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Acoustic DNA Detection for Cancer Diagnosis

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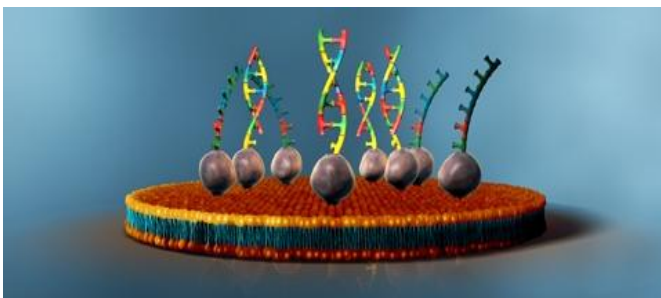
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Dimitris Stratiotis

SUMMARY

In 1948 Mandel and Metais discovered the presence of Nucleic Acids in the human circulatory system (cNAs). This finding gained medical importance, since it was shown that, even at the onset of tumor diseases, and during cancer progression, circulating tumor DNA (ctDNA), which carries genetic and epigenetic information, could be detected at the blood of cancer patients, as a portion of the total cell-free circulating DNA (cfcDNA). CfcDNA is present at both healthy individuals and patients; nevertheless the total cfcDNA concentration is increasing during cancer development. It is known that ctDNA is responsible for the total cfcDNA concentration increase. This implies that ctDNA except for a useful qualitative biomarker, is also a good quantitative biomarker, which could be used for cancer early diagnosis, monitoring as well as an indicator of the therapy efficiency in tumor malignancies, and these reasons underscore the efficiency and the accuracy of ctDNA as a cancer biomarker. Despite the importance of ctDNA, analysis of patients sample is currently based on methods like real-time PCR, digital PCR and methylation specific PCR, and the preparatory steps of the samples can affect the clinical results. Biosensors are electronic devices able to detect and analyze DNA interactions and DNA hybridization processes in real-time, in a label-free way with high accuracy and the examples of these uses, in the field of acoustic biosensor development, are abundant in the scientific literature. In this experimental process Quartz Crystal Microbalance (QCM) acoustic sensors were used to detect small concentrations of DNA (concentrations that resemble ctDNA concentrations at the onset of the disease and later stages) as a mean to study the potential use of QCM sensors in integrative diagnostic platforms for applications in cancer early diagnosis and monitoring. Since the DNA mass is not causing a signal after adsorption on the sensor's surface I used liposomes as amplifying elements of the acoustic signal. The results show the ability of QCM-D biosensors to detect fM concentrations of ssDNA analytes.

Keywords: Quartz Crystal Microbalance (QCM-D), cancer, circulating tumor DNA (ctDNA), cell-free circulating DNA (cfcDNA), point of care, diagnostic integrated platforms.

ΠΕΡΙΛΗΨΗ

Το 1948 οι Mandel και Metais ανκάλυψαν την ύπαρξη κυκλοφορούντων νουκλεϊκών οξέων(cNAs) στο ανθρώπινο κυκλοφορικό σύστημα. Τα ευρήματα αυτά απέκτησαν μεγάλη ιατρική σημασία αργότερα, όταν δείχτηκε ότι οι ασθενείς που πάσχουν από καρκίνο εμφανίζουν κυκλοφορούν DNA που προέρχεται από τα καρκινικά κύτταρα (ctDNA), ως κλάσμα του συνολικού κυκλοφορούν DNA (cfcDNA). Το ctDNA μπορεί να δώσει πληροφορίες που αφορούν την γενετική σύσταση των κυττάρων καθώς και τις επιγενετικές αλλαγές που απαντώνται σε αυτό κατά την καρκινογένεση. Το cfcDNA συναντάται τόσο σε ασθενείς αλλά και υγιείς ανθρώπους, ωστόσο η συγκέντρωση του σε ασθενείς είναι σημαντικά αυξημένη και η αύξηση αυτή συμβαίνει από την αρχή της καρκινογένεσης και ανεβαίνει ανάλογα με την εξέλιξη της νόσου, λόγω κυρίως της αύξησης της συγκέντρωσης του ctDNA. Τα παραπάνω χαρακτηριστικά καθιστούν το ctDNA έναν πολλά υποσχόμενο βιομάρτυρα για την πρόγνωση και διάγνωση του καρκίνου καθώς και για την παρακολούθηση της εξέλιξης της νόσου κατά τη διάρκεια της θεραπείας. Η ανάλυση του ctDNA βασίζεται προς το παρόν σε τεχνικές όπως η PCR, η real-time PCR, η digital PCR που απαιτούν έχουν υψηλό κόστος και μεσολαβεί μεγάλο χρονικό διάστημα από την απομόνωση του ctDNA μέχρι την ανάλυση του, με αποτέλεσμα να επηρεάζεται η ποσότητα και η ποιότητα του δείγματος και συνεπώς το τελικό αποτέλεσμα. Οι QCM-D βιοαισθητήρες είναι ηλεκτρονικές συσκευές ικανές να ανιχνεύουν σε αληθινό χρόνο, χωρίς τη χρήση ακριβών και επικίνδυνων ανιχνευτών, αλληλεπιδράσεις του DNA με άλλα στοιχεία καθώς και αντιδράσεις υβριδοποίησης. Η χρήση του QCM-D σαν βασικό στοιχείο ανάλυσης του cfcDNA σε διαγνωστικές πλατφόρμες είναι κάτι παραπάνω από επιθυμητό καθώς έτσι θα μειωνόταν ο χρόνος της διάγνωσης καθώς και της προετοιμασίας του δείγματος. Στην παρούσα μεταπτυχιακή εργασία, QCM-D βιοαισθητήρες χρησιμοποιήθηκαν για την ανίχνευση μικρών συγκεντρώσεων DNA (συγκεντρώσεις στην κλίμακα του ctDNA) για να εξετασθεί η ικανότητα τους στην ποσοτική ανάλυση του ctDNA. Επειδή οι συγκεντρώσεις που χρησιμοποιήθηκαν δεν είναι ικανές να παράγουν ακουστικό σήμα, το σήμα ενίσχυθηκε με τη χρήση λιποσωμάτων. Τα αποτελέσματα δείχνουν ότι το σύστημα μπορεί να ανιχνεύσει συγκέντρωση μονόκλωνου DNA στην τάξη του fM.

1. INTRODUCTION

Cancer is one of the most fatal diseases globally, resulting in high patients' mortality in both developing and developed countries ¹ as shown in Figure 1. Until now there is not an effective way to eradicate the disease, but there is great effort paid to unravel the basic biology of the disease, drug discovery and development of novel tools for the prognosis, diagnosis and monitoring the progress of the tumor malignancies.

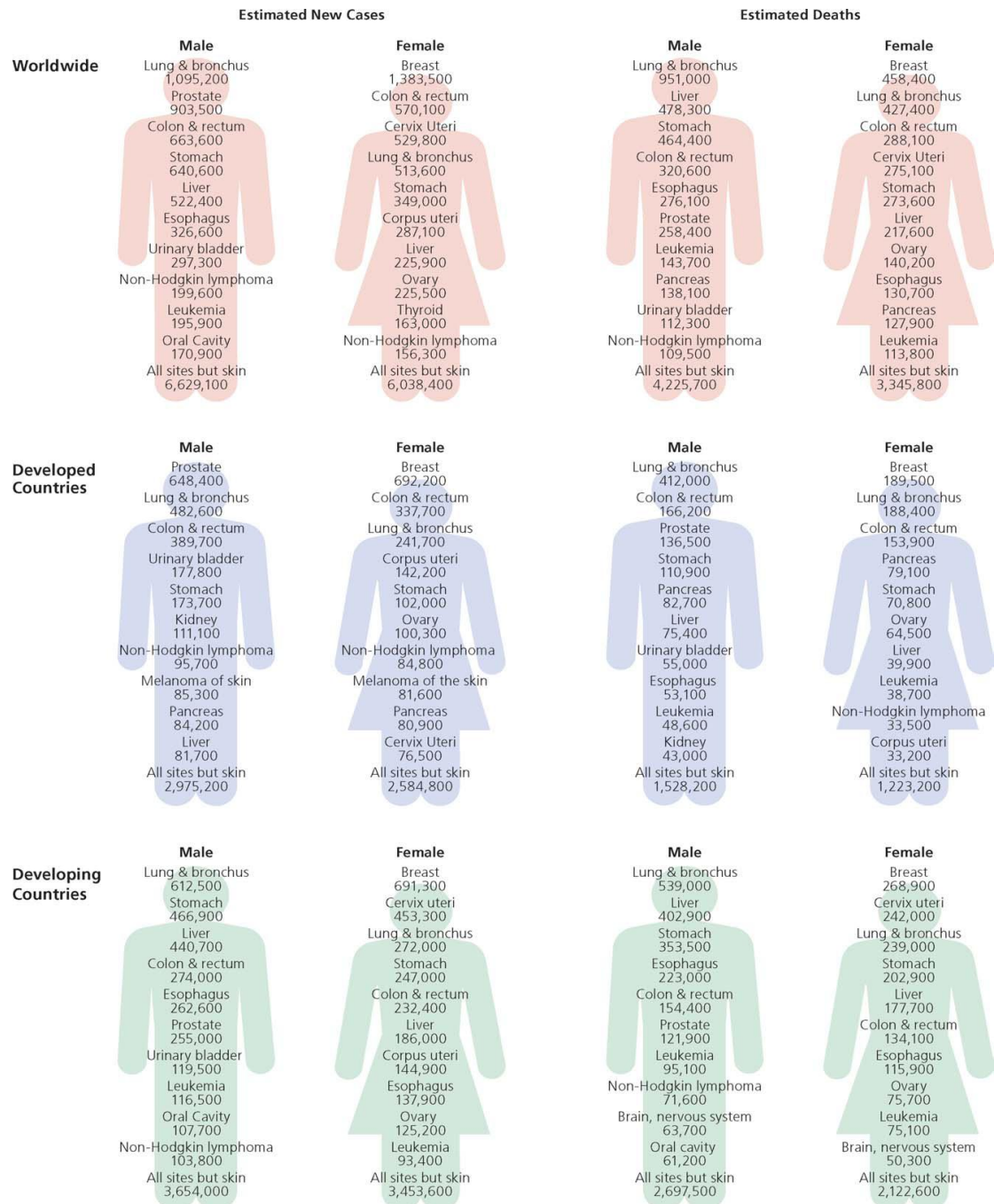


Figure1: Global cancer statistics for the leading cancer types according to the patient sex and the economic development. By Jemal et al.

The field of biomarkers for prognosis and early diagnosis of carcinogenesis and cancer progression is currently based on protein biomarkers. Even though protein biomarkers are quite accurate the ability to distinguish the different tumor cells is not evident, while it is even more difficult to distinguish the different tumor cells' subtypes of a single tumor. For this reason circulating tumor Nucleic Acids (ctNAs) has emerged as promising biomarkers of tumor malignancies onset and development, providing useful qualitative and quantitative information².

More than 60 years ago Mandel and Metais have described the presence of nucleic acids in the human blood³. These cNAs are cell free circulating DNA (cfcDNA) and circulating RNA (cRNA). This finding gained more interest many years later when normal and mutated variants from single-copy gene RAS, which is known to be implicated in cell proliferation and cancer development, were identified in the circulating DNA of cancer patients⁴. This was the first indication that posed the question about the use of cfcDNA as biomarker for tumor diseases.

Nowadays we know cNAs are blood components of both normal individuals and patients of several diseases like cancer⁵. Concerning the cancer patients, cfcDNA includes a small portion of cfcDNA originating directly from the tumor and is referred as circulating tumor DNA (ctDNA). In cancer patients' cfcDNA concentration is significantly increased, consistently with the progression of the disease and this elevation is ought to the increase of the concentration of ctDNA. Thus ctDNA quantitative analysis can highlight accurately the stage of the disease, and this has both prognostic and diagnostic importance. Moreover ctDNA levels change after cancer treatment and showed that ctDNA is also an appropriate biomarker which provides a clear image concerning the efficiency of the treating means or drug resistance⁶.

The mechanisms underlying cfcDNA release in the circulation are still unclear. Different cellular processes have been proposed to act as mechanisms of cfcDNA generation. Cellular apoptosis and necrosis were considered to be the main source of release at both healthy individuals and patients⁷. In later studies the contribution of these cellular processes was rather underestimated, since many studies underscored the important involvement of active DNA release from the cells⁸⁻¹⁰. The last process is consistent with the observation that ctDNA concentration is significantly elevated in cancer patients and that is ought to the active release of cNAs from the tumor cells immediately after tumor vascularization. Figure 2 is a depictive review about DNA release process from the paper of Schwarzenbach et al¹¹.

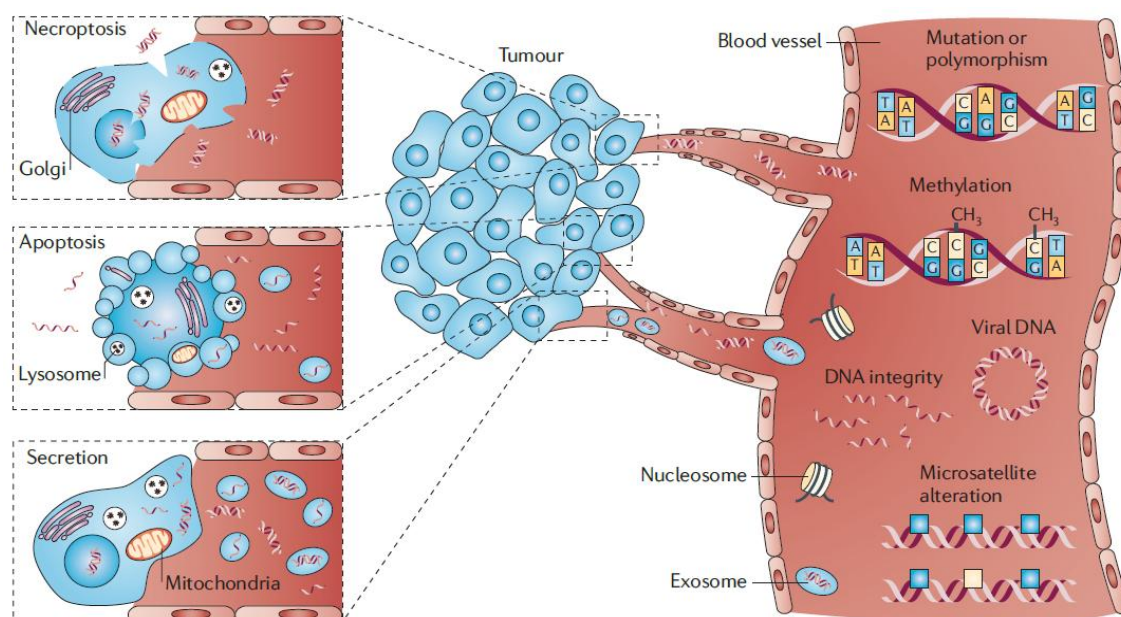


Figure 2: The three major cellular processes involved in ctDNA release in the circulation.

CtDNA does not provide only information about the development of the tumor disease in a quantitative way. Qualitative information can also be extracted by ctDNA analysis concerning mutations occurring at the normal cells and transit them to a pathologic tumor cell. This has been mentioned before in the example of RAS variants isolation from single copy cancer patients. Similar researches are carried out and many tumor specific mutations have been identified. Epigenetic and genomic size alterations can also be identified. Epigenetic alterations and especially DNA methylation play a vital role in the dynamics of gene expression. Methylation of promoters has been reported to be associated with specific tumors even though methylation profile is not considered yet a good biomarker^{12,13}. Finally information concerning microsatellite size variants, which is known to play a significant role in loss of heterozygosity and other genomic instability abnormalities, are also detectable¹⁴.

All the qualitative and quantitative information which could be gained from ctDNA underscore its potential use as an accurate non-invasive biomarker for the prognosis and early diagnosis of tumor malignancies. Nevertheless ctDNA analysis is currently based on methods like PCR, real-time PCR, digital PCR, methylation specific PCR and DNA sequencing. These methods are high-cost, time-consuming and the preparatory steps of sample treatment may affect the final result. Thus it would be very pleasing to find new tools able to analyze ctDNA in shorter time period, without the need of complicated sample preparation, and able to uncover all the qualitative and quantitative information from the ctDNA of cancer patients.

Acoustic biosensors are electronic devices able to analyze DNA interactions in real-time, in a label-free way and provide qualitative and quantitative results. Processes of DNA interactions and on sensor hybridization^{15,16}, single-base mismatch detection¹⁷⁻²⁰ and allele variants investigation^{16,21} are abundant in the scientific research literature. The abilities of acoustic biosensors make them an ideal tool as core components of diagnostics on-chip platforms that could be used as tools for prognosis or diagnosis of tumor diseases using liquid biopsies. In the borders of a long term ambitious plan, these devices could be “handle-held”, providing to pathology doctors a tool to daily examine cancer onset and progression, giving economic and social advantages due to the immediate diagnosis and treatment of patients²².

The purpose of this work was to study and analyze the sensing ability of Quartz Crystal Microbalance with Dissipation detection technology (QCM-D) to detect low DNA concentrations similar with those of ctDNA in liquid samples. As mentioned earlier ctDNA concentration is relatively low compared to the total cfcDNA concentration. QCM-D biosensors have a mass detection limit at the scale of some nanograms per ml, while ctDNA is under this point. For this reason the use of acoustic signal amplification elements is a necessity. The amplification elements used in this study were liposomes of different diameters size. A previous study has reported the use of liposomes as amplifiers of the acoustic signal of analyte DNA²³. Nevertheless, in that study the analyte DNA length was relatively small (27 nts), compared to the length of ctDNA fragments, which are constituted by small fragments ranging from 70bps to 200bps and large fragments even to a length of 21kbp²⁴. In my study two different lengths of DNA molecules and specifically 50nts and 157nts were used as analytes. These two DNA molecules have different physicochemical properties since their length is shorter or equal to the persistence length of a double stranded DNA molecule, respectively²⁵. Consequently these differences are expected to affect the hybridization process and/or the amplification process and thus the final acoustic signal.

As a preclinical validation of the principle described, some experiments included the hybridization process of complementary DNA strand in the complex matrix including Fetal Bovine Serum. The goal is to use plasma and serum as liquid biopsies in the integrated diagnostic platforms, so for that reason a system able to carry the ctDNA analysis without previous extraction from the plasma is desired. Moreover this ability provides the advantage of avoiding the preparatory steps of ctDNA extraction and purification. As a result better DNA quality and higher

quantity is expected. Previous works focusing on DNA hybridization efficiency in complex matrixes have been carried and the outcome describes that DNA hybridization is less efficient compared to “in-buffer” hybridization assays²⁶⁻²⁸.

This study provides proof of principle of dsDNA immobilization acoustic signal and DNA hybridization acoustic signal amplification with liposomes in simple and complex matrixes. The results show that the detection limit of the system for a 50bps DNA detection is this of amplifying an acoustic signal by a hybridization process of $1,2 \times 10^{-12}$ M complementary analyte, which is consistent with the findings of previous similar studies²³ in buffer solution. Consistent results were also obtained for the complex matrixes when the hybridization efficiency is a restriction factor strongly affecting the detection limit but it is still observable that the detection limit of the method in complex matrix is higher compared to the simple buffer matrix.

Attention must also be paid to the means used for the immobilization of dsDNA or ssDNA probe, since greatly influence the overall result. In this study two methods were used for DNA immobilization. The first method of immobilization depends on the interaction between neutravidin and its ligand biotin. Neutravidin is a derived protein of avidin that is de-glycosylated, but retains the binding affinity to biotin. Biotin, also known as vitamin D, is a chemical compound that naturally interacts strongly with avidin and avidin derivatives. The neutravidin - biotin interaction is one of the strongest found in nature with a dissociation constant $K_d = 10^{-15}$. Neutravidin was selected as an immobilization anchor between the surface and the DNA since it exhibits low non-specific interaction with liposomes. The details about the process will be given later in the Materials and Methods.

The second method of DNA immobilization is based on the semi-covalent bond between the gold substrate and thiol-groups. This method is widely used for the formation of Self-Assembled Monolayers in biosensors technology^{29,30}. With this method thiol-modified DNA molecules can form a SAM on the sensor surface. While the thiol-group-gold interaction is relatively as strong as the affinity interaction between neutravidin and biotin, SAM formation is a time-consuming process (at least 15 hours), but exhibits the desired feature of no non-specific interaction with liposomes, as observed during this study.

Summarizing, this detailed study investigated the ability of QCM-D biosensors to analyze DNA hybridization and dsDNA detection at low concentrations compared

with ctDNA concentration of normal and cancer patients to provide information about their use in diagnostic platforms.

2.MATERIALS AND METHODS

2.1 Buffers and Reagents

Phosphate Buffered Saline (PBS)

PBS was used as experimental buffer. In these experiments PBS tablets formulated by Sigma Aldrich were used to prepare 1X PBS, which is prepared by dissolving a tablet in 200mL ddH₂O. The typical 1X PBS used in these experiments contains 10mM phosphate buffer (Na₂HPO₄), KCl 2,7mM, NaCl 137mM.

PBS-EDTA 10mM

PBS-EDTA10mM was used as the buffer for the dilution of thiol-modified DNA and the formation of Self Assembled Monolayers. The buffer was prepared to a final volume of 50ml with 49.9mL 1X PBS and 100μl EDTA 0.5M and stored at 4°C.

Sodium Dodecyl Sulfate 2% (SDS 2%)

SDS 2% was used for the cleaning of the set-up apparatus and the sensors' surfaces. The reagent was used at concentration 2% w/v (2g SDS per 100ml ddH₂O). For all the uses the SDS reagent buffer was filtered to remove impurities with a 0.20μm filter. The stock SDS was supplied by Sigma Aldrich.

10X TBE Buffer

10X TBE buffer was prepared as stock buffer for preparing 0,5X TBE buffer used in agarose gel electrophoresis. 10X TBE buffer contains 27g Tris, 12,75g Boric Acid and 10ml EDTA 0,5M in a final volume of 250mL. TBE 0,5X is prepared by adding 50ml of 10X TBE in 950mL ddH₂O.

Other Buffers and Reagents

During the experiments I used several chemical reagents: Chloroform, Ethanol (EtOH 95%) and others were all supplied by the IMBB-FORTH stock house.

2.2 DNA Probes and Sequences

In these experiments, 50nt and 70nt single stranded DNA probes with different 5'-end or 3'-end modifications were used, which were all supplied by Eurogentec. An 157 bps dsDNA used, was a product of PCR with modified primers. The 157bps DNA is part of the exon 20 of the BRCA1 gene. The sequences of the probes and the primers as well as their modifications are presented below.

157bps DNA primers

Forward Primer

5'-ThiolMC6-D-TCC-TGA-TGG-GTT-GTG-TTT-GG-3'

Reverse Primer

5'- Cholesterol TEG-TGG-TGG-GGT-GAG-ATT-TTT-GTC

50 nt Probes sequences

F50 (DNA:1)

5'-Biotin-ATT-TCA-GAG-AGG-AGG-AGA-GAG-CGG-TGC-GGT-AGG-AGA-GAG-AGA-GGA-GGA-TC-3'

R50 (DNA:2)

5'-GAT-CCT-CCT-CTC-TCT-CTC-CTA-CCG-CAC-CGC-TCT-CTC-CTC-CTC-TCT-GAA-TT-3'

R50Ch5 (DNA:3)

5'-Cholesteryl-TEG-GAT-CCT-CCT-CTC-TCT-CTC-CTA-CCG-CAC-CGC-TCT-CTC-CTC-CTC-TCT-GAA-TT-3'

70nt Probe sequence

F70Thiol (DNA:4)

5-ThiolMC6-D-TCC-TGA-TGG-GTT-GTG-TTT-GGA-ATT-CAG-AGA-GGA-GGA-GAG-AGC-GGT-GCG-GTA-GGA-GAG-AGA-GAG-GAG-GAT-C-3'

2.3 Neutravidin Stock and Experimental Mix preparation

Neutravidin (Neu) was supplied by Life Technologies in the form of powder. The stock is prepared by adding sterile PBS 1X until the final Neu concentration is 5mg/ml. For use in the acoustic experiments the Neu sample is prepared by dissolving 4 μ l of stock Neu in 1961X PBS buffer (Final concentration of Neu100ng/uL).

2.4 POPC Liposomes preparation

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) lipids were supplied by Avanti Polar Lipids Inc. suspended in chloroform in a total amount of 2mg and stored at -4°C. To prepare a stock liposome mix 2mg are needed. In a glass vessel cleaned with chloroform 2mg of POPC lipids mix is added and immediately dry in a stream of nitrogen until the lipids form a hardly transparent membrane and there are no liquid remnants. Dry for 1 hour and 30mins in the stream of nitrogen. Then 1mL of PBS buffer is added and the membrane is dissolved by shaking at 600rpm for 1hour and 30mins using a vortex machine to form large vesicles. The vesicles are filtered through an Avanti membrane of the proper pore diameter at least 21 times to form the liposomes of the desired diameter. The lipids are stored at 4°C for 10 days after preparation.

2.5 QCM-D sensors

Gold coated 5MHz Quartz Crystal Microbalance (QCM-D) sensors were supplied by Q-Sense (Biodyn Scientific Inc, Sweden). QCM-D's core component is an AT-cut quartz crystal sandwiched by two gold electrodes. One of the electrodes is capped by chromium and subsequently coated with gold to form the sensor's surface. Due to the piezoelectric properties of the quartz crystal, a current applied on the sensor electrodes causes an in-plane oscillation of the crystal. The acoustic waves propagate vertically to the sensor's surface. Mass deposition causes a shift to the frequency of the wave. At the interface the acoustic wave undergoes a diminishing propagation and there is energy loss which is affected by the viscosity and the density of the liquid phase or deposited biomolecular film.

2.6 QCM-D E4 qSenseSet up

The E4-qSense set up is composed by a four-chamber stage with temperature control. Each chamber hosts one QCM-D sensor. The sensors are stabilized inside the chamber by two O-rings which prevent air and liquid leakage. The flow rate in the microfluidic set up, which contains the inlet outlet tubes and the 40ul chamber volume, is controlled by an Ismatec Pump. The temperature applied and the real time operation monitoring is executed by the Q-soft software on the personal computer. Data can be obtained in Microsoft Excel format using QTools software.

2.7 QCM-D Operation

The experimental measurements were carried at the 7th overtone of 35MHz. During the experiments I used small molecules that form a thin viscoelastic film. Frequency changes (ΔF) occurring during the experiment are proportional to the mass deposited on the sensor's surface as the Sauerbrey equation describes: $\Delta m = -\Delta F \frac{C}{n}$, where Δm is the mass deposited on the film, ΔF is the frequency shift, C is the sensitivity constant of the sensor which in that case is 17,7ng/Hz cm² and n is the vibrational mode of the sensor. Energy dissipation (ΔD) is extruded by the analysis of the decay of the vibrational amplitude with time. $\Delta D = \frac{E_{dissipated}}{2\pi E_{stored}}$, where ΔD is the Dissipation shift, $E_{dissipated}$ is the Energy dissipated during vibration, E_{stored} is the energy stored in the vibrational system. The energy dissipation depends on the viscosity and the elasticity of the film deposited on the sensor surface.

2.8 QCM-D sensors and set up preparation and cleaning

Gold coated QCM-D sensors were rinsed sequentially with Acetone 100%, ddH₂O, Bulk 95% Ethanol, ddH₂O. Then they underwent plasma cleaning at 600mTorr (HI setting) for 3 minutes. After the experiments the set up including the QCM-D sensors was washed with 20mL 2% SDS, 20mL ddH₂O, 20mL 100% or 95% Ethanol and 20mL ddH₂O. The sensors after the set up cleaning were further cleaned by immersing them for at least 30 minutes and the cleaning steps are same as in the preparatory cleaning and underwent the same plasma cleaning.

2.9 Self-Assembled Monolayers Formation on QCM-D sensors

Preparatory Steps

In the case of SAM formation the steps of QCM-D preparation require a pre-cleaning process. The sensors are soaked in 2% SDS for 30minutes. Then they are thoroughly rinsed with ddH₂O. Afterwards the standard preparatory steps (described before) were followed. The plasma cleaning must be done at 600mTorr for 9 minutes, to remove SDS remnants.

SAM formation mix

For each QCM-D sensor I used 100ul of the SAM formation mix which contains: 1μM DNA:4, 4μM 157 bps DNA forward primer (final thiolate-groups concentration 5μM) in 95ul 1X PBS-EDTA 10mM.

SAM formation

Immediately after plasma cleaning the sensors were placed in a Petri Dish. 100ul of the SAM formation mix were added on the sensor surface. The dish must be closed with lid to avoid evaporation of the SAM formation mix. The sensors were incubated with the SAM formation mix at room temperature for at least 15 hours. After the incubation and before the experiment each sensor was rinsed with 1X PBS buffer and ddH₂O, to stop SAM formation and remove unbound remnants of the mix. The sensors were then dried in a stream of N₂.

2.10 Polymerase Chain Reaction (PCR)

PCR was used for the acquisition of dsDNA 157bps. The dsDNA molecules were acquired by the thermocycling program which is described next.

PCR thermocycling program

START CYCLE	95°C for 5 minutes
40 Cycles	
Denaturation	95°C for 10 seconds
Annealing	62,5°C for 10 seconds
Extension	72°C for 10 seconds
Final Extension	72°C for 3 minutes
STORE	Store forever at 4°C

The PCR reaction was carried in 100ul SARSTEDT tubes.

Reactants	Volumes
2X KapaHotSTART buffer (containing polymerase)	5ul
Template DNA (Human genomic DNA, 1pmol/ul)	1ul
Forward Primer (10pmol/ul)	1ul
Reverse Primer (10pmol/ul)	1ul
MgCl ₂ 25mM	0,5 ul
ddH ₂ O	1,5 ul

Isolation and Cleaning of PCR Products

The PCR products were either cleaned after PCR reactions or isolated by the electrophoresis agarose gel and subsequently cleaned. For the cleaning of PCR products we used the NucleoSpin Gel and PCR Clean Up Kit.

Immediate PCR products clean-up

- Adjust the PCR products volume, if needed, to 100ul by adding ddH₂O.
- Add 200ul NT1 buffer. Mix well and load the sample to the Kit DNA extraction column.
- Centrifuge for 30 secs, 11.000 x g at RT.
- Discard the flow through and load 700ul NT3 buffer to wash the silica membrane in the column.
- Centrifuge for 30 secs, 11.000 x g at RT.
- Discard the flow through liquid.
- Repeat the Wash step once.
- Dry the silica membrane in the column by centrifuging for 1 min, 11.000 x g at RT.
- Elute DNA by adding 30ul NE buffer and incubate for 1 min at RT.
- Centrifuge for 1min, 11.000 x g at RT.

PCR products extraction from agarose gel.

- With a clean scalpel remove the desired DNA fragment from the agarose gel and remove excess agarose. Determine the weight of the gel slice. Mix the slice with NT1 buffer. NT1 is added according to the weight of the gel slice. Add 200ul NT1 per 100mg of gel slice.
- Incubate the slice with NT1 at 50°C for 5-10 minutes until the gel slice is dissolved.
- Mix well and load the sample to the Kit DNA extraction column.
- Centrifuge for 30 secs, 11.000 x g at RT.
- Discard the flow through and load 700ul NT3 buffer to wash the silica membrane in the column.
- Centrifuge for 30 secs, 11.000 x g at RT.
- Discard the flow through liquid.
- Repeat the Wash step once.
- Dry the silica membrane in the column by centrifuging for 1 min, 11.000 x g at RT.
- Elute DNA by adding 30ul NE buffer and incubate for 1 min at RT.
- Centrifuge for 1min, 11.000 x g at RT.

2.11 Agarose gel Electrophoresis

Agarose gel electrophoresis was used to identify PCR products according to their molecular size. The gel electrophoresis was done using 2% agarose gel. The agarose gel is prepared with agarose and 0,5X TBE buffer.

2% Agarose gel preparation

- Weight 2 gr of agarose and add them in 100mL 0,5X TBE Buffer in a 500mL glass flask.
- Dissolve the agarose in the TBE Buffer with thermal heating for 2 mins and 30 secs in a microwave oven.
- After heating we add 10ul GelRed to the liquid mix. GelRed is used instead of ethidium bromide (EtBr). Like EtBr, GelRed intercalates in the backbone of the nucleic acids and fluoresces after exposure to UV light.

- The gel in the liquid form is poured in a plastic tray with well-formative, to coagulate and form the gel. After coagulation we remove the well-formative.

DNA samples preparation

For gel electrophoresis 2ul of sample DNA (or the appropriate volume in order to have 50ng or more of the DNA fragment interested) is mixed with 2ul 6X Loading Buffer and ddH₂O to final volume 12ul.

Gel electrophoresis

- Add the gel in the electrophoresis tank in an orientation were sample loading is near the negative pole. The negative charge of DNA during electrophoresis causes the DNA shift inside the gel.
- The electrophoresis is done in 100mV or 100mA for 30 minutes.

2.12 Experimental Methods

dsDNA immobilization on the surface via biotin-neutravidin interaction

Description of the acoustic assay: The experimental steps are described below in bullets. The experimental conditions that are stable through the experiments are given in the following table.

STANDARD CONDITIONS	
Temperature	25.0 °C
Flow rate	50ul per minute
QCM Vibrational Mode measurement	7 th overtone (Frequency=35MHz)

- Equilibration of the acoustic signal by rinsing with 1X PBS (Frequency≈0 Hz, Dissipation≈0 x 10⁻⁶).
- Neutravidin Injection: [Neutravidin] = 100ng/ul in 1X PBS, Final Sample Volume= 200ul.
- Rinsing with 1X PBS until the acoustic signal is steady.
- dsDNA Injection: the desired concentration diluted in 1X PBS, Final Sample Volume= 100ul.
- Rinsing with 1X PBS until the acoustic signal is steady.

- Liposomes Injection: Add 50ul of stock Liposomes in 450 ul 1X PBS, Final Sample Volume= 500ul.
- Rinsing with 1X PBS until the acoustic signal is steady.

On sensor dsDNA hybridization with probe immobilized via biotin-neutravidin interaction

Description of the experimental assay: The experimental steps are described below in bullets. The experimental conditions that are stable through the experiments are given in the following table.

STANDARD CONDITIONS	
Temperature	25.0 °C
Flow rate	50ul per minute
QCM Vibrational Mode Measurement	7 th overtone (Frequency=35MHz)

- Equilibration of the acoustic signal by rinsing with 1X PBS (Frequency=0 Hz, Dissipation $\approx 0 \times 10^{-6}$).
- Neutravidin Injection: [Neutravidin] = 100ng/ul in 1X PBS, Final Sample Volume= 200ul.
- Rinsing with 1X PBS until the acoustic signal is steady.
- ssDNA Probe Injection: 50 pmols of DNA.:1 diluted in 1X PBS, Final Sample Volume= 100ul.
- Rinsing with 1X PBS until the acoustic signal is steady.
- ssDNA Complementary Injection: the desired concentration of DNA.:2 diluted in 1X PBS, Final Sample Volume = 100ul. In the case of serum matrix experiments in the 10ul or 1ul of FBS was present at the sample.
- Rinsing with 1X PBS until the acoustic signal is steady.
- Liposomes Injection: Add 50ul of stock Liposomes in 450 ul 1X PBS, Final Sample Volume= 500ul.
- Rinsing with 1X PBS until the acoustic signal is steady.

Ds DNA detection in Self Assembled Monolayers

Description of the acoustic assay: The experimental steps are described below in bullets. The experimental conditions that are stable through the experiments are given in the following table.

STANDARD CONDITIONS	
Temperature	25.0 °C
Flow rate	50ul per minute
QCM Vibrational Mode Measurement	7 th overtone (Frequency=35MHz)

- Equilibration of the acoustic signal by rinsing with 1X PBS (Frequency=0 Hz, Dissipation $\approx 0 \times 10^{-6}$).
- Liposomes Injection: Add 50ul of stock Liposomes in 450 ul 1X PBS, Final Sample Volume= 500ul.
- Rinsing with 1X PBS until the acoustic signal is steady.

On sensor hybridization after probe immobilization on SAM

Description of the acoustic assay: The experimental steps are described below in bullets. The experimental conditions that are stable through the experiments are given in the following table.

STANDARD CONDITIONS	
Temperature	25.0 °C
Flow rate	50ul per minute
QCM Vibrational Mode Measurement	7 th overtone (Frequency=35MHz)

- Equilibration of the acoustic signal by rinsing with 1X PBS (Frequency=0 Hz, Dissipation $\approx 0 \times 10^{-6}$).
- ssDNA Complementary Injection: the desired concentration of DNA.:2 diluted in 1X PBS, Final Sample Volume = 100ul.
- Rinsing with 1X PBS until the acoustic signal is steady.
- Liposomes Injection: Add 50ul of stock Liposomes in 450 ul 1X PBS, Final Sample Volume= 500ul.
- Rinsing with 1X PBS until the acoustic signal is steady.

2.13 On Bench Hybridization for dsDNA 50bps formation

ds DNA 50bps is assembled by hybridization of ssDNA probes.

- Mix 10 pmol of DNA.:1 with 100pmol of DNA.:3 in PBS, Final Volume=100ul or 10ul.
- Denaturate with thermal heating at 95°C for 5 minutes.

- Mix well the volume with pipette.
- Incubate on bench under light limited conditions* for at least 1hr and 30mins.

*Biotin is affected by light presence so light limiting conditions ensure higher quality of the biotin modification.

3.RESULTS

All the methods described at the materials and methods were able to detect DNA adsorption or hybridization and acoustic amplification was successful using every method. Nevertheless the detection limit and the sensitivity vary between the methods. It is also demonstrated that the size of liposomes plays also an important role, and can affect both the detection limit of a method and the sensitivity.

Concerning the non-specific interactions that may lead to false-response, it was observed that Self Assembled Monolayers is a good surface for the operation of the amplification event, since liposome addition causes neither Frequency nor Dissipation shifts in the absence of analyte.

All of the measurement results that will be given below are those which exceed the experimental noise. The experimental noise was small in the majority of the experiments.

3.1 Detection of dsDNA molecules immobilized via neutravidin- biotin interaction.

The method background: In these experiments, dsDNA concentration varying from some picograms to milligram per 100ul, was immobilized on the sensor surface via the neutravidin-biotin interaction. Neutravidin is a complex of four subunits with a total molecular weight approximately 60KDa. The protein has four biotin-binding sites with a $K_d=10^{-15}$ M. This protein operates as molecular anchor between the DNA and the surface. Neutravidin adsorbs in a flat-on conformation on gold surfaces that restrict the availability of at least two binding sites at a monolayer film. dsDNA can be anchored on the sensor surface due to the '5-end biotin-modification of one strand. After anchoring the complementary strand which carries a 5'-end cholesterol modification is able to interact with liposomes.

Liposomes interaction with the DNA is based on the affinity of cholesterol to the hydrophobic region of lipid membranes. Upon liposomes addition this affinity interaction is used to attract the liposomes and bind liposomes on the sensor surface. Obviously the number of the anchored liposomes is almost proportional to the number of DNA molecules immobilized on the sensor surface. Since the molecular weight is above the detection limit of the sensor and the viscosity of the liposomes is greater than the viscosity of the DNA, a shift in the Frequency and the Dissipation occurs. Thus it is easy to assume that liposomes amplify the DNA signal proportionally. Figure 3 is a descriptive depiction of this experimental principle.

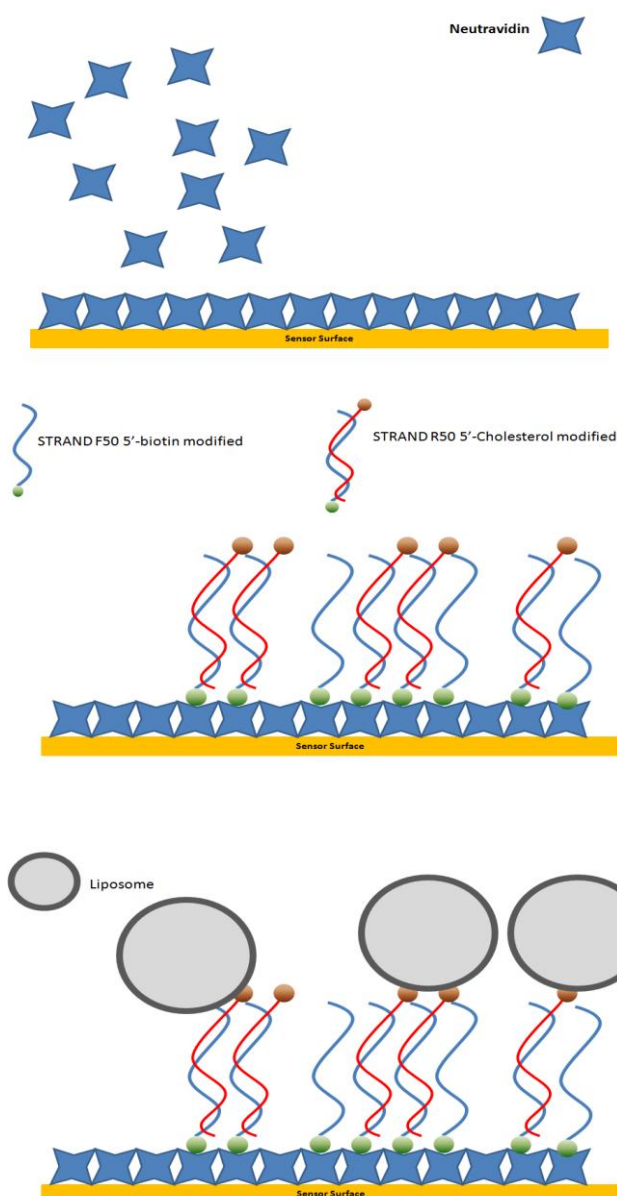


Figure 3: Schematic overview of the dsDNA detection via DNA binding on the sensor surface and amplification with liposomes:

a) The neutravidin adsorbs on gold and forms a rigid film.

b) dsDNA with biotin modified 5-end binds on the film via the biotin-neutravidin interaction.

c) Addition of liposomes. Liposomes anchor on the DNA molecules via the lipid-cholesterol affinity interaction.

The representation is not in scale.

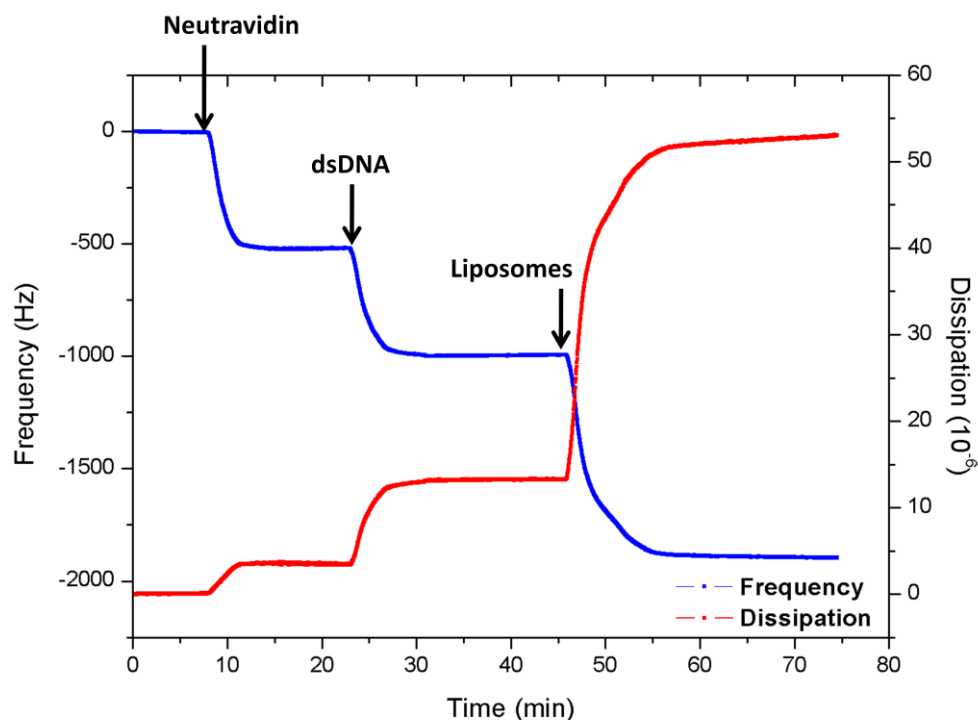


Figure 4: A typical real time graph of the experimental process of dsDNA detection on a neutravidin film. The first step is neutravidin adsorption on the sensor surface. Then the dsDNA-cholesterol modified is injected and binds onto the neutravidin film via their biotin group. Finally liposomes are injected, interacting with the dsDNA via the cholesterol – lipid and anchoring to the surface.

With this experimental method I investigated the amplification of the presence of two dsDNA molecules. The first dsDNA had 50bps and was obtained by hybridization as described at the Materials and Methods. The second had 157 bps and was obtained by PCR as described previously. Figure 4 shows a typical real time graph of the experimental process.

3.1.1 dsDNA 50 bps - 50nm Liposomes

dsDNA injection on the neutravidin film leads to an initial rapid adsorption, which implies that the biotin-modification is sufficient to lead the DNA molecules from the flow phase to the sensor's surface. DNA samples with a concentration less than 10ng/200ul produce no acoustic response. Taking under consideration that the QCM-D is able to detect DNA at the ng scale, is not in the imagination scale to assume that a smaller amount of DNA molecules can transport from the flow phase to the detection limit area and interact with the neutravin layer.

In all the experiments the D/f ratio, which is the fingerprint of a DNA molecule according to its length and its intrinsic viscosity, had constantly values near the theoretical value $D/f = 0,0175 \cdot 10^{-6}/\text{Hz}^{31}$. Figure 5 shows the DNA D/f versus DNA Frequency Plot which is indicative of DNA conformation. In high Frequency Shifts (that are related to great mass deposition) there is a proper D/f value as mentioned.

In lower Frequency shifts the D/f value changes due to the inability of mass

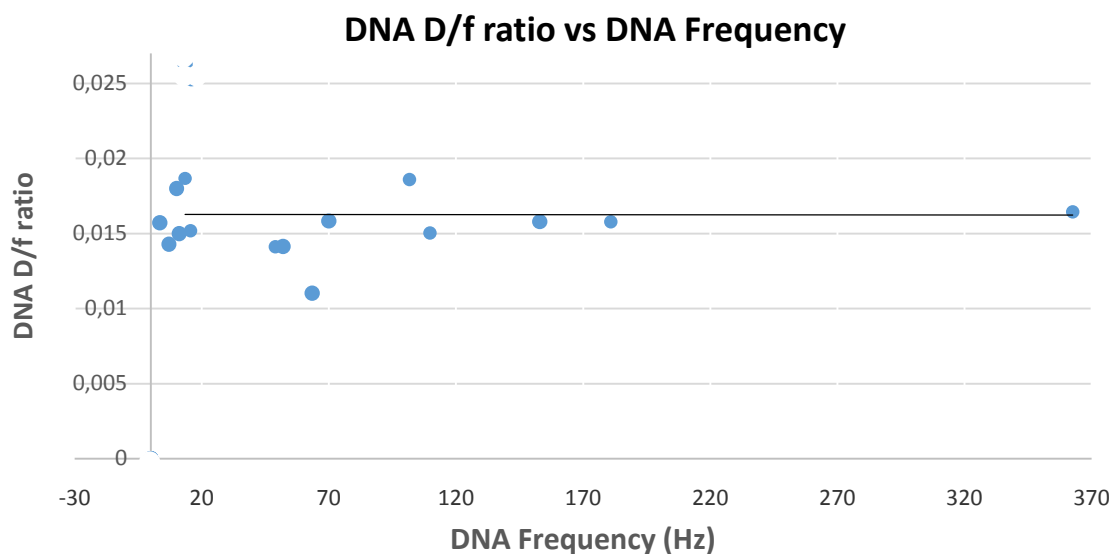


Figure 5: The ΔF vs D/f ratio plot. The trendline of 50 bps dsDNA intercept is 0,0165 near the theoretical D/f value expected.

deposited to cause significant frequency shift while the dissipation factor is stable.

Neutravidin adsorption was also examined by the D/F ratio, and similarly the adsorbed neutravidin forms a rigid film with expected D/F ratio value (data not shown). Table 1 shows the ΔF , ΔD and D/F ratio of 50nm Liposomes amplification step according to the DNA concentration in the sample. Non-specific interactions ($\Delta F=0\text{Hz}$, $\Delta D=0,12 \times 10^{-6}$).

Taking into account the values presented, it is obvious, that with the amplification step QCM-D sensors are able to detect DNA concentration more than 3 orders of magnitude lower compared to processes without the amplification step. As stated earlier, DNA samples with concentration lower than 10ng/200ul, were not able to produce ΔF and ΔD signals during DNA sample injection. By using 50nm POPC liposomes we were able to detect as low as 0,005ng/200ul. These results underscore the importance of the amplification step for lowering the detection limit of QCM-D when used in DNA detection experiments.

DNA Concentration	ΔF Liposomes 50nm	ΔD Liposomes 50nm	D/f Liposomes 50nm
ng/200ul	Hz	10^{-6}	$10^{-6}/\text{Hz}$
1551	942 \pm 0	24,5 \pm 0	0,0260
1419	845 \pm 0	23,58 \pm 0	0,0279
825	476,5 \pm 45,96	16,63 \pm 1,153	0,0349
100	67 \pm 11,31	2,82 \pm 0,46	0,0421
66	287,5 \pm 71,42	11,96 \pm 2,022	0,0416
50	423,5 \pm 88,39	14,30 \pm 2,369	0,0337
20	326 \pm 29,7	13,05 \pm 0,134	0,0400
10	10,5 \pm 0,7	0,18 \pm 0,056	0,0171
5	30 \pm 2,8	0,87 \pm 0,247	0,0291
1	28 \pm 5,6	0,58 \pm 0,035	0,0208
0,5	6 \pm 0	0,05 \pm 0,077	0,0091
0,1	6 \pm 0	0,27 \pm 0	0,045
0,05	9,75 \pm 2,4	0,25 \pm 0,014	0,0256
0,01	6 \pm 0	0,2 \pm 0	0,0333
0,005	11,5 \pm 7,7	0,17 \pm 0,035	0,0152

Table 1: Liposomes 50nm ΔF , ΔD and D/f acoustic values according to 50bps dsDNA concentration in the sample volume (n \geq 3).

One of the system's desired features is sensitivity. The term describes the ΔF or/and ΔD shifts units caused in response to DNA molecules deposition on the sensor's surface. The ideal sensitivity required for our system would be the ability to detect ΔF and ΔD response after the deposition of one DNA molecule. To analyze the sensitivity of the system I use the DNA concentration versus $\Delta F/\Delta D$ plot.

Figure 6 presents the two plots. The changes of ΔF and ΔD are shown to follow a rather sigmoid trend. Specifically we can observe that from DNA concentrations ranging from 0,005ng/200ul to 10 ng/200ul, there is a small ΔF and ΔD shift in response to DNA concentration increase, forming a lag phase on the plot. From concentrations ranging from 10ng/200ul to 1200ng/200ul there is the sigmoid phase and great ΔF and ΔD shifts can be observed in response to little DNA concentration increase. For higher sample concentrations there is no significant ΔF or ΔD response indicating that the surface is near saturation there is no space for further liposome's anchoring. It is noteworthy to mention that in concentrations near 100ng/200ul it is observed that both the ΔF and ΔD shifts do not follow exactly the trend described. More over the error bars show great deviation from the average value, since there was great dispersion between the experimental values. In these concentrations it is also observed that the ΔF and ΔD of the experiments form clusters of lower and higher values with almost no intermediate, thus the result is an average value with great error bars (Data shown at Supplementary Appendix).

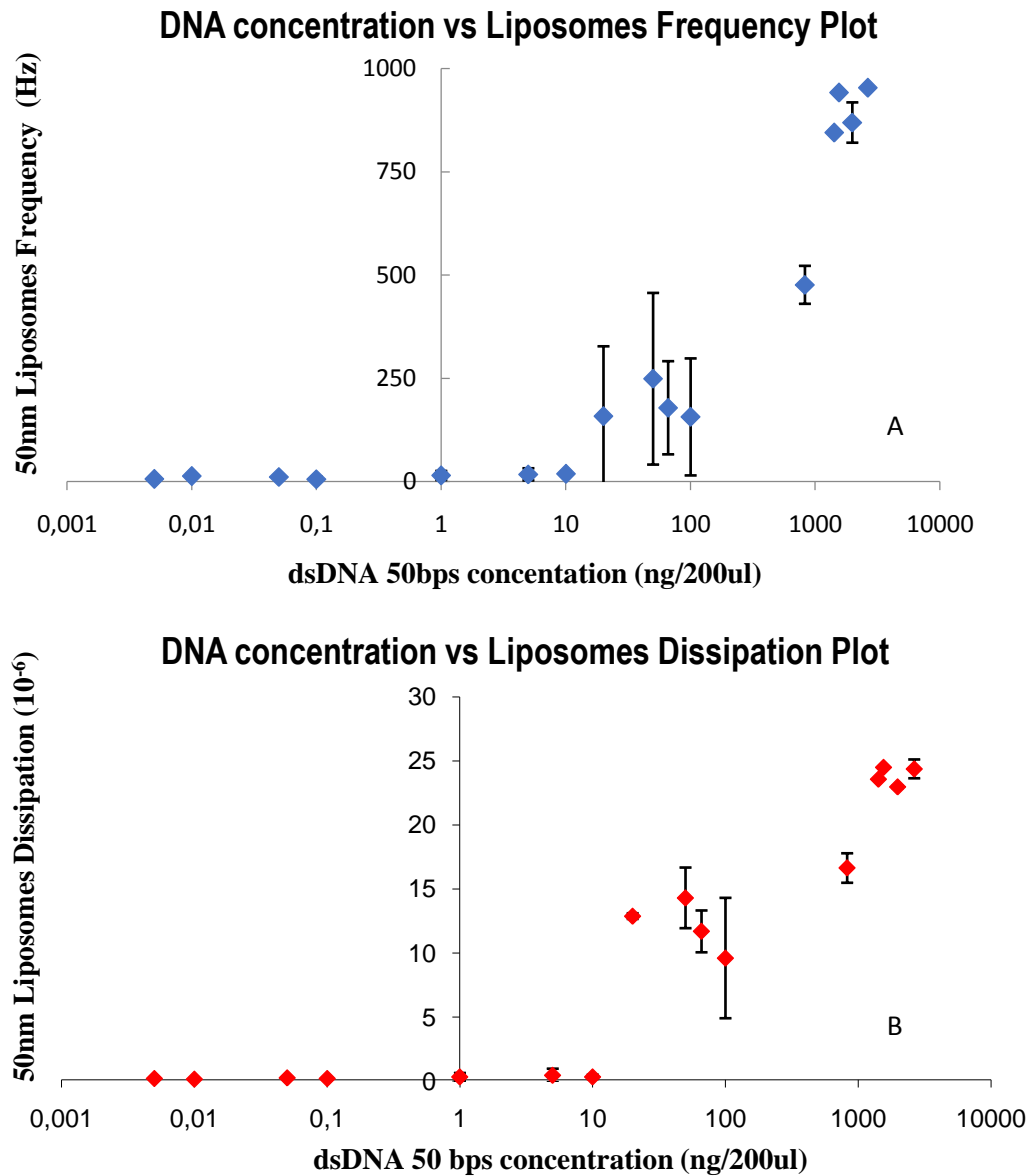


Figure 6: A) The ΔF shift caused by liposomes versus the the ds DNA concentration plot. B) The ΔD shift caused by liposomes versus the the ds DNA concentration plot. The DNA concentration axis is at logarithmic scale.

Concerning the D/f ratio, which is the fingerprint of the interfacial phenomena we observe that the D/f ratio produced and measured by ΔF and ΔD shifts of liposomes' anchorage varies according to the DNA immobilized on surface but the values do not follow a specific trend that could be used for accurate DNA quantification (See values at Table 1).

Summarizing the system is suitable for detecting dsDNA for concentrations at 7,5pM. The system is more sensitive for dsDNA samples with concentration ranging from 1,65nM to 215nM.

3.1.2 dsDNA 157 bps - 200nm Liposomes

During these experiments the neutravidin and DNA adsorption was suitable as mentioned at the previous chapter concerning the experiments with 50bps DNA. In these experiments I used a longer dsDNA obtained by PCR method as described at the section of Materials and Methods. The D/F vs Frequency plot of the DNA reveals that the acoustic ratio D/F is approximately $0,028 \times 10^{-6}/\text{Hz}$, near the expected theoretically $D/F=0,0324 \times 10^{-6}/\text{Hz}$ (Figure 10).

The experiments of with this combination of DNA length and liposome size was carried once, for that reason there is no error indicated at the Table 2 which presents the acoustic values of liposomes anchoring according to DNA concentration.

DNA concentration ng/200ul	Liposomes ΔF Hz	Liposomes ΔD 10^{-6}	Liposomes D/f $10^{-6}/\text{Hz}$
110	90	11,06	0,1228
11	13	2,44	0,1876
1,1	5	1,26	0,252
0,11	0	0,2	**
0,011	7	0,71	0,1014

Table 2: Liposomes 200nm ΔF , ΔD and D/f acoustic values according to 157bps dsDNA concentration in the sample volume (n=1).

The values show that with the use of 200nm liposomes it is possible to probe at least 3 orders of magnitude of DNA compared to experiments without the amplification step. Moreover taking into account that both ΔF and ΔD values at the lowest concentration detected is significantly higher compared to the lowest concentration detected at the experiments with 50bps DNA with 50nm Liposomes, it is not farfetched to conclude that the detection of dsDNA 157bps at lower order of magnitude is highly possible.

Concerning the sensitivity the DNA concentration versus ΔF and ΔD of Liposomes Plots present an image similar with the one obtained with the 50bps DNA with 50nm Liposomes amplification. ΔF and ΔD plots reveal a sigmoid pattern during amplification. For concentrations lower than 1,1 ng/100ul the amplification acoustic values follow a lag phase and the shifts in response to DNA concentration increase are low resulting in low sensitivity. For concentrations in the range of 1,1 to 11 ng per 100ul, the ΔF value of amplification is still following the lag phase of lower concentrations but ΔD shift changes in an initiating sigmoid trend. For

concentrations in the range of 11ng to 110 ng per 100ul both ΔF and ΔD amplification values follow the sigmoid trend, providing high sensitivity for DNA concentration increase. Due to the lack of experiments for higher DNA concentration, I cannot say if this value is at the saturation point.

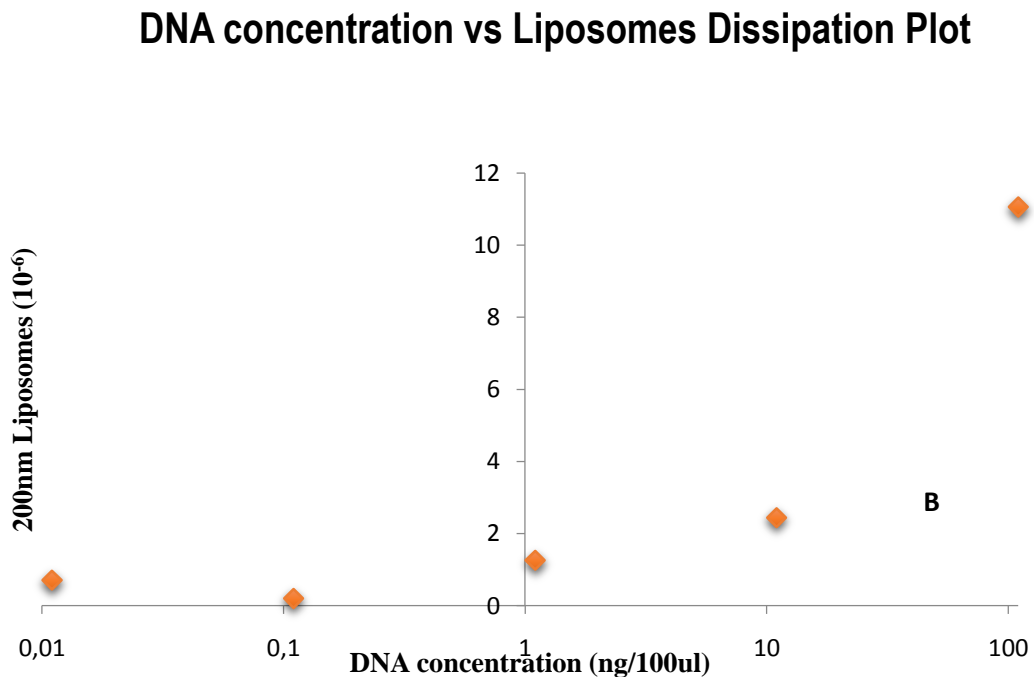
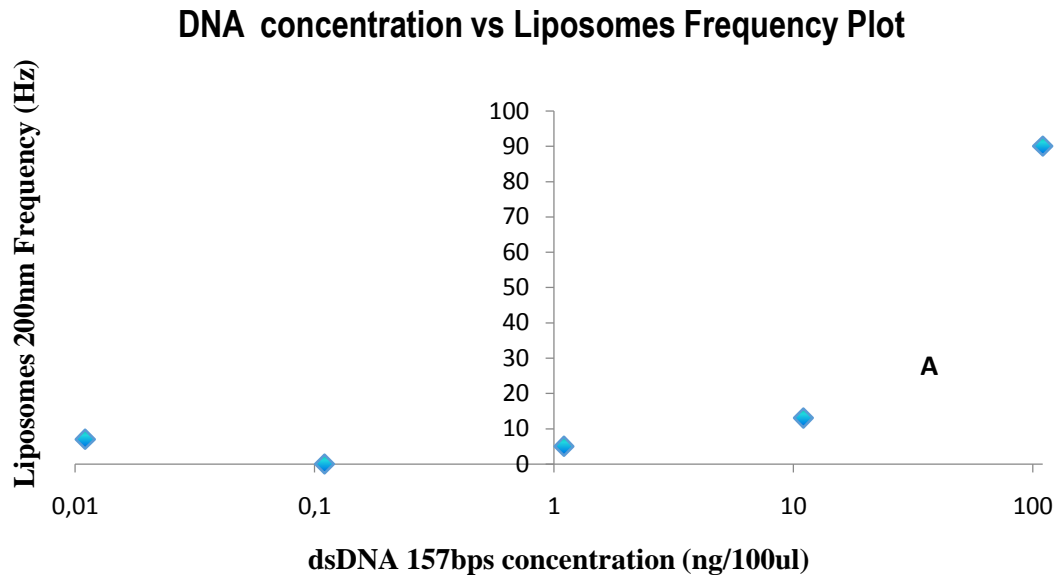
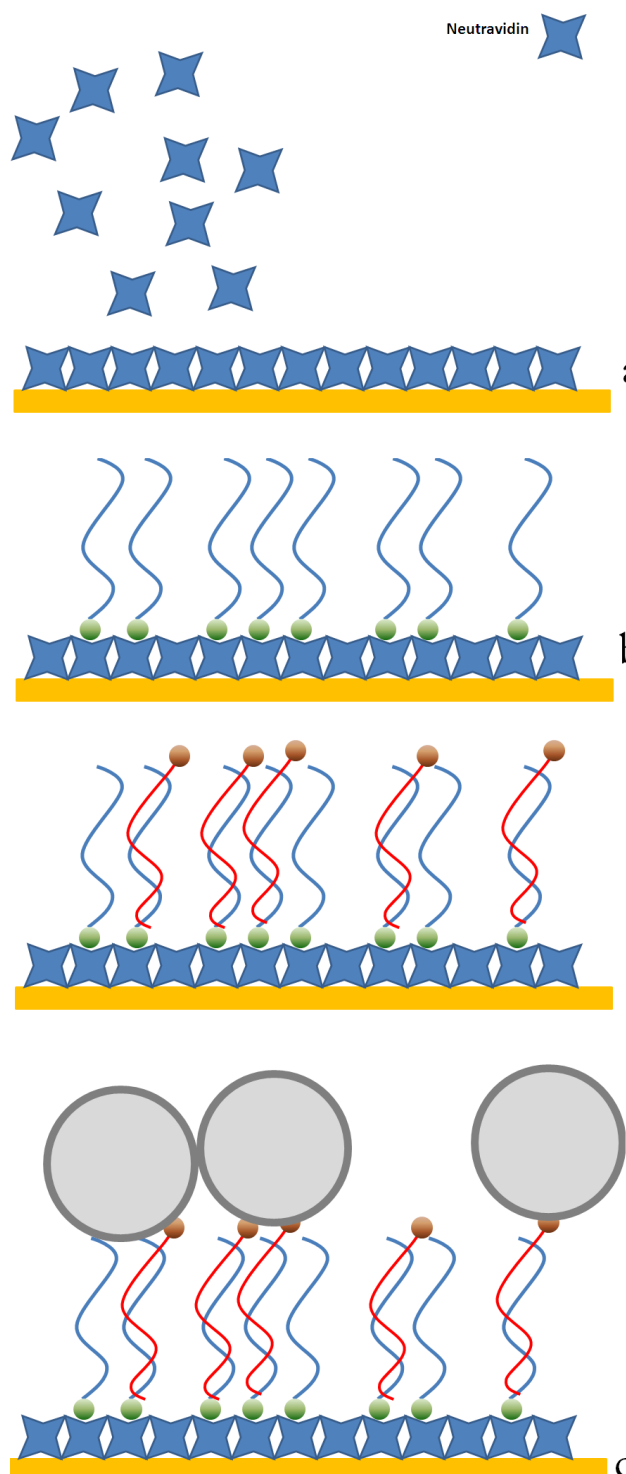


Figure 7: A) The ΔF shift caused by liposomes versus the the ds DNA concentration plot. B) The ΔD shift caused by liposomes versus the the ds DNA concentration plot. The DNA concentration axis is at logarithmic scale.



3.2 ssDNA immobilization via neutravidin - biotin interaction followed by surface Hybridization

The method background: This method is similar to the aforementioned method, only in this method the dsDNA molecules assemble on the sensor surface and the hybridization process can be monitored. In these experiments after neutravidin adsorption on the sensor surface, a 5'-end biotin-modified ssDNA probe is immobilized. At the next step a complementary 5'-end cholesterol TEG- modified ssDNA analyte is added. Due to sequence complementarity between probe and analyte the hybridization event leads to the formation of dsDNA molecules on the sensor surface. Then the addition of liposomes is proportional to the dsDNA molecules, since ssDNA probes cannot interact with liposomes. As a result liposomes again cause ΔF and ΔD shift which acts as an acoustic amplifier of the analyte binding.

Figure 8: Schematic overview of the ssDNA detection via DNA hybridization on the sensor surface and amplification with liposomes:

- The neutravidin adsorbs on gold and forms a rigid film.
- ssDNA probe with biotin modified 5-end binds on the film via the biotin-neutravidin interaction.
- 5-end cholesterol modified ssDNA analyte hybridizes with immobilized probe molecules, and forming of dsDNA molecules on surface
- Addition of liposomes. Liposomes anchor to the dsDNA molecules via the lipid-cholesterol affinity interaction.

The representation is not in scale.

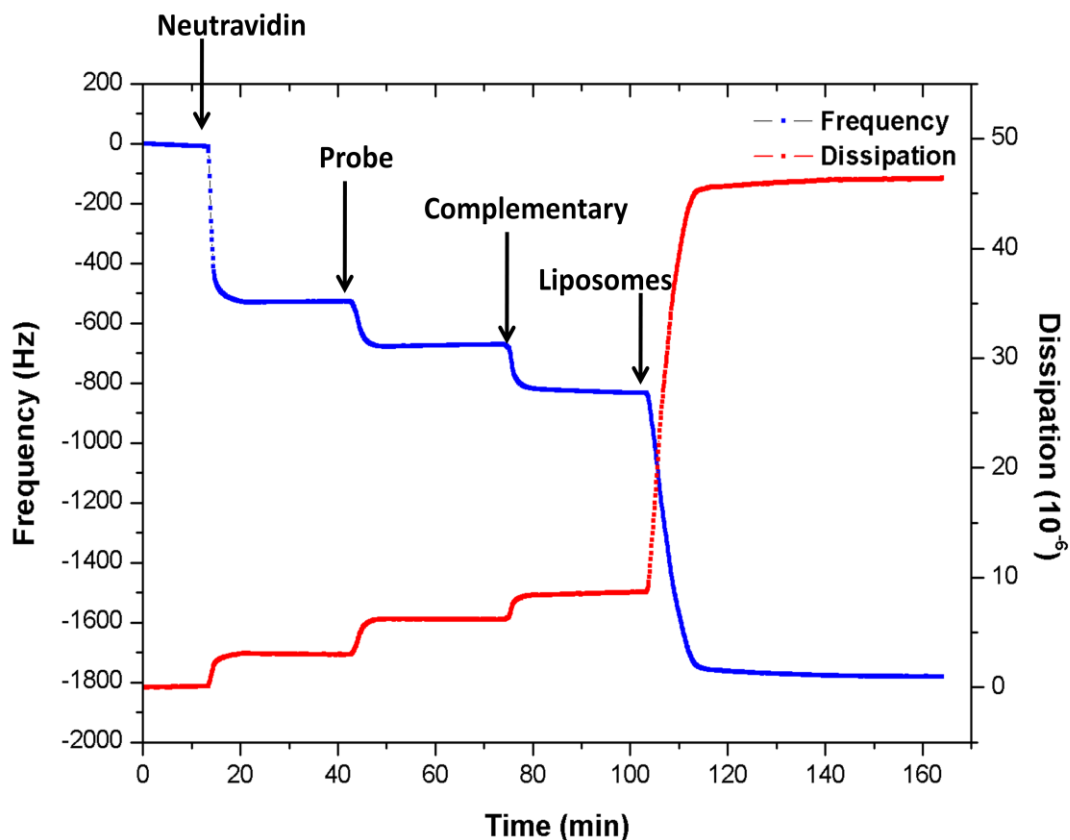


Figure 9: A typical real time graph of the experimental process. After initial equilibration with PBS rinsing neutravidin is added and forms a film. Then 50pmol of the 50nts ssDNA biotin modified probe is injected and adsorbs onto the neutravidin film via the biotin-neutravidin interaction. Then the analyte ssDNA Cholesterol modified is added which hybridizes with the immobilized probe. Finally the liposomes are injected and anchor on the dsDNA formed on the sensor surface via the lipid-cholesterol interaction.

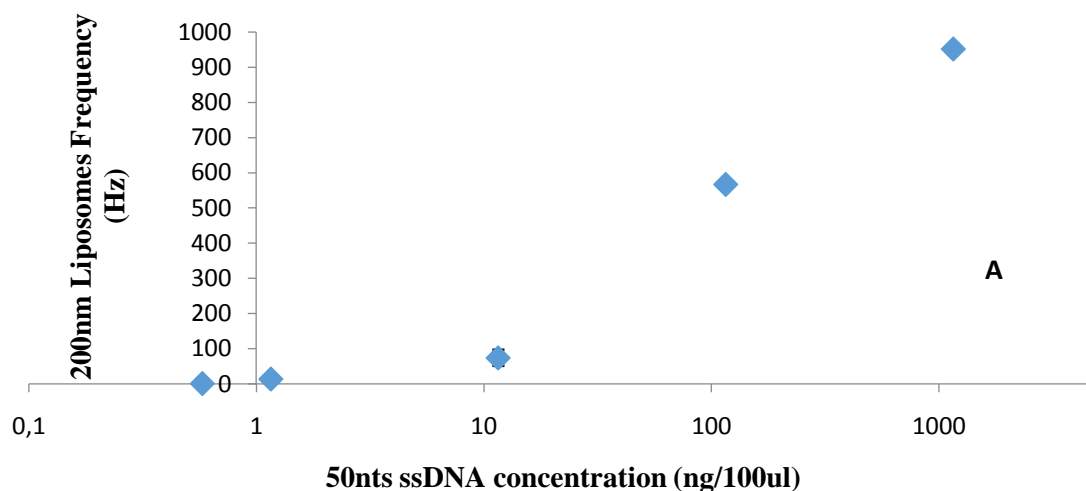
The hybridization processes are ideal for detecting DNA molecules in a sample using acoustic biosensors. Liposomes were used to amplify the acoustic detection of ssDNA molecules after hybridizing with a complementary probe immobilized on the sensor's surface via the neutravidin-biotin interaction. Specifically, DNA1 (50pmol) was immobilized on the neutravidin film and afterwards the desired amount of DNA3 was added and hybridized with the complementary DNA1. Then the liposomes are added and allowed to interact with the cholesterol tag of DNA3. Non-specific interaction ($\Delta F=0\text{Hz}$, $\Delta D=0,12 \times 10^{-6}$) are observed.

The D/f ratio of DNA hybridization was near the expected $D/f= 0,0175 \text{ } 10^{-6}/\text{Hz}$ high concentrations, but in low concentrations the D/F varies due to the excess of ssDNA probe (data not shown). Table 3 presents the acoustic values obtained after liposomes anchoring according to the concentration of the analyte (DNA3).

DNA concentration (ng/100ul)	Liposomes ΔF Hz	Liposomes ΔD 10^{-6}	Liposomes D/f $10^{-6}/\text{Hz}$
1158	952 \pm 0	37,77 \pm 0	0,039674
115,8	567 \pm 0	34,35 \pm 0	0,060582
11,58	74 \pm 22,62	5,97 \pm 4,42	0,080676
1,158	13,5 \pm 2,12	1,32 \pm 0,38	0,097778
0,579	1 \pm 0	0,43 \pm 0,32	0,43

Table 3: Acoustic values of 200nm Liposomes adhesion according to DNA3 concentration in the hybridization process(n=2).

ssDNA concentration vs Liposomes Frequency Plot



ssDNA concentration vs Liposomes Dissipation Plot

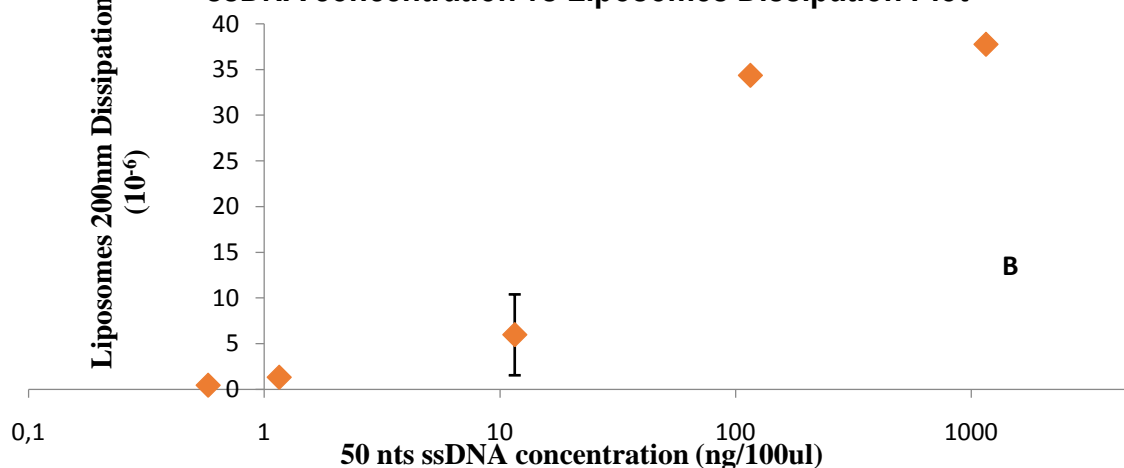


Figure 10: A) The ΔF shift caused by liposomes versus the ssDNA concentration plot. B) The ΔD shift caused by liposomes versus the ssDNA concentration plot. The DNA concentration axis is at logarithmic scale .

The results of these experiments show that the lowest concentration of analyte which could be detected after the hybridization process is 0,579 ng/100ul. This detection limit is two orders of magnitude higher compared to the detection limit of the 50 bps dsDNA.

In the figure 10 are presented the ΔF and ΔD versus analyte concentration plots.

The detection limit of this experimental procedure for detecting 50nts ssDNA after hybridization using 200nm liposomes was relatively high, but the ΔF and ΔD vs analyte concentration plots show that this method has a relatively higher sensitivity than the experimental processes using dsDNA. Both ΔF and ΔD plots follow a sigmoid trend but in both graphs we can see that the measurements obtained for different orders of magnitude concentration of the analyte significantly differ from each other out of the experimental error. The experimental error is also a great advantage of this method since it is observed that there is low deviation and the values obtained for each measurement ($n=2$) are reproducible. The only exception is the measurement for detecting 11,58 ng.

3.3 dsDNA immobilized in a SAM layer and 50nm & 100nm Liposomes

The method background: In this method dsDNA was immobilized on the sensor surface via a thiol-gold covalent bond as part of Self Assembled Monolayer. The 5'-end alkane-thiol modified dsDNA (5'-HS-(CH₂)₆-DNA-3') was produced with PCR using thiolated primers. Alkane-thiols have a great affinity for metals such as gold³⁰ and form compactly assembled films on metallic surfaces. In these experiments mixed SAMs were made by immobilizing 157 bps DNA Thiol-modified forward primer and the desired concentration of 5-end thiol modified 157 bps DNA with 5-end cholesterol modification on the complementary strand molecules. Upon liposome addition, liposomes anchor on the 157bps dsDNA molecules almost proportionally to the number of dsDNA molecules causing a ΔF and ΔD acoustic signal. Since the dsDNA is part of the SAM this method is not an amplification of DNA acoustic signal but rather a proof of principle used to quantify the dsDNA immobilized in the SAM.

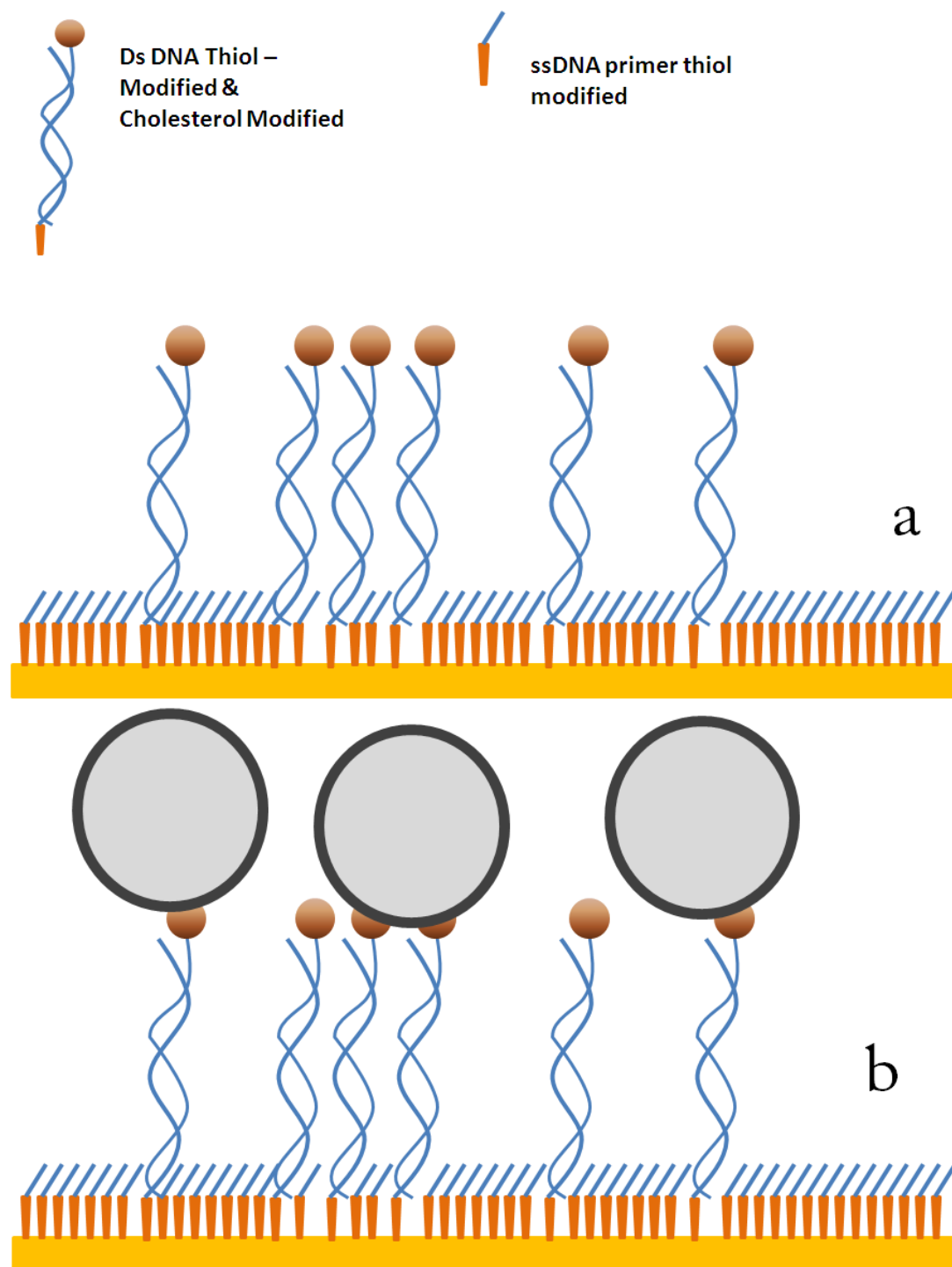


Figure 11: dsDNA detection immobilized within a SAM. a) The surface by SAM formation contains dsDNA cholesterol modified. B) After liposomes addition, liposomes anchor on the dsDNA and cause a Frequency and Dissipation shift proportional to the number of dsDNA molecules immobilized on the sensor's surface.

Representation not in scale.

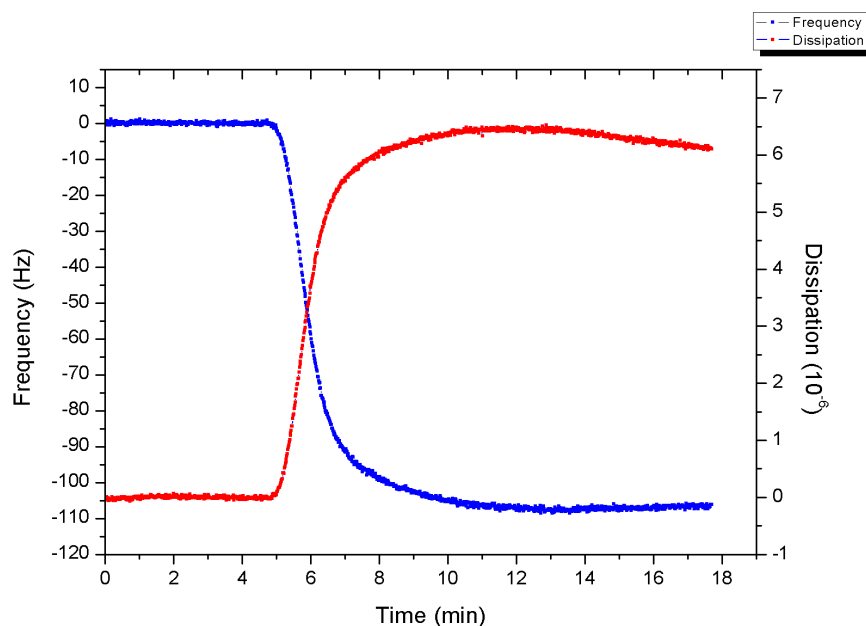


Figure 12: A typical real-time graph of the experimental process. After the initial signal equilibration of the surface, containing the dsDNA in a desired concentration the only step is the addition of liposomes, which anchors proportionally to the dsDNA immobilized on the sensor surface.

	DNA Concentration (ng on surface)	ΔF Liposomes Hz	ΔD Liposomes 10^{-6}	Liposome D/f $10^{-6}/\text{Hz}$
50nm Liposomes	80	105,6 \pm 1,15	5,96 \pm 0,43	0,0564
	40	39 \pm 2,64	3,35 \pm 0,46	0,0858
	20	20,5 \pm 3,53	1,45 \pm 0,07	0,0707
	10	7,3 \pm 3,21	0,46 \pm 0,09	0,0627
	5	3,5 \pm 1,5	0,22 \pm 0,10	0,0641
100nm Liposomes	100	80 \pm 5,5	6,68 \pm 0,38	0,0835
	80	86,6 \pm 19,5	6,14 \pm 0,85	0,0708
	40	41,2 \pm 12,1	2,90 \pm 1,14	0,0703
	20	12,6 \pm 4,5	1,34 \pm 0,23	0,1060
	10	17,3 \pm 15,6	1,24 \pm 1,31	0,0715
	5	4,3 \pm 0,5	0,38 \pm 0,02	0,0892
	1	1,8 \pm 0	0,24 \pm 0	0,1283

Table 4: The acoustic values of dsDNA mixed in SAM detection with 100nm & 50nm Liposomes.

With this method the DNA is previously deposited on the sensor surface during the formation of the SAM. The only step is the injection of liposomes which leads to the detection of DNA on the sensor surface. This is rather an acoustic detection process than an amplification process, since DNA is deposited before the experimental measurements. This method also provides the proof of principle of detecting dsDNA molecules on the sensor surface. I used the 5'-thiol modified/ 3'-Cholesterol Modified 157 bps dsDNA obtained with PCR, as described previously. To unravel differences between different liposome sizes I used 50nm Liposomes and 100nm Liposomes.

The values of the acoustic experiments with both liposome sizes are presented at Table 4.

Using 100nm liposomes it is possible to detect lower concentrations of dsDNA. Even though the ΔF value of the lowest concentration detected with 50nm Liposomes is 3,5Hz indicating the fact that lower concentrations can be also detected, ΔD value is relatively small with great error bar. It must be noticed that SAM layers do not interact non-specifically with liposomes thus lower ΔD values are acceptable. The experiments with lower DNA concentrations, specifically 1ng produced no acoustic signal, and as a result the 5ng is the detection limit of the method if 50nm Liposomes are used.

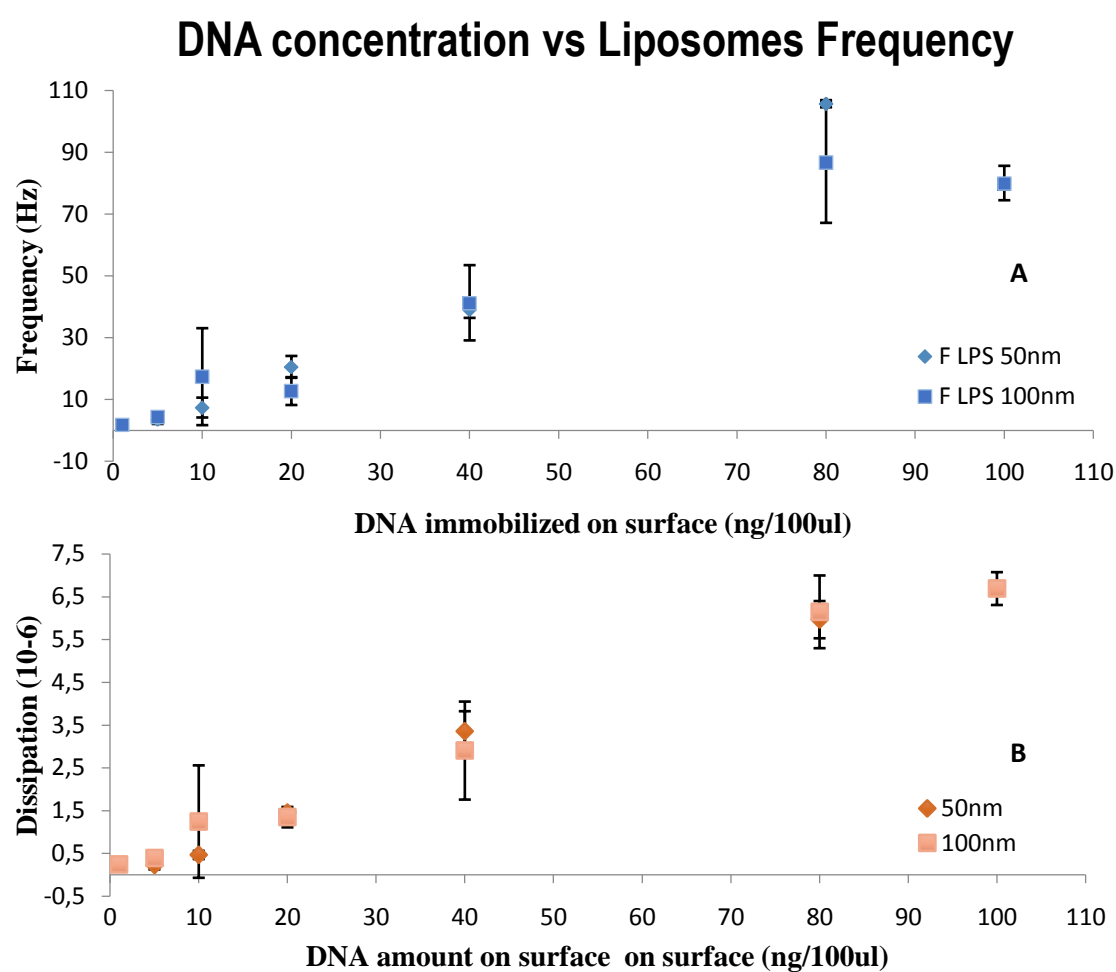


Figure 13: A) The ΔF shift caused by liposomes versus the dsDNA concentration plot. B) The ΔD shift caused by liposomes versus the dsDNA concentration plot.

On the other hand by increasing the liposome diameter to 100nm, 1ng of DNA was successfully detected and both ΔF and ΔD values indicate that lower DNA concentrations can be detected. In an experiment of immobilizing 100pg of DNA there was no acoustic signal. For that reason 1ng is considered the detection limit

of the method if using 100nm liposomes. Figure 16 represents the ΔF and ΔD versus DNA concentration plots.

As revealed by the plots there is little difference between the two liposomes sizes in small concentrations, although the initial expectation is that larger diameter liposomes have a more viscous nature, so they cause greater energy dissipation compared to liposomes with smaller diameter. This is not always the case at high analyte concentrations, but the ΔD was useful for probing dsDNA at low concentrations. At analyte concentrations equal or lower than 10ng ΔD caused by 200nm liposomes is higher compared to ΔD caused by 50nm Liposomes. Moreover, using 200nm liposomes, 1ng of dsDNA was detected while using 50nm Liposomes the detection limit was 5ng as mentioned before.

Concerning the sensitivity of the method it is obvious that from the detection limit to the concentration of 80ng the system is highly sensitive, while at higher concentrations both ΔF and ΔD values are not consistent with the almost linear relationship which can be identified for ΔF and ΔD values at both plots for both liposome sizes.

3.4 Hybridization and ssDNA detection on SAM functionalized surface using 50nm & 200nm Liposomes.

The method background: This method is similar with the “on sensor hybridization” described previously, except for the fact that in this case the probe is immobilized on the sensor surface via a SAM layer formed by alkane-thiol modification (5'-HS-(CH₂)₆-DNA-3') it carries, in a mixed SAM with the 157bps DNA thiol-modified primer. DNA recognition sequences extend out of the DNA sequences formed by the 157 DNA thiol-modified forward primer and can hybridize with target complementary molecules. The complementary molecules are 5-end cholesterol modified and as a result dsDNA molecules derived by hybridization occurring on the sensor surface can become anchors for liposomes due to the cholesterol-lipids interaction. Liposomes addition leads to ΔF and ΔD acoustic signal, which is proportional to the dsDNA molecules formed on the sensor surface. This method has the major advantage of eliminating non specific interactions since Liposomes cannot interact non-specifically neither with the DNA probes nor with the rest components of the self assembled monolayer.

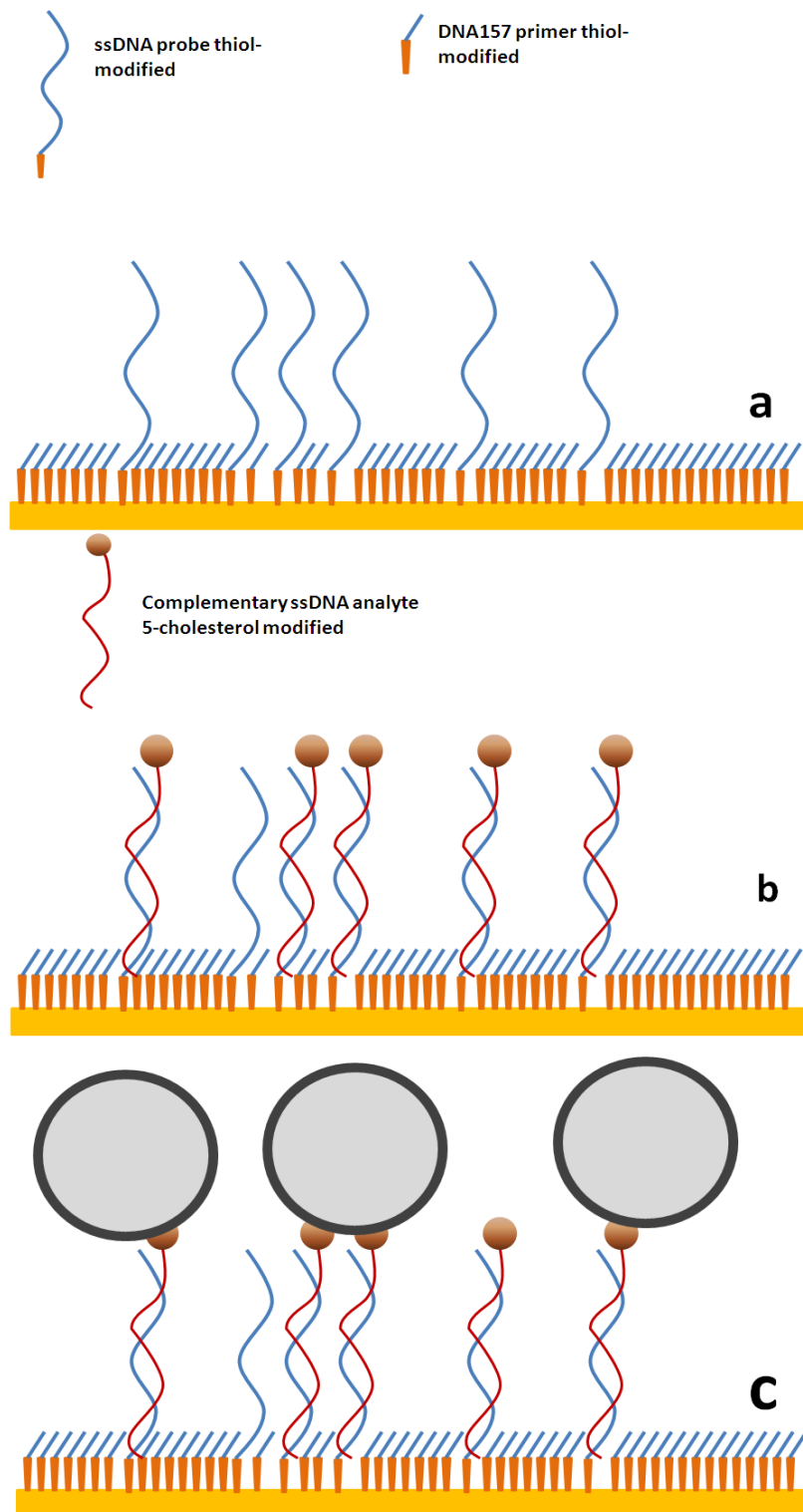


Figure 14: On sensor hybridization

a) the surface contains the probe immobilized within a SAM.

b) Complementary analyte ssDNA 5-end cholesterol modified hybridizes with the probe.

c) Liposomes anchor on the dsDNA formed on the sensor's surface due to the lipid-cholesterol interaction and amplify the acoustic signal of hybridization.

Representation is not in scale.

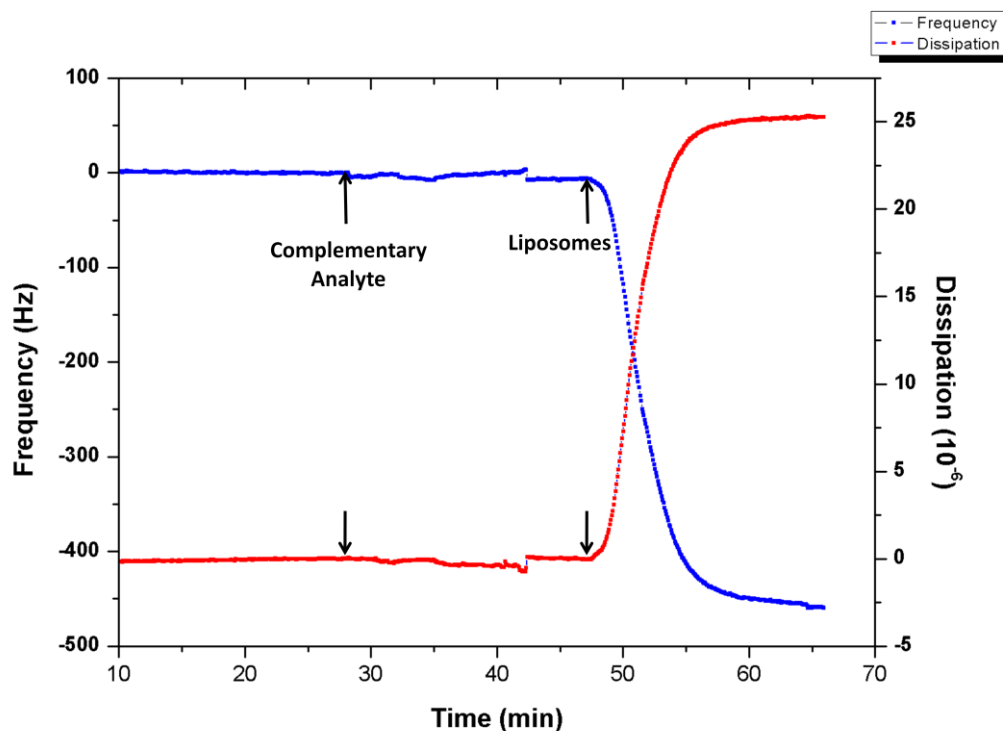


Figure 15: A typical real-time graph of the experimental process containing two steps. The injection of the ssDNA analyte which hybridizes with the complementary probe and the injection of liposomes which anchor proportionally on the dsDNA molecules on the sensor's surface.

The method is similar to the one described at the subchapter 3.2 of the Results, with the only difference being the immobilization method since in this experimental process the ssDNA is part of a SAM. Specifically the DNA probe (DNA4) is 70nts, 20nts accounting for the SAM formation and 50nts part which is complementary to the target analyte (DNA3). The experimental process includes two steps: a) the injection of the complementary analyte and hybridization of the target analyte and b) the injection and anchoring of liposomes proportionally to the dsDNA formed after hybridization on surface.

In these experiments the amplification of the on sensor hybridization was done using liposomes of 50nm and 200nm diameter size.

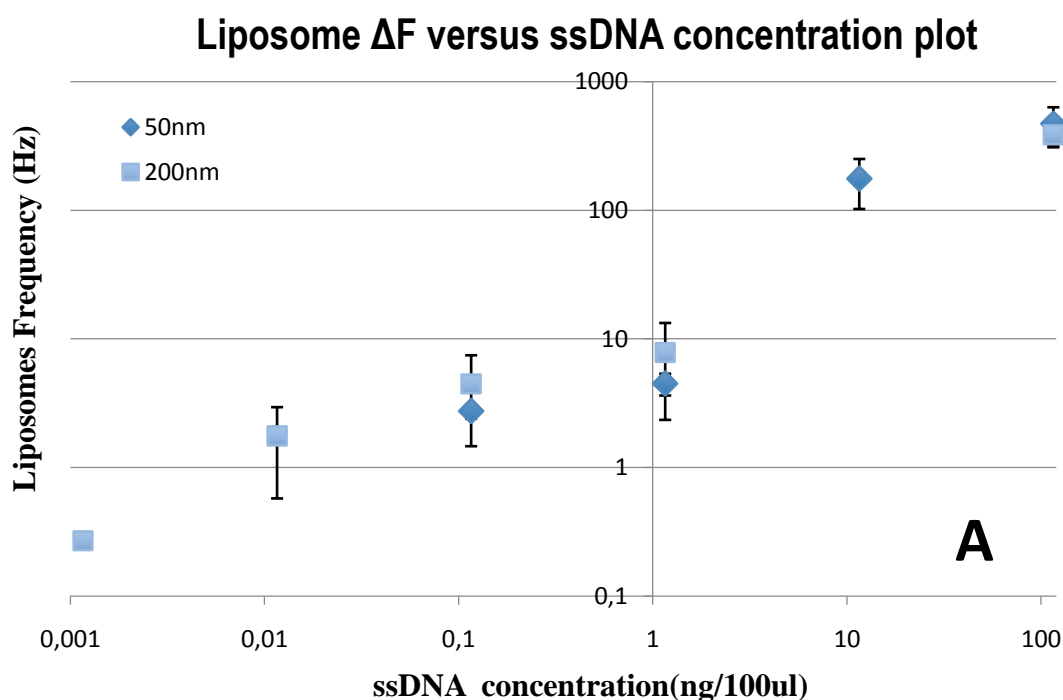
At the table 5 I present the acoustic values after 50nm and 200nm liposomes adhesion on the sensor surface according to the ssDNA analyte concentration which leads to DNA hybridization and dsDNA formation.

	DNA concentration (ng/100ul)	Liposomes ΔF (Hz)	Liposomes ΔD (10^{-6})	Liposomes D/f ($10^{-6}/\text{Hz}$)
50nm Liposomes	1158	347 \pm 157	15,62 \pm 5,93	0,0450
	115,8	471,3 \pm 161	22 \pm 4,82	0,0466
	11,58	176,6 \pm 74	10,84 \pm 2,63	0,0613
	1,158	4,5 \pm 0,8	0,30 \pm 0,16	0,0674
	0,1158	2,75 \pm 0,3	0,12 \pm 0,01	0,0436
200nm Liposomes	115,8	387 \pm 72	25,48 \pm 0,41	0,0658
	1,158	7,8 \pm 5,4	0,84 \pm 0,63	0,1072
	0,1158	4,4 \pm 3	0,28 \pm 0,10	0,0641
	0,01158	1,75 \pm 1,1	0,06 \pm 0,03	0,0382
	0,001158	0,27 \pm 0,02	0,04 \pm 0,007	0,1296

Table 6: The acoustic values of the amplification step of 50nts analyte DNA hybridization on the SAM functionalized surface.

Observing the acoustic values of liposomes anchoring it is easy to conclude that the detection limit of method using 50nm liposomes is 0,1158ng/100ul and the detection limit using 200nm liposomes is 0,001158ng/100ul. Using 200nm liposomes we could detect DNA concentrations of two orders of magnitude lower than using 50nm liposomes. It is also observed that using the hybridization method of forming dsDNA on a SAM the detection limit using 50nm liposomes is more than one order of magnitude lower compared to the detection of dsDNA which is previously immobilized during SAM formation as part of it.

At the figure 16 are presented the liposome ΔF and ΔD versus analyte ssDNA concentration plots for both liposome diameter sizes. Note that both axes are at logarithmic scale.



Liposomes ΔD versus ssDNA concentration plot

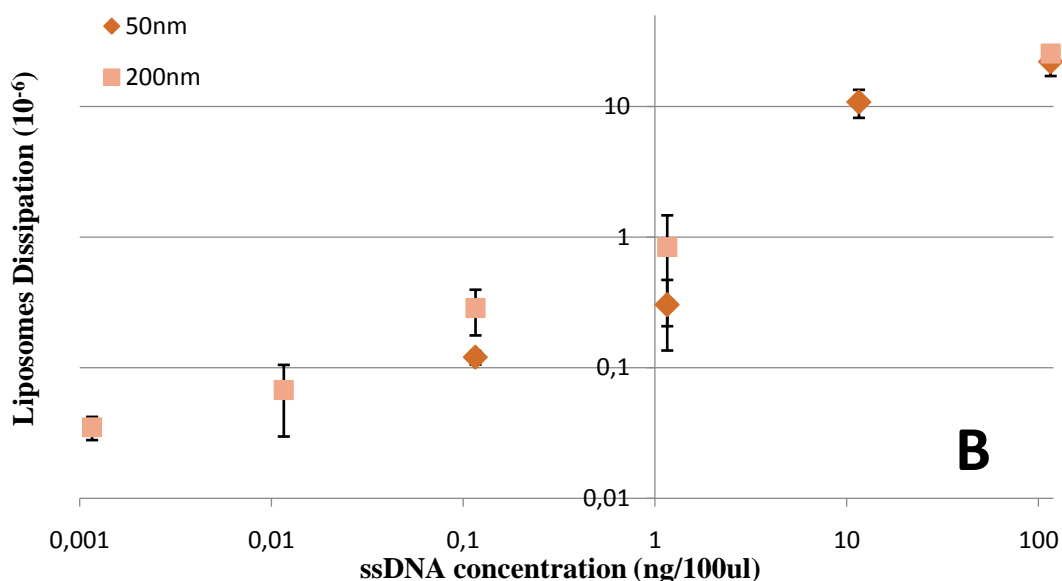


Figure 16: A) The ΔF shift caused by liposomes versus the ssDNA concentration plot. B) The ΔD shift caused by liposomes versus the ssDNA concentration plot. The DNA concentration axis is at logarithmic scale.

The sensitivity of the method is sufficient enough to discriminate DNA concentration in one order of magnitude in both cases either using 50nm or 200nm liposomes, but is enhanced for ssDNA concentrations ranging from 1ng/100ul to 100ng/100ul. Observing the ΔD versus ssDNA concentration plot it is obvious that using 200nm liposomes the ΔD values gained are out of the error bar of lower or higher order of magnitude concentration points. This finding shows that even though ΔF values using this method is a sufficient tool to detect and approximately quantify low concentrations of DNA, ΔD is a powerful tool that provides a better quantification scale.

3.5 Amplified detection of Hybridization Processes in complex - serum containing medium.

Detection of ssDNA diluted in complex matrixes such as human serum is major challenge in biosensors development. The success of such processes provides an outcome concerning the conditions which limit pre-acoustic preparatory steps which affect both the quality and the quantity of the analyte. In these experiments I used 50nts probe immobilized on surface via neutravidin-biotin interaction to capture the analyte DNA3 diluted either in 1% v/v FBS, or 10% v/v FBS. For the amplification of the hybridization event I used 200nm Liposomes. The experimental process includes four steps: a) The neutravidin adsorption, b) the probe

immobilization, c) the analyte addition and subsequent hybridization, and d) the addition and proportional anchoring of liposomes on dsDNA molecules assembled after the hybridization process. A real time graph of the experimental process is depicted at the Figure 17.

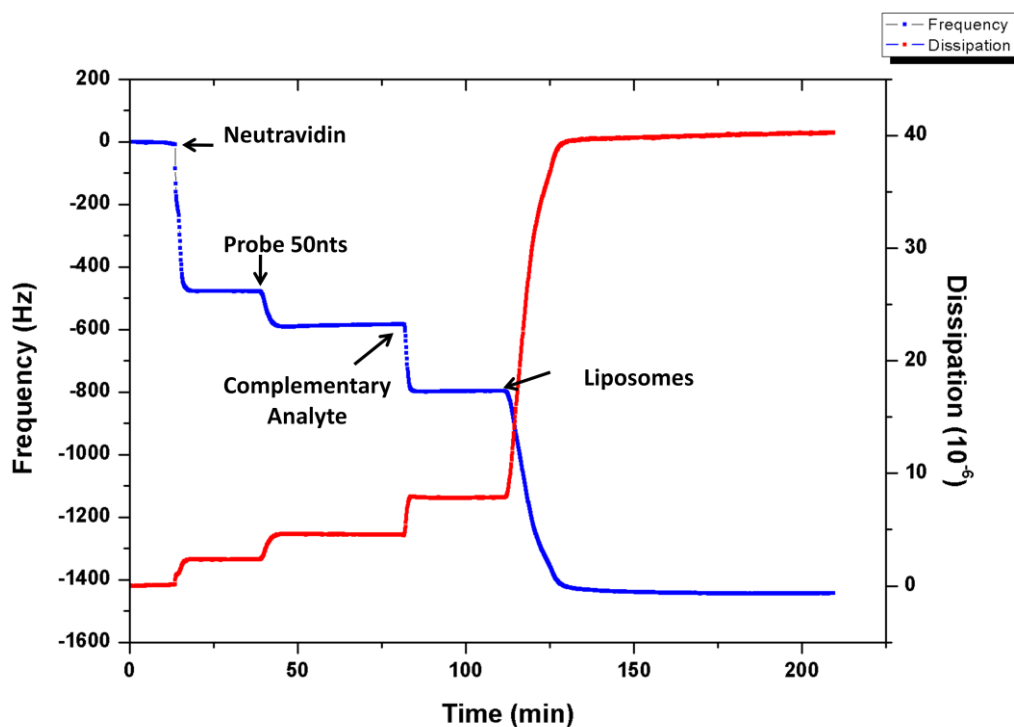


Figure 17: Real time graph of amplified acoustic detection of hybridization in serum. The process includes the adsorption of neutravidin, the addition of 50pmols 50nts Probe, the addition of the analyte and the anchoring of liposomes on dsDNA molecules formed after the hybridization process.

	DNA concentration (ng/100ul)	ΔF Liposomes (Hz)	ΔD Liposomes (10^{-6})	Liposomes D/f ($10^{-6}/\text{Hz}$)
10% Serum	1158	66	6,59	0,0998
	115,8	82 \pm 32	7,44 \pm 3,04	0,0907
	11,58	11 \pm 5	2,6 \pm 0,25	0,2363
	1,158	9	2,04 \pm 0,023	0,2261
1% Serum	1158	560	28,16	0,0502
	115,8	260	16,42	0,0631
	11,58	55	7,2	0,1309
	1,158	7	0,75	0,1071
	0,1158	1	0,83	0,8300
	0,01158	3	0,06	0,0200

Table 7: The acoustic values of the amplification step of 50nts analyte DNA hybridization in the presence of 1%v/v and 10%v/v FBS.

Table 7 presents the acoustic values of liposome anchoring on the dsDNA assemblies. The detection limit of the method in the presence of 10%v/v FBS is 1,158ng per 100ul, while the detection limit in the presence of 1%v/v FBS is two orders of magnitude lower, 0,01158ng per 100ul. It must be noticed that experiments in PBS without serum, the detection limit is 0,0579ng per 100ul, and this DNA concentration is five times higher than the detection limit of the same DNA at the presence of 1%v/v FBS. This result underscores two main facts, a) the detection limit of ssDNA immobilized on neutravidin film, needs further improvement, and b) the presence of 1%v/v FBS at the assay buffer, does not affect strongly the hybridization efficiency.

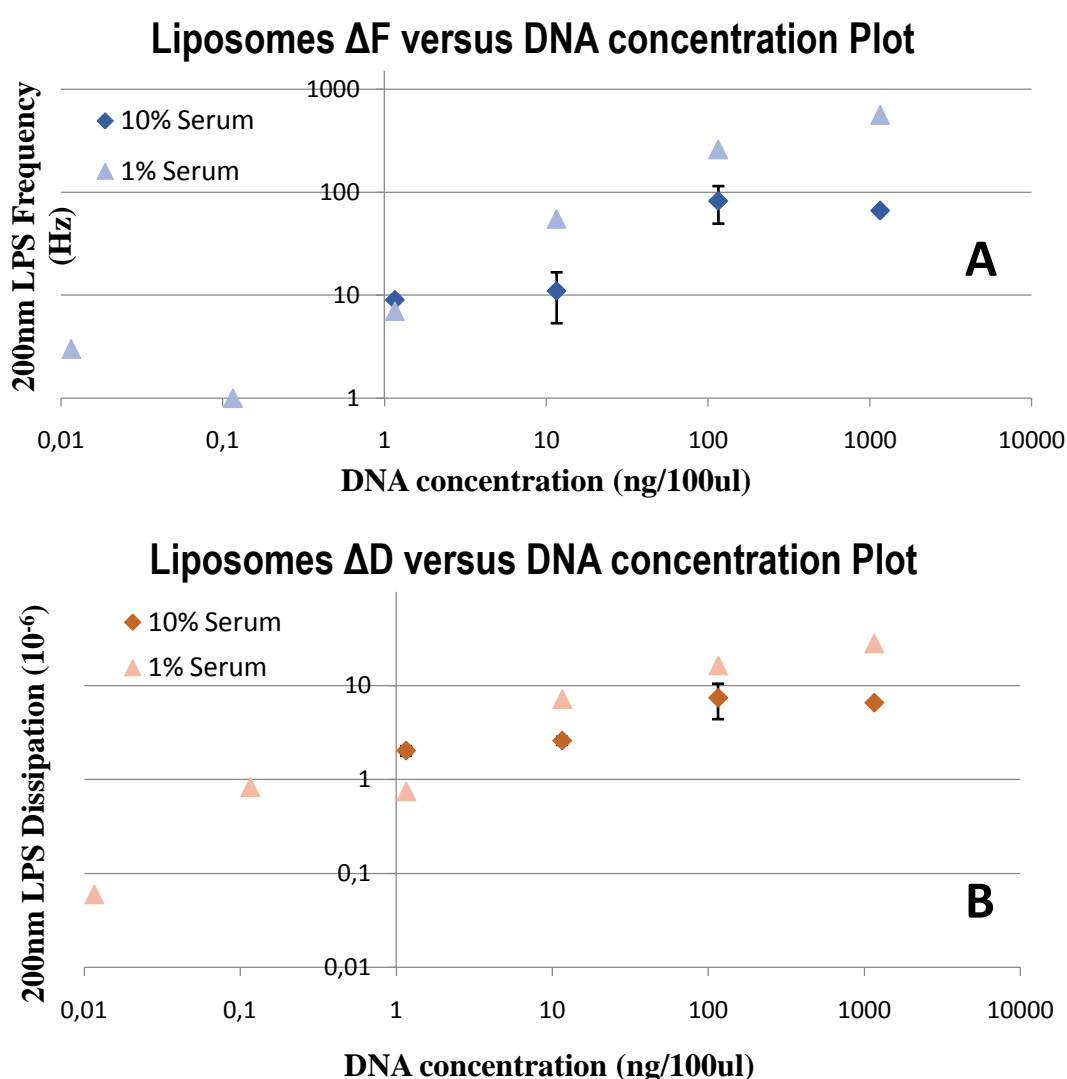


Figure 18: A) The ΔF shift caused by liposomes versus the ssDNA concentration plot in presence of 1% or 10% v/v FBS. B) The ΔD shift caused by liposomes versus the ssDNA concentration plot in presence of 1% or 10% v/v FBS. Both axes are at logarithmic scale.

At the figure 18 are presented the ΔF of liposomes versus DNA concentration and ΔD of liposomes versus the DNA concentration plots. The sensitivity of this experimental process in the presence of 10%v/v FBS is rather low for

concentrations ranging from 1,158ng per 100ul to 11,58 ng per 100ul while is enhanced for concentrations higher than 11,58 ng per 100ul till 115,8ng per 100ul which is considered the plateau. For higher concentrations there is no difference neither in ΔF nor in ΔD values as can be noticed by both the acoustic values and the plots.

On the other hand the sensitivity of the method in the presence of 1% v/v FBS is enhanced from all the concentrations tested. The paradox of lower ΔF at the concentration of 0,1158 ng per 100ul at the plot is considered as an experimental error due to the fact that an expected value for ΔD was obtained in the same experiment.

Collectively and in accordance with the bibliography it is observed that in the presence of high concentrations of serum the hybridization process is less efficient. In addition the results show that not only the efficiency, but also the sensitivity of the method diminishes while increasing the serum concentration.

Another interesting fact is that in high analyte DNA concentration, there is less serum removal from the surface and in the absence of analyte DNA (control) there is almost total serum removal. These findings show that serum proteins strongly interact with the DNA, hence increasing the analyte concentration there is lower serum removal, and maybe higher liposomes-serum remnants non-specific interaction. In other words the amplification step contains a non-specific liposome anchoring on the serum remnants. Therefore much more experiments should be done in the future to optimize the DNA detection and amplification in complex matrices.

4. DISCUSSION

The results presented show that using liposomes it is possible to amplify the acoustic signal of ssDNA or dsDNA immobilized on surface when its concentration is less than the required to produce acoustic signal. The one-step amplification process in all the experiments at different conditions and with differences in the analyte DNA and in the liposome size, could produce acoustic signals for at least two orders of magnitude lower than the non-amplified DNA detection. This underscores that this advance could be used for ctDNA analysis. Even though previous studies used an amplification step using micro-gravimetric QCM these results are valid in terms of Frequency shift (ΔF) after liposome anchorage²³. For the first time, in this study it is shown that energy dissipation (ΔD) values of

liposomes anchorage could be used to amplify the acoustic detection of DNA when there is not an observable ΔF shift after liposomes addition. Moreover in the same study referred earlier the amplification elements were liposomes containing cholesterol and oligonucleotides for analyte detection, with diameter $180 \pm 40 \text{ nm}$. The detection limit which was carried in buffer solution was 10^{-12} M while the detection limit in this study's similar experimental condition using 200 nm Liposomes and following the hybridization process on a SAM layer is $723 \times 10^{-11} \text{ M}$. Our detection limit is higher but taking under consideration that cholesterol usage and oligonucleotides increase the ΔF response due to their larger mass (compared to the liposomes used in this study) and single target detection, it is not far from the imagination to assume that using liposomes containing cholesterol as amplification elements, and by using both ΔF and ΔD values as means of detection we can achieve a lower detection limit using QCM-D biosensors.

Nevertheless the restriction factors for the usage in hand-held diagnostic platforms are abundant.

Single subtype cancer cells' ctDNA concentration is present at zM concentration scale while our detection limit is 723 fM , five orders of magnitude higher than the desired. Given these conditions, ctDNA analysis requires previous extraction from human blood and sequence amplification before tested on the QCM-D. Recently Moschou et al.,³² presented the usage of low-power consuming, portable microfluidic-based μPCR chip device for the rapid DNA amplification. The μPCR chip is small in size so, its incorporation on a diagnostic platform for pre-acoustic sequence amplification of the sample is possible. For 1 zM of DNA, 30 amplification cycles are sufficient for increasing the concentration to 800 fM which is our current detection limit. Assuming that the efficiency of PCR is $e=1$, then the number of cycles to amplify 1 zM DNA to 800 fM is calculated to 30.

Another desired feature of this system would be to enhance the sensitivity and lower the detection limit by adding a second or more amplification steps. Dendritic amplification is an experimental process used to amplify the acoustic signal of the first amplification elements and has been used in previous study, where oligonucleotide functionalized nanoparticles were used as first and second amplification elements³³, and in another study liposomes were used as first and second amplification elements¹⁹. Both of these studies have been carried using microgravimetric QCM sensors and as a result the amplification process has not been studied in terms of Energy Dissipation (ΔD). During my study I carried an experiment to estimate the value of using dendritic amplification (experimental

process information is given at the appendix). I used a second amplification step with liposomes as amplifiers of the first amplification signal for the detection of 115,8ng per 100ul. The result is presented at the Figure 19.

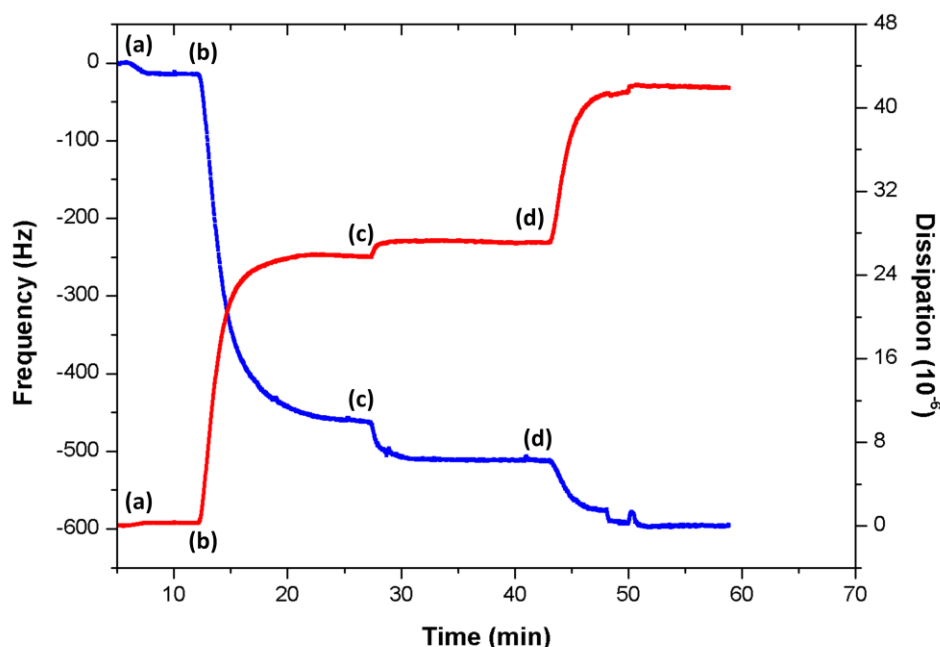


Figure 19: Hybridization on SAM. Probe DNA 4. Steps: a) Complementary ss DNA3, b) addition of 50nm Liposomes functionalized with Oligonucleotide 50bps 5'-end Biotin/3'-end Cholesterol (probe sequence), c) Neutravidin, d) Liposomes same as in (b).

The acoustic values of the first amplification step were $\Delta F=450(\text{Hz})$, $\Delta D=25,53(10^{-6})$ and $D/f=0,0567(10^{-6}/\text{Hz})$. After the addition of neutravidin as bridge for the binding between the second amplification liposome and the first amplification liposome, anchored on the surface, the second signals (without the neutravidin response) were $\Delta F=83(\text{Hz})$, $\Delta D=14,42(10^{-6})$ and $D/f=0,1737(10^{-6}/\text{Hz})$. The difference in the D/f ratio implies that during the second amplification step the liposomes anchored on the first liposomes and not directly on the surface. Moreover neutravidin does not interact with the SAM layer non specifically (data not shown) and this can be observed by the $D/f=0,0296(10^{-6}/\text{Hz})$, while the D/f ratio of the neutravidin adsorption on the sensor surface is $D/f=0,0050(10^{-6}/\text{Hz})$. Summarizing with this method the amplification response was increased by adding one second amplification step mostly in terms of ΔD . Thus, adding a second amplification step would be beneficial, providing the ability to detect lower DNA

concentrations, especially using ΔD values, since as mentioned earlier in this study ΔD has greater potential than ΔF in DNA detection and amplification.

The sensitivity comprises a major challenge in some of these experiments especially in those of the detection of dsDNA molecules. Obviously in all cases either using neutravidin or SAM to immobilize dsDNA molecules on the sensor surface it was shown that the detection limit is higher compared to the hybridization respective. It must be reminded that the sensitivity is the term describing the changes in the acoustic signals ΔF and ΔD in response to molecular deposition on the sensor's surface. In a homogeneously sensitive system there must be a unit of mass that causes a specific ΔF (and ΔD according to the molecules viscoelasticity). QCM-D surface is not characterized by homogenous sensitivity through the sensor surface, contrarily it is known that the sensitivity is maximum at the center of the disk and diminishes towards the periphery³⁴. This is considered the factor of the great error bars observed in relatively high concentrations of dsDNA molecules on the neutravidin surface. The manner by which the molecules diffuse and bind on the surface is still unclear but it is highly possible, especially in low DNA concentrations, that the molecules bind on the first biotin binding sites, which happens to be at the periphery of the disk, and as a result both the detection limit and the sensitivity are affected. A good solution to this problem would be to use a patterned surface unable to bind neutravidin, DNA or liposomes at the periphery and leave a gold surface site accessible at the center of the disk. For example PEG-modified surface could be the ideal non adhesive coating at the periphery and restrict the gold surface only to the center where the sensitivity is maximized. Another useful tool, to examine if this feature of QCM-D sensors has indeed an effect on the method's sensitivity, is the use of fluorescent liposomes. Since liposomes use DNA molecules as anchorages to the surface their distribution on the sensor surface is based on the DNA distribution and that could provide the information about their diffusion on the sensor surface during the experiments and their final placement.

As long as the use of QCM-D in diagnostic hand-held platforms there is another important issue that must be noted. CtDNA extraction and purification from human serum is a time-consuming work and, until now, the only way to extract it is based on kits provided by several suppliers. Nevertheless, it would be very pleasing to fabricate surfaces that do not interact non-specifically with components of the human blood, in order to specifically detect responses caused exclusively by DNA binding. In this study, and many studies carried previously reported both the non-

specific interaction of serum components with sensor surface³⁵ and also the effect of serum on the hybridization efficiency^{26,28}. Glycol-based layers are able to minimize serum non-specific interactions with the surfaces^{26,28,35}, but the remaining non-specific interaction can produce false-positive results, and this was estimated by the results of this study.

The restriction effect on the hybridization process efficiency caused by the serum's presence is an issue of vital importance since the concentration of the analyte molecules is low and even a partial restriction of the hybridization process, could provide false-negative results. Moreover serum contains DNases, with DNaseI to be the most abundant. DNaseI is an enzyme implicated in the mechanisms of cellular apoptosis, which is responsible for the digestion of DNA³⁶⁻³⁸. Thus the presence of DNases leads to the degradation of ctDNA. Consequently false-negative results could be produced. A previous study has demonstrated the use of SDS as means to deactivate the serum components and improve the hybridization process²⁷. SDS usage was not evaluated in this study, nevertheless, SDS or other detergent use is considered essential for improving the efficiency of hybridization.

A major challenge is the selectivity of the probes and their hybridization exclusively with target ssDNA molecules. The experiments performed during this project included the use of probes and complementary analytes that bear base-repeats, and the hybridization occurred may involved partially ssDNA assemblies due to hybridization mis-positioning. Moreover the mismatch selectivity was not tested. Both of these problems require immediate solution since partial mismatch and partial dsDNA assembly are phenomena that restrict the analysis of ctDNA. In the case of many tumors the difference between normal and tumor DNA fragments may differ only in a single base. The absence of single base selectivity may lead to false-positive results. Hairpin-DNA probes are ssDNA molecules that bear a target-recognition sequence flanked by two complementary sequences near at 5'-end and 3'-end. These regions force the molecule to adopt a stem-loop structure in the absence of target analyte, showing selectivity of a single-base mismatch³⁹⁻⁴¹. If this type of probe is used the cholesterol modification would be non-accessible to the liposomes unless the target is present and hybridizes without mismatches with the probe. Concluding the use of pinhead-DNA probes could provide the desired feature of selectivity to avoid false-positive results.

The results showed that the D/f ratio of liposomes adsorption is increased by increasing the DNA length and/or the liposomes size. Nevertheless in experiments carried but not demonstrated in the results it was observed that the amplification

process cannot occur for the detection of 657bps DNA. This result underscores the importance for studying, in the future, which is the longest possible DNA length which optimizes the detection and amplification methods used in the present study in terms of detection limit, sensitivity and selectivity. It must also be noticed that liposomes are not the only amplification elements which could be used. Nanoparticles, nanotubes, proteins and DNA-structures could be used as amplification elements as has been demonstrated by several previous studies^{42,43}.

5. CONCLUSION

The present study has addressed a novel method to amplify the acoustic detection of dsDNA and ssDNA molecules by the use of liposomes, for application in ctDNA analysis, cancer diagnosis and monitoring. Moreover, it was demonstrated the use of Energy Dissipation shift (ΔD) as means for studying the amplification process of acoustic DNA detection. Interestingly, ΔD as means for studying the DNA sensing process could improve the detection limit, compared to Frequency Shift (ΔF) which was used in previous studies. It was also demonstrated that D/f ratio of Liposomes adsorption increases either with increasing the DNA length or/and the liposome size. The study of hybridization processes provided knowledge concerning the hybridization efficiency, and thus the method's detection limit in cases where preparatory steps of ctDNA samples are avoided. It was also discussed the possible future perspective for improving the method to show the desired features of low-detection limit, increased sensitivity and selectivity in amplified acoustic DNA detection.

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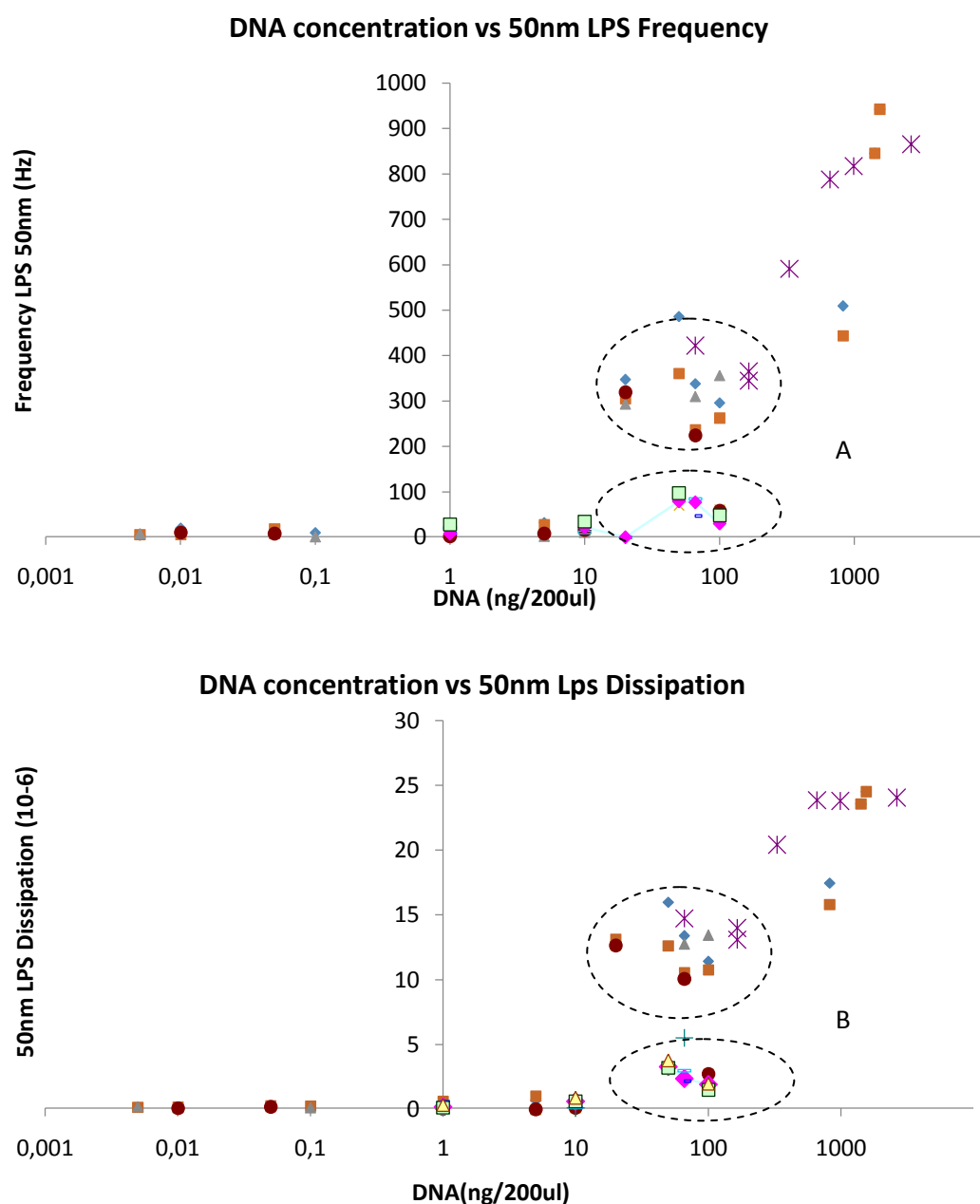
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In the following paragraphs I present some details concerning the experiments indicated that have more information available at the main PhD thesis.

1. dsDNA 50 bps – 50nm Liposomes on Neutravidin film

The Liposomes' ΔF versus DNA concentration and Liposomes' ΔD versus DNA concentration plots were presented but in the concentrations ranging from 20ng per 100ul to 100ng per 100ul the error bars of the acoustic values are much greater than expected. As mentioned this is caused by the great deviation from the average values. Moreover it has been shown that the great deviation is ought to the production two acoustic signals forming clusters near the limit of the error bars. The same plots are presented next at ApFif1 A and B. containing the acoustic values obtained by different experimental process. Clusters of acoustic values are highlighted by circle.



ApFig1. A) Liposomes' ΔF vs DNA conc. Plot, b) Liposomes' ΔD vs DNA conc. Plot.

This phenomenon is probably ought to the positioning of dsDNA as mentioned at the discussion. The sensitivity is not homogenous through the sensor surface, but is maximized at the center of

the disk and diminishes towards the periphery. dsDNA positioning near the periphery results in lower ΔF and ΔD shifts compared to the values of their positioning near the center of the disk.

2. Dendritic amplification of 115,8ng 50nts ssDNA with 200nm Liposomes.

The DNA probe(100pmol) was immobilized on the sensor surface as part of SAM. The complementary ssDNA addition was the next step. Upon ssDNA addition hybridization occurs as can be observed by the real time graph presented at the Discussion. The liposomes used were POPC 200nm that have been incubated along with 50nts DNA bearing a 5'-end biotin modification and a 3'-end Cholesteryl TEG modification. Its nucleotide sequence was the same with F50 (DNA :1). The addition of these liposomes could not produce non-specific acoustic signal. The liposomes are anchoring on the dsDNA assemblies via the cholesterol modification of the target analyte. Next Neutravidin is added (same concentration of this used for forming a Neu film). The D/f ratio produced is by far more different from this observed during neu adsorption on surface and as a result we can assume that the neutravidin interacted solely with the biotiny modifications of the oligonucleotides assembled on the liposomes. At the third step the same liposomes incubated with the same liposomes are added and interact with other accessible biotin binding sites of the neutravidin protein molecules. As a result the second liposomes have anchored on the first using the nucleotides and the Neu molecules as bridges. The D/f ratio produced from the second liposome underscores the difference in their adhesion pattern compared to the liposomes of the first amplification step which produced a different D/f ratio. The acoustic values of the experiment are given at the following table.

STEP	ΔF (Hz)	ΔD (10^{-6})	D/f ratio ($10^{-6}/\text{Hz}$)
Complementary	14	0,27	0,0192
Liposomes 50nm with Oligonucleotide Biotin/Cholesterol modified	450	25,53	0,0568
Neutravidin	50	1,48	0,0296
Liposomes 50nm with Oligonucleotide Biotin/Cholesterol modified	83	14,42	0,1717