (B) liposomes bound after centrifuge.

3.3 Quantification of encapsulated DNA

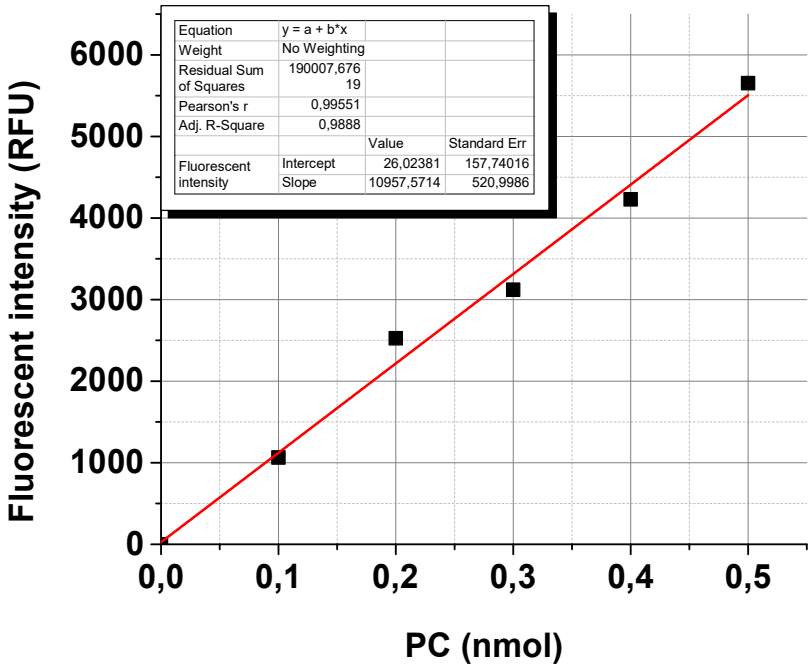
The quantification of encapsulated DNA was realized by qPCR after lysis of the liposomes (ddH_2O – Triton 0.1 %). The resulting solution of the lysis was purified and concentrated according to the protocol of DNA purification (Nucleospin Gel and PCR Clean-up, MACHEREY-NAGEL). The DNA mass of DNA-loaded liposomes was calculated from the standard curve for DNA (Graph 3). The standard curves were fitted with serial 10-fold dilutions of the initial known DNA concentration for each experiment and were linear in the range tested (R^2=0.99). The slope of the standard curve was -3.054 which is considered to be the ideal slope for 10-fold dilutions (meaning that in every cycle the DNA is doubled). The aforementioned results showed that the DNA concentration in the liposome preparation was 0.3 ng/µl.



Graph 3. Construction of the standard curves for DNA. The standard curves were constructed with serial 10-fold dilutions of the DNA. Each standard dilution was amplified by real-time quantitative PCR. The cycles were plotted against the logarithm of the known DNA mass.

3.4 Quantification of liposomes

Lipid concentration in the liposome preparation was measured by Phosphatidylcholine Assay Kit described in M&M (§2.6). A typical Phosphatidylcholine (PC) standard curve constructed using fluorometric reading is shown in Graph 4. The slope of the standard curve was 10,957.57.



Graph 4. Typical Phosphatidylcholine standard calibration curve using fluorometric reading.

The concentration of PC (nmol/µl) in the test samples was calculated using the standard curve shown in Graph 3. The equation used for the calculation of [PC] is described below:

$$[PC] = \left(\frac{A}{B}\right) * D \qquad \text{(Eq. 1)}$$

Where:

A = Amount of PC in the sample well (nmol) from the standard curve

B = Sample volume added into the reaction well (µl)

D = Dilution factor

Our results showed that the PC concentration in the sample was 0.364 nmol/µl, so 2.19×10^{14} POPC molecules. The total number of lipid molecules in the sample (taking into account the 1% of b-DOPE lipids) was 2.22×10^{14}. To determine the quantity of liposomes in the sample, the number of lipid molecules per liposome was calculated. The equation used in order to calculate the number of lipid molecules per liposome is described below, considering liposomes as spheres of 100 nm of diameter , the thickness of bilayer 5 nm (Liu, Yan and Eisenthal, 2001) , and the POPC head area per molecule 0.71 nm^2 (Lantzsch *et al.*, 1996):

$$\text{Ntot} = \frac{[4\pi\left(\frac{d}{2}\right)^{2-} + 4\pi\left[\frac{d}{2} - h\right]^2]}{a} \qquad \text{(Eq. 2)}$$

Where:

d: Diameter of the liposome

h: Thickness of the bilayer

a: Phosphatidylcholine head group area

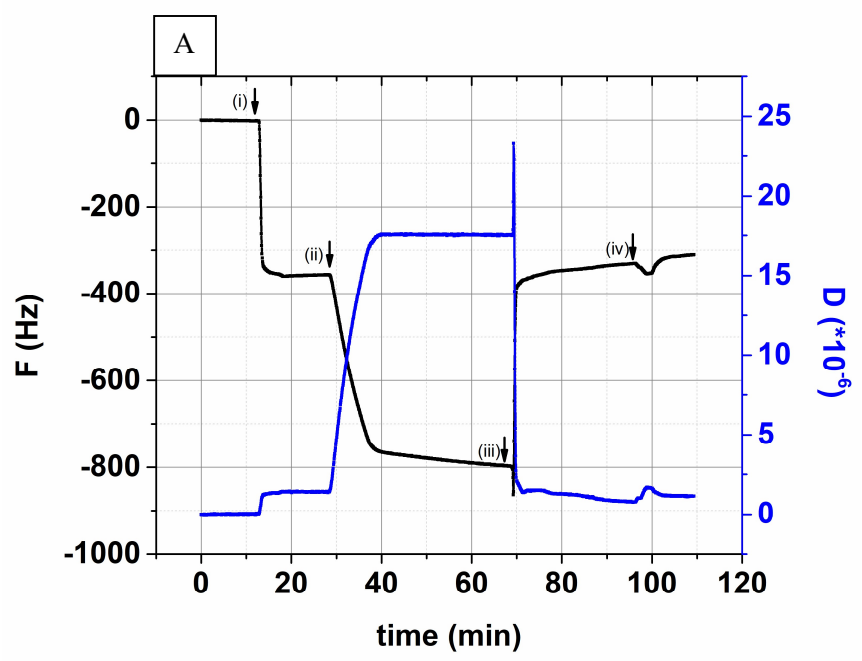
Thus, the total number of lipids in a 100 nm size liposome is ~80,047. The total number of lipids in 1 µl of the sample was 2.22×10^{14} so the total number of liposomes in 1 µl of the sample was $\frac{2.22\times10^{14}}{80{,}047} = 2.77\times10^{9}$.

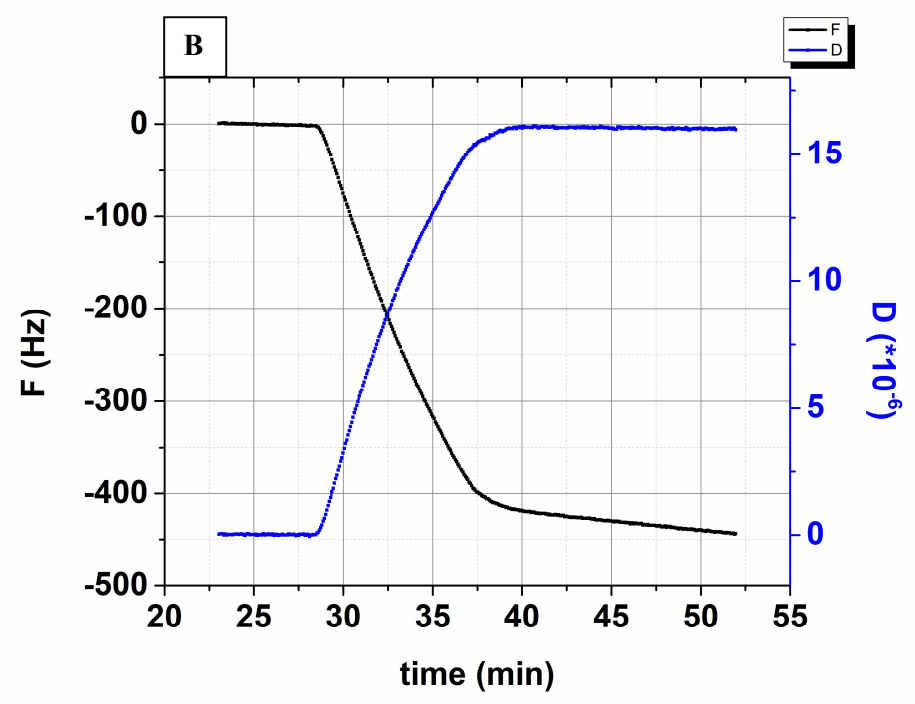
3.5 Determination of encapsulation efficiency

The quantification of the encapsulated DNA along with the lipid quantification provided the basis for the calculation of DNA mass/Liposome ratio. DNA concentration into the liposomes was 0.3 ng/µl. Also, the total number of liposomes was 2.77×10^{9} in 1 µl of the sample. Thus, the ratio of DNA mass/Liposome is 1.07×10^{-10} which can be translated into 1.13 DNA molecules per liposome. This ratio was calculated to be used in QCM-D experiments for determining the number of liposomes bound on the biosensor surface.

3.6 QCM-D experiments: quantification of bound liposomes

In order to quantify the number of liposomes that were bound on the neutravidin-coated biosensor surface, a suitable immobilization protocol for QCM-D was used. As shown is the sensogram (Graph 5), neutravidin adsorbed on the surface (Step i) and then liposomes with biotinylated lipids were injected and bound to neutravidin (Step ii). The next step (Step iii) was the injection of ddH_2O-Triton 0.1% for the lysis of bound liposomes and the final step (Step iv) included rinsing with PBS for the system to be re-equilibrated.



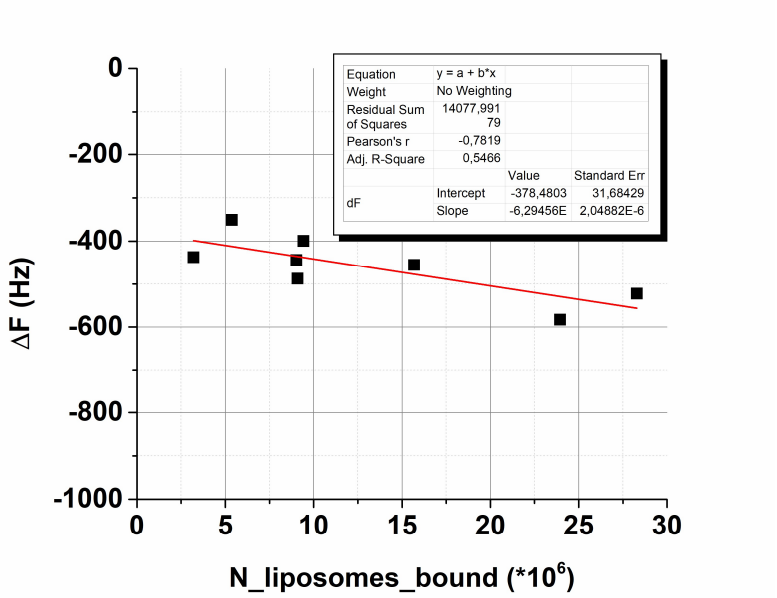


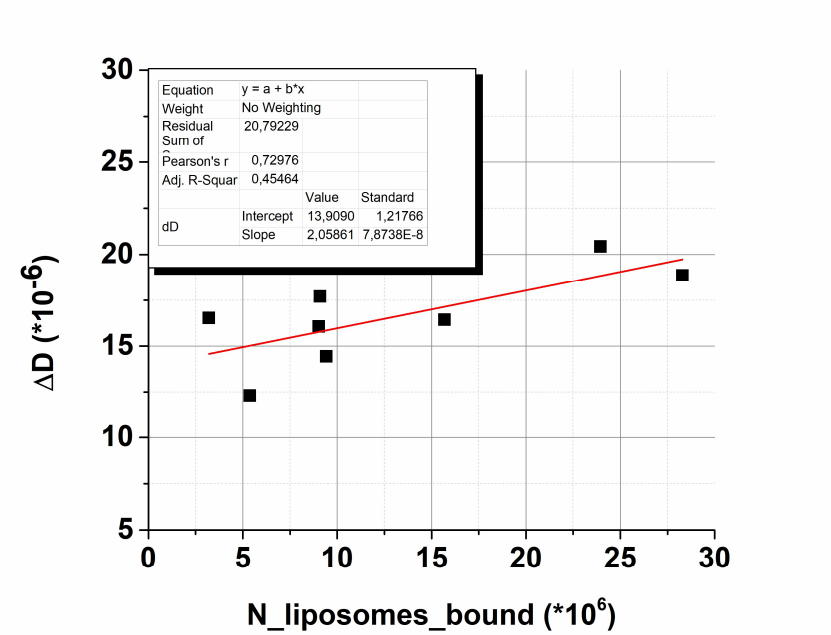
Graph 5. Measurement of energy dissipation (ΔD) and frequency changes (ΔF) of bound liposomes in a typical sensogram. Graph A includes the different steps with (i) be the neutravidin adsorbion on the surface, (ii) be the liposome bound to neutravidin, (iii) the injection of ddH_2O-Triton 0.1% for the lysis of bound liposomes and the final step (iv) the rinsing with PBS for the system to be re-equilibrated. Graph B includes a zoom in of step (ii).

As shown in Graph 5 (B), liposomes were bound on the surface provoking a change of ΔF= -444.16 Hz and a change of ΔD = 16.05 $\times 10^{-6}$. For the quantification of surface bound liposomes two quantification methods were used and compared. The first one, in which the quantification was performed indirectly, the wastes of the sensors were collected to be purified and concentrated 13 times and then to be quantified using qPCR. The unknown DNA mass of bound liposomes was calculated from the standard curve of DNA, as described earlier (Graph 3). From the ratio of DNA mass/Liposome calculated above and from the measurement of the unknown DNA concentration, the number of liposomes previously bound on the surface was determined. The second method, in which the quantification was performed directly, the wastes of the sensors were collected and the liposomes were measured by Phosphatidylcholine Assay Kit.

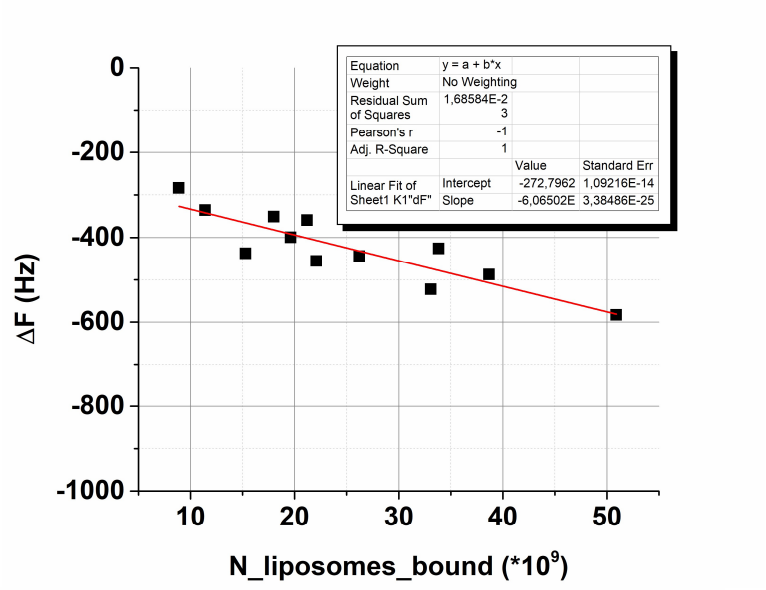
Graph 6 and 7 show the ΔF and ΔD changes provoked by the surface bound DNA loaded liposomes versus the number of liposomes bound. In Graph 6 this number has been calculated according to the first method and in Graph 7 according to the second method.

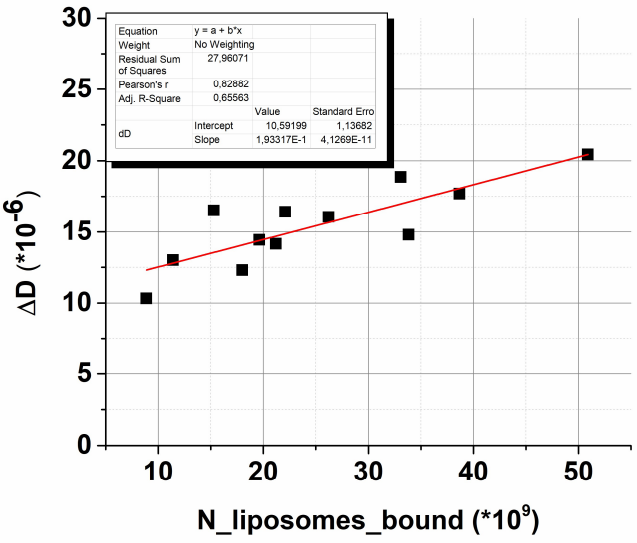
Also, acoustic ratio of energy losses per unit mass, ΔD/ΔF was determined and was ~0.04 10^{-6}/Hz in both of the quantification methods of bound liposomes (Graph 8/Graph 9), that is in great agreement with previous studies in the lab (Unpublished data).



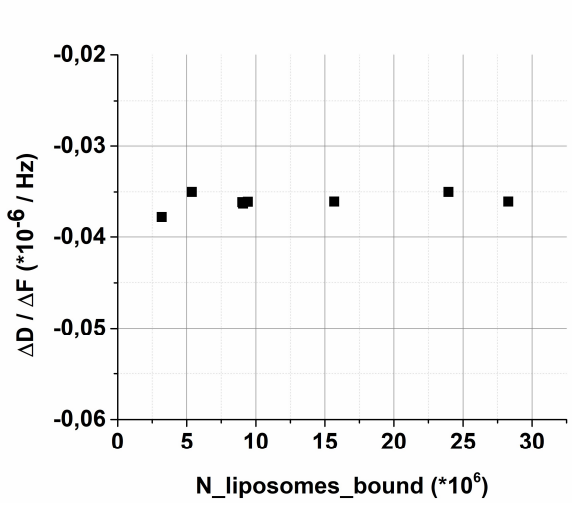


Graph 6. Correlation of number of surface bound liposomes with changes in Frequency (ΔF) and Dissipation (ΔD) according to the first quantification method of liposomes.

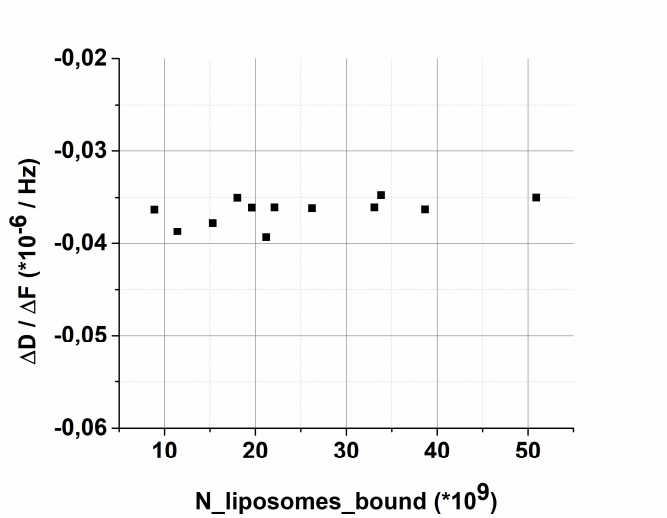




Graph 7. Correlation of number of surface bound liposomes with changes in Frequency (ΔF) and Dissipation (ΔD) according to the second quantification method of liposomes.



Graph 8. Correlation of number of surface bound liposomes with acoustic ratio of energy losses per unit mass, $\Delta D/\Delta F$ (10^{-6}/Hz) according to the first quantification method of liposomes.



Graph 9. Correlation of number of surface bound liposomes with acoustic ratio of energy losses per unit mass, $\Delta D/\Delta F$ (10^{-6}/Hz) according to the second quantification method of liposomes.

Chapter 4 – Discussion

In this work, we tried to establish a methodology for quantifying DNA-loaded carriers immobilized on a biosensor surface. For this purpose, we used two different approaches; a) quantification based on qPCR and PC hydrolysis measurements defining the encapsulation efficiency of the carriers and b) measurement of the lipids present in QCM-D waste after disrupting the liposomes and rinsing. The first one aimed at an indirect quantification. The determination of DNA mass/Liposome permitted the correlation of the number of bound liposomes with the acoustic signal obtained each time (Graph 7). Despite the reproducible results as far as the lipid quantification is concerned, DNA molecules were also detected in the control experiments. This observation led us to conduct further studies. In order to evaluate the method described above, the lipids of the waste have been directly quantified using the PC hydrolysis kit and the number of liposomes previously bound on the surface has been calculated.

Comparing the two methods, we observe that in both cases Δf values of the acoustic signal decreased, while ΔD values of the acoustic signal increased as the number of bound liposomes increased (Graph 6/Graph 7). Also, the results of the direct quantification were more reproducible (by studying either the Δf or the ΔD shifts of the acoustic signal); and thus we concluded that the number of liposomes bound on the biosensor surface was underestimated using the first method. A possible explanation to this observation might be that DNA molecules were trapped in the tubing and therefore not coming out to the collected waste. Even a low amount of DNA molecules in combination with the very sensitive quantification method of qPCR could have affected the final result. Nevertheless, the acoustic ratio ΔD/ΔF was calculated to be ~0.04 10^{-6}/Hz using both quantification methods (Graph 8/Graph 9). This indicates that the liposomes are intact on the biosensor surface and is in great agreement with previous studies in Biosensors lab. Overall, the direct quantification *via* lipid quantification seems to provide better results and is also the method including less experimental handling. Both methods can be further optimized in order to eliminate as efficiently as possible any experimental errors or contaminations, the most important being the one of DNA molecules detected in our control experiments. However the two methods could be useful in the future for many applications depending on the probe or the carrier that would need to be quantified.

Chapter 5 - Bibliography

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