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NOVEL LIPOSOME FORMULATION FROM ALGAE MEMBRANES AS CARRIERS FOR BIOACTIVE COMPOUNDS

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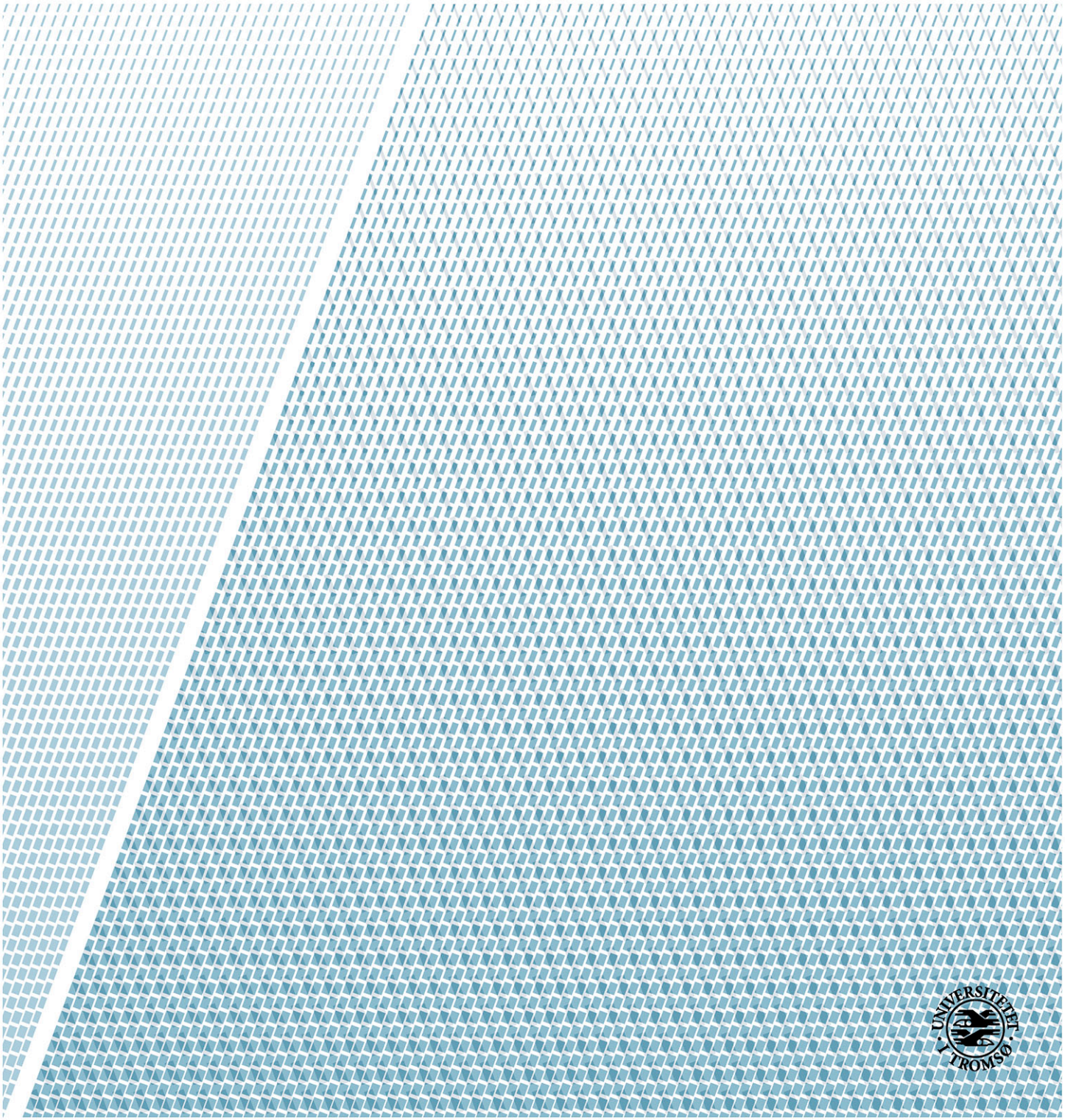
Thesis for the degree Master of Pharmacy May 2014



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FOREWORD

The work presented in this master thesis was performed at the University of Florence (UniFI), Physical Chemistry Department. The analytical work was carried out during the period from October 2013 til May 2014.

Dr. Sandra Ristori at the department of physical chemistry, UniFI, was the main supervisor. Professor Natasa Skalko-Basnet, Drug Transport and Delivery Research Group, Department of Pharmacy, was the internal supervisor at University of Tromsø, UiT.

ACKNOWLEDGEMENTS

First of all I would like to express my gratitude to my supervisor Dr. Sandra Ristori that had the main responsibility for the practical work, carried out in Italy. Her support, help and guidance throught this master thesis work have been priceless, and made my stay in Italy unforgettable. All the experience and practice that I received during this work is irreplaceable and I hope to take advantage of all the acquired knowledge in my future work. I am also very grateful for the all the help, advices and supervision of Professor Natasa Skalko-Basnet. I would like to thank her for introducing me to biopharmaceutics, nanotechnology and nanomedicin as these are very exiting research fields.

Then I would like to thank all of the other people that were collaborating with us and have taken part in this work. I am grateful for all their effords, assistance and encouragement through the laboratory work. Following people have taken part in this work: Cristina Gonnelli, Ilaria Colzi, Agnese Magonio (Dipartment of Biology), Giuseppe Pieraccini, Riccardo Romoli (CISM Mass Spectrometry Centre), Brunella Perito, Enrico Casalone, Cristina Indorato (Dipartment of Biology), Claudiu Supuran, Fabrizio Carta, Daniela Vullo (Department of Chemistry "Ugo Shiff"). If it wasn`t for their collaboration, none of this work would be possible.

Not least, I would like to thanks all of the great people I met during my stay, especially the students of University of Florence, which made my stay extra memorable.

Finally I would like to thank my family, especially my parents for their love and support through the years of my education. They have always stood by my side, believed in me and gave me encouragement. I will endeavor to do the same for you!

Tromsø, May 2014

Anna N. Troyan

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ABSTRAKT

Liposomer er lipid baserte partikkel systemer, som kan inkorporere både hydrofile og hydrofobe substanser. På den måten kan de anvendes som bærersystemer innenfor levering av legemidler. Liposomer består av et dobbelt lag samensatt fosfolipider. Disse systemer er svært fleksible i komposisjon, og har vist stor potensiale i forbedring av levering og aktiv målretting av aktive substancer til cellene. De har vist økt biotilgjengelighet av bioaktive forbindelser som generelt har dårlig absorpsjon i kroppen. Konvensjonelle liposomer bestående av ulike typer fosfolipider har allerede blitt analysert som potensielle legemiddelbærere, og viser lovende resultater. I tillegg har det vært utført flere studier angående struktur og sammensetning av naturlig forekommende microorganismer, hovedsakelig ulike typer bakterier og alger, og deres potensiell anvendelse innenfor farmasøytisk teknologi og legemiddelutvikling. Det er blitt identifisert flere typer fettstoff i membraner av disse naturlige microoganismer. På den måten gir dette et grunnlag for å kunne anvende disse i fremstilling av liposomer. Dermed er målet med dette prosjektet om å vurdere mulig bruk av lipider utvunnet fra organiske membraner, nemlig fra cyanobakterier og ikke-svovel purpurbakterier, til å opptre som potensielle bærere for bioaktive substanser. Ett av stoffene vi er interesserte i, er kurkumin, et naturlig polyfenol og ekstrakt fra roten av gurkemeie planten. Kurkumin har et bredt spekter av biologiske aktiviteter of farmakologiske virkninger. Dessverre har det vært vanskelig frem til nå å dra nytte av disse effektene, der lav oral biotilgjengelighet av kurkumin utgjør det største problemet. I denne studien ble de fysisk-kjemiske egenskapene av liposom-kurkumin kompleks klarlagt hovedsakelig ved UV/VIS Spektrometri og dynamisk lysspredning teknikker. Flere studier har allerede rapportert at liposomer har evne til å øke oppløseliget og dermed forbedre biotilgjengelighet av lipofile legemidler. Med dette i bakhodet ønsket vi i tillegg å undersøke om organiske liposomer har evne å motvirke andre aktuelle terapi- relaterte problemer, blant annet antibiotikaresistens. Det introduserer et problem for den nåværende terapeutisk behandling, siden stadig flere bakterier utvikler resistens og etterlater oss med færre behandlingsalternativer av eksisterende infeksjoner. Introduksjon av antibakterielle midler gjennom nanopartikler, inkludert liposomer, har vist til å være en av de mest lovende strategier for å bekjempe ulike resistensmekanismer. På den måten ønsker vi å karakterisere antibiotika inkorporert i liposomer, tillaget fra komponenter funnet i bakterier, fra fysiko-kjemisk standpunkt. I tillegg ønsket vi å evaluere deres antibakteriell effekt på levende bakteriekulturer, for å klargjøre om liposomer kan bidra til bedre aktivitet og overgå noen av eksisterende mekanismer for antibiotika resistens.

ABSTRACT

Liposomes are lipid-based systems which have the ability to incorporate various chemical substances and thus can be used for drug delivery purposes. Liposomes are composed of phospholipids, flexible in composition, and have shown great potential to improve delivery and targeting of bioactive substances which otherwise have poor bioavailability in the body. Conventional liposomes composed of various types of phospholipids have already been analyzed as potential drug carriers and show promising results. There also have been investigations on the composition of naturally found organisms, mainly different kinds of bacteria, and their potential to be used in pharmaceutical applications. Various types of lipids are found in the membranes of these natural organisms. This fact provides a basis for possible employment of these in liposome preparation. Thus the goal of this project was to evaluate the possibility of using liposomes derived from algae membranes, namely Gram-negative purple non-sulfurbacteria and cyanobacteria, to act as potential drug carriers for bioactive substances. One of the model substances was curcumin, a natural polyphenol derived from rhizomes of dietary spice turmeric. Curcumin exhibits a range of biological activities and pharmacological actions; however, these are hard to take advantage of, because of its low oral bioavailability. In this study, the physico-chemical properties of liposome-curcumin complex was mainly determined by UV/VIS Spectrometry and Dynamic Light Scattering techniques. Through numerious studies liposomes have already shown the ability to improve poor solubility and consequently increase the bioavailability of lipophilic drugs. Additionally, there is a great interest of investigating the ability of liposomes to overcome other therapy-related problems. One of currently existing drawback issues in the therapeutical treatment is the antimicrobial resistance. It has become a major problem, since more and more bacteria are becoming resistant to antimicrobial agents used today, leaving us with no means of treating existing infections.(1) One of the most promising strategies for enhancing drug delivery and combating various resistance mechanisms is administration of microbial agents by nano delivery systems, including liposomes. Therefore, it was of our interest to characterize liposomal antibiotics from physico-chemical point of view, and investigate their antibacterial activity on living bacterial cultures compared to free antibiotics and antibiotics loaded on conventional liposomes.

LIST OF ABBREVIATIONS

Amp	Ampicillin
API	Atmospheric pressure ionization
AR	Antibiotic resistance
B. subtilis	*Bacillus subtilis*
CA	Carbonic anhydrase
D (or CCY110)	Cyanothece CCY0110
DC	Drug carrier
DLS	Dynamic light scattering
E. coli	*Escherichia coli*
ESFA	Ethylaminobenzenesulfonamide
ESI	Electrospray ionization
LC	Liquid chromatography
LTQ	Linear Trap Quadrupole
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
OD	Optical density
P (or 42OL)	*Rhodopseudomonas palustris* 42OL
PenG	Penicillin G
S (or VI22)	Cyanothece VI22
SAXS	Small angle X-ray scattering
SFA	Sulfanilamide

1 INTRODUCTION

Nanoscience is a science that is currently one of the fastest growing research fields. It is a study of structures and materials on a nanometer scale. Many researchers worldwide have had their focus on employing principles of nanoscience to different technological applications. One of many applications of nanoscience is the use of nanotechnology in medicine, for instance use of nanoscale drug deliverysystems. These systems show promising results in delivering various pharmaceutical agents by different application routes. Their use offers improved pharmacokinetic properties, controlled and sustained release of drugs and, more importantly, lower systemic toxicity provided by biocompatibility and biodegradability of these systems. Nanoparticles and nanoencapsulation have been proposed for medical imaging, targeting cancer cells, drug delivery to tumors and penetration of the BBB, delivery and protection of insulin and other bioactive compounds.(2,3) Some of these delivery systems are micelles, nanocapsules, dendrimers, nanorods and liposomes. Generally, phospholipids offer means for improvement of poor solubility and consequently low bioavailability of lipophilic drugs.(4,5,6) In the first part of this project we focused on lipophilic model compound, curcumin. Several research groups have already proposed complexes of curcumin and phospholipids, such as patented drug Meriva ®.(7) Similarly, other nanosystems containing phospholipids, such as liposomes, have been proposed suitable for curcumin delivery. Numerous studies have already shown high loading capacity and improvement of curcumin bioavailability by using conventional liposomes both *in vivo* and *in vitro*.(8,9,10) We went a step further and formulated liposomes prepared from lipids extracted from naturally occurring bacteria, earlier classified as algae. In general, stability of liposomes depends on the lipid composition, particle size of incorporated material and vesicle diameter, where app. 200 nm or less gives better dispersibility and practical use.(11) Thus the focus will be on characterizing the liposome-curcumin complex by considering physico-chemical properties, in particular liposomal size.

As mentioned earlier liposomes can incorporate various kinds of materials and because of their flexibility may overcome different pharmaceutical issues.(12) One of greatest health challenge worldwide is occurrence of new infectious diseases and their limited treatment options. Despite of advanced and innovative technologies, fast development of microorganisms presents a growing issue. The most important class of compounds

against bacterial microorganism is represented by antibiotics, and has been used for decades as medical treatment, both by oral and systemic delivery.(13) However, the effectiveness of these drugs increasingly and inevitably reduces with the occurrence of microbial resistance. This creates a major obstacle in clinical practice, and serious treats for public health and great economical expenses. The failure of antibiotic therapy annually causes thousands of deaths. Development of multiple drug resistance and adverse side effects are some of the drawbacks provided by conventional antibiotics, which are currently treated by administrating antibiotics in higher doses, multiple drug administration and, longer and repetitive therapies.(14) This generates intolerable toxicity and creates the need for a continuous development of new drugs, which requires significant investment of money, labour and time.(14) There is a relatively low rate of novel antibiotic discovery and rapid development of bacteria, with the probability to become pathogenic. These factors limit the option for addressing microbial resistance by developing new antibiotics.(15) Therefore, the most promising and efficient strategy is to enhance the activity of already existing antibiotics, and thus counter the problem of antibiotic resistance.(16) There are several known mechanisms of bacterial resistance, and some of them can be prevented or overcome by already existing application methods, for instance administration of antimicrobial agents encapsulated in nanoparticles, such as liposomal drug carriers.(17,18,19) Liposomes can protect antibiotics against environmental deactivation, enhance their transport through physiological barriers and offer target delivery, thus increasing drug bioavailability and limiting side effects.(20,21,22) In addition, liposomal carriers exhibit low toxicity, high therapeutic efficiency and the ability to deliver poorly water-soluble drugs.(23)

Photosynthetic bacteria, such as sulfur- and cyano-bacteria are one valuable source that can offer membrane lipids of natural origin, which can be used for lipid preparation. Thus in this work we have considered unconventional liposomal nanovectors, assembling them through a “top-down” procedure using these biodegradable natural-origin lipids. Our aim was to evaluate the possibility of assembling liposomes directly from lipid membranes isolated from those organisms and acquire basic information about their structural properties. Additionally, their ability of acting as potential drug carriers for bioactive substances and effectiveness of liposomal antibiotics on cell cultures was evaluated by commonly used physico-chemical methods.

1.1 Antimicrobial therapy

1.1.1 Antibiotics and resistance

Antimicrobial drugs have been an important therapy to treat bacterial infections for decades. At the same time evolution of microbial organisms and antibiotic resistance (AR) is a natural phenomenon that occurs due to continuous exposition to antimicrobial drugs, overuse of antibiotics in human and animal feed, mutation and other natural occurring mechanisms.(24) AR is defined as a resistance of microorganisms to standard antibiotic therapy to which they were originally sensitive. Thus, AR is a growing problem, reducing the effectiveness of the existing treatment with antibiotics and causing continuous search for new efficient drugs. AR posesses an increasingly large threat to global public health and causes serious economic consequences.(25) A current study reports that the rate of adaptive mutations in *E. coli* is "on the order of 10^{-5} per genome per generation, which is 1,000 times as high as previous estimates".(26) One of the most promising strategies for enhancing drug delivery and combating AR is the administration of microbial agents by nanoparticles, including lipid based drug carriers such as liposomes.

There are several emerged mechanisms of action that result in resistance to antibiotics. Numerous studies have shown that liposomes can fight several of these resistance mechanisms exhibited by some microorganisms. Those include decreased uptake and increased efflux (27,28,29) of drug from the microbial cell, biofilm formation (30) and intracellular location of bacteria.(31) The lipid bilayer of a liposome-antibiotic complex can make a quick fusion with the plasma membrane of the microbial cell. After fusion a high concentration of drug can be released directly into the cell, thus bypassing the resistance mechanism of decreased uptake. For instance, liposomes can be small enough to be phagocytosed by host phagocytes, which contain intracellular microbes. Once inside the host cell, the carrier can release drugs that then combat these intracellular bacteria. Additionally, by using drug targeting, liposome carriers can overcome resistance issues by bringing higher doses of the drug directly to the infection site. Moreover, there is a possibility to load liposome carrier with multiple antimicrobial agents, thus making the resistance more unlikely to occur.(32) Consequently, optimal

effect can be achieved faster, resulting in a shorter time of treatment and exposure to antibiotics.

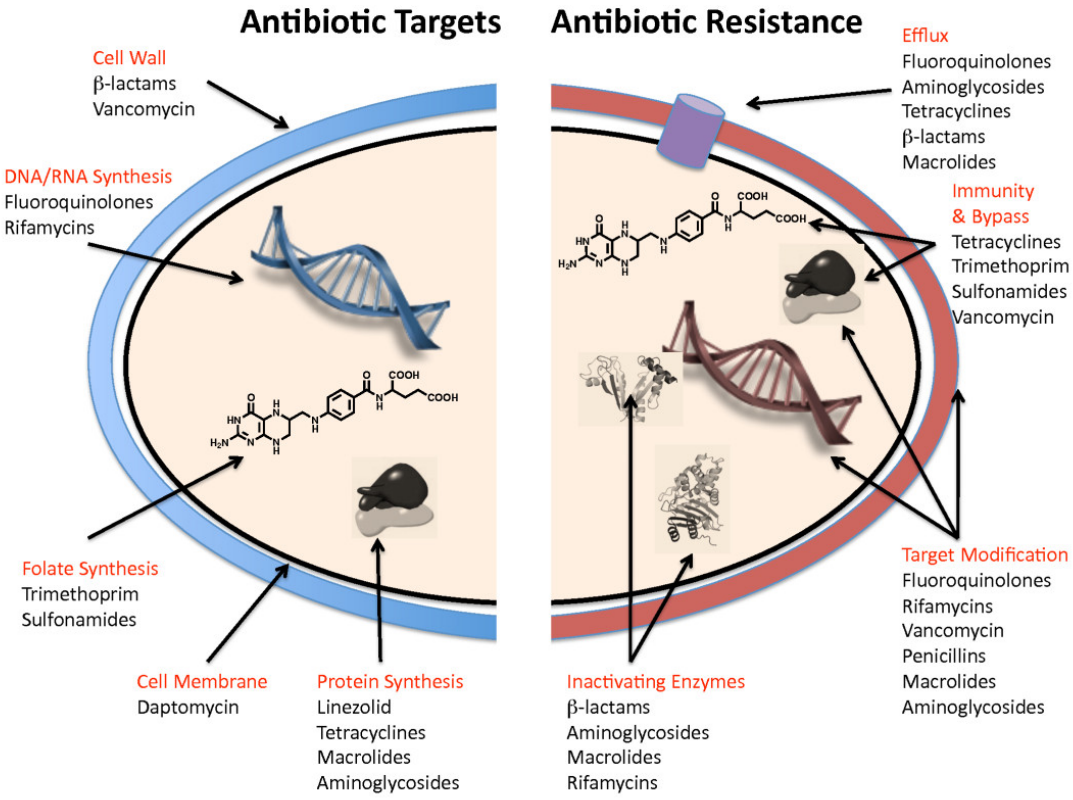


Figure 1: Overview of antibiotic targets and mechanisms of resistance (33)

1.1.2 Ampicillin (Amp)

Ampicillin, also referred to as Aminobenzylpenicillin, is an orally active, semi-synthetic broad- spectrum antibiotic.(34) Amp is a beta-lactam antibiotic, structurally related to penicillin which shares the same mechanism of action as the other penicillins. It works by inhibiting bacterial cell-wall synthesis through binding to one or more penicillin-binding proteins of actively dividing cells. Amp may be both bactericidal and bacteriostatic. That will depend on the drug concentration and the type of microorganism involved. Amp is clinically effective not only against the gram-positive organisms but also against a variety of gram-negative organisms. It is stable in the presence of gastric acid and is well absorbed from the gastrointestinal tract. It diffuses readily into most of the body tissues and fluids.(35) Oral bioavailability of Amp on fasting state is reported to be 50%.(36)

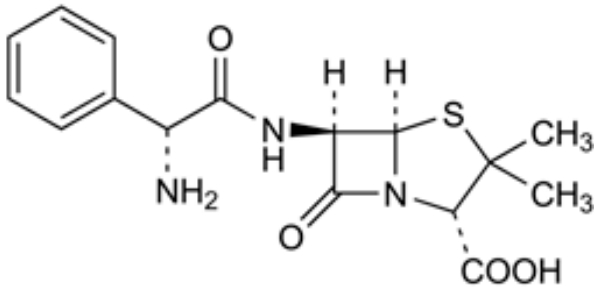


Figure 2: Ampicillin molecule

1.1.3 Penicillin G (PenG)

Benzylpenicillin is commonly known as PenG, and is a hydrophobic, narrow spectrum β-lactam antibiotic produced by *Penicillium spp*. (37) As an antibiotic it inhibits synthesis of bacterial cell wall, hence it is bactericidal. PenG is given via intravenous or intamuscular route as a treatment against various types of infections caused by susceptible, usually gram-positive, organisms. Additionally, a small range of Gram-negative organisms are also reported to be susceptible to PenG. Parenteral administration is preferred due to PGs instability under acidic stomach conditions. Consequently, a high tissue concentration can be achieved, resulting in an increased antibacterial activity. Despite reportedly increased resistance among many types of bacteria, PG is still widely used.

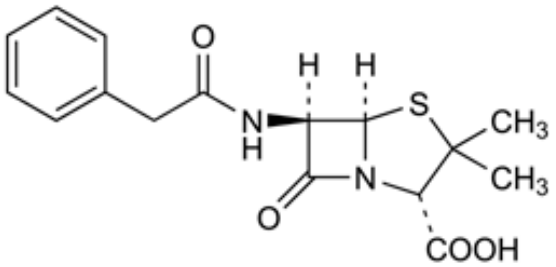


Figure 3: Benzylpenicillin molecule

1.1.4 Sulfanilamide (SFA)

SFA is a molecule consisting of sulfonamide functional group attached to an aniline group. It is an antibacterial compound which acts as competitive inhibitor that inhibits enzymatic reactions involving dihydropteroate synthetase (DHPS).(38) This prevents production of folic acid and hence synthesis of amino acids which are important for growth of microorganisms. Hence SFA and its derivatives are bacteriostatic, inhibiting growth and multiplication of bacteria. However, mainly because of its adverse effects, SFA-based antibiotics have been replaced by less toxic alternatives.(39)

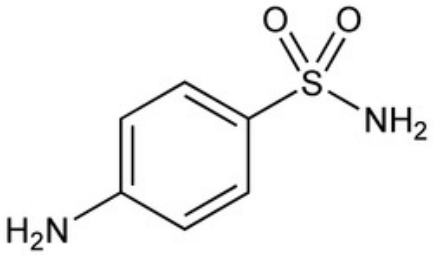


Figure 4: : Sulfanilamide molecule

1.1.5 Ethylaminobenzenesulfonamide (ESFA)

This compound is a synthetic antimicrobial agent, and is one among many available derivatives of sulfanilamide, which contain same functional groups. ESFA completely inhibits the synthesis of folic acid in microbes.

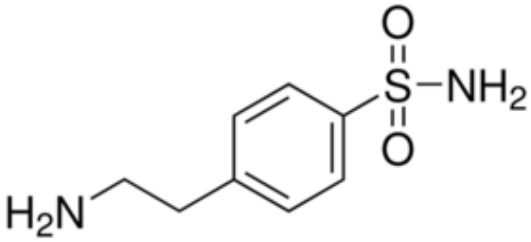


Figure 5: 4-(2-Aminoethyl)benzene sulfonamide molecule

1.1.6 *Escherichia coli* (*E. coli*)

E. coli is a gram-negative bacterium of the genus *Escherichia*, which is a large and diverse group of bacteria. Most of *E. coli* strains are harmless, and are commonly found in nature as a part of the normal flora in the gut. However, some serotypes can be pathogenic, causing serious food poisoning, diarrhea, urinary tract infections and pneumonia.(40) Sensitivity of *E. coli* strains is varying widely. *E. coli* infections may be treated with amoxicillin and other semisynthetis penicilles. Being gram-negative bacteria it is generally resistant to antibiotics used against gram-positive bacteria. Additionally, *E. coli* and other enterobacteria are major contributors to transferable antibiotic resistance to other species of bacteria, mainly by horizontal gene transfer. *E. coli* carries multiple drug-resistance plasmids which can be readily transferred to other species. Equally important is the fact that *E. coli* is a frequent member of biofilms. *E. coli* interact closely with other microorganisms, enabling an easy plasmid transfer among them.(41)

1.1.7 *Bacillus subtilis* (*B. subtilis*)

B. subtilis is a Gram-positive bacterium of the genus *Bacillus*. This species is commonly found in soil, but also is a part of normal human gut flora. *B. subtilis* bacteria are non-pathogenic. However, other species of *Bacillus* can cause anthrax and food poisoning. The bacteria are easy to handle and often used as an experimental model.

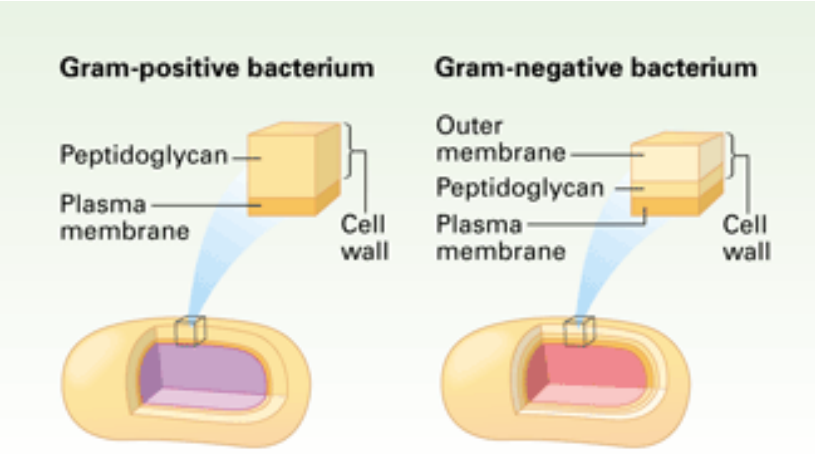


Figure 6: Membrane of gram-positive and -negative bacteria (42)

1.2 Drug carriers

Basically a drug carrier is a system that can improve the delivery and effectiveness of a drug. An optimal carrier should be stable, reproducible and applicable for different purposes. The following aims can be defined for a drug carrier:

- control release of a drug
- prolong *in vivo* drug actions
- enhance pharmacological effects
- protect the drug against degradation
- prolong drug presence in the circulation
- enhance poor permeability through various membrane barriers
- reduce drug toxicity
- increase the effectiveness of drug delivery to the target sites

Various types of drug carriers can be found, and liposomes are among the most commonly systems used nowadays.

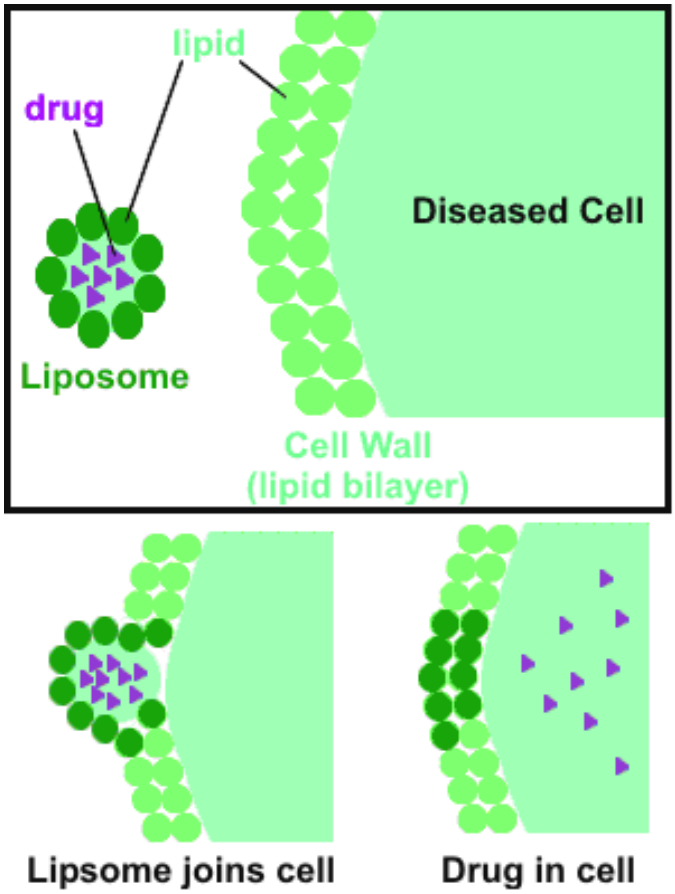


Figure 7 : Direct drug targeting by fusion with microbial cell (43)

1.2.1 Choice of a drug carrier and its role in improved therapy

Drug carriers can play an important role in drug delivery and the outcomes of a therapy, especially when the drug itself has poor bioavailability and can not be administered by its pure form. The method by which a drug is delivered can have a significant effect on its efficacy. Some of the purposes of using a drug carrier are to target delivery to a particular cell or receptor, prolong therapeutic effect by sustained release of a drug, enhance bioavailability and solubility, minimize drug toxicity and of prolong its shelf life. Various nano-carriers have been purposed for the delivery purposed. Some of these are micelles, polymeric vesicles, nanocapsules and liposomes, and show great promise as drug delivery systems for a variety of compounds.

When a drug carrier is developed several factors has to be taken into account. The main goal is to obtain a drug delivery system which has optimized loading capacity and release properties. A delivery vehicle has to have the ability to load and deliver an amount of a drug in the concentration range that gives satisfactory effect and does not provide any toxicity. It is also important to create a biodegradable and biocampatible system to reduce any toxicity provided by the drug and the carrier itself. The development of a drug carrier that can not only protect the drug itself from different environments present in the body and to increase its systemic bioavailability by minimizing its degradation and loss, but also to bring the drug directly to the therapy required zone.

Targeted delivery is believed to improve efficacy of a therapy, and can also be used for diagnostics. The fact that the drug is released directly at the target site or can accumulate in the pathological area, can reduce its side effects and enhance treatment efficacy. Another important factor is that the carrier can not only bring the drug to a target site, but also is able to release it in a defined manner. Controlled drug relase can be obtained by various means depending on the target site position in the body. Potential release mechanisms can involve desorption of adsorbed drugs and drugs bound to the surface of a carrier, diffusion through the carrier wall/matrix or the erosion of the matrix. The controlled release can be carried out by rate-programmed drug delivery systems, or activated drug release by physical, chemical or biochemical

processes (pH, light, temperature). Additionally a rate of drug releaes by a carrier can depends on the administration route. (44,45,46)

Drug carriers can be applied for drugs or bioactive compounds that have not yet been used for therapeutical treatment, improve therapeutic efficacy and limit side effects of older drugs. Aditionally drug carriers can be used to improve patinent safety and use, or reduce manufacturing costs. The choice of a drig carrier is dependentent on several factors, for instance, the nature and character of a drug, administration route and desired effect.

1.3 Liposomes

Liposomes are lipid-based vesicle systems and are widely employed biodegradable carrier systems. These containers are composed of a lipid bilayer made of phospholipids, which have both hydrophilic and lipophilic parts. The building blocks spontaneously form a vesicle, bilayer membrane that contains an aqueous core. Liposomes can be designed to entrap both water soluble and lipid soluble materials. A particular drug can be entrapped within or in the membrane layer for drug delivery purposes. The lipid bilayer of liposomes is similar to the outer surface of a cell. This characteristics enable the liposome passage through the cell membranes and binding with the exterior of cells possible, ensuring that the drug reaches the interior of cell and hence the site of action. Attaching different functional groups to the outer layer can modify liposome system.(47)

Present applications of liposomes are in the immunology, dermatology, vaccine adjuvant, eye disorders, brain targeting, infectious disease and in tumour therapy.(48) As mentioned above, liposome carrier- systems can be employed for various materials. In this project we have focused on antibiotics and curcumins. The main focus was to evaluate the ability of liposomes to act as drug-carriers for thess types of substances. Although antibiotics and curcumins belong to different classes of drugs and have different chemicalproperties, their structure bears some similarities; for instance both possess benzene rings and contain a variety of different functional groups in their structure . In addition, both substances do not have the typical amphiphile structure, therefore they are not taken up easily by phospholipid bilayers.

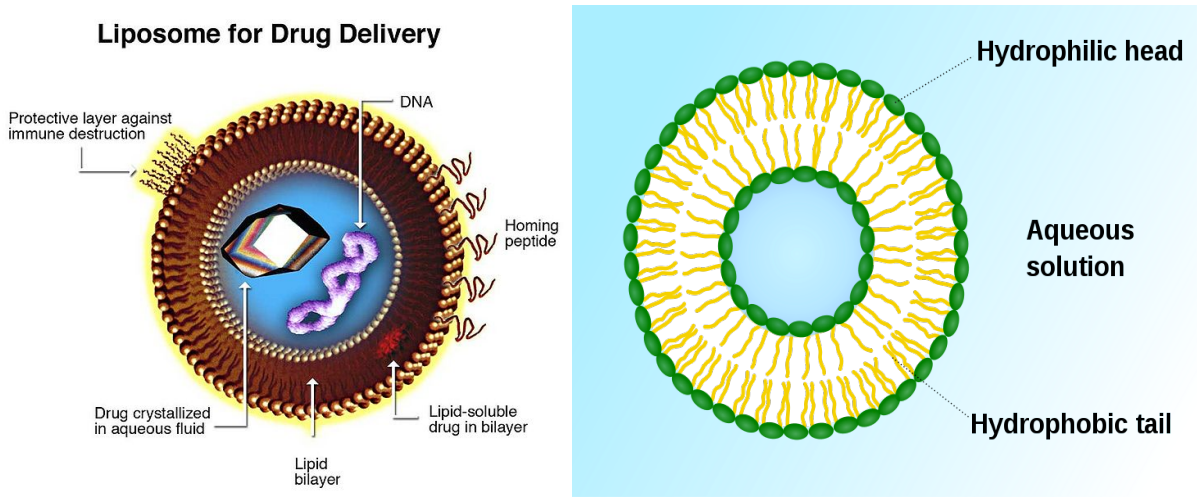


Figure 8: Structure of liposome drug-carrier (49)

1.3.1 Liposome-entrapped antibiotics

Numerous studies of liposomes as antibiotic carriers have shown that they can significantly enhance drug distribution, increase antibacterial activity, improve pharmacokinetics, reduce drug toxicity and other side effects of antibiotics. However, it has also been observed a range oflimitations, in particular the low encapsulation efficiency for many antibiotics.(50) Thus there is still a room for improvement in this delivery system's technology. The success of liposomes as drug carriers is reflected in a number of liposome-based formulations, which are already commercially available or currently undergoing clinical trials. The insight gained from clinical use of liposome drug delivery systems can now be integrated to design liposomes that can be targeted to different tissues, cells or intracellular compartments.(51)

Lately, the main focus of a number of studies has been on antibiotic treatment since it is not always successful, especially against severe infections. Targeting of antibiotics to infected tissues or cells by encapsulation in liposomes is under investigation and may be of importance in the treatment of different types of infections. Liposomal encapsulation of antibiotics has demonstrated improved therapeutic index and reduced toxicity in some of the antibiotic treatments.(52)

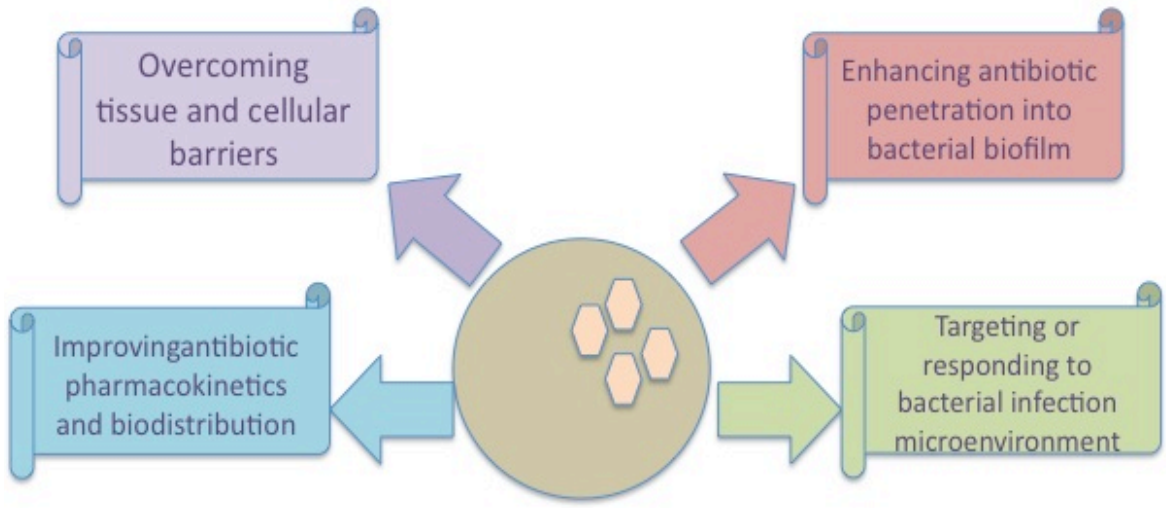


Figure 9: Advantages of antibiotic-loaded polymeric particles

1.4 Bacteria

Photosynthetic bacteria, such as sulfur- and cyanobacteria, offer a valuable source of natural origin membrane lipids, which are a subgroup of the wider class of biosurfactants.(53,54) These bacteria reproduce quickly and can be easily and cost-effectively cultivated for mass lipid production. Thus these organisms represent an attractive source for lipid extraction. Moreover, plasma membranes of these bacteria possess some similarities to the membranes of pathogen bacteria, which are currently treated with antibiotics. This similarity can promote the interaction between liposomes loaded with antimicrobial agents and the target microbial cells.
Being prokaryotic organisms, they have relatively simple assembled membranes. Consequently, the lipid fractions obtained from membranes of these bacterial cells are expected to be relatively homogeneous. There is a considerable diversity of phospholipid types present in various bacterial strains, but most abundant type is glycerolipids containing two fatty acid chains.(55)

The microorganisms chosen for the aim of this work are specific strains of cyanobacteria and purple sulfur bacteria. These are already utilized in various biotechnological processes.(56,57) By considering them in this work, a further value can be added to their exploitation.

1.4.1 Cyanotece VI22

1.4.2 Cyanotece CCY0110

The cyanobacteria, also referred to as blue-green algae, are photoautotropic prokaryotic organisms. Cyanobacteria are a major and phylogenetically coherent group of Gram-negative prokaryotes, and include a large variety of species of widespread occurrence and with diverse morphological, physiological and biochemical properties. These bacteria are characterized by a great morphological diversity, unicellular as well as filamentous species being included with a cell volume ranging from over more than five orders of magnitude. Because of their properties they are of particular interest for molecular biological studies.(58,59)

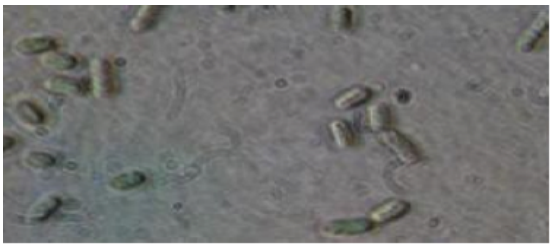


Figure 10a: VI22

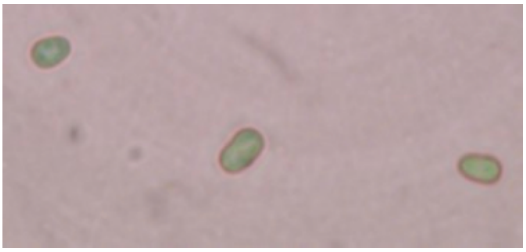


Figure 10b: CCY0110

1.4.3 *Rhodopseudomonas palustris* 42OL

R. palustris is a purple photosynthetic Gram-negative bacterium that belongs to the alpha proteobacteria family, with an outer membrane mainly composed of lipopolysaccharides. The bacteria are widely distributed in nature. These are found in diverse places such as swine waste lagoons, earth- worm droppings, marine coastal sediments and pond water. They are rod-shaped to ovoid, sometimes slightly curved. The dimensions are 0.6-0.9 x 1.2-2.0 µm. Their motility is assured by a subpolar flagella.(60,61)

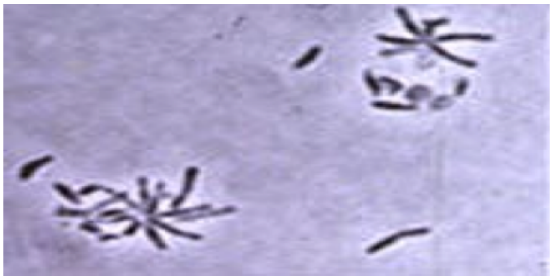


Fig 11: 42OL

1.5 Curcumin

Curcumin is an active natural polyphenoic constituent of dietary spice tumeric. This yellow coloured phenolic pigment is obtained from powdered rhizome of the plant *Curcuma longa*. (62) Curcumin is included in the group curcumioids, were other two are desmethoxycurcumin and bis-desmethoxycurcumin. Curcumin is a bis-α,β-unsaturated β-diketone, which can exhibit two tautomeric forms. Keto form exists in acidic and neutral solutions and the stable enol form predominate in alkaline medium.(63) This naturally derived component holds a high place in Ayurvedic medicine, but its role in the treatment of various conventional diseases is also well established.(64)

Extensive scientific research on curcumin has demonstrated a wide spectrum of therapeutical effects such as anti-inflammatory (65,66), antioxidant, antibacterial (67), antiviral (68), antifungal (69), antitumor (70), antispasmodic (71) and hepatoprotective(72). Also antitumor and antiangiogenesis effects were observed *in vivo*.(73) The pharmacological safety and efficacy of curcumin makes it a potential compound for treatment and prevention of a wide variety of human diseases. Although curcumin shows promising pharmacological effects, there is still a clinical struggle caused by its low bioavailability. Major reasons contributing to the low plasma and tissue levels of curcumin appear to be poor absorption, rapid metabolism, and rapid systemic elimination. Curcumin undergoes rapid degradation first by hydrolysis, which is then followed by molecular fragmentation.(74) Poor aqueous solubility (~20 µg/mL) is a major challenge. To improve the bioavailability of curcumin, numerous approaches have been undertaken, including liposome incorporated curcumin and curcumin nanoparticles.

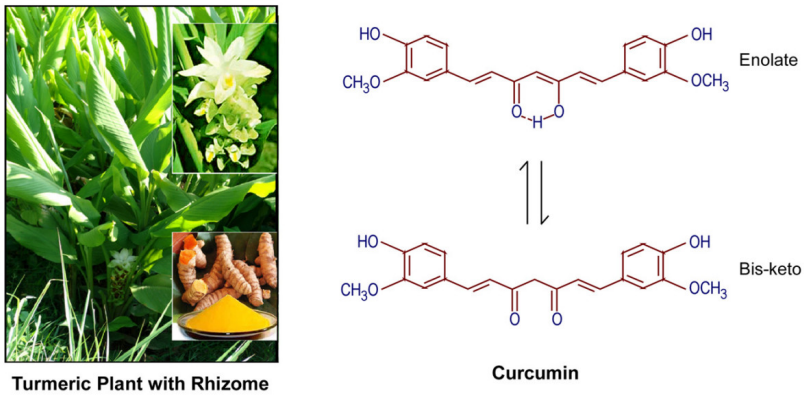


Figure 12: Curcumin and its tautomeric forms (75)

1.5.1 Liposome-curcumin complex

Despite the low bioavailability, therapeutic efficacy of curcumin against various human diseases, including cancer, cardiovascular diseases, diabetes, arthritis, neurological diseases and Crohn´s diseases, has been documented.(76) Studies that have already been carried out and huge interest for curcumin assures its promising future.

A wide variety of carriers have been tried as a mean to improve the bioavailability of curcumin, where liposomes provided promising results.(77,78,79) A liposome-encapsulated curcumin permits also its intravenous administration. One study showed that the liposome-curcumin complex suppresses growing and induces apoptosis of human pancreatic cells in vitro.(80) In a recent study, oral liposome-encapsulated curcumin (LEC) prepared from lecithin was examined in Sprague-Dawley (SD) rats by administration of 100mg/kg doses. High bioavailability of curcumin was evident in the case of LEC. Moreover a faster rate and better absorption of curcumin were observed as compared to free drug and curcumin/Lecithin mixture.(81) However, conventional liposomes may be relatively unstable under physiological conditions typically found in the gastrointestinal tract. Thus a liposome preparation may require surface modification of liposomes. One of such modification method is chemical linkage of poly(ethylene glycol)s (PEG) to hydrophilic amino termini liposomes. PEG has shown to provide structural stabilization of liposomes as well as efficient steric protection of encapsulated drug.(82) Some other modification techniques applied on liposome-curcumin complex involves coating it with with silica particles or cationic lipid/polymer (HPTMA). Surface modifications of liposomes have shown to protect liposomes fom degradation, improve their cellular entrance and prolong circulation time. (83,84)

1.5.2 Cetyl trimethylammonium bromide (CTAB)

In order to enhance effectiveness of curcumin and improve its bioavailability, liposomal carriers with desired properties have been designed. Furthermore, in this project we were investigating the possibility to use CTAB as adjuvant in the process of curcumin uptake by liposomes. In fact, it has been reported that CTAB is able to form a complex with curcumin, whose stability and spectral properties have been investigated by absorption and fluorescence spectroscopy. (85,86,87)

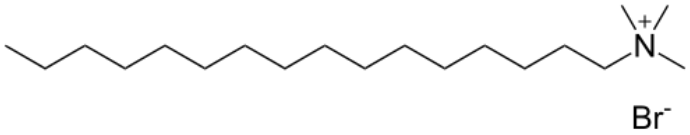


Figure 13: CTAB molecule

1.6 Charaterization of drug carrier systems

1.6.1 UV/VIS Spectrometry

Spectrophotometric analysis performs quantitative measurement on how much a chemical substance absorbs light by passing a beam of light through the sample. Use of electromagnetic spectrum to analyze different molecules is the main principle applied in spectrometry. Information gained during UV/VIS analyze comes from ultraviolet (190-380nm) and visible region (380-750nm) of electromagnetic spectrum.(88)

Qualitative analysis can be carried out by comparing the spectrum from analyzed sample with the standard spectrum of known compounds. Additionally, by measuring the absorbance at specific wavelengths, one can detect impurities in the analyte. Another useful characterization can be performed for quantitative purposes of the compounds that absorb UV radiation. This determination is based on the Beer-Lambert Law:

$$A = \log I_0 / I_t = \log 1/T = -\log T = \varepsilon bc$$

where

A is absorbance

ε is molar absorbtivity (L mol^{-1} cm^{-1})

b is the path length of the cuvette(cm)

c is the concentration of the compound in solution (mol L^{-1})

In this work Lambda 35 Perkin UV/VIS Spectrometer was used for determination of phospholipid amount present in liposomal samples prepared from bacterial cultures. Phospholipids have an expected absorbance at 820 nm.

Lambda 35 double beam spectrometer offers high stability, high accuracy and reproducibility, and performs in the range 190 nm-1100 nm.(89)

1.6.2 Mass Spectrometry (MS)

MS is a sensitive method, used for both quantitative and qualitative analysis, during which the analyte is ionized and separated according to the mass/charge ratio (m/z), either from directly injected samples or samples eluted from a liquid chromatograph. A general MS experiment is best fitted for quantitative analysis of known compounds. During this work, the analysis was performed on Thermo Finnigan LTQ, an advanced analytical instrument. The detector consists mainly of a syringe pump, a divert/inject valve, an atmospheric pressure ionization (API) source and a MS detector. LTQ detector is a linear ion trap mass spectrometer type equipped with an electrospray ionization (ESI) source external to the mass analyzer. ESI is useful instrument that produce ions for MS detection, especially from macromolecules. ESI offers little fragmentations and an advantage in that the molecular ion is always observable. However, little structural information is available from the MS obtained using ESI. (90) Since our interest was mainly in mass quantification of samples containing ampicillin and penicillin, electrospray ionization mass spectrometry (ESI-MS) was chosen for this purpose.

LTQ can be operated in either positive or negative ion polarity mode. In this work, positive ions were transmitted to the analyzer for mass determination. The sample solution was introduced into API source by a syringe pump from injecting valve fitted with a loop and an LC pump. A LC/MS analyze was performed, during which the sample was injected onto an LC column, where it was separated into different components. These then proceed into the MS detector where they are analyzed.

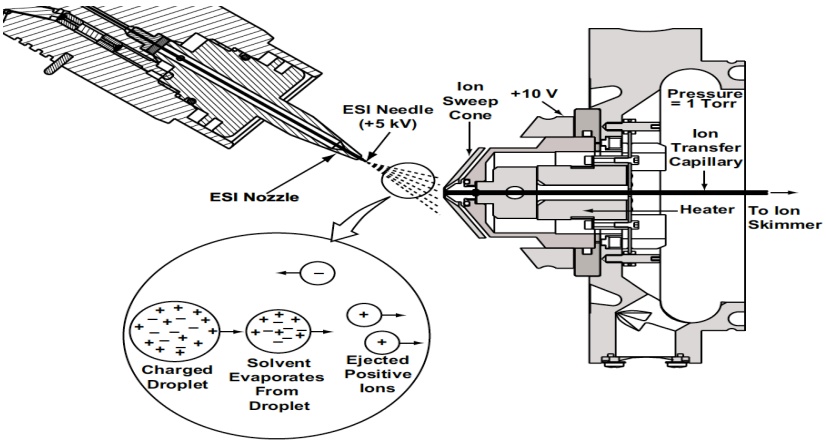


Figure 14: ESI process in the positive ion polarity mode (91)

1.6.3 Dynamic Light Scattering (DLS)

DLS analysis was performed on a Malvern Zetasizer Nano S (92) (Malvern Instruments, Southborough, MA) ZEN1600 model equipped with a 4.0 mW, He-Ne laser operating at 633 nm and with a back-scattering detector (173°). The detector operates in 0.6 nm to 6 µm hydrodynamic diameter.

DLS method enables us to determine particle size, which is the size of the sphere that diffuses at the same speed as the particle that is analyzed. Zetasizer is using DLS to measure the Brownian motion of the particles and thus determining their size. Brownian motion is defined as:

"The random movement of particles in a liquid due to the bombardment by the molecules that surround them".

The particle movement in liquid is random and the speed of this movement is used to determine particle size. Small particles move quickly and large ones move slowly in colloidal system.

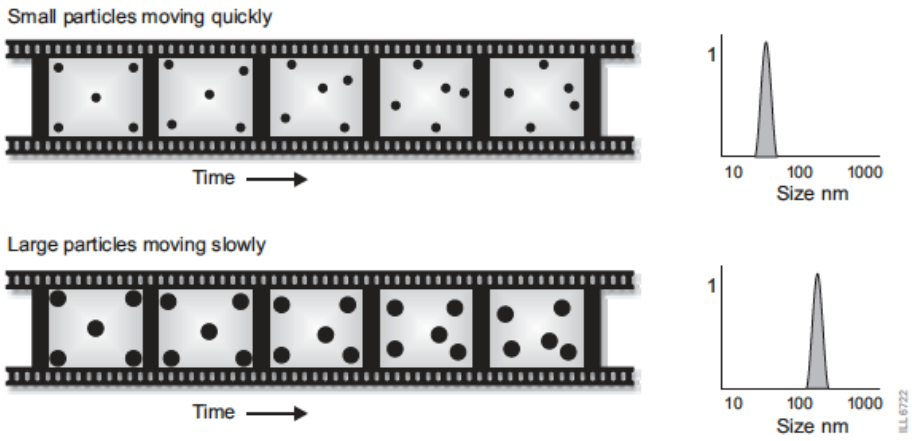


Figure 15: movement of particles regarding to size (86)

DLS provides four main types of information:

1) zeta potential: a potential that exists between a surface of a solid particle and dispersing liquid around it, which varies according to the distance from the particle surface

2) standard deviation: displays 1 standard deviation of the zeta distribution around the mean - result, in (mV)

3) conductivity: the ability of a sample to conduct electrical current

4) peak means: displays the mean zeta potential for up to three peaks within the result

DLS is a useful tool in determining the size and size distribution of plain and loaded liposomes in this work. In the case of liposomal drug delivery these properties are known to influence liposome stability, drug entrapment and delivery efficacy.(93,94,95) However, to get more detailed information on the liposome properties, such as their lamellarity and bilayer thickness, a detailed investigation by Small Angle X-ray scattering should be applied.

Finally, the time autocorrelation functions are analyzed by the cumulant method to obtain the polydispersity index (PDI), which is a measure of homogeneity in the size of scattering objects. Small values of PDI (< 0.2) indicate homogeneous populations, while PDI > 0.4 indicates high heterogeneity. An inverse Laplace transform algorithm (CONTIN) is used to obtain the mean size and size distribution of particles present in the samples.

1.6.4 Biological Assays

Sensitivity assay is often carried out to evaluate the response of various bacterial cultures to liposomal antibiotics. The main principle of sensitivity assay is to incubate a bacterial strain of interest, also called inoculum, and mix it with different concentrations of antibacterial samples. An easy way to evaluate the effectiveness of antibiotic samples is to analyze the growth of the bacterial strains. For this purpose a microtiter plates can be applied. After filling the wells of the plate with defined amounts of inoculum, liposomal antibiotic and free antibiotic, the optical ensity (OD) is recorded and nominated (t_0). Following, the plate is incubated for 24 hours, and OD is recorded again (t_{24}). Since OD increases with increased number of microbial cells present in the well, it is possible to evaluate if the growth of cells is inhibited or not. This is done by calculating the difference between the two OD readings ΔOD (t_{24}-t_0). Also, the minimum inhibitory concentration (MIC) can be evaluated by using antimicrobial samples in different concentrations. MIC is then defined as the lowest observed concentration of the antimicrobial that inhibits growth of the microorganism. Additionally, it can be of interest to examine what kind of effect an antimicrobial execute on the bacterial cell.

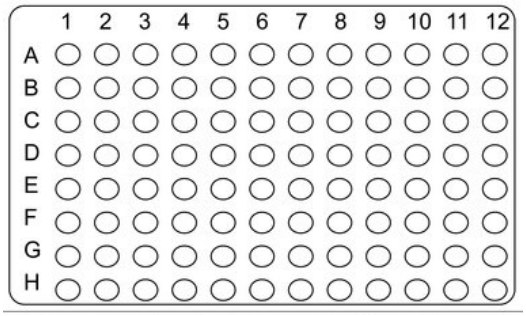


Figure 16: The microtiter plate

Bactericidal effect is the ability of an agent to kill bacteria. In contrast, bacteriostatic effect is observed when an agent inhibits bacterial reproduction. For this purpose *spread-plate method* can be applied used. The purpose of this method is to grow and isolate bacterial colonies. It is a quantitative technique that allows determination of the number of bacteria in the sample. The principle of this technique is to transfer a sample of bacterial culture to a Petri dish filled with agar medium, which offers nourishment for successful growth. First of all, the bacterial cells are treated with either free or liposome entrapped antibiotic in the liquid media. Then the scalar dilutions of the samples are made, and the required amount of each sample is transferred to the surface of a Petri dish. The inoculum is then spread on the

surface of the agar medium using sterilized L-rod. Finally the plate is incubated in inverted position at 37 ˚C in a heat cabinet. After incubation, the colony forming units (CFU) are counted, and comparison of final title to initial title is made to estimate viable bacterial colonies. Thus plating assay allows the counting of viable cells recovered after treatment of microbial cells with the antibiotic. The assay can show whether liposomal antibiotic exhibited bactericidal properties. Furthermore, the growth of the colonies on the agar plate can demonstrate if bactericidal effect is concentration dependent.

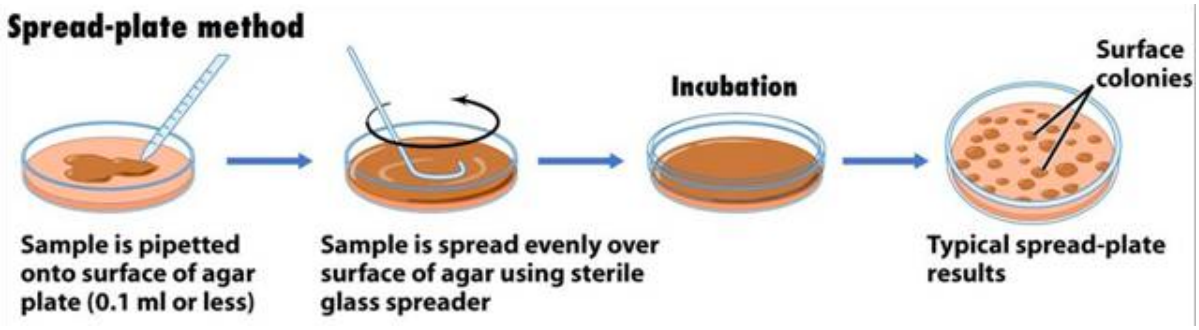


Figure 17: Spread-plate method (96)

1.6.5 Enzymatic Assays

The analysis of samples containing conventional liposomes and sulfanilamides was carried out by *stopped-flow* method, which is a wellknown technique used for analyzing the kinetics of reaction in a solution, prepared by mixing together the reactant solutions. Stopped-flow method is used for studying mechanisms with a fast reaction rate (1ms-100s). The main principle of this method is that reactant solutions are simultaneously injected and rapidly mixed in a mixing chamber. Then the flow of the mixed solutions passing through a spectrophotometer cell, is quickly stopped at observation cell. The arrest of the flow triggers monitoring by photometric device which can register changes occurring during milliseconds.

Thus the progress of the reaction is followed by recording the absorbance at a specific wavelength as a function of time, of either the reactant or product.(97) Thus by analysing the change in absorbance one can acquire information about complexity of reaction mechanisms and determine reaction rates. Additionally, parameters affecting the kinetics of a reaction, such as temperature, pH or reagent concentration, can be analyzed.(98)

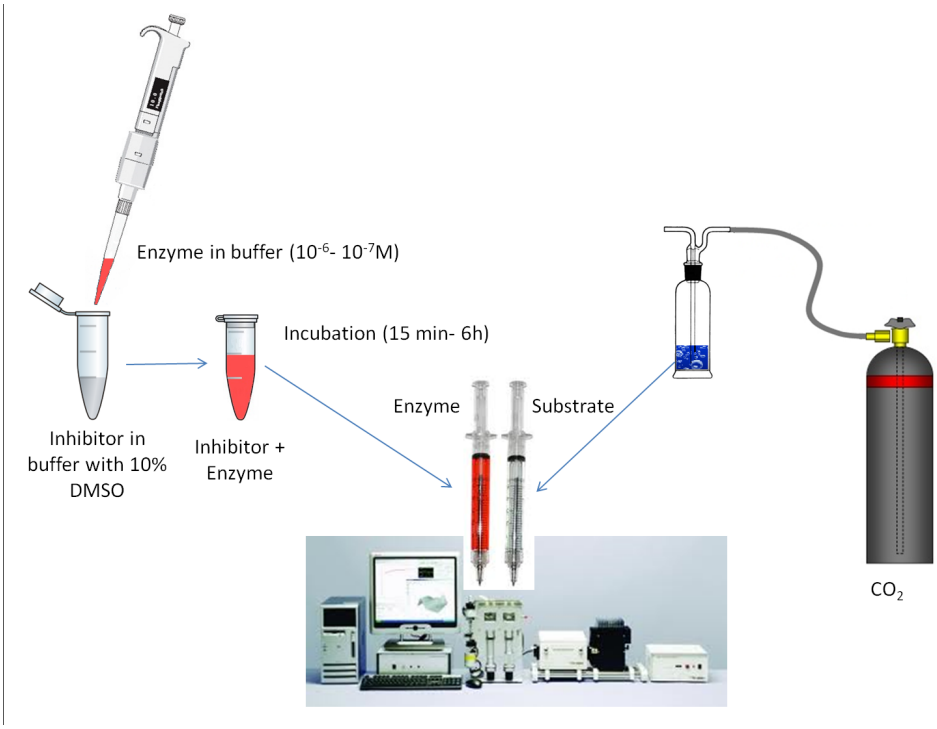


Figure 18: diagram of the stopped-flow apparatus

In this work an Applied Photophysics stopped-flow instrument has been used for assaying the Carbonic Anhydrase (CA) catalyzed CO_2 hydration activity.

CA form a family of enzymes (α, β, γ, δ, ε), classified as metalloenzymes, since the active site of most of them contain zinc ion. These enzymes catalyze hydration of carbon dioxide and conversion to bicarbonate. Thus they are important in cellular metabolism and vital for organism survival. The primary enzyme function is regulation of internal pH (acid-base balance) and contribution with transport of carbon dioxide out of the blood and tissues.

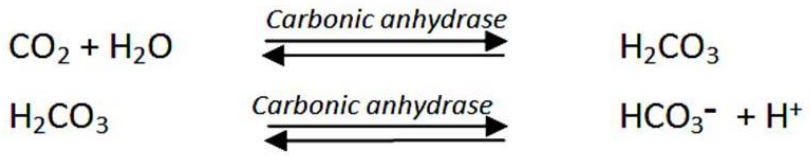


Figure 19: carbonic anhydrase catalyzes reactions. (99)

Carbon dioxide, bicarbonate, carbonic acid, and carbonate are key metabolites in all living systems, and the equilibrium of these different forms in living cells is important for proper physiological functioning.(100) Therefore, a range of pharmacological compounds are aimed to suppress CA activity for different purposes. CA inhibitors are thus a class of pharmaceuticals with different applications. Some of their clinical uses are established as diuretics, antiepileptics, antiglaucoma and antimicrobial agents.(101,102,103) In this work we were considering liposome-incorporated and free- sulfur compounds. These compounds contain the sulphonamide (SO2NH4) functional group. Sulphonamides have shown the ability to inhibit CA.(104)

1.6.6 Small-angle X-ray scattering (SAXS)

The X-rays can be used to investigate the structural properties of various materials: solids, liquids or gels. SAXS is a technique where the elastic scattering of X-rays by a sample which has inhomogeneities in the nm-range, is recorded at very low angles. The SAXS experiment can provide information about the fluctuations of electronic densities in heterogeneous matter, and provide information about the shape and size of marcomolecules. (105)

2 AIMS OF THE STUDY

The aim of this thesis was to develop, optimize and evaluate liposomal drug carrier model for bioactive compounds such as curcumin, penicillin G and ampicillin. The focus was on the potential of organic liposomes to improve the bioavailability and increase pharmacological action of biological active substances. The main focus was on extraction of the lipid fraction from algae membranes, namely sulfur- and cyano-bacteria, preparation of liposomes and incorporation of bioactive compounds of interest. In this project the formulations of plain and loaded liposomes were extensively characterized from the structural point of view, using analytical techniques such as Dynamic Light Scaterring. Additionally MS and UV/VIS were used for analysing the lipid composition and evaluating entrapment efficiency of liposomes. Furthermore, the biological activity and anti-proliferative effects of loaded liposomes against bacterial microorganisms were also evaluated in order to establish the correlation between biological activity and physicochemical properties. Characterization of plain and loaded liposomes, especially regarding their size, is important since the size of liposomes has been shown to be an important factor in the efficient drug delivery, targeting and biological response.

3 MATERIALS AND METHODS

3.1 Chemicals and solutions

The microorganisms chosen as starting material for the present work are currently exploited in the removal of heavy metals from aqueous solutions.(106,107) These are the unicellular N2-fixing *Cyanothece* sp. strains CCY0110 and VI22, isolated from marine and saline habitats respectively (108,109), and the purple, non-sulfur photosynthetic bacteria, *Rhodopseudomonas palustris*, strain 42OL (110).

Table 1: List of materials used for various preparations

Preparation of liposomes from bacterial cultures	**Preparation of conventional liposomes**	**Preparation of curcumin solutions**
• *Cyanotece VI22* dry matter (=S) • *Cyanotece CCY0110* dry matter (=D) • *Rhodopseudomonas palustris* 42OL dry matter (=P) • Folch solution = $CHCl_3$:CH_3OH (2:1) • NaCl 0.9% solution • distilled/sterile H_2O • nitrogen gas flow • liquid nitrogen	• Cholesterol • DOPE • DOPC • DCCHOLHCL • soybean lipids • chloroform ($CHCl_3$) • methanol (CH_3OH)	• curcumin dry powder • ethanol (CH_3CH_2OH) • CTAB solution (1 mg/mL in ethanol) • CTAB solution (2 mg/mL in ethanol)
Preparation of antibiotic solutions	**Determination of total phosphorus**	**Sensitivity essay**
• dimethyl sulfoxide (DMSO) • acetone ($(CH_3)_2CO$) • methanol (CH_3OH) • ampicillin dry powder • penicillin G dry powder • sulfanilamide dry powder • ethylaminobenzenesulfonamide dry powder	• chloroform ($CHCl_3$) • methanol (CH_3OH) • 8.9 N H_2SO_4 aqueous solution, 10% ascorbic acid ($C_6H_8O_6$) solution, 2.5 % ammonium molybdate (VI) tetrahydrate solution, hydrogen peroxide (H_2O_2) solution	• E. coliXL1Blue • *E. coli* XL1Blue/pUC18 • B. subtilis 168 • Lysogeny Broth (LB) medium • Nutrient Broth (LB) medium

3.2 Equipment

Table 2: List of equipment

Analytical balance	_
Vortex mixer	_
Thermometer	_
Heating oven/magnetic stirrer	_
Vacuo-Term vacuum oven	Selecta
Probe sonicator	_
Thermo container for liquid nitrogen	_
Lipid extruder	_
Avestin polycarbonate mebranes	200nm
Cuvettes	Quarts (QS)
UV/VIS Spectrophotometer Lambda	35 Perkin Elmer
Malvern Zetasizer Nano S for DLS	Malvern Instruments, Southborough, MA; ZEN1600 model.
Synchrotron. (European Synchrotron Radiation Facility	ESRF
Instrument Thermo for MS	Finnigan LTQ
Applied Photophysics stopped-flow instrument	
Agar plates	_
Microtiter plates	_
Spectrometer	Imunelle
BioPhotometer	Eppendorf

3.3 Cultivation of bacterial cultures

Bacterial strains of CCY0110, VI22 and 42OL were grown in ASN-III, AMA (enriched seawater) and RPN media, respectively. Cultivation media consisted of base media solution and solution of trace elements. All required compounds were mixed in a 1L non-autoclaved bottle.

Table 3: Base media for RPN

Components	Per 1L
DL-malic acid	2.0 g
NH_4Cl	0.5 g
K_2HPO_4	0.5 g
KH_2PO_4	0.3 g
$MgSO_4.7H_2O$	0.4 g
NaCl	0.4 g
$CaCl_2.2H_2O$	0.075 g
Ferric citrate	0.005 g
Yeast extract	0.4 g
Trace elements *	10 mL
Distilled water	1.0 L

Table 4: * Trace elements for RPN

Components	Amounts
$ZnSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.03 g
H_3BO_3	0.3 g
$CoCl_2.6H_2O$	0.2 g
$CuCl_2.2H_2O$	0.01 g
$NiCl_2.6H_2O$	0.02 g
$Na_2MoO_4.2H_2O$	0.03 g
$NaWO_4.2H_2O$	3 mg
$NaSeO_3$	3 mg
$FeSO_4.7H_2O$	0.3 g

Table 5: Base media for AMA

Components	Per 1L
Sea salt	33.3 g
$NaNO_3$	1.5 g
Citric acid	0.003 g
Ferric Ammonium Citrate	0.003 g
EDTA (disodium salt)	0.0005 g
A5 trace minerals**	2.0 mL
K_2HPO_4	0.04 g
$NaHCO_3$	0.1 g
Distilled water	1.0 L

Table 6: Base media for ASN-III

Components	Per 1 L
NaCl	25.0 g
$MgSO_4 \cdot 7H_2O$	3.5 g
$MgCl_2 \cdot 6H_2O$	2.0 g
$NaNO_3$	0.75 g
$K_2HPO_4 \cdot 3H_2O$	0.75 g
$CaCl_2 \cdot 2H_2O$	0.5 g
KCl	0.5 g
$NaCO_3$	0.02 g
Citric acid	3.0 mg
Ferric Ammonium Citrate	3.0 mg
Mg EDTA	0.5 mg
Vitamin B_{12}	10 g
A5 trace minerals**	1.0 mL
Distilled water	1.0 L

Table 7: ** Trace elements for AMA and ASN-III

Components	Amounts
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 g

Figure 20: Light cultivation of bacterial culture

Figure 21: obtaining dry bacteria pellet by centrifugation

3.4 Extraction of lipid fraction from bacterial cultures

The first step of the procedure was the preparation of dry cell samples. The bacterial cells were harvested at mid- to late- growth phase by centrifugation (20 min, 4000 rpm, 20 ˚C). The bacteria were used right away after the drying or frozen for later use. To obtain cell-free extracts, to the pellets 10 mL of EtOH was added and left in the heating cabinet at 30 ˚C overnight to evaporate any liquid residues. After the pellets were completely dried, 50 mg of dry material per sample was used for lipid extraction.

The second step in the preparation of the samples was extraction of the lipid fraction from dry samples of bacterial cultures. After weighting the samples, the pellets were gently crushed with a metal rod to improve solubility of the components. Then 5 mL of Folch solution was added to each sample. Further on the samples were washed with 1ml of aqueous 0.9% NaCl, 3 times. After each washing step the samples were vortexed and the upper liquid phase was removed. On the end, all of the solid residual matter was also removed from each sample. Samples were then left for drying in the fume cupboard. For faster evaporation of the solvent, the samples were hand-rotated while drying under a nitrogen gas flow.

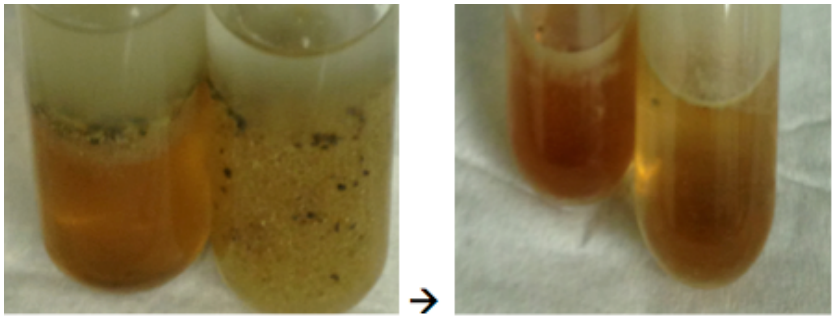
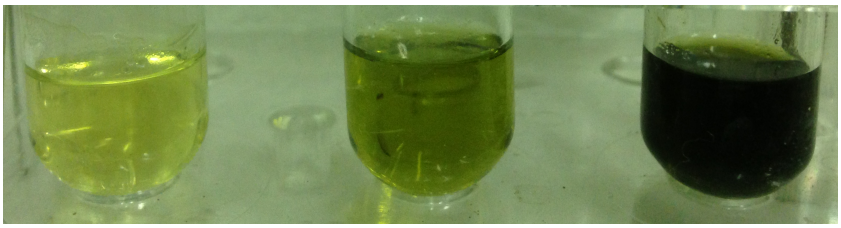


Figure 22: washing P samples, with NaCl, removal of upper layer and solid matter

Clean samples



D S P

Figure 23: Lipid solutions before obtaining the dry lipid lipid film

3.5 Preparation of plain liposomes from lipid fraction

For all liposome preparations the lipid film hydration method was used. A few modifications of the standard method were made for our procedure. For instance, the rotary evaporation was done manually with nitrogen gas flow. Before the hydration, the samples were also placed in the vacuum oven for final drying (30 min, 35 ˚C). After the rehydration, the samples were homogenized by eight freeze-thaw cycles and downsized by sonication (5 cycles, 4 min/cycle).

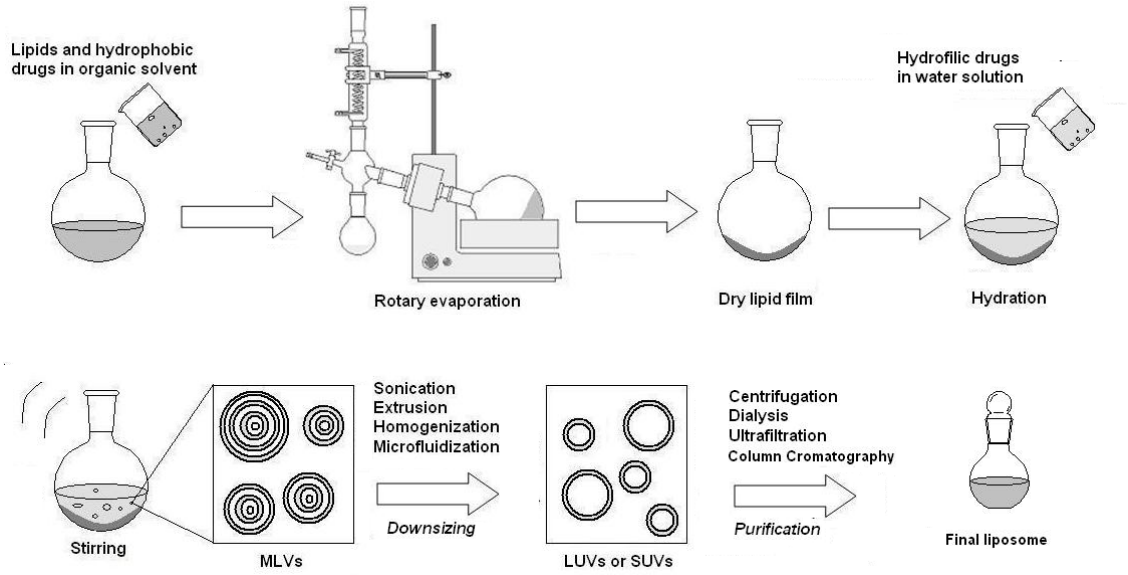


Figure 24: Liposome production by lipid film hydration method (111)

Vesicle formation obtained by lipid hydration followed by vortexing and freeze-thawing generates MLV and MLV/LUV respectively. While vesicle size reduction by sonication results mainly in SUV.

Table 8: Composition of prepared samples with following technique

S_1, S_2	lipids from *Cyanotece VI22*
D_1, D_2	lipids from *Cyanotece CCY0110*
P_1, P_2.	lipids from *Rhodopseudomonas palustris* 42OL

The procedure used to obtain the lipidic fraction was as described previously. After the addition of Folch and NaCl solutions, the samples were left for complete drying. Any liquid phase left was dried under the nitrogen gas flow in the fume cupboard to avoid any oxidation. Afterwards, the samples were put under vacuum (Selecta Vacuo-Term, 30 min, 70 cmHg, 35 ˚C). Rehydration was performed by adding 3 mL of distilled H_2O and vortexing of the samples for ≈ 30 seconds. Obtained liposomal suspension was then processed by freeze-thaw procedure (8 cycles, freezing T=-200 ˚C, heating T=50 ˚C). Each cycle consisted of following steps:

a) Freezeing of the sample tube in liquid nitrogen and removal
b) Gently heating of the sample in water bath at ≈ 50 ˚C until it is completely melted
c) Vortexing the sample for ≈ 15 seconds
d) Repetition of steps a-c

For further homogenization, sonication technique was appllied (5 cycles, 4 min/cycle). To prevent overheating each sample was cooled under the water after each sonication cycle.

Figure 25: Vacuum Freeze-Thaw: liquid nitrogen; hot bath; vortexing Sonication

Figure 26: S_2 D_2 P_2

3.6 Preparation of liposomes loaded with curcumin

Table 9: Composition of prepared samples with following technique

SC	lipids from *Cyanotece VI22* + curcumin
DC	lipids from *Cyanotece CCY0110* + curcumin
PC	lipids from *Rhodopseudomonas palustris* 42OL *+ curcumin*

The procedure of extraction of lipid fraction was as described above. Additionally, 1 mL of curcumin solution (4mg/mL in ethanol) was added after the cleaning step with 0.9% NaCl. After the addition of curcumin solution, the procedure described for the Preparation of plain liposomes from lipid fraction was applied.

3.6.1 Loading of curcumin in the presence of CTAB

Table 10: Composition of prepared samples with following technique

SC+	lipids from *Cyanotece VI22* + curcumin + CTAB
SC++	lipids from *Cyanotece VI22* + curcumin + 2xCTAB
DC+	lipids from *Cyanotece CCY0110* + curcumin + CTAB
DC++	lipids from *Cyanotece CCY0110* + curcumin+ 2xCTAB
PC+	lipids from *Rhodopseudomonas palustris* 42OL *+ curcumin* + CTAB
PC++	lipids from *Rhodopseudomonas palustris* 42OL *+ curcumin* + 2xCTAB

The procedures used were as described above. After the addition of curcumin solution , 0.5 ml CTAB solution (1mg/mL in ethanol) was added to to SC+, DC+, PC+. While 0.5 ml of CTAB solution (2mg/mL in ethanol) was added to SC++, DC++, PC++ samples.

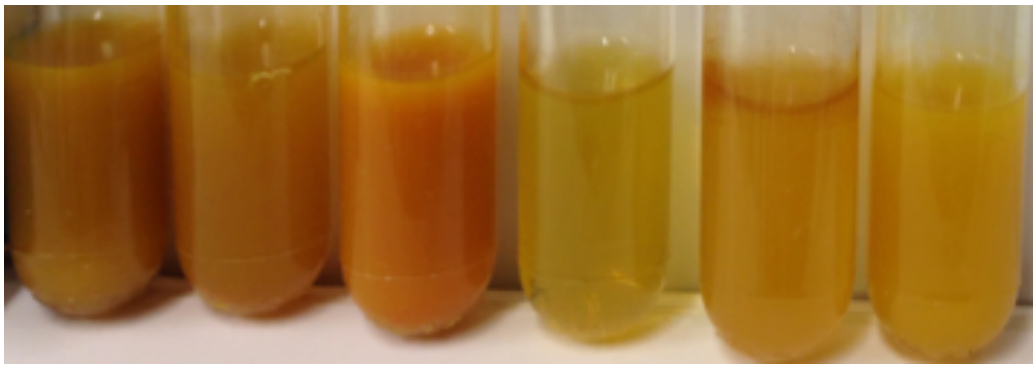


Figure 27:

DC DC+ SC SC+ PC PC+

Figure 28:

SC++ DC++ PC++

3.7 Preparation of liposomes loaded with antibiotics

Dimethyl sulfoxide (DMSO) solution was chosen as a solvent for ampicillin and penicillin G. DMSO is a poorly volatile solvent and thus presents a rate limiting step in the procedure since the samples have to be completely dry to obtain lipid film. Thus a dissolution test was performed. The aim of the test was to find the lowest amount of DMSO alone or combined with another solvent needed to fully dissolve antibiotics used in this work.

Table 11: Dissolution testing for antibiotics

Solute		Solvent		Results
Ampicillin	20 mg	CH_3OH	5 mL	Precipitation
Ampicillin	20 mg	2:1 $CHCl_3$:CH_3OH	5 mL	Precipitation
Ampicillin	20 mg	C_3H_7OH	5 mL	Aggregates
Ampicillin	20 mg	1:1 CH_3OH:C_3H_7OH	5 mL	Aggregates
Ampicillin	20 mg	DMSO	5 mL	Fully dissolved
Ampicillin	20 mg	$(CH_3)_2CO$	5 mL	Not fully dissolved
Ampicillin	20 mg	1:1 DMSO:$(CH_3)_2CO$	5 mL	Fully dissolved
Ampicillin	**20 mg**	**1/3:2/3 DMSO:$(CH_3)_2CO$**	**5 mL**	**Fully dissolved**
Amoxicillin	20 mg	1:1 DMSO:$(CH_3)_2CO$	5 mL	Fully dissolved
Penicillin G	20 mg	1:1 DMSO:$(CH_3)_2CO$	5 mL	Fully dissolved
Penicillin G	20 mg	1/4:3/4 DMSO:$(CH_3)_2CO$	5 mL	Not fully dissolved
Penicillin G	20 mg	1/5:4/5 DMSO:$(CH_3)_2CO$	5 mL	Not fully dissolved
Penicillin G	20 mg	2/5:3/5 DMSO:$(CH_3)_2CO$	5 mL	Fully dissolved
Penicillin G	**20 mg**	**1/3:2/3 DMSO:$(CH_3)_2CO$**	**5 mL**	**Fully dissolved**

The amount of DMSO part in the solvent solution corresponding to less than ≈ 33 % of total volume of the solution, showed that the solute was not fully dissolved indicating that either precipitate, crystal formation or cloudy suspension. Thus for both ampicillin and penicillin G the final solvent was composed of DMSO in range 33-40% and acetone in range 60-67% of total volume of the solution.

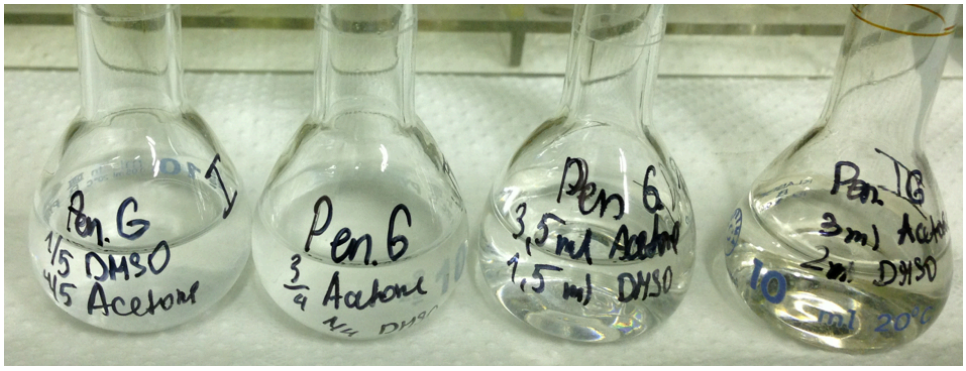


Figure 29: Dissolution testing of penicillin G

3.7.1 Ampicillin (A)

Table 12: Composition of prepared samples with following technique

S	lipids from *Cyanotece VI22*
S_A	lipids from *Cyanotece VI22* + ampicillin
D	lipids from *Cyanotece CCY0110*
D_A	lipids from *Cyanotece CCY0110* + ampicillin
P	lipids from *Rhodopseudomonas palustris* 42OL
P_A	lipids from *Rhodopseudomonas palustris* 42OL + ampicillin

To prepare ampicillin stock solution (4 mg/mL, ≈ 10^{-2} mol/L) ≈ 20 mg ampicillin dry powder was dissolved in a solution of ≈ 3 mL $(CH_3)_2SO$ (Dimethyl sulfoxide; DMSO) and ≈ 2 mL acetone to a final solution of 5 mL. The procedure described for the preparation of liposomes loaded with curcumin was slightly modified. For instance, after completing the washing step with 0.9% NaCl, the samples were placed in hot water bath (temperature) until app. one half of the solution evaporated. This was done to shorten the time of evaporation, because of DMSO's low melting point. Then the samples were cooled to ambient temperature for app. 5 minutes. Afterwards, the desired amount of newly prepared ampicillin solution (200 - 600 µL) was added to each of the samples. The samples were vortexed and dried under the fume cupboard. The same lipid film hydration method for liposome preparation was used as for the curcumin loaded liposomes. Additionally, after the preparation, all liposomal antibiotics were further purified with filtration by syringe filters (0.2 µm pore size) before proceeding with biological trials.

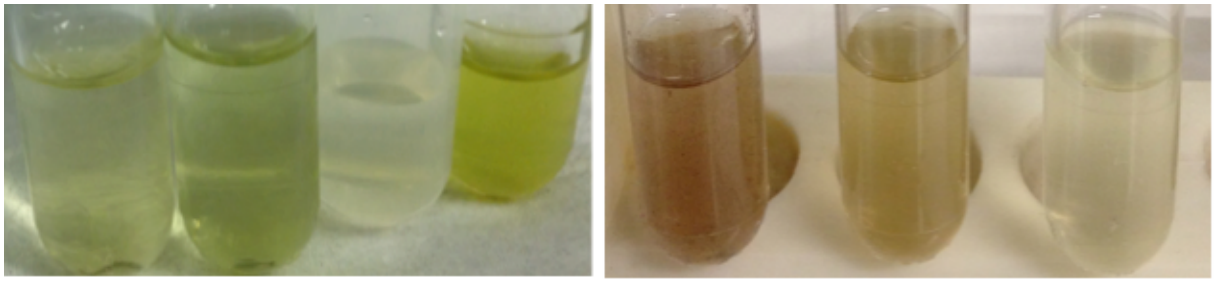


Figure 30: Trial 1 Trial 2

S S_A D D_A P_A S_A D_A

3.7.2 Penicillin G (PenG)

Table 13: Composition of prepared samples with following technique

S	lipids from *Cyanotece VI22*
S_{PG}	lipids from *Cyanotece VI22* + PenG
D	lipids from *Cyanotece CCY0110*
D_{PG}	lipids from *Cyanotece CCY0110* + PenG
P	lipids from *Rhodopseudomonas palustris* 42OL
P_{PG}	lipids from *Rhodopseudomonas palustris* 42OL + PenG

The desired amount of PenG stock solution (200 - 600 µL) was added after the plain lipid suspension was heated in water bath and cooled to room temperature to decrease the sample volume.

For preparation of liposomes loaded with PenG exactly the same procedure as for ampicillin loaded liposomes was used.

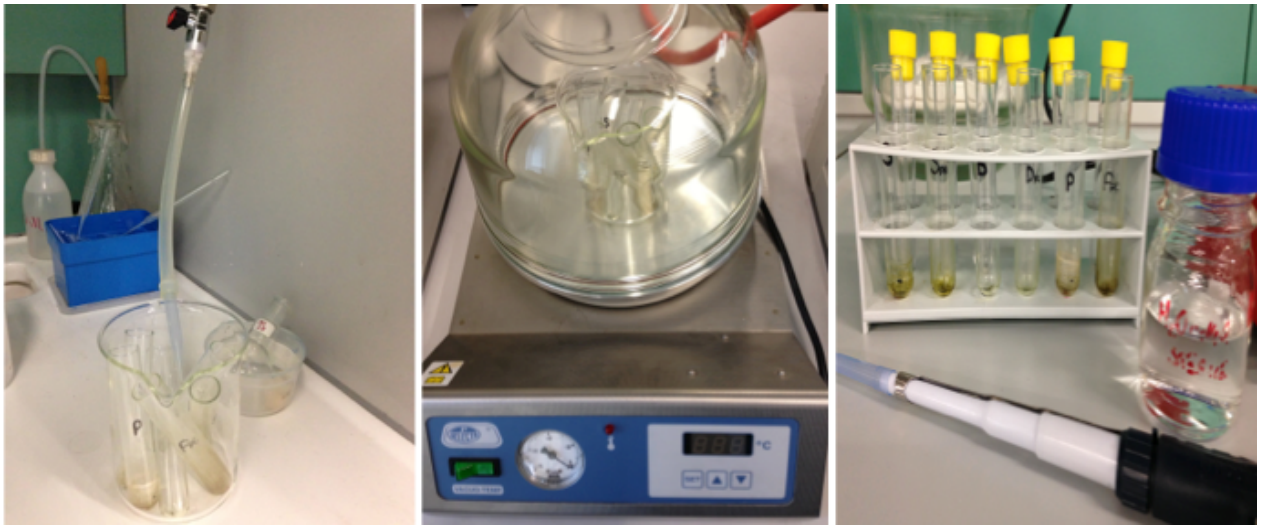


Figure 32: Rotary drying Vacuum oven Rehydration

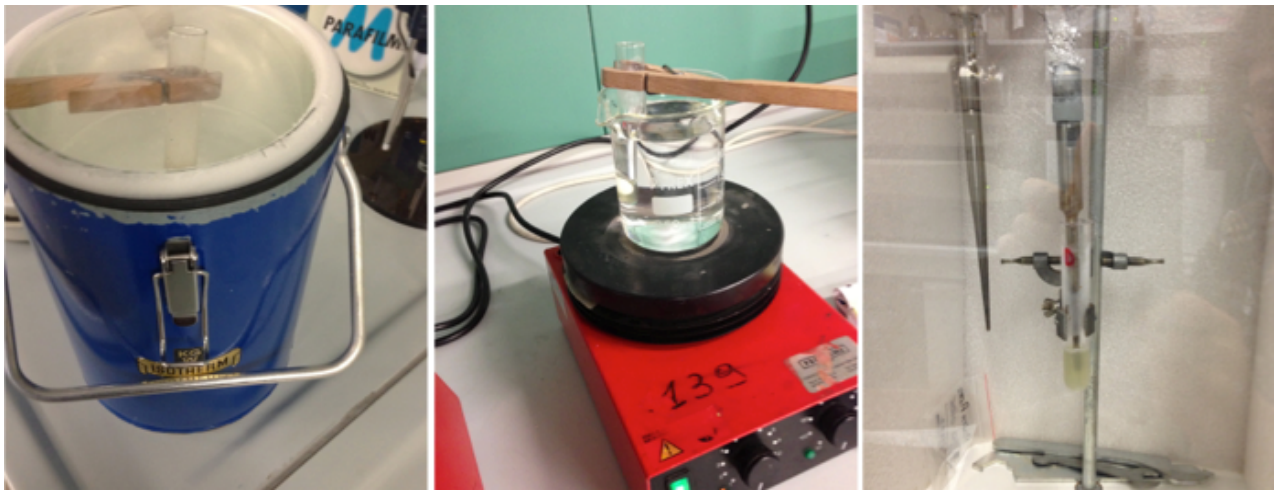
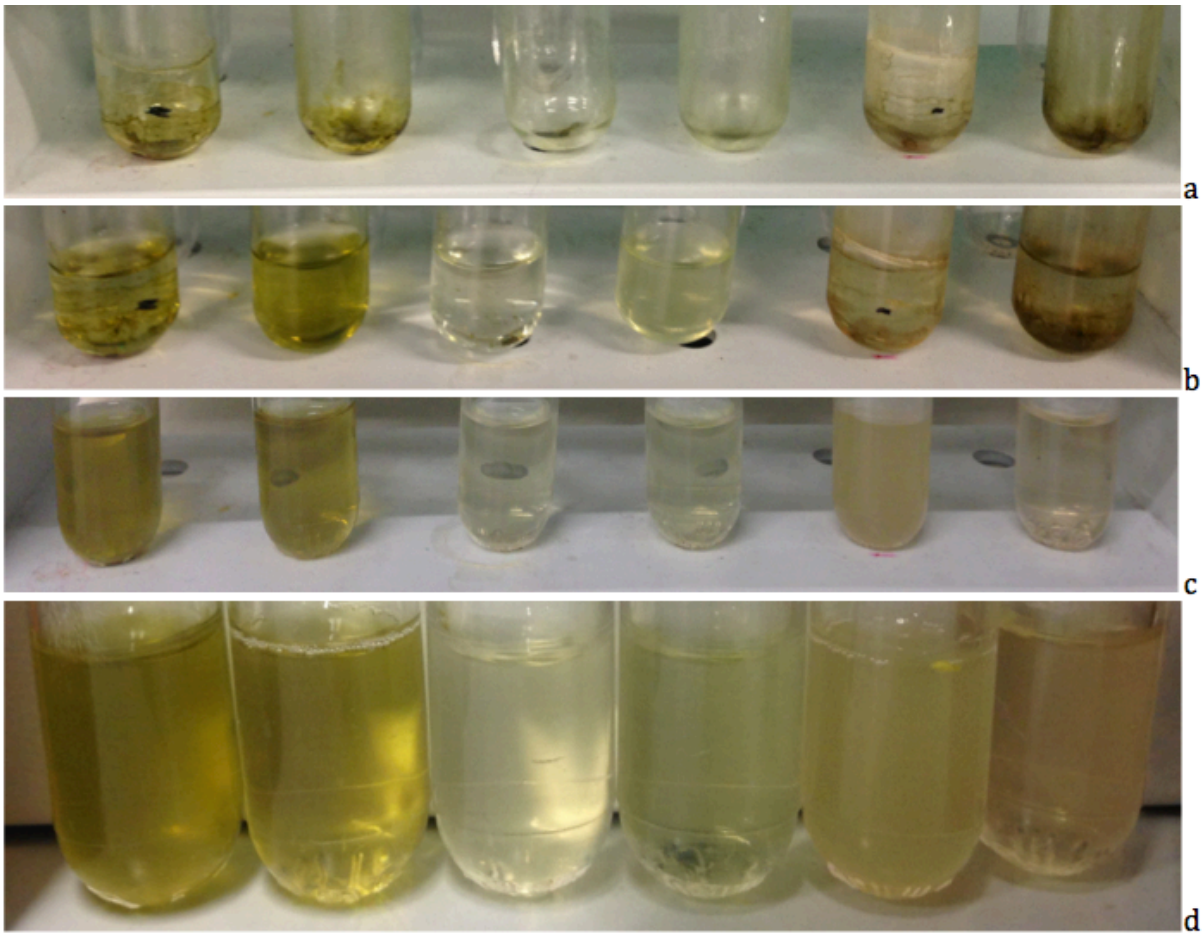


Figure 33: Freeze-thawing Sonication



S S_{PG} D D_{PG} P P_{PG}

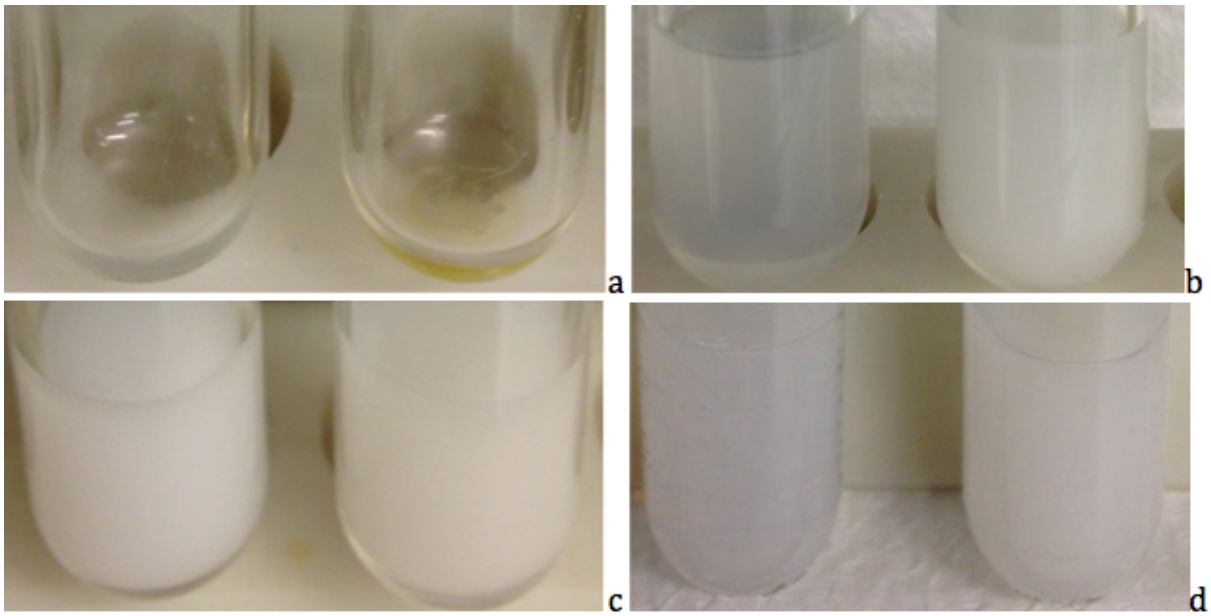
Figure 34: a) lipid dry film after vacuum
b) rehydration with distilled water
c) samples after freeze-thaw procedure
d) samples after sonication procedure

3.8 Preparation of plain conventional liposomes

Table 14: Composition of prepared samples with following technique

L_1	CHOL, DOPE, DOPC, DCCHOLHCL
L_2	Soybean lipids, CHOL

Stock solutions with lipids concentration of 40 mg/mL were made by dissolving the desired amount of lipids in (3:1, v/v) chloroform:methanol solution. L1 was prepared by mixing together 10 wt% DCCHOLHCL, 20 wt% CHOL, 30 wt% DOPE and 40 wt% DOPC. L2 was prepared by mixing together 20 wt% CHOL and 80 wt% soybean phospholipids (SPC). The samples were placed under vacuum (Selecta Vacuo-Term, 30 min, 70 cmHg, 35 °C) and rehydrated with 4 mL of distilled H_2O. Liposomal suspension was then subjected to freeze-thaw (8 cycles, frozen at ≈ -200 °C, heated at ≈ 50 °C). For further homogenization and vesicle size reduction, liposomal suspensions were processed with extrusion through polycarbonate membranes (Avestin polycarbonate membrane, 200 nm, 27 passages). This technique results in LUV and SUV types of liposomes. After the preparation, all samples were filtered with syringe filter with 0.2 μm pore size.



L_1 L_2 L_1 L_2

Figure 35: a) dry lipid film
b) after rehydration with sterile water
c) after freeze-thawing
d) after extrusion

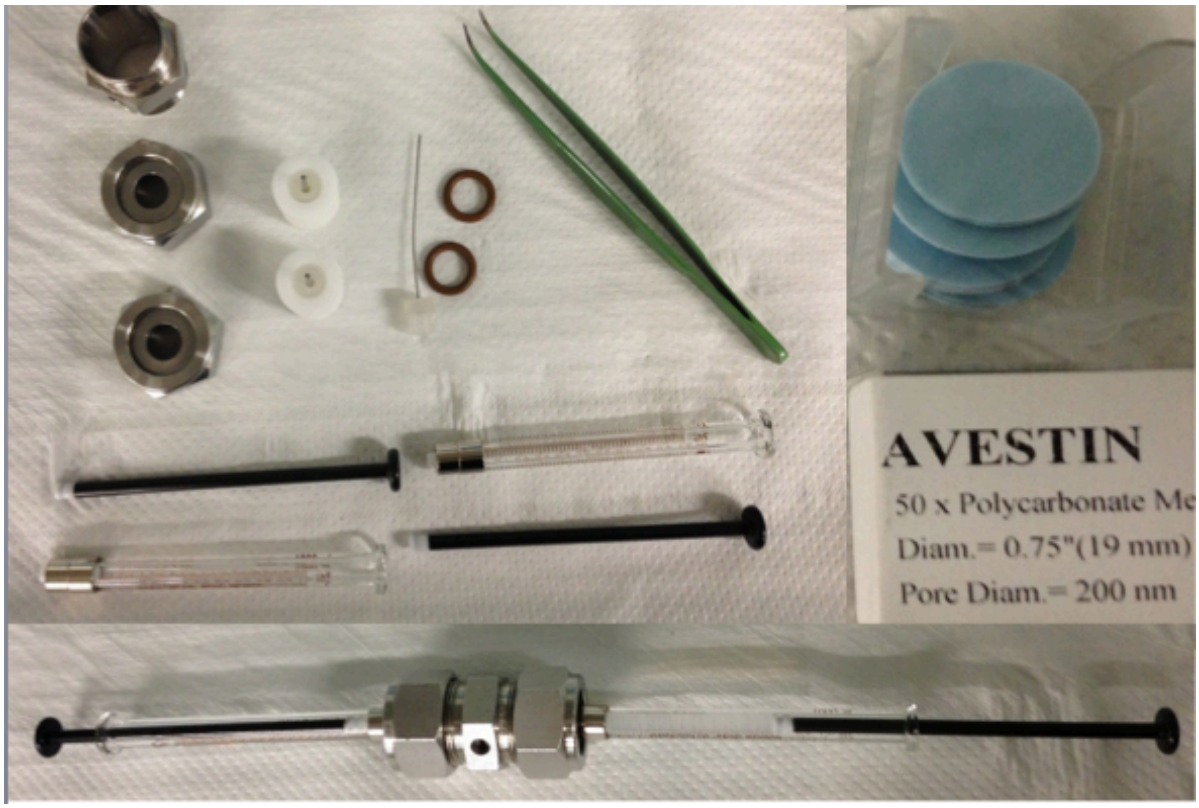


Figure 36: Lipid extruder

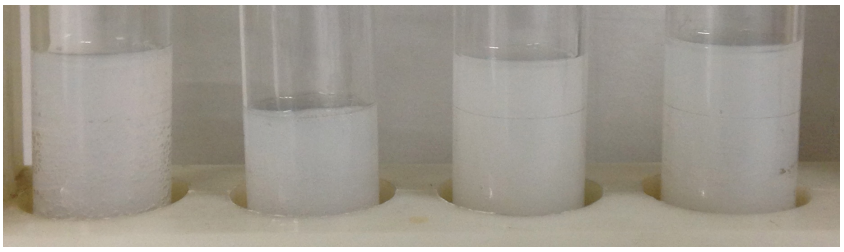
3.9 Preparation of conventional liposomes loaded with antibiotics

3.9.1 Penicillin G (PenG)

Table 15: Composition of prepared samples with following technique

L_1G	CHOL, DOPE, DOPC, DCCHOLHCL + PenG
L_2G	Soybean lipids, CHOL + PenG

We used PenG stock solution with concentration of 4mg/mL. Loading of conventional liposomes with PenG was carried out by the same technique as described in the Preparation of plain conventional liposome. The desired amount of PenG stock solution (800 µL) was added to 1 mL of lipid mixtures L1 and L2 right after the lipid mixture was prepared, thus obtaining a PenG concentration of 0.8 mg/mL.



L_1 L_1G L_2 L_2G

Figure 37: plain and penicillin G loaded conventional liposomes after freeze-thawing

3.9.2 Ampicillin (Amp)

Table 16: Composition of prepared samples with following technique

L_1A	CHOL, DOPE, DOPC, DCCHOLHCL + Amp
L_2A	Soybean lipids, CHOL + Amp

We used Amp stock solution with concentration of 4mg/mL. Loading of conventional liposomes with Amp was carried out by the same technique as described in the Preparation of plain conventional liposome. The desired amount of stock solution Amp (800 µL) was added to 1 mL of lipid mixtures L1 and L2 right after the lipid mixture was prepared.

L_1 L_1A L_2 L_2A

Figure 38: plain and ampicillin loaded conventional liposomes

3.9.3 Sulfanilamide (SFA)

Table 17: Composition of prepared samples with following technique

L_1S	CHOL, DOPE, DOPC, DCCHOLHCL + SFA
L_2S	Soybean lipids, CHOL + SFA

Following samples were prepared: L_1S, L_2S. Stock solution with molar concentration 10^{-2} mol/L was prepared by dissolving ≈ 8.6 mg sulfanilamide dry powder in (1:1, v/v) acetone:methanole solution to a final volume of 5 mL. Loading of conventional liposomes with SFA was carried out by the same technique as described in the preparation of plain conventional liposome. The desired amount of SFA (400 µL) stock solution was added to 1 mL of lipid mixtures L1 and L2 right after the lipid mixture was prepared.

3.9.4 Ethylaminobenzenesulfonamide (ESFA)

Table 18: Composition of prepared samples with following technique

L_1ES	CHOL, DOPE, DOPC, DCCHOLHCL + ESFA
L_2ES	Soybean lipids, CHOL + ESFA

Following samples were prepared: L_1ES, L_2ES. Stock solution with molar concentration 10^{-2} mol/L was prepared by dissolving ≈ 10 mg ESFA dry powder in (1:1, v/v) acetone:methanole solution to a final volume of 5 mL. Loading of conventional liposomes with ESFA was carried out by the same procedure as described in the preparation of plain conventional liposomes. The desired amount of ESFA (400 µL) stock solution was added to 1 mL of lipid mixtures L1 and L2 right after the lipid mixture was prepared.

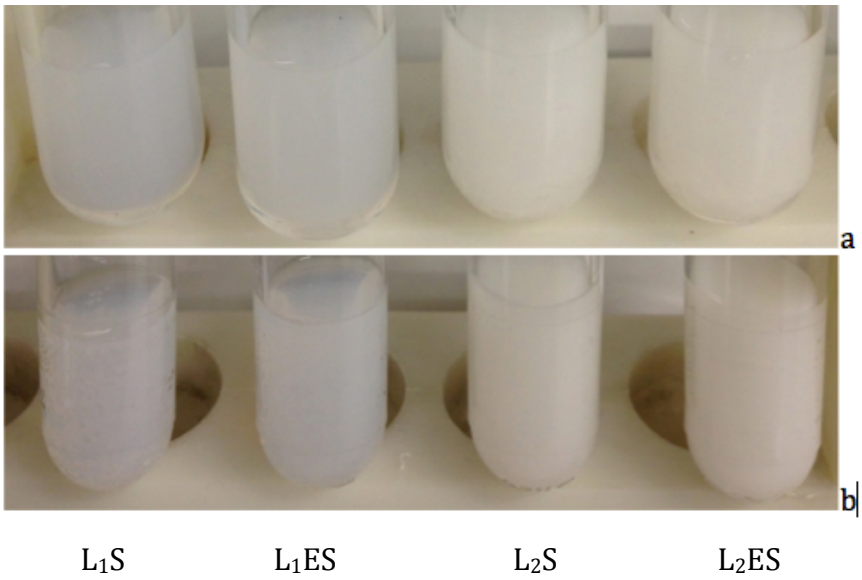


Figure 39: a)plain and penicillin G loaded conventional liposomes after rehydration
b)plain and penicillin G loaded conventional liposomes after freeze-thawing

3.10 Determination of total phosphorus content

Table 19: Composition of samples used according to following procedure

S_1, S_2	lipids from *Cyanotece VI22*
D_1, D_2	lipids from *Cyanotece CCY0110*
P_1, P_2.	lipids from *Rhodopseudomonas palustris* 42OL

Total phosphorus analysis experimental set up was as described in determination of total phosphorus protocol"(112,113) with a few modifications. Dry liposome samples were dissolved in 1:1 (v/v) of methanol:chloroform to a total volume of 25 mL. There have been made a standard curve (x=amount of phosphorus in mg; y=absorbance at 820 nm) by considering different volumes of a solution with a phosphorus concentration of 1mg/mL. To be able to calculate the amount of phosphorus present in our samples, we had to choose a volume o we had to define a reference value on the standard curve. It was decided to use a sample volume of 175 µL. Thus 175 µL of each sample was taken out and transferred into a screw cap glass tubes, and heated in water bath (100 ˚C, 5 min). Before further processing, the sample tubes were removed and cooled to ambient temperature. Further,450 µL H_2SO_4 was added to the bottom of each sample tube. Afterwards ,450 µL H_2SO_4 was added, and then the samples were gradually heated in vaselin oil (190°C, 20 min). Once again the sample tubes were removed and cooled to ambient temperature. Further, a volume of 150 µL H_2O_2 was added, samples were heated up in vaselin oil (190 °C, 30 min) and cooled for app. 5 minutes. Then 3.9 mL of distilled H_2O and 0.5 mL ammonium molybdate (VI) tetrahydrate solution was added. Finally, the sample tubes sealed by screw caps were heated in water bath (100 °C, 7 min).

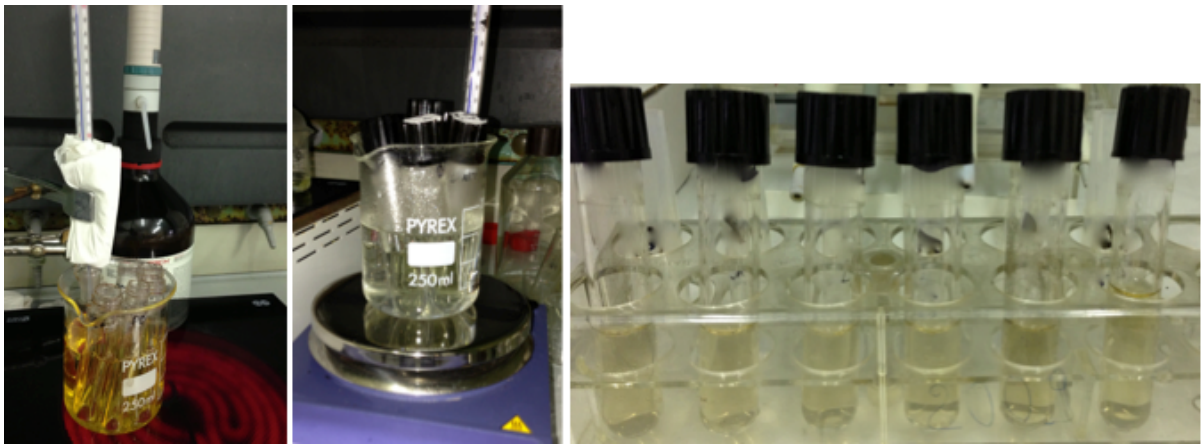


Figure 40: Heating in vaselin oil/ water bath; S S_A D D_A P P_A

The modified procedure for determining total phosphorus content was applied for samples in Table 20.

Table 20: Composition of samples used according to following procedure

SC	lipids from *Cyanotece VI22* + curcumin
SC+	lipids from *Cyanotece VI22* + curcumin + CTAB
DC	lipids from *Cyanotece CCY0110* + curcumin
DC+	lipids from *Cyanotece CCY0110* + curcumin+ CTAB
PC	lipids from *Rhodopseudomonas palustris* 42OL *+ curcumin*
PC+	lipids from *Rhodopseudomonas palustris* 42OL *+ curcumin* + CTAB
S	lipids from *Cyanotece VI22*
S_A	lipids from *Cyanotece VI22 + Amp*
D	lipids from *Cyanotece CCY0110*
D_A	lipids from *Cyanotece CCY0110 + Amp*
P	lipids from *Rhodopseudomonas palustris* 42OL
P_A	lipids from *Rhodopseudomonas palustris* 42OL + Amp

A volume of 100 µL was withdrawn from each sample and transferred in separate glass tubes. Then the samples were left overnight for drying at 30 °C. After drying, 750 µL chloroform:methanol (1:1, v/v) solution was added to the sample tubes. Afterwards, 175 µL of each sample solution was taken out and transferred into a screw cap glass tubes. From this point and further on the same steps were followed as described above.

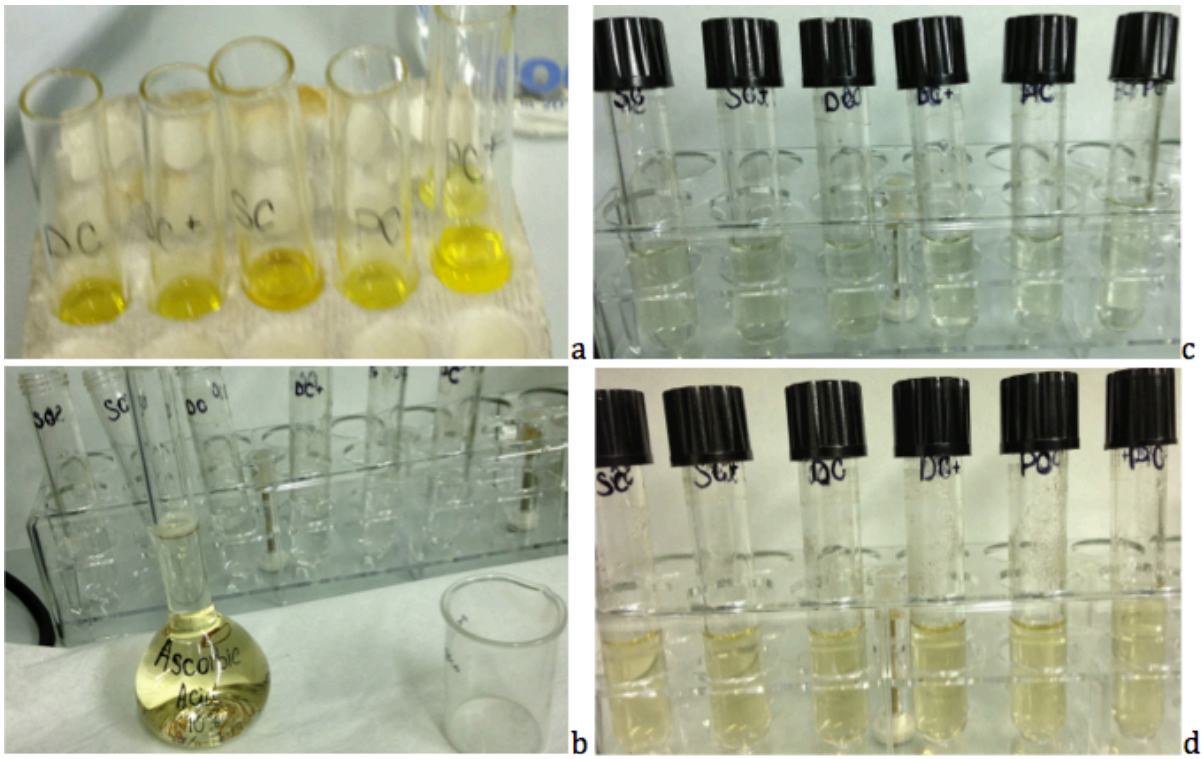


Figure 41: a) rehydration of dry overnight samples with chloroform:methanol solution
b) ascorbic acid stock solution used in the procedure
c) after addition of 450 µL H_2SO_4
d) ready samples for UV/VIS analyze

The determination of total phosphorus was carried out for following samples:
S_1, S_2, D_1, D_2, P_1, P_2 ; S, S_A, D, D_A, P, P_A ; SC, SC+, DC, DC+, PC, PC+
Absorbance at 820 nm was determined by the help of spectroscopic UV/VIS analyzer (Perkin Elmer Lambda Spectrophotometer). The conditions were as follows: range 750 - 1100 nm, speed 60 nm/sec, QS cuvettes, sample volume2000 µL).

3.11 Biological assays

In order to evaluate effect of liposomes loaded antibiotics on living cells, several sensitivity assays with bacterial microorganisms were carried out. There we tested both plain liposomes and liposomes containing either ampicillin or penicillin G. These were tested and compared against nonentrapped antibiotics. *Escherichia coli XL1* Blue strain (Tet^R) was used for sensitivity assay of both ampicillin and penicillin G loaded liposomes. Additionally, E. coli (XL1Blue /pUC 18 (Tet^R, Amp^R)) strain containing a plasmid carrying Amp resistance gene was tested. The resistance gene encodes a periplasmic β-lactamase, which causes the breakdown of ampicillin inside the periplasm of the Gram-negative bacterial cell. Furthermore the effect of ampicillin loaded liposomes was evaluated on *Bacillus subtilis* strain. Moreover, in the last part of this work, conventional liposomes loaded with ampicillin were tested against *E. coli* XL1Blue. Finally, the experiments involving penicillin G loaded liposomes to evaluate its bacteriocidal/bacteriostatic effect on E. coli were carried out.

E. coli strains were cultured in Luria Bertani (LB) broth, composed of 10g tryptone, 5g yeast extract and 10g NaCl per liter. Nutrient Broth (NB) media was used as inoculate for *Bacillus subtilis.* After growth under incubation at 37 °C, bacterial cells were collected in the exponential growth phase. Thereafter the inoculum was diluted in LB 2x until OD_{600nm} = 0.05, corresponding to a cell density of app. $2x10^6$ cfu/ml. This was done to increase the sensibility to antibiotics. Absorbance was recorded at 600 nm by BioPhotometer (Eppendorf), which was zeroed against LB. The scalar dilutions to be tested of antibiotic loaded liposomes and free antibiotic solution were made with sterile distilled water. Two fold serial dilutions of the additives were prepared and added to an equal volume of bacterial suspension in LB 2x in a final volume of 250 µL. Control for free antibiotic solution for comparison, positive (inoculum + H_2O) and negative (LB diluted + H_2O) controls were included as well. At least two independent experiments were performed in duplicate for each test. After distribution in microtiter plates, the absorbance at t_0 was recorded by Imunelle Spectrometer plate reader at 595 nm. Thereafter the cells were incubated at 37 °C, under continuous shaking at 100 rpm for 24 h. The absorbance was then recorded after incubation at t_{24}. By calculating ΔOD it

was possible to evaluate if bacterial cells were sensitive to encapsulated antibiotic and showed inhibited bacterial growth.

The minimum inhibitory concentration (MIC) values for the bacterial strain under study were determined by means of broth microdilution in microtiter plates. The lowest antibiotic concentration at which no increase in absorbance was observed, corresponding to complete growth inhibition, was taken as the MIC value. Growth control consisting of LB medium with no test compounds, LB medium with plain liposomes, at the same concentration at which they were present in the tests, and sterility control were included in the study.

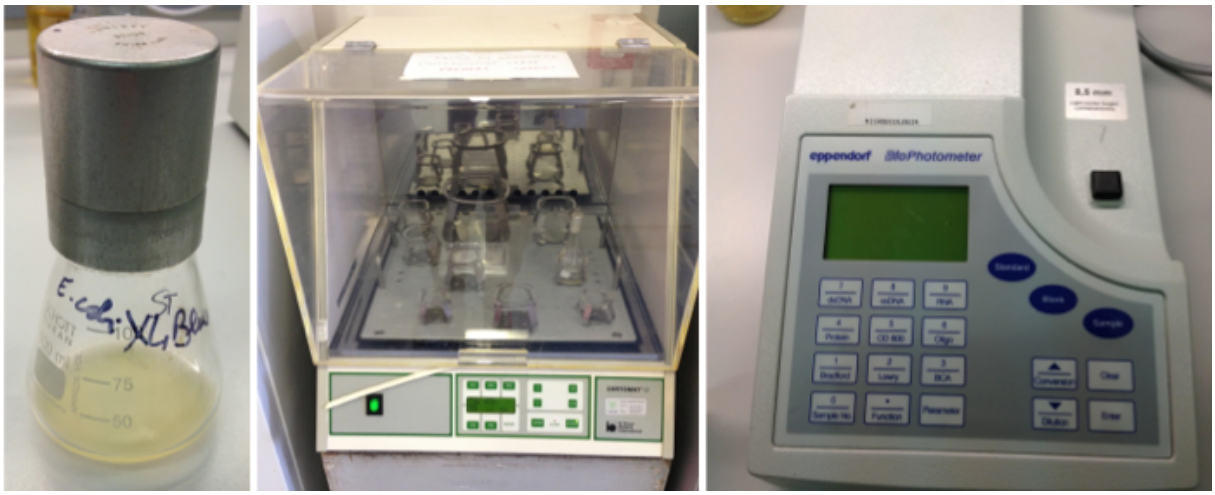


Figure 42: Inoculate of E. coli XL Blue; incubation; recording of optical density

Liposomal samples where positive inhibitory effect was observed were used for further analysis by a spead plate method to evaluate if their effect on bacterial cultures was bacteriocidal or bacteriostatic. First a viable title at t=0 had to be determined. This was done bypreparing the serial dilutions (10^{-2}, 10^{-3},10^{-4}) of physiological solution of *E. coli* in LB 2x (=inoculum). Later. these were used for agar plate culturing to determine colony forming units (CFU). Culturing was performed by taking a small volume of prepared bacterial suspension and distributing it evenly across the surface of an agar plate using a smooth sterilized spreader. Thereafter these plates were incubated for 24 hours at 37°C.

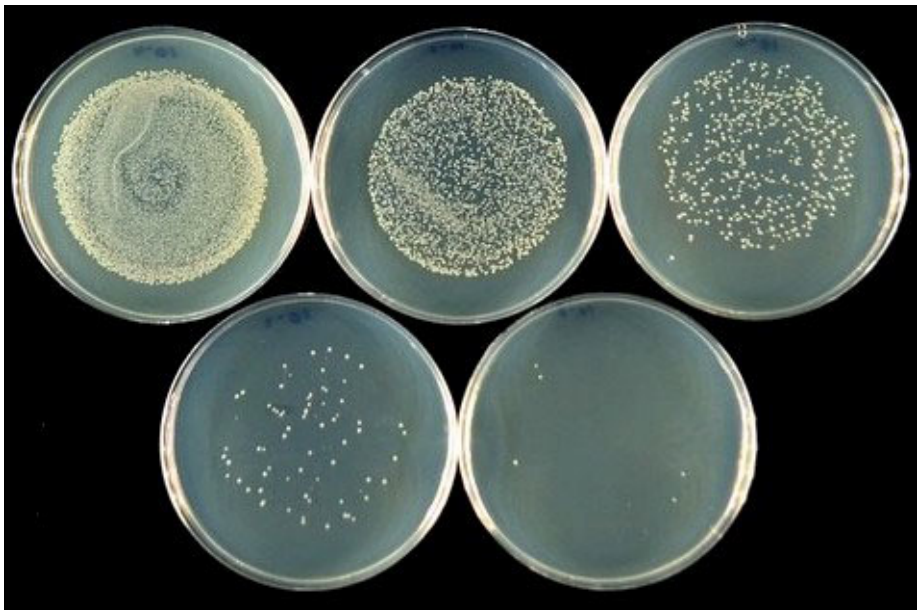


Figure 43:growth of E. coli used for counting CFU units (114)

Sterilized petri dishes were prepared by filling empty dishes with app. 15-20 mL of newly prepared LB 1x liquid medium. The thickness of the agar was roughly 4 mm. It took app. 15 minutes for them to solidify. Thereafter, they were placed in the heating oven for drying (50 °C,30 min). Prior to plating they were cooled to room temperature.

The analysis of bactericidal activity was done by extracting 100 µl of each sample taken from the microtiter plate, and plating directly on the petri dishes, followed by 24 h incubation at 37 °C. Afterwards, the bacterial colonies (CFU) were counted. The plates with CFU up to 300 could be used to calculate CFU/mL of the original sample.

3.12 Dynamic light scattering (DLS)

DLS is a technique used for evaluation of the mean size and size distribution of isolated nanosize particles in a solution. In this work DLS method was used to estimate the particle size of loaded liposome with various bioactive compounds compared to plain liposomes. DLS was performed on a Malvern Zetasizer Nano S instrument equipped with a 4.0 mW, He-Ne laser (λ = 633 nm) and operating in the backscattering modality with detection angle at 173° with respect to the incident light. Temperature was set at 25°C. The obtained atocorrelation functions were analyzed in term of monomodal or bimodal size distribution. instrument.

Before the analysis all samples were diluted with distilled water. We performed 3 analyses (11runs/analyze; 10sec/run) for each sample to ensure reproducibility.

3.13 Enzymatic assay

As mentioned earlier we have applied a stopped-flow method to evaluate ability of liposomal sulfanilamides to suppress carbonic anhydrase activity.

In this experiment, phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 Mm Hepes buffer (pH 7.4) and 20 mM $NaClO_4$ for maintaining constant ionic strength, following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s, at 20 °C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water and dilutions down to 0.01 nM were made thereafter with distilled-deionized water. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes were obtained from

Lineweaver–Burk plots and represent the mean from at least three different determinations. The results are presented in Tables: 32A-37A.

3.14 Mass Spectrometry (MS)

We have used MS, because UV/VIS spectroscopy was not a sensible enough method for the quantitative determination of antibiotic incorporated by our systems. All MS analyzes were performed with plain liposomes, liposomal antibiotics and corresponding free antibiotic solution as the reference. The obtained spectra were compared with a library of mass spectra for ampicillin an penicillin G.

The liposomes were disrupted in advance with methonol to free incorporated antibiotics and to make them less optically dense. In fact, optical density of tested substances can be challenging for MS analysis. In the case of bacteria-derived liposomes, this problem could not be overcome by dilution with water, therefore the dilution was made with methanol. Thus all the samples were diluted in MeOH 1:1 (v/v), sonicated (5 min) and centrifugated (10 min) in advance. Greater dilution with methanol was needed for samples containing conventional liposomes to overcome the initial turbidity of the samples. Prior to examination of our samples, an analysis of matrix effect was performed to evaluate if it introduced significant error in the mass detection. However it was concluded that in our case matrix effect was negligible.

The analysis was performed in positive ion mode with a full MS scan, and the expected spectra range was extracted. A full scan provided a full mass spectrum of analyzed compounds. In theory, full scan type provides more information about an analyte or a parent ion than does selected ion monitoring. However, it does not offer the same sensitivity.

3.15 Small-angle X-ray scattering

The fitting of SAXS intensity diagrams was performed with the Global Analysis Program (GAP), written by G. Pabst (115), which allows reproducing the scattering profiles of mono and multilamellar liposomes. By this procedure relevant structural properties of the membranes can be obtained. The distributions of electron density in the polar and apolar regions are also accessible.

The experimental SAXS diagrams were recorded at the high brilliance ID02 beamline of the ESRF (European Synchrotron Radiation Facility, Grenoble, France) (116). The wavelength and energy of the incoming beam were 0.995 Å and 12.46 keV, respectively. Temperature at the sample holder was 25 ± 0.1 °C. Sample to detector distance was 1 m, and the q-range covered was 0.0103-0.547 $Å^{-1}$, where q is the scattering vector modulus given by $q = (4\pi/\lambda) \sin\theta$, and 2θ is the scattering angle. Therefore, if $2\pi/q$ is taken as an estimation of the corresponding distances, the maximum length scale probed was ~ 600 Å. The samples were placed in 1.5 mm diameter glass capillaries and at least 3 curves were recorded at different positions along each capillary. The measured profiles were normalized to an absolute scale using a standard procedure reported in the litterature (117,118). The background scattering of the capillary filled with water was subtracted from the sample intensity and the resulting quantity was denoted *I(q).*

The Global Analysis Program (GAP) version 1.3 was provided by Dr Georg Pabst of the Austrian Academy of Sciences, Graz (115). This software models the SAXS pattern of bilayer-based structures by the following equation:

$$I(q) = \frac{(1 - N_{diff}) S(q) P(q) + N_{diff} P(q)}{q^2}$$

Equation 1

where N_{diff} is the fraction number of positionally uncorreraled bilayers (i.e. those forming non interacting vesicles), *S(q)* is the structure factor defining the spatial distribution of scatteres and describing the inter-particle interactions and *P(q)* is the

the absolute square of the bilayer form factor. The electron density is modelled by a thre-Gaussians profile (119,120), representing the polar head groups, placed at ± z_H, and the hydrocarbon core. The standard deviation of these electron distributions are σ_H *and* σ_c, respectively. The terminal methyl group in the bilayer center corresponds to the minimum of the electron density profile, as sketched in Figure 44. The amplitude (always negative) of the hydrophobic tails with respect to the headgroup is termed r_H.

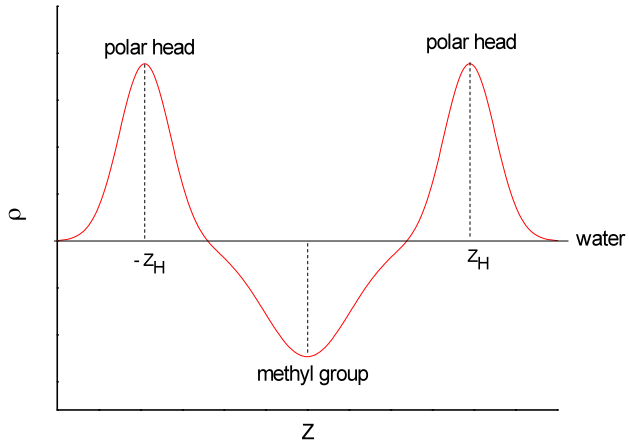


Figure 44: Electron density profile as a function of the distance from the bilayer center, modeled by the sum of three gaussian distributions.

3.16 Statistical evaluation

In this work we have considered new kind of liposomes prepared from lipids of various bacteria. Therefore our main focus was on optimizing the preparation rather than running a throughout statistical analysis. Each preparation step was adjusted over and over during the whole work period. Thus the statistical evidence of our results have still to be evaluated. Additionally, in the various parts of this work, we followed different criteria for the procedure. For example, the determination of phospholipid content was done in triplicate and the error was quite small (±1-2%). That was done before the initiation of this work. For the mass quantification, the testing of standards showed (±4%) deviation.

For DLS, the samples were interpreted by considering the autocorrelation curves in terms of bimodal size distributions. Thus we relied on cumulant analysis, and DLS results are presented as polydispersity indexes with zeta-averaged diameters.

4 RESULTS AND DISCUSSION

The method of liposome preparation used in this work is a top-down technique by reverse-phase evaporation. This method is based on the replacement of organic solvent by an aqueous media, and it is one of most commonly applied methods of liposome preparation.(121) The lipid hydration method consists of dissolving the lipids in suitable organic solvent, chloroform: methanol mixture, as we used in this work. The solvent was then removed under the drying and reduced pressure until a thin film was formed. Afterwards the lipid film was hydrated in an aqueous medium, above the phase-transition temperature, resulting in the formation of MLV liposomes. Although this is the simplest method of liposome vesicle formation on laboratory scale, there has been reports on low entrapment efficiency for entrapment of hydrophilic molecules.(122,123) Therefore, the method used for liposome preparation should be modified to improve the entrapment but at the same time remain simple, and possibly applicable for a large-scale production. Besides that, liposome carrier systems should retain the property of easy size regulation to remain within 100-200 nm for drug delivery applications. Furthermore, it is desirable to have the possibility of loading liposomes with flexible amounts of bioactive compounds to obtain therapeutic doses.

4.1 Characterization of liposomes

4.1.1 UV/VIS Spectrometry

Spectroscopy as a method is particularly useful in determining the percentage of phospholipids present in the samples.

Table 21 : Phospholipid Standard Curve

x= amount of phosphorus(mg), y=absorbance at 820 nm

Volume of P standard solution (µl)	Amount of P (mg)	A (at 820 nm)
350	7.053	1.0579
250	5.038	0.7408
175	3.526	0.5259
100	2.015	0.3064
50	1.008	0.1872

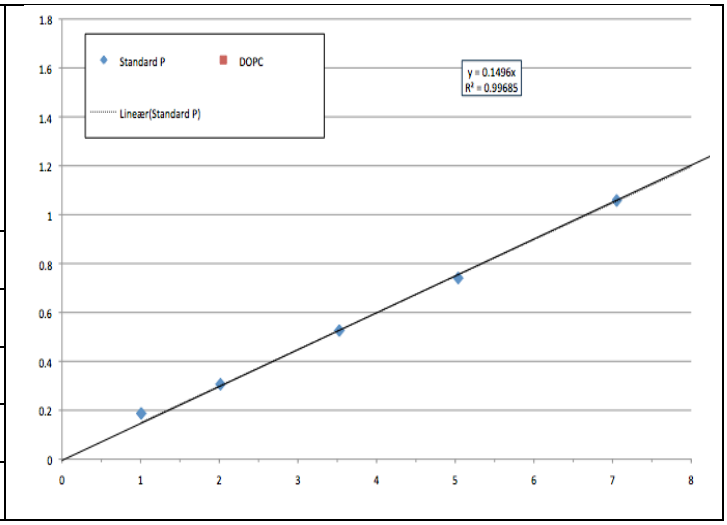


Table 22: Phospholipid content in plain liposomes and liposome containing curcumin

Sample	Absorbance (at 820 nm)	Phospholipids (%)
S_1	0.3183	55.57
S_2	0.3578	61.62
D_1	0.3914	67.49
D_2	0.3096	53.38
P_1	0.3514	60.59
P_2	0.3662	63.14
SC	0.267	61.03
SC+	0.2732	62.84
DC	0.2785	64.39
DC+	0.267	61.03
PC	0.2708	62.14
PC+	0.2784	64.36
S	0.2768	63.89
S_A	0.2687	61.52
D	0.2863	66.66
D_A	0.2817	65.32
P	0.2722	62.55
P_A	0.2611	59.31

Table 22 show that the amount of phospholipids in samples S, D and P is around 60 %. Since we used 50mg ± 0.03 of dry bacteria for each sample preparation, the phospholipid content in each sample is roughly 30 mg, which corresponds to a lipid concentration of 10 mg/mL.

The amount of total phosphorus detected in the samples was in a line with those reported in the literature.(124) About 60% of phospholipids were found to be present in the membranes of some cyanobacteria. Palmitic and linoleic acid are among the most abundant fatty acids in most cyanothece strains.(125) However, it is important to remember that there are many different strains of bacteria, which can contain various types of lipids in different concentrations in their membranes. Therefore, the precise amount of phospholipids in dry bacterial matter, that we have been using, is hard to define even in the literature.

In addition it was attempted to use UV/VIS spectrometry for quantification of the amount ampicillin present in the samples. However, the graphs presented in Table 4A are scattered and hard to interpret, possible due to lipid aggregates in the solutions. In addition, P samples exhibited particular behavior as compared to other samples, maybe due to the fact that NaChol had induced the formation of mixed aggregates, micelles or small liposomes, which scattered the light at high frequency. Thus this technique showed to be unsuitable for analyze of our samples.

4.1.2 Mass Spectrometry (MS)

The MS spectra obtained for S_A, D_A and P_A liposomes followed typical pattern for ampicillin as the reference sample and the spectra pattern described in the library database. Table 5A-6A. The base peak for ampicillin is expected at m/z 350.1 (characteristic positive ion). The results indicated that the ampicillin was present in the samples in significant amount. For further quantification and determination of amount of antibiotics incorporated by liposomes, additional analyses with reference samples containing various ampicillin concentrations have been run. At 350 and 160 m/z we found a possible interference with the peaks of the base choline, present in many polar head of phospholipids. Since peak at 174 m/z had less interference, it was decided to use it for further analyze and calculations.

MS analysis was also run for conventional liposomes containing ampicillin and penicillin G. These samples initially represented a challenge because of their turbidity and slightly precipitation of L2 samples, causing unpredictable interferences within the system. The samples were filtered through 0.2 μm syringe filters and diluted 1:10 (v/v) with methanol to combat this problem. After several trials, it was possible to determine the amount of penicillin G present in the samples, however the amount of ampicillin measured by MS was 2-3 orders of magnitude higher than the theoretical value. Thus another protocol has to be developed and applied to characterize conventional liposomes loaded with ampicillin.

Table 23: Calculated amount of antibiotics present in the liposome samples by MS

Sample	Calculated amount (ppm)	Initial amount (ppm)	Entrapment efficiency (%)
First trial			
S	-	M_A = 1.025 x 10^{-2} mol/L C_A = 232 ppm	34
S_A	79.194		
D	-		51.8
D_A	120.128		
P	-	M_A = 0.996 x 10^{-2} mol/L C_A = 238.7 ppm	50.9
P_A	121.521		
Second trial			
S	-	M_A = 1.007 x 10^{-2} mol/L C_A = 705.3 ppm	56.6
S_A	399.4		
D	-		56.3
D_A	397.3		
P	-		47.7
P_A	336.5		
S	-	M_{PG} = 1.238 x 10^{-2} mol/L C_{PG} = 828 ppm	3.5
S_{PG}	29.356		
D	-		32.8
D_{PG}	271.586		
P	-		2
P_{PG}	16.634		
L1	-	M_{PG} = 1.224 x 10^{-2} mol/L C_{PG} = 818.4 ppm	21.5
$L1_{PG}$	175.99		
L2	-		27
$L2_{PG}$	220.86		
L1	Not analyzed	M_{PG} = 1.009 x 10^{-2} mol/L C_{PG} = 706 ppm	Not analyzed
$L1_A$			
L2			
$L2_A$			

Table 23 shows the quantification of ampicillin and penicillin G detected in liposome samples. In respect to the entrapment for ampicillin in D and P liposomes it appears that P and D liposomes are able to entrap similar amounts of ampicillin (50 %), slightly higher as compared to S liposomes. (30 %). However, with an increased amount of ampicillin added to initial lipid solution, from app. 200 to 700 ppm, the entrapment efficiency increased for almost 20%. It would be also interesting to conduct MS analysis of conventional liposomes incorporating ampicillin. Regarding the penicillin G entrapment the percentage was much lower as compared to entrapment of ampicillin, especially in liposomes P. Samples with conventional liposomes L1 and L2 showed greater potential for penicillin G entrapment compared to S and P liposomes. Liposomes D showed greatest entrapment efficiency for penicillin G, slightly over 30%. This could

be due to the lipid membrane properties of Cyanothece CCY0110. However, more extensive research needs to be done regarding the composition of membrane lipids from current bacteria we were using, to gain a deeper understanding on the composition of our liposomes, and their interaction with incorporating materials. The further procedure steps involved in the preparation of loaded liposomes need to be discussed and possibly modified to increase the loading capacity of the drugs within liposomes.

4.1.3 Dynamic Light Scattering (DLS)

Dynamic Light Scattering is a technique used for evaluation of the mean size and size distribution of isolated nanosize particles in a solution. In this work DLS method was used to estimate the particle size of loaded liposome with various bioactive compounds and compare those to plain liposomes. DLS was performed on a Malvern Zetasizer Nano S instrument. All samples were diluted 1:40 v/v with distilled water before the analysis. The results are discussed below and the obtained final data for plain and liposomal antibiotics are reported in Tables 8A-12A.

DLS modelises all of the particles in a solution as spherical and allows to measure the their corresponding spherical diameter. In order to get more detailed information on the liposome properties, such as lamellarity and bilayer thickness, a detailed investigation by Small Angle X-ray scattering was initiated and is currently in progress. (Table 28A)

According to the results presented below the liposome vesicles containing bacterial lipids had an average size distribution between 100-300 nm, making them appropriate for use as drug carriers.

4.1.3.1 Curcumin loaded bacterial liposomes

Particles in samples S and D decreased in size when curcumin was incorporated, but an increase was observed after the addition of CTAB. The addition of both curcumin and CTAB induced an increase of particle size in sample P, while regarding S and D samples, the size decrease by addition of curcumin, and increases again after addition of CTAB. The increase of size after addition of CTAB can indicate that CTAB molecules are succesfully inserted in the lipid bilayer of liposomes. Differences in the behaviour of S,D and P samples after curcumin insertion, could suggest that liposomes of lipids from cyanobacteria get more compact when curcumin is added, while liposomes of type P are originally larger. These differences could also be dependent on the amount of curcumin incorporated in the liposomes, and should be investigated further. (Table 24)

In addition, we have been investigating the possibility of using Cetyltrimethylammonium bromide (CTAB) as adjuvant in the process of curcumin incorporation in liposomes. In fact, it has been reported that CTAB is able to form a complex with curcumin, whose stability and spectral properties have been investigated by absorption and fluorescence spectroscopy.(126) The CTAB concentration we used in the samples was under the CMC (1mM at 25 °C) in all final solutions. This means that CTAB did not form micelles and its only effect was due to insertion in the phospholipid bilayers.

Table 24: Particle size of liposomes containing curcumin

Type	Peak 1	Peak 2	Z-Average(nm)
S	58	234	75
SC	44	219	57
SC^{+}	108	-	88
SC^{++}	188	4937	152
D	342	98	168
DC	154	38	74
DC^{+}	121	-	111
DC^{++}	180	31	97
P	143	-	116
PC	174	-	135
PC^{+}	176	-	110
PC^{++}	210	44	144

+ = CTAB (4.58 x 10-4 mol/L)
++ = CTAB (9.14 x 10-4 mol/L)

In series of S liposomes the most striking effect on the size distribution was contributed to CTAB, which is able to induce the formation of a remarkably monodisperse liposomes, whose mean diameter was 100 nm and 200 nm for CTAB 4.58 x 10-4 M (+) and 9.14 x 10-4 M (++), respectively. These size values are well within in the optimal range for drug carriers, which in most cases is not higher than 200nm. (127) (Table 9A)

The DLS results obtained for CCY110 bacteria (D) were similar to those of VI22 bacteria (S), in agreement with the common *Cyanothece* strains. The main difference resides in the fact that when CTAB is present at higher concentration (i.e. 9.14 x 10-4 M) an aggregate population of very small particles (25-30 nm) is detected, which indicates that micelles were coexisting with liposomes. It is possible that in this case the CTAB amount was too high to be incorporated in the liposomes bilayer, thus inducing a sort of micro-phase separation for the lipid/surfactant system under study.

The filtered P sample contained a single population of liposomes, which was very little affected by curcumin association with the phospholipid bilayer. (Table 9A) Addition of CTAB induced the formation of a second liposome population with smaller size or the above described micro-phase separation, depending on the CTAB concentration. (Table 9A)

4.1.3.2 Ampicillin loaded bacterial liposomes

Results below (Table 25) indicate different behavior of S, D and P samples. For instance particles in the sample P were apparently smaller in size after the addition of ampicillin, unlike S and D samples. This characteristic behavior observed in P sample can be promising for further *in vitro* testing. However, results indicate large aggregates and broad size distribution in sample S and D upon addion of ampicillin. (Table 8A)

Table 25: Size distributions of plain liposomes and liposomes containing ampicillin

Type	Peak 1	Peak 2	Z-Average(nm)
S	305	55	197
S_A	237	4276	176
D	74	440	127
D_A	319	4635	239
P	928	186	433
P_A	602	116	726

In the case of cyanobacteria, the antibiotics presence affected the size distribution, while decreasing the overall liposome dimension. On the other hand, only the latter of these effects was observed for liposomes prepared from membranes of sulfur bacteria. It is probable that in all preparations a bi-modal size distribution corresponds to unilamellar liposomes coexisting with oligolamellar liposomes.

4.1.3.3 Penicillin G loaded bacterial liposomes

Table 26: Size of plain and antibiotic loaded liposomes

Liposome type	Antibiotics	Mean diameter[a] distrib1 (nm)	weight distrib 1	Mean diameter distrib2 (nm)	weight distrib 1	PDI
CCY-lipo	-	440±30	45 %	75±20	55%	0.61
CCY-lipo	Amp	320±20	100 %	-	-	0.38
CCY-lipo	PenG	400±20	100%	-	-	0.38
VI22-lipo	-	300±40	70 %	60±15	30%	0.69
VI22-lipo	Amp	230±20	100 %	-	-	0.38
VI22-lipo	PenG	750±50	55%	85±15	45%	0.48
42OL-lipo	-	900±50	72%	185±20	28%	0.55
42OL-lipo	Amp	200±50	84 %	70±10	16%	0.48
42OL-lipo	PenG	700±50	70%	80±15	30%	0.49

Plain liposomes showed two size distributions, suggesting that large multilamellar structures and smaller monolamellar liposomes were formed by the lipid mixtures extracted from the chosen bacterial strains of both cyanobecteria and purple non-sulfur bacteria. The high polydispersity index (PDI) confirmed that heterogeneous liposome populations were present in these samples. The mean diameter values are dependent on the spontaneous curvature exhibited by lipid bilayers. The diameter increases after addition of both penicillin G and ampicillin in the following order VI22-lipo (S) > CCY-lipo (D) > 42OL-lipo (P).

The presence of ampicillin induced more uniform size distributions in all samples. Indeed, PDI became invariably lower. Additionally, the size distribution became mono-modal for liposomes obtained from cyanobacteria membranes. While in the case of 42OL liposomes the addition of ampicillin did not change the size distribution, which was still comprised of a population of both large and smaller aggregates. The size of aggregates became somewhat smaller, and the weight of the aggregates with the size around 200 nm increased from 28 to 84%. In contrary, after addition of PenG, the mean size of the scattering objects remained high and the population of these was still considerably polydisperse.

4.1.3.4 Sulfanilamide loaded conventional liposomes

Samples of plain conventional liposomes of type L1 and liposomes containing sulfanilamide showed mono-modal size distributions with a mean diameter in a range 100-200 nm. (Table 10A) On the contrary, L2 liposomes, containing soybean lipids, were much greater in size. (Table 11A) The mean diameter of particles in the plain liposome sample and sample containing ethylaminobenzenesulfonamide was somewhat similar, around 400 nm. (Table 27) The L2 liposomes with sulfanilamide showed bi-modal size distributions. This behavior was not as expected since all samples were extruded through filters of 200 nm pore size. The reason for that could be the fact that L2 sample series contained visible precipitation even after the extrusion, indiacting that larger lipid aggregates could be formed. Another factor that could be influencing this particular increase in size of L2 series is the actual extrusion process, as there might be a small chance that the filters could tear during the procedure.

Table 27: Size distributions of conventional liposomes and liposomes containing ethylaminobenzenesulfonamide (ES) and sulfanilamide (SA)

Type	Peak 1	Z-Average (nm)
L1	169	160
L1ES	166	156
L1SA	166	158
L2	430	569
L2ES	431	478
L2SA	913	812

4.1.3.5 Penicillin G loaded conventional liposomes

Samples of plain conventional liposomes of type L1 and liposomes containing penicillin G showed mono-modal distribution with a mean diameter in a range 100-200 nm. (Table 28) This was expected since liposomes were extruded with the polycarbonate filters of 200 nm pore size. The same was also true for L2 samples. However, L2G samples exhibited bi-modal distribution, indicating a possible coexistence of both small liposomes and larger aggregates.

Table 28: Size distributions of conventional liposomes and liposomes containing penicillin G

Type	Peak 1	Peak 2	Z-Average(nm)
L1	170	-	162
L1G	175	-	161
L2	182	-	159
L2G	162	5166	162

4.1.4. Biological assays with antibiotic-loaded liposomes

Gram-negative bacteria, such as *E. coli,* are generally more resistant to a large variety of antibiotics, β-lactams included, in comparison to Gram-positive bacteria. One of the reasons is the presence of a broad-specificity drug-efflux pump, and the outer membrane (OM), which provides an effective barrier for antimicrobial agents by restricting influx.(128) Thus OM constitutes a first-line defense against antimicrobial challenge, unlike Gram-positive organisms, which lack OM and hence lack the front-line defense.(129) Porins found in the outer membrane provide a path through the OM for small hydrophilic substances, for instance β-lactam antibiotics.(130) Ampicillin and PenG diffuse across the OM of *E. coli* through the OmpF channel. In this work we postulated that liposomes made from an organic source, in this case bacterial membranes, could improve the influx of penicillin through the OM of *E. coli.* With this aim, we compared the activity of ampicillin and penicillin G loaded liposomes to those of free antibiotics.

The β-lactam antibiotics efficiently inhibit the bacterial transpeptidases in the periplasmic space, which results in weakly cross-linked peptidoglycan, and makes the growing bacteria highly susceptible to cell lysis and death.(131) Thus, we proposed that bacterial liposomes could additionally protect the antibiotic from degradation by β-lactamase in the periplasmic space. To confirm this, we tested liposomal Amp and PenG against *E. coli* XL1Blue (pUC18).

All samples tested in biological assays were filtered through syringe filters with 0.2 μm pore diameter. Liposomes made from the following bacteria were used; D (*Cyanotece CCY0110)* P (*Rhodopseudomonas palustris 42OL*) and S (*Cyanotece VI22*). Bacterial strains used to test the liposomal antibiotics included *E. coli XL1Blue, E. coli XLBlue/pUC18* and *Bacullus subtilis 168.*

It has been reported earlier that cyanobacteria and purple non-sulphur bacteria can exhibit antibacterial and antifungal activity towards microbial organisms, including *E. coli.* (132,133) However, the results obtained in all biological assays that we performed, did not indicate any antimicrobial activity of plain liposomes.(Tables 13A, 23A) This could be due to either too low lipid concentration of particular lipids in our liposomal systems, or the fact that the effective lipid part was not present in liposomes.

4.1.4.1 Ampicillin loaded bacterial liposomes

Several assays with liposomal ampicillin in various concentrations were carried out to compare their effect to free ampicillin solutions. The results from one of the first assays are presented in Tables 13A, 14A. We can observe that D_A samples (*Cyanotece CCY0110* + ampicillin) in concentration up to 58 μg/mL, did not show growth inhibition of neither *E. coli XL1Blue* nor *E. coli XLBlue/pUC18*, having resistance gene to ampicillin. Neither did free ampicillin inhibit the growth of ampicillin resistance strain of *E. coli.* However, MIC of free ampicillin against not resistant strain was ≤ 7 μg/mL. The D_A samples (explain what they are) were tested using a spread plating method, which showed a growth of unknown microorganism. Thus the poor effect of D_A against non-resistant strain of *E. coli* could be explained by either too low ampicillin concentration or the fact that the samples were contaminated with unknown microorganism. Thereafter we used liposomes containing higher ampicillin concentration. The results in Table 15A show that free and liposomal ampicillin in samples D_A (*Cyanotece CCY0110* + Amp*)* P_A (*Rhodopseudomonas palustris 42OL* + Amp) S_A (*Cyanotece VI22* + Amp) in the concentration of approx. 200 μg/mL did not inhibit the growth of the bacterial strain that has been used in the testing. That was rather as expected as the used strain of *E. coli XL Blue* (pUC 18) contains a plasmid with ampicillin resistance gene.
In conclusion, liposomes can help antibiotics to enter the bacterial cell through periplasmatic membrane of the cell wall, and allow them to exert their effect. However, liposomes do not protect antibiotic from degradation through the enzymatic cleavage inside periplasmatic space in bacterial cell.

Samples of liposomes loaded with ampicillin and tested against *E. coli XL Blue* were found to be as active as free ampicillin exhibiting a MIC value of 14.5 μg/mL for all liposome preparations and free ampicillin (data not shown).

In later experiments, the samples D_A, S_A and P_A were tested against *Bacillus subtilis 168* strains incubated in nutrient broth (NB) medium. The results are summarized in Tables 16A, 18A. *Bacillus subtilis* is a Gram-positive bacterium sensitive to ampicillin. Free ampicillin showed a MIC of 200 μg/mL, which is much higher compared to liposomal ampicillin in all samples, which had a MIC of 58 μg/mL. (Table 29)

Table 29: MIC values obtained from sensitivity assay for D_A, S_A ,P_A and free Amp against B. subtilis 168, based on Table 18A

Samples	S_A	P_A	D_A	Amp
MIC(μg/mL)	58	58	58	200

4.1.4.2 Penicillin G (PenG) loaded bacterial liposomes

We carried out several trials with *E. coli XL Blue* and liposomal PenG using liposomes extracted from S, D and P bacteria. The trials are summed up in Tables: 20A-25A. Summarized, the results indicate that D samples loaded with PenG had a MIC around 29 μg/mL, while S and P containing samples had clearly lower MIC of 3.6 and 1.8 μg/mL, respectively. In comparison, free PenG had a MIC > 29 μg/mL. Thus PenG loaded in our liposomal systems, especially the P samples, were more effective against *E. coli* XL Blue than free PenG and PenG mixed with plain liposomes. (Table 30)

Table 30: MIC values obtained from sensitivity assay for D_{PG}, S_{PG}, P_{PG} and free PenG against E. coli XL Blue (Tet^R), based on Table 22A

Samples	D_{PG}	S_{PG}	P_{PG}	PenG
MIC(μg/mL)	29	3.6	1.8	>29

Additional analysis showed that PenG loaded in D, P and S liposomes did not inhibit bacterial growth of *E. coli XL Blue (pUC18)* compared to PenG. Thus both AMP and PenG-loaded liposomes tested at the highest concentrations inhibited *E. coli* XL1blue strain, but did not inhibit the growth of *E. coli* XL1Blue (pUC18).

Table 31: *In vitro* activities (MIC values in μg/mL) of free ampicillin (Amp) and penicillin G (PenG), and Amp-loaded and PenG-loaded liposomes against *E. coli* XL1Blue (pUC18) strain.

Free	PenG-loaded liposomes			Free	Amp-loaded liposomes		
PenG	D	S	P	Amp	D	S	P
> 1000	> 136	> 14.5	> 8.5	> 1000	> 199	> 200	> 168

Table 31 summarizes the results of sensitivity assay of *E. coli* to PenG and Amp loaded liposomes. To exclude any contribution to inhibitory effect from liposomes as carrier, PenG mixed with plain liposomes were tested as well. The concentration of plain liposomes corresponded to the concentration present in the loaded liposomes. The same MIC values were found for free PenG and PenG mixed with unloaded liposomes indicating that liposomes did not exhibit extra inhibitory effect on *E. coli*. (Table 32)

Table 32: *In vitro* activities (MIC values in μg/mL) of free penicillin G (PenG), free PenG plus unloaded liposomes, and PenG-loaded liposomes against *E. coli* XL1Blue strain.

Free PenG	Free PenG + liposomes			PenG-loaded liposomes		
	D	S	P	D	S	P
58	58	58	58	29	7.2	3.6

Further analysis using a spread plate technique was applied for the samples which showed inhibition of bacterial growth, namely P_{PG} (8.5 μg/mL), S_{PG} (14.5 μg/mL) and D_{PG} (68 μg/mL). *E. coli XL Blue* was considered for this experiment. The results showing CFU count and calculations of initial title (CFU/mL at t_0) and final title (CFU/mL at t_f) are summarized in Tables 29A-32A. The bactericidal effect was considered positive when the number of viable cells was <0.1% (1×10^{-3}). According to Table 32A the lowest concentration of PenG at which the bactericidal effect was observed was 58 μg/mL for free PenG, ≥ 29 μg/mL for D, 3.6 μg/mL for S and ≥ 1.8 μg/mL for P samples, respectively. Thus all of our liposomal preparation show bactericidal effect at lower concentration compared to free PenG solution. Sample P showed lowest MIC and expressed their bactericidal effect at lowest concentration compared to all other samples. Noteworthy is the fact that P samples had initially the lowest incorporation efficiency, as determined by MS. This could be possibly explained by the lipid composition in the membranes of *Rhodopseudomonas palustris 42OL.* This needs to should be further examined in the future. Another interesting finding worth mentioning was that, despite the fact that free PenG was tested in the same concentration that initially showed inhibition of bacterial growth, the plating method showed strong colony growth. One of the possible explanations for this could be the contamination of the samples. Thus the experiment should be repeated to confirm these observations and whether free PenG indeed exhibits bactericidal effect at lower concentrations.

E. coli is a Gram-negative bacterium naturally resistant to PenG. Thus *E. coli* is not necessarily the best candidate for PenG sensitivity essay. In general,
Gram-negative bacteria, even without penicillin resistance genes, tend to be less affected by beta-lactam antibiotics such as PenG compared to Gram-positive bacteria. However, our results indicate that PenG entrapped within liposomal systems was more effective in inhibiting *E. coli* growth compared to free PenG, and PenG mixed with unloaded liposomes.

4.1.4.3 Penicillin G loaded conventional liposomes

Conventional liposomes loaded with PenG were tested to examine their effect on *E. coli XL Blue*. According to results present in Table 27A, L1 liposomes loaded with PenG inhibited bacterial growth at concentration ≤ 44 μg/mL, while MIC for L2 liposomes was ≤ 55 μg/mL. The growth inhibition of free PenG was not confirmed, and might occur at a concentration greater than 100 μg/mL. Based on previous experiments we found that our samples inhibited bacterial growth in a rank as following: DPG ≥ 29, SPG ≥ 3.6, PPG ≥ 1.8, PG ≥ 58. Therefore, it seems that our liposomes of type S,D and P exhibited lower MIC compared to conventional liposomes. However, these results have to be confirmed and evaluated further.

Even though liposome samples were diluted with water and even further diluted 1:1 (v/v) with inoculum, they were still optically dense. The turbidity of the sample solutions made the reading of optical density challenging. The OD at T_0 was rather high, making the evaluation of bacterial growth complicated. To confirm this, additional reading of OD was initiated for plain liposome samples, which were diluted with LB in the same manner as the PenG liposome samples. In that way we would get the reference values that could help with the interpretation of the results.

As seen in Table 28A, the initial OD for the samples was quite high even though the samples were only diluted with LB and did not contain inoculum. Thus the results of MIC determination of conventional liposomal containing PenG are challenging. Another assay

method should be applied in this case. It has been suggested to test conventional liposomes by spread plate technique.

4.1.4.4 Summary of biological assays

Outer membrane (OM) is unique for Gram-negative bacteria, and it acts as a selectivity barrier for a variety of different compounds. β-lactam antibiotics are known to overcome the permeability barrier of the OM by using porin channels.(117) These diffusion channels are expressed in the OM and allow a non-specific transport of charged and zwitter-ionic nutrient molecules.(134) As mentioned earlier, Amp and PenG also diffuse through the same channels. It has recently been reported that permeability of Amp is four times higher than PenG.(135) Correspondingly, we found that *E. coli* XL1 Blue tends to be more resistant to free PenG (MIC = 58 μg/mL) than to free Amp (MIC =14,5 μg/mL). On the contrary, liposomal PenG showed increased inhibitory activity against *E. coli* XL1 Blue cells. It was found that the MIC of free PenG was reduced 2, 8 and 16 fold when PenG was loaded in D, S and P liposomes, respectively. As mentionedearlier, any independed inhibitory effect of liposomes was excluded.

Our results indicate that liposomes can help PenG to enter the bacterial cell through outer membrane of Gram-negative bacteria. After diffusion into the periplasmic space, the antibiotics are released and can exhibit their inhibitory activity on transpeptidase enzymes. In addition, liposomes enhanced the inhibitory activity of PenG. The type P liposomes, made from *R. palustri*, showed greater effectiveness, followed by S and D liposome from *Cyanothece* sp. From these results we can conclude that the chemical and structural characteristics of the lipid fraction extracted from the different strains of bacteria, and used for the preparation of liposomes, influence the ability of the latter in enhancing the antibacterial activity of PenG against *E. coli* cells. Unlike for PenG, we could not observed any enhanced antibiotic activity of liposomal Amp compared to freeAmp. The different behavior of liposomal PenG and Amp can be explained by the different permeabilities of these two antibiotics through OM of *E. coli.* Unlike PenG, the permeation of Amp was not further enhanced by liposomal vectors.

However, at this stage our liposomal systems loaded with the same concentration of PenG and Amp that showed inhibition of *E. coli* XL1 Blue strain growth , did not show any effect on *E. coli* XL1 Blue with pUC18 plasmid. Thus all three kinds of liposome vectors failed to protect antibiotic from enzymatic cleavage by β-lactamase inside periplasmatic space. All these results suggest that liposomes as drug carriers are indeed able to overcome physiological barriers of Gram-negative bacteria, facilitate transport through outer membrane, even more effectively than free antibiotic, and allow antibiotic release into the periplasmic space. However, at this stage, the mechanism by which this facilitated diffusion through the outer membrane happens, remains unclear. Based on the observation that liposomes were not able to protect the antibiotic against enzymatic degradation, the most likely pathway would be that the antibiotic is entering the periplasm not entrapped (free) and protected by the liposomal vector. It has been discussed that the delivery of antibiotics probably happens through fusion of liposomes with OM. Some of the antibiotic resistance mechanisms possessed by microbial cells is the decreased uptake of drug and increased efflux of drug out of the bacterial cell. It is known that liposomes can bypass these resistance mechanisms by fusing with the OM or plasma membrane, followed by release of high enough drug concentration that overcomes efflux by transmembrane pumps.(17) The transport of antibiotics using a liposomal vector has been earlier applied in several studies. The delivery of drug in high concentration to the periplasm due to liposome fusion with OM of Gram-negative bacteria, and corresponding decrease of MIC and better antimicrobial activity, have been observed for various liposome entrapped antibiotics and reported by several authors.(19,136) Similarly, we observed reduction of MIC for PenG entrapped by all three liposomal types.

Our results suggest that liposomes prepared from the lipid fraction of naturally occurring bacteria, are able to act as successful vectors for PenG. Liposomes can help to overcome the physiological barrier of the outer membrane of a bacterial cell, in this case *E coli.* As a results the drug is released from the carrier and can enter the bacterial periplasm, where it exert its inhibitory effect on transpeptidase activity. Very likely the drug transport occurs through liposome fusion with the OM and hence release of nonliposomally protected content into the periplasm. Thus the liposomes do not offer

further drug protection inside the periplasm, as indicated by the fact that the antibiotics underwent degradation through enzymatic cleavage by β-lactamase.

4.1.5 Enzymatic inhibition potential

In this experimental part the activity of liposomal antibiotics on the cytosolic carbonic anhydrase enzymes was analysed and compared to the activity of free antibiotics and acetazolamide (Diamox®), which is a patented carbonic anhydrase inhibitor. This diuretic is used for medical treatment of glaucoma, hypertension and epileptic seizures. (137)

Summarized results in Table 33 are presented as quantitative measures of half maximal inhibitory concentration (IC_{50}), which indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process by half.

Table 33: Enzymatic assay (expressed as IC_{50})

	IC_{50}	Enzyme type
L1	n.d	CA I
L1SA	4.7×10^{-6}	CA I
L1ES	3.1×10^{-6}	CA I
L2	n.d	CA I
L2SA	4.4×10^{-6}	CA I
L2ES	3.2×10^{-6}	CA I
SA	3.3×10^{-7}	CA I
ES	6.3×10^{-7}	CA I
AAZ	9.5×10^{-8}	CA I
L1	n.d	CA II
L1SA	9.7×10^{-7}	CA II
L1ES	6.3×10^{-8}	CA II
L2	n.d.	CA II
L2SA	9.5×10^{-7}	CA II
L2ES	8.9×10^{-7}	CA II
SA	5.1×10^{-7}	CA II
ES	7.9×10^{-8}	CA II
AAZ	3.1×10^{-8}	CA II
n.d = not detected, AAZ= acetazolamide, SA = sulfanilamide, ES = ethylaminobenzenesulfonamide		

In the experiment two types of carbonic anhydrase enzyme, namely CAI and CAII were used. In all assays the plain conventional liposomes (L1, L2) did not inhibit carbonic anhydrase, as expected. Lower value of IC_{50} indicates that less of the compound is needed to inhibit the enzyme; therefore the compound will be more active. Thus the results (Table 33) indicate that acetazolamide, being a drug, showed lower IC_{50} values as compared to free sulfanilamide (SA) and ethylaminobenzenesulfonamide (ES). L1 and L2 liposomes loaded with SA and ES did not exhibit higher activities, just a slightly lower IC_{50} was measured for ES loaded liposomes. However, this slightly improved activity of ES can be explained by the fact that theoretical concentration of liposomal SA was 230 ppm, while ES concentration was 267 ppm. Additionally, all liposomal sulfanilamides showed less activity compared to both free antibiotics and acetazolamide. It was also observed that all tested samples showed greater inhibition activity against carbonic anhydrase type II (Table 33). The L2SA sample used in this experiment contained precipitates, which could be easily dispersed upon shaking, and did not affect the results.

The current analysis is just the starting phase of a larger research project. It was performed to evaluate possible effect of liposomal sulfonamides, and compare the effect to free sulfanilamide and ethylaminobenzenesulfonamide. However, we yet do not know the actual drug amount that was encorporated by the conventional liposomes. Therefore it have to be clarified in future analysis.

The positive inhibitory effect on carbonic anhydrase by both liposomal SA and ES was observed, although lower than the effect of free sulfanilamides. It would be possible to proceed with biological trials to determine how liposomal sulfanilamides behave when acting on real cell cultures. It has been already discussed that sulfonamides and their derivatives can be used as antibiotics by acting on carbonic anhydrase in microbial cells.(138) ”Bacterial CAs represent at this moment a very promising targets for obtaining antibacterials devoid of the resistance problems of the clinically used agents.”(139) Furthermore, there are no reports on the use of liposomes for delivery of sulfanilamides. Thus this delivery approach can represent a new, innovative and possibly more efficient delivery of these types of drugs to bacterial cells.

4.1.6 Small-angle X-ray scattering

In our experiments no system showed quasi long-range order, which allowed to infer that multilamellar liposomes were not present in these samples. Indeed, the SAXS signal of multilamellar (or oligolamellar) structures is stronger than the pattern of monolamellar liposomes and their presence is unambiguosly revealed by Bragg peaks, even if there is coexistance of the two types of aggregates and monolamellae are by far the major component (e.g. 98-99%).
The scattering diagrams of plain and antibiotic-loaded CCY110 liposomes are shown in Table 39A. The corresponding best fit parameters are reported in Table 34.

These diagrams showed that the liposomes obtained from the membrane of CCY110 bacteria had similar characteristics to more conventional liposomes. In particular, the bilayer head-to-head thickness ($2Z_H$ = 46 Å) was only slightly larger than the values measured for DOPC/DOPE liposomes ($2Z_H$ = 39.6 Å)(140) and DMPC liposomes ($2Z_H$ = 34.7 Å). Moreover this value is very close to the thickness reported for DPPG bilayers at 25 °C ($2Z_H$ = 44 Å)(119), which is in line with the widespread use of glycerolipid to build model bacteria membranes(141). The insertion of antibiotics in CCY-liposomes showed that the bilyaer undergoes a contraction, but its electron densities are only slightly varied. This variation being detected throughout the membranes and not in a particular region, suggests that the antibiotic molecules are interacting with both the polar heads and the hydrophobic core.

Similar results were obtained for 42OL-liposomes (Table 40A; Figure 1) therefore we concluded that the insertion modality of Ampicillin and penicillin G in the membranes derived from these capsule-free bacteria was similar. The best fit parameters obtained for these samples are also reported in Table 34.

The liposomes prepared from VI22 bacteria had a different structure. In fact in this case the polar layer was markedly thicker, probably in consequence of some molecules composing the outer polyglycan envelope being retained in the final liposomes. The insertion of antibiotics seemed to increase the thickness of the outer layer, though a complete fitting of the scattering pattern was not possible for loaded liposomes and only

the position of the first maximum could be correctly reproduced for these systems, also because of their low SAXS intensity. To illustrate this point, Figures 2 and 3 in Table 40A show the experimental diagrams of samples VI22-lipo and VI22-lipo + PenG, respectively.

Table 34. Structural and electron density parameter of plain and antibiotic-loaded liposomes obtained by the fitting of SASX intensity diagrams

Liposome type	Antibiotics	Z_H	s_H	r_C	s_C
CCY-lipo	-	23.0	0.10	-1.1	0.50
CCY-lipo	Amp	11.5	0.12	-1.2	0.70
CCY-lipo	PenG	13.0	0.12	-1.3	0.65
VI22-lipo	-	54	0.55	-0.7	0.38
VI22-lipo	Amp	80	0.18	-0.1-	0.55
VI22-lipo	PenG	75	0.22	-0.1	0.70
42OL-lipo	-	21.0	0.09	-0.8	0.42
42OL-lipo	Amp	17.0	0.09	-0.6	0.7
42OL-lipo	PenG	12	0.10	-0.8	1.0

5 CONCLUSION

In this work we have considered a new method of liposome preparation, where we used lipid extractions from bacterial cultures. We also evaluated the possibility of these organic liposomes to incorporate bioactive compounds such as curcumin and β-lactam antibiotics. The preparation and loading procedures were optimized throughout the whole work period, and we successfully achieved relatively easy techniques for such liposomal preparations. Both plain and loaded liposomes were extensively characterized from the structural point of view, using various analytical techniques. We were also able to determine the amount of entarapped ampicillin and penicillin G. Although the incorporation efficacy of penicillin G in liposomes was relatively poor, the liposomes still showed very encouraging results in the sensetivety assay against *E. coli*.

Also results from DLS show somewhat different behaviour of different liposome types. However, in overall, the bacterial liposomal vesicles show an average size distribution between 100-300 nm. Additionally the size tends to decrease or remain small enough even after addition of bioctive compounds. Therefore they are appropriate for use as drug carriers. Even though further research have to be carried out, already at this stage our results indicate that there is a full potential of using lipids derived from bacterial cultures in liposome preparation and using them as drug carriers for bioactive compounds. Especially promising anti-proliferative effects are observed for liposomal penicillin G. This can open new and exiting arenas for employment of organic biodegradable material.

6 FUTURE PERSPECTIVES

By experimental determination, we were able to establish that the total phosphorus content in all bacterial samples was around 60%. However, there numerous strains of bacteria can be found in nature, which vary both in structure and lipid composition. Additionally, it has to be kept in mind that all the studies available on bacteria derived lipids, involve only laboratory experiments and there is a lack of information on technological, economic and applicative aspects of these polymers. The next appropriate step would be to determine the exact phospholipid composition in the particular strains that we have been using. This could provide us with even more understanding on loading capacity of the bacterial liposomes and interactions between the phospholipid bilayers and the incorporated material.

Concerning liposomal curcumin, the anti-inflammatory properties have still to be evaluated. There will be initiated *in vitro* testing at the Department of Pharmacy in Faculty of Health Sciences of University of Tromsø. If positive results will be obtained, this delivery mechanism by bacterial liposomal can possibly also be applied for delivering of other pharmacological interesting phytochemicals. Conclusively, it would open new avenues for use of bacterial liposomes in food delivery industry as well as pharmaceutical industry. Even though liposomal encapsulation technology can offer promising drug delivery approaches, additional innovative research is also needed to address the cost-effectiveness and long-term safety of this delivery system.

To evaluate the effectiveness of our liposomal carriers, a comparison study with conventional liposomes that we started have to be continued. Additionally, the enzymatic assay that we carried out with conventional liposomal sulphonamide and its derivative, show promising results. Effectiveness of liposome incorporated sulphonamides have not been reported before. This work will continue with sensitivity assay of these liposomal systems on living microorganisms. If it shows positive effects, it will be possible to proceed with liposomes made of bacterial lipids. This can offer a completely new delivery system that can be used to possible prevent one or several mechanisms for antibiotic resistance, which is a great issue nowdays.

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APPENDIX (A)

UV/VIS Spectrometry

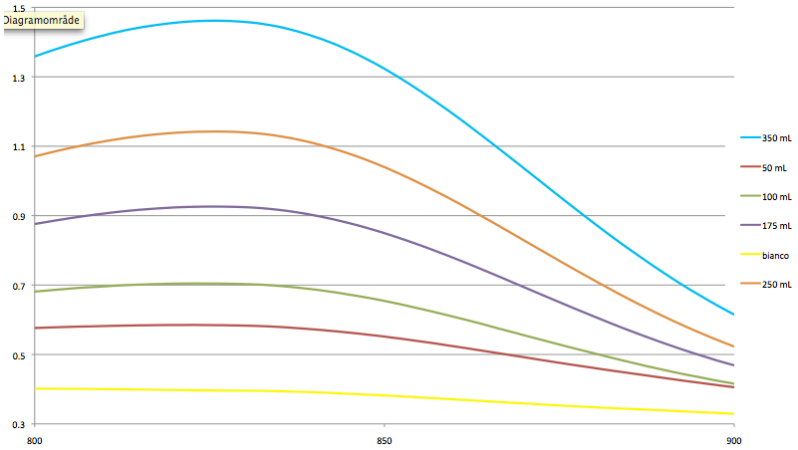
Determination of total phosphorus by UV/VIS Spectrometry

Table 1A: UV/VIS results for preparation of standard curve

(performed before this thesis was initiated)

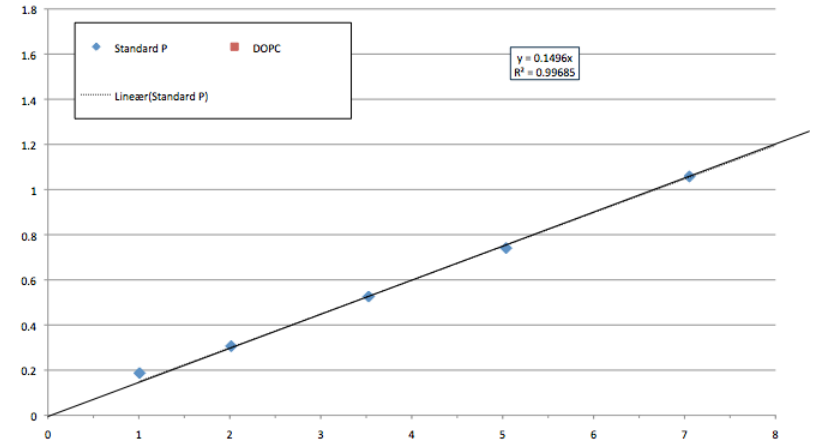
Testing of standards with various phosphorus concentrations.

Data was translated at point 1078 with 0.295
Standard P solution C = 20.15 mg/mL



Curve	A (820nm)	A - bianco
350 mL	1.4556	1.0579
250 mL	1.1385	0.7408
50 mL	0.5849	0.1872
100 mL	0.7041	0.3064
175 mL	0.9236	0.5259
bianco	0.3977	0
	ml of standard P	mg of P
	350	7.0525
	250	5.0375
	50	1.0075
	100	2.015
	175	3.52625
	bianco	0

Standard curve (y=0.1496x) used later for calculation of total phosphorus present in liposome samples

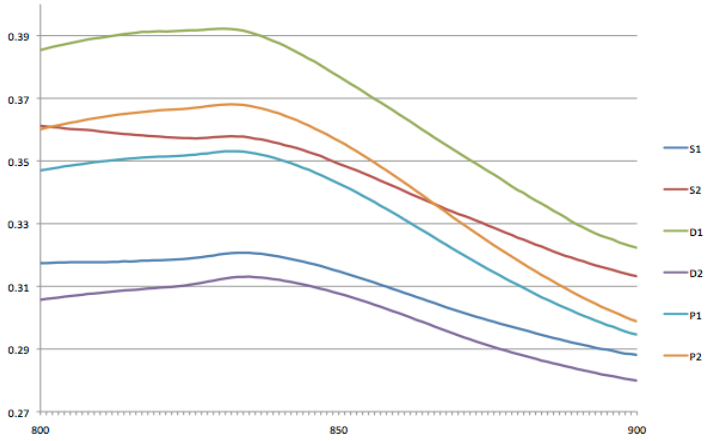


standard	mg of P	A (820nm)
350 mL	350	1.0579
250 mL	250	0.7408
50 mL	50	0.1872
100 mL	100	0.3064
175 mL	175	0.5259
bianco	0	0
	DOPC	A (820nm)
	350	1.1059
	250	0.6966
	50	0.1476
	100	0.3162
	175	0.5781
	bianco	0

Table 2A: determination of total phosphorus from plain in loaded liposomes

S_1, S_2, D_1, D_2, P_1, P_2

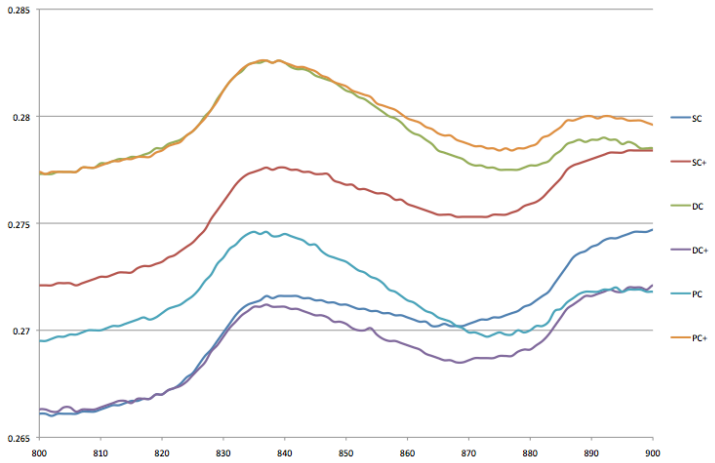
Data was translated at point 1078 with 0.295



Sample	A (820 nm)	Ratio to 175 mL	% P (MW =700)
S1	0.3183	2.866	55.6
S2	0.3578	2.584	61.6
D1	0.3914	2.360	67.5
D2	0.3096	2.983	53.4
P1	0.3514	2.628	60.6
P2	0.3662	2.522	63.1

SC, SC+, DC, DC+, PC, PC+

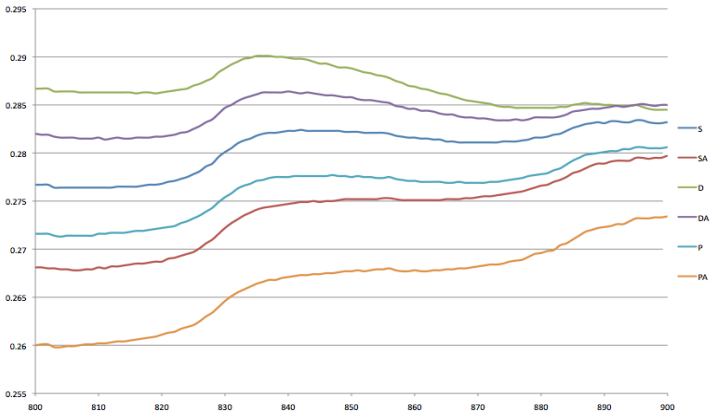
Data was translated at point 1078 with 0.295



Sample	A (820 nm)	Ratio to 175 mL	% P (MW =700)
SC	0.267	3.459	61.03
SC+	0.2732	3.381	62.84
DC	0.2785	3.316	64.39
DC+	0.267	3.459	61.03
PC	0.2708	3.411	62.14
PC+	0.2784	3.318	64.36

S, SA, D, DA, P, PA

Data was translated at point 1078 with 0.295

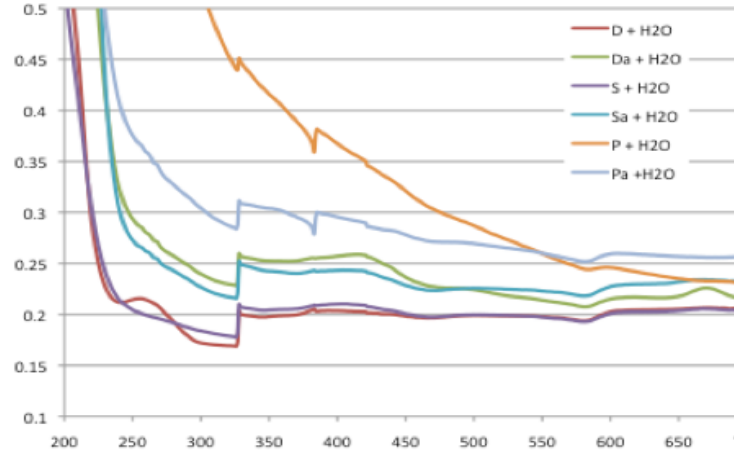
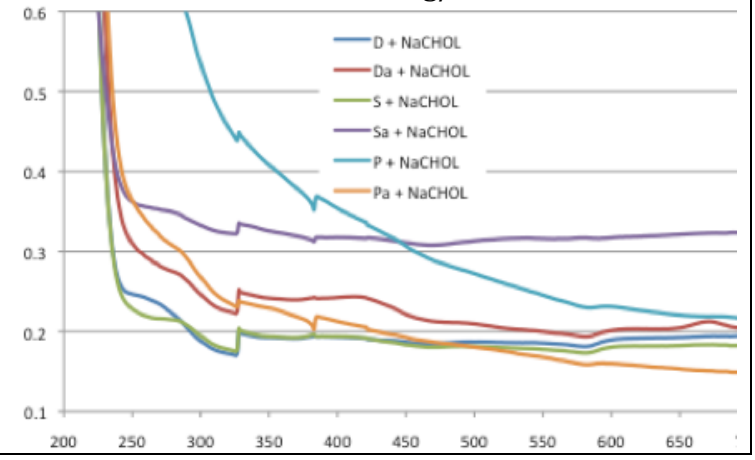


Sample	A (820 nm)	ratio to 175 mL	% P (MW = 700)
S	0.2768	3.337	63.89
SA	0.2687	3.437	61.52
D	0.2863	3.226	66.66
DA	0.2817	3.279	65.32
P	0.2722	3.393	62.55
PA	0.2611	3.537	59.31

Table 3A: UV/VIS analyze conditions

Sample	Amount of sample (mL)	Analyze range (nm)	Cuvette quarts (QV)
S_1	2	750-1100	QV
S_2	2	750-1100	QV
D_1	2	750-1100	QV
D_2	2	750-1100	QV
P_1	2	750-1100	QV
P_2	2	750-1100	QV
SC	2	750-1100	QV
SC+	2	750-1100	QV
DC	2	750-1100	QV
DC+	2	750-1100	QV
PC	2	750-1100	QV
PC+	2	750-1100	QV
S	2	750-1100	QV
S_A	2	750-1100	QV
D	2	750-1100	QV
D_A	2	750-1100	QV
P	2	750-1100	QV
P_A	2	750-1100	QV

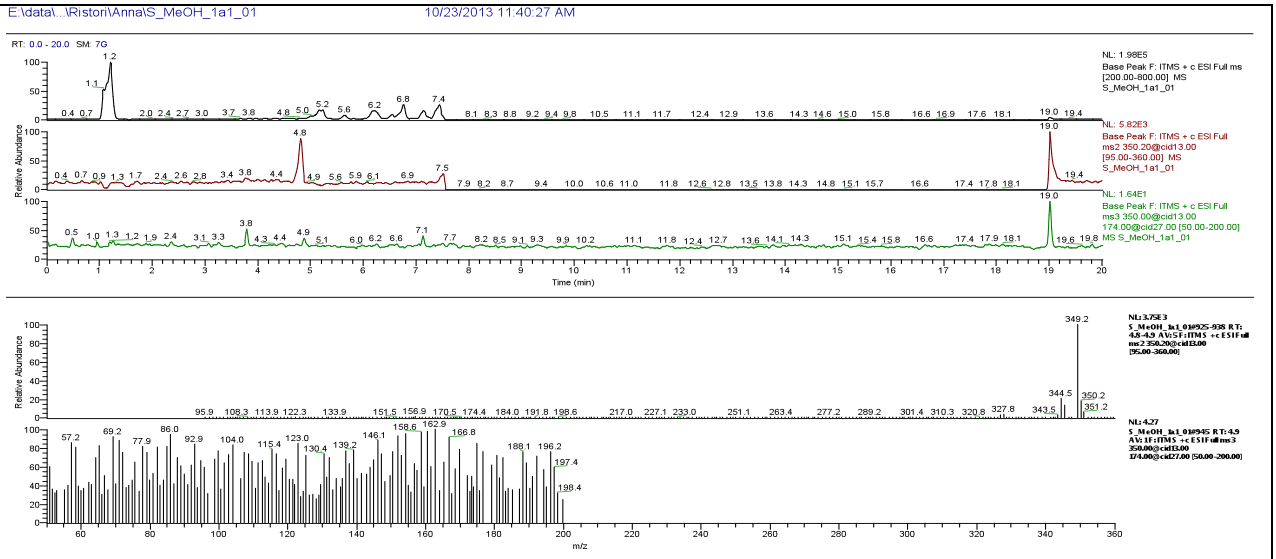
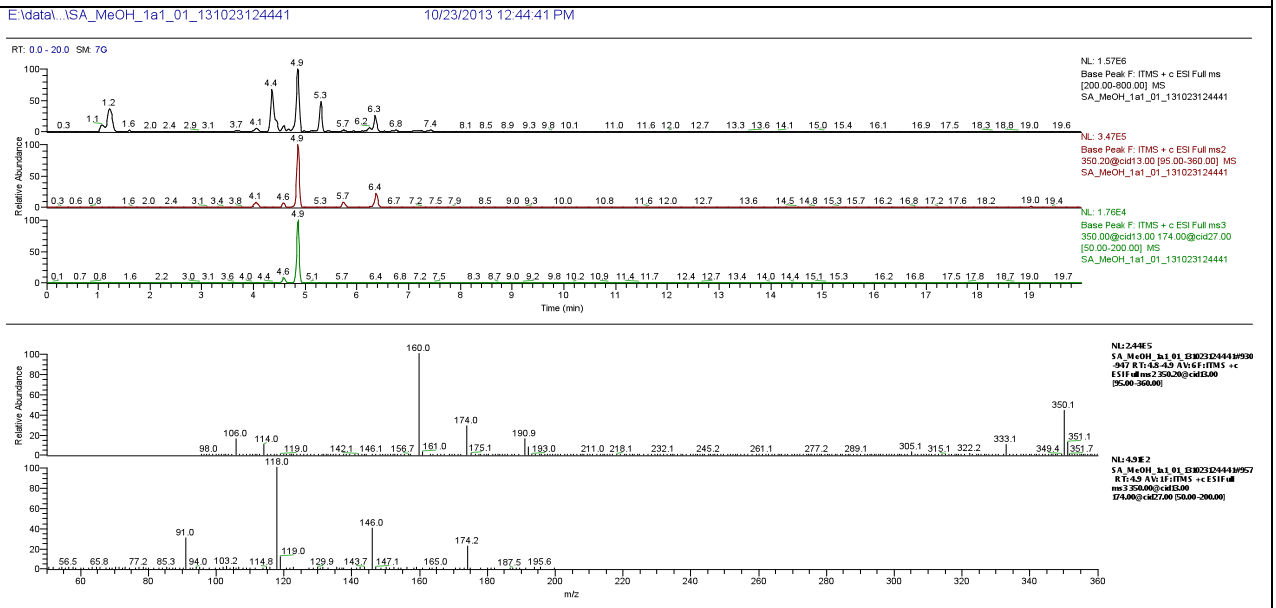
Table 4A: UV/VIS determination of ampicillin in liposome samples
Analyze was carried in quart (QV) cuvettes at a range 200-700 nm

S S_A D D_A P P_A	diluted 1:10 with water 
S S_A D D_A P P_A	Diluted 1:10 with NaChol 20mg/mL soln. 

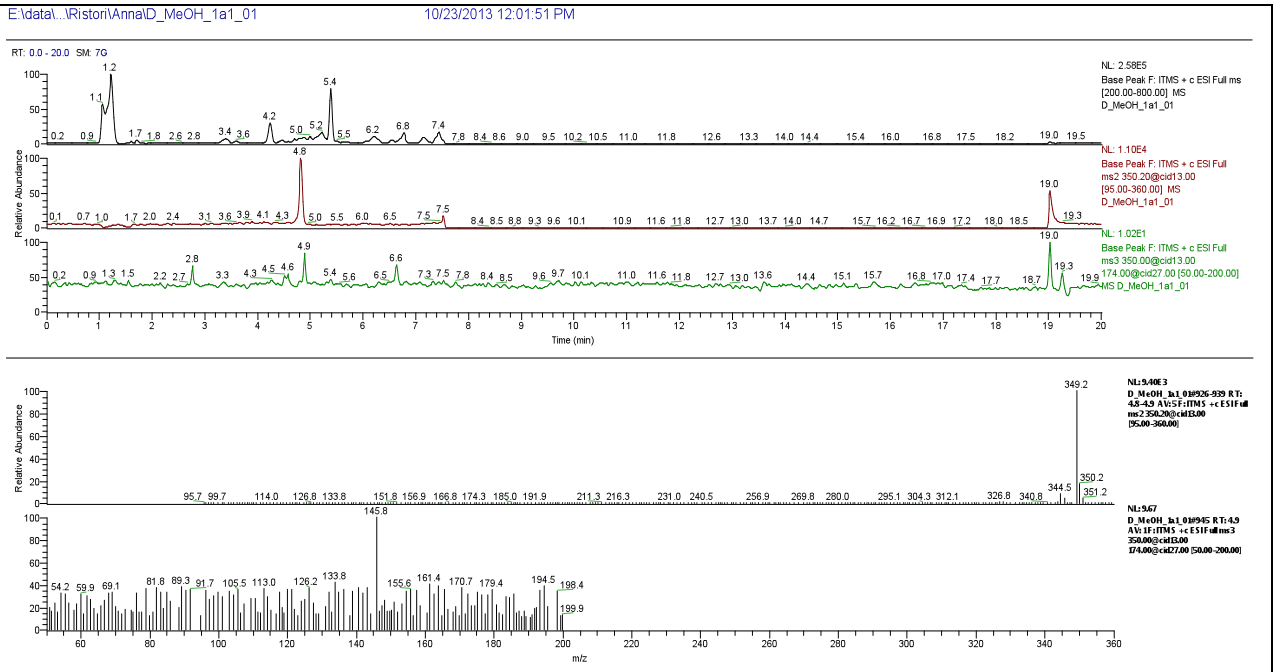
Mass Spectrometry

Mass spectrometry results for determination of amount of ampicillin in liposome samples

Table 5A: MS results for plain liposom samples and liposomes containing ampicillin

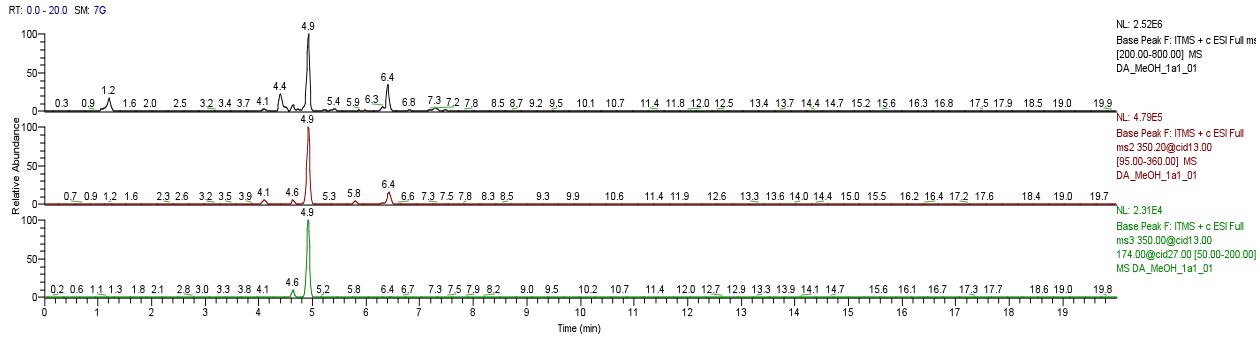
S	
S_A	

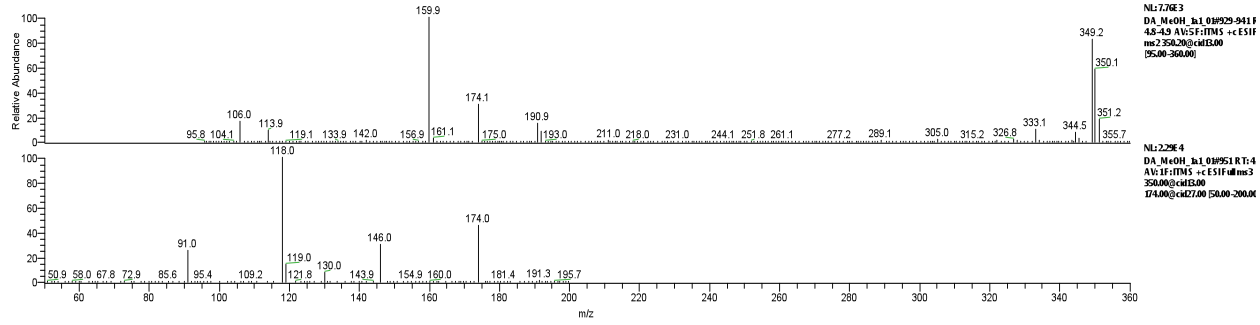
D


E:\data\...\Ristori\Anna\D_MeOH_1a1_01 10/23/2013 12:01:51 PM
RT: 0.0 - 20.0 SM: 7G
NL: 2.58E5
Base Peak F: ITMS + c ESI Full ms
[200.00-800.00] MS
D_MeOH_1a1_01
NL: 1.10E4
Base Peak F: ITMS + c ESI Full
ms2 350.20@cid13.00
[95.00-360.00] MS
D_MeOH_1a1_01
NL: 1.02E1
Base Peak F: ITMS + c ESI Full
ms3 350.00@cid13.00
174.00@cid27.00 [50.00-200.00]
MS D_MeOH_1a1_01
Relative Abundance
Time (min)

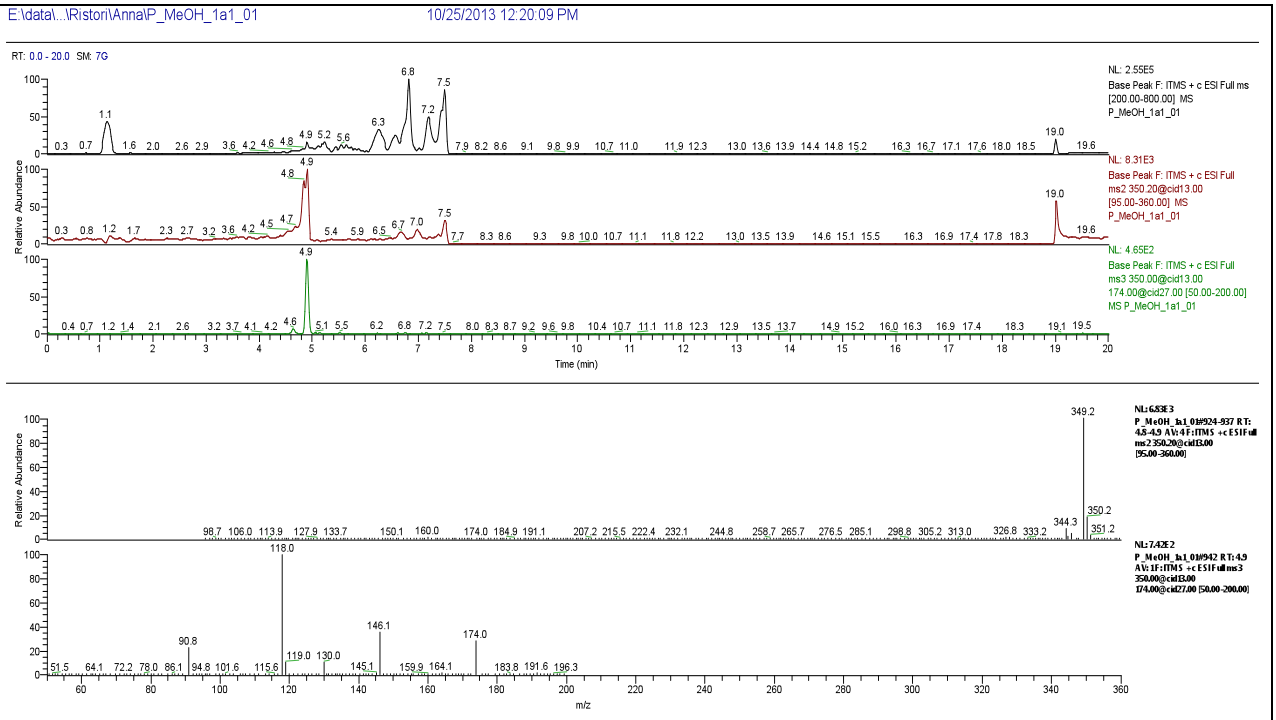
NL: 9.40E3
D_MeOH_1a1_01#926-939 RT:
4.8-4.9 AV: 5F: ITMS + c ESI Full
ms2 350.20@cid13.00
[95.00-360.00]
NL: 9.67
D_MeOH_1a1_01#945 RT: 4.9
AV: 1F: ITMS + c ESI Full ms3
350.00@cid13.00
174.00@cid27.00 [50.00-200.00]
Relative Abundance
m/z

D_a

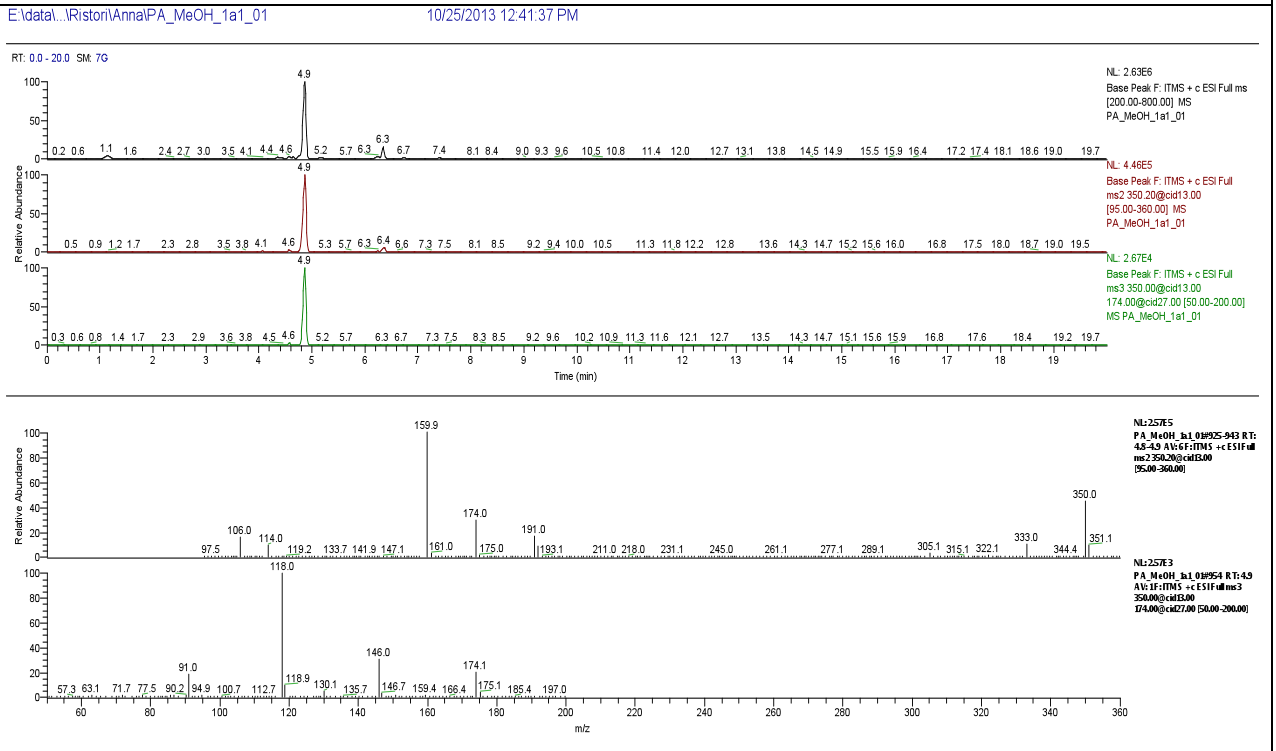

E:\data\...\Ristori\Anna\DA_MeOH_1a1_01 10/23/2013 1:27:31 PM
RT: 0.0 - 20.0 SM: 7G
NL: 2.52E6
Base Peak F: ITMS + c ESI Full ms
[200.00-800.00] MS
DA_MeOH_1a1_01
NL: 4.79E5
Base Peak F: ITMS + c ESI Full
ms2 350.20@cid13.00
[95.00-360.00] MS
DA_MeOH_1a1_01
NL: 2.31E4
Base Peak F: ITMS + c ESI Full
ms3 350.00@cid13.00
174.00@cid27.00 [50.00-200.00]
MS DA_MeOH_1a1_01
Relative Abundance
Time (min)


NL: 7.76E3
DA_MeOH_1a1_01#929-941 F
4.8-4.9 AV: 5F: ITMS + c ESI F
ms2 350.20@cid13.00
[95.00-360.00]
NL: 2.29E4
DA_MeOH_1a1_01#951 RT: 4
AV: 1F: ITMS + c ESI Full ms3
350.00@cid13.00
174.00@cid27.00 [50.00-200.00
Relative Abundance
m/z

P


E:\data\...\Ristori\Anna\P_MeOH_1a1_01 10/25/2013 12:20:09 PM
RT: 0.0 - 20.0 SM: 7G
NL: 2.55E5
Base Peak F: ITMS + c ESI Full ms [200.00-800.00] MS P_MeOH_1a1_01
NL: 8.31E3
Base Peak F: ITMS + c ESI Full ms2 350.20@cid13.00 [95.00-360.00] MS P_MeOH_1a1_01
NL: 4.65E2
Base Peak F: ITMS + c ESI Full ms3 350.00@cid13.00 174.00@cid27.00 [50.00-200.00] MS P_MeOH_1a1_01
Relative Abundance
Time (min)
NL: 6.53E3
P_MeOH_1a1_01#924-937 RT: 4.5-4.9 AV: 4 F: ITMS + c ESI Full ms2 350.20@cid13.00 [95.00-360.00]
NL: 7.42E2
P_MeOH_1a1_01#942 RT: 4.9 AV: 1 F: ITMS + c ESI Full ms3 350.00@cid13.00 174.00@cid27.00 [50.00-200.00]
m/z

P_A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RT: 0.0 - 20.0 SM: 7G
NL: 2.63E6
Base Peak F: ITMS + c ESI Full ms [200.00-800.00] MS PA_MeOH_1a1_01
NL: 4.46E5
Base Peak F: ITMS + c ESI Full ms2 350.20@cid13.00 [95.00-360.00] MS PA_MeOH_1a1_01
NL: 2.67E4
Base Peak F: ITMS + c ESI Full ms3 350.00@cid13.00 174.00@cid27.00 [50.00-200.00] MS PA_MeOH_1a1_01
Relative Abundance
Time (min)
NL: 2.57E5
PA_MeOH_1a1_01#925-943 RT: 4.5-4.9 AV: 6 F: ITMS + c ESI Full ms2 350.20@cid13.00 [95.00-360.00]
NL: 2.57E3
PA_MeOH_1a1_01#954 RT: 4.9 AV: 1 F: ITMS + c ESI Full ms3 350.00@cid13.00 174.00@cid27.00 [50.00-200.00]
m/z

Table 6A: Detection of Ampicillin amount present in the samples, based on Table 5A

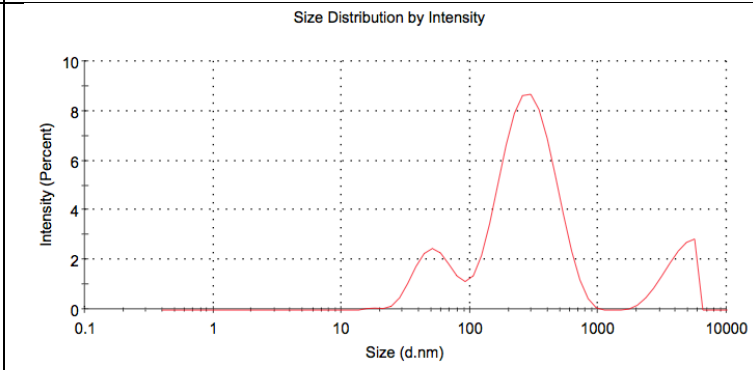
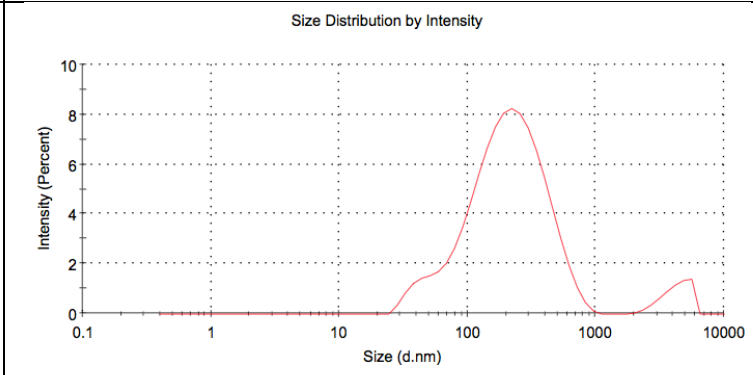
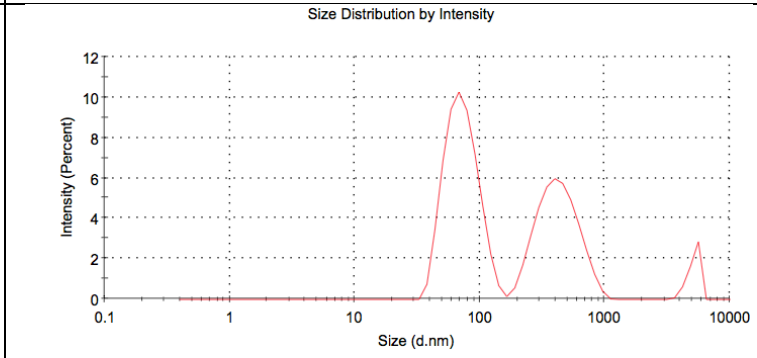
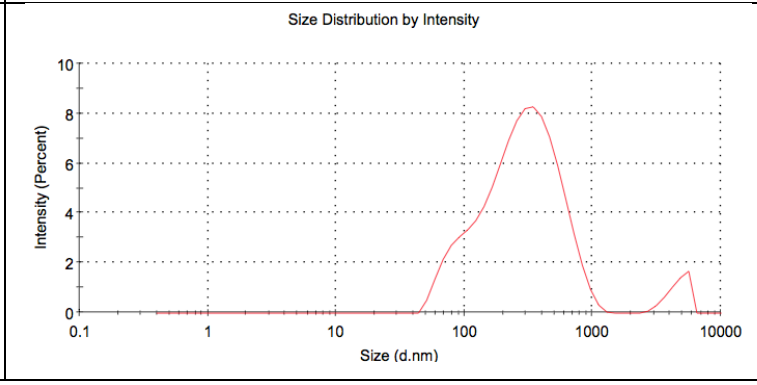
Sample	Specified amount (ppm)	Calculated amount (ppm)
1) Ampicillin 2µL 1000 diluted 200 µL (Standard)	10.000	9.595
2) Ampicillin 2µL 1000 diluted 200 µL (Standard)	10.000	10.405
S dil. MeOH	-	0.003
S_A dil. MeOH	-	79.194
D dil. MeOH	-	-
D_A dil. MeOH	-	120.128
P dil. MeOH	-	1.884
P_A dil. MeOH	-	121.521

Dynamic Light Scattering

Table 7A: Abbreviations

S	plain liposomes from VI22
D	plain liposomes from CCY110
P	plain liposomes from 42OL
L1	Conventional liposomes of type L1
L2	Conventional liposomes of type L2
C	Curcumin
A	Ampicillin
S	Sulphanilamide
ES	Ethylaminobenzenesulfonamide
+	CTAB (4.58×10^{-4} mol/L)
++	CTAB (9.14×10^{-4} mol/L)

Table 8A: DLS results for plain and ampicillin containing samples

	Peak 1	Peak 2	Z-Aver. (d.nm)	
S	305.0	54,79	197.1	
S_A	237.1	4276	176.2	
D	73.76	439.5	127.3	
D_A	318.9	4635	239.1	

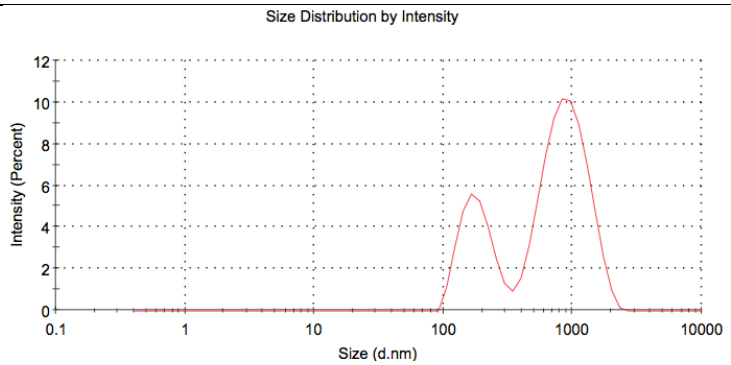
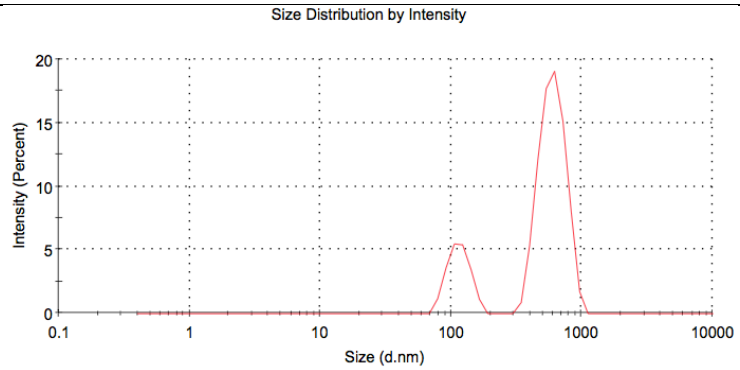
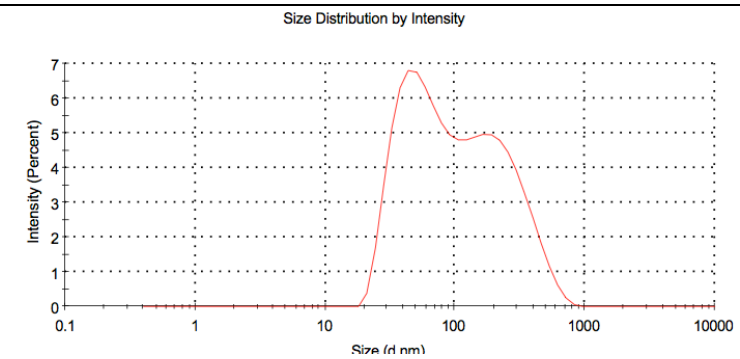
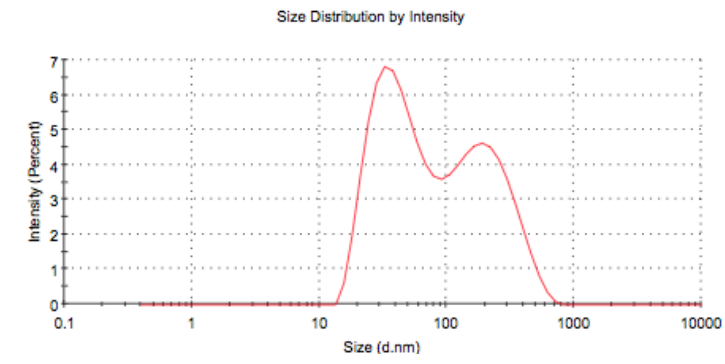
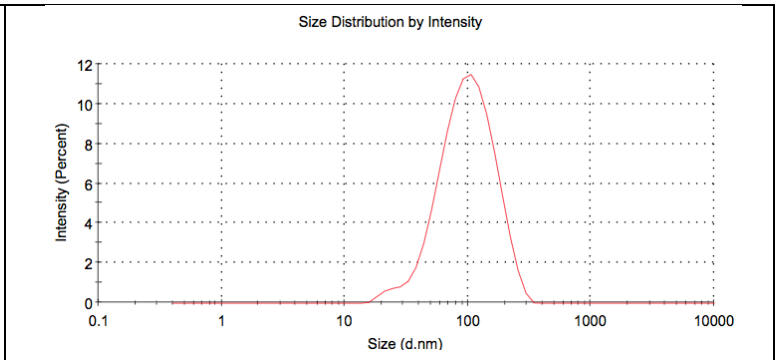
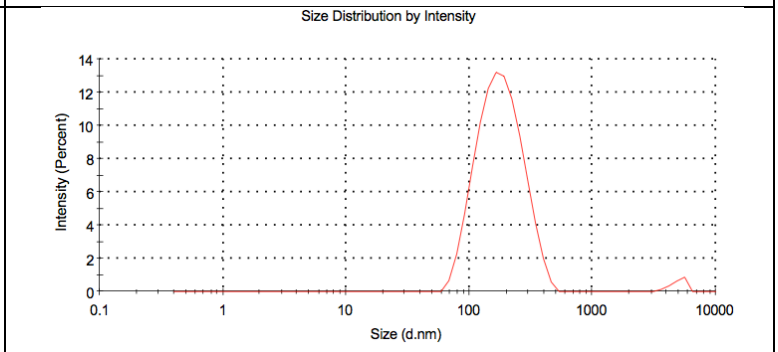
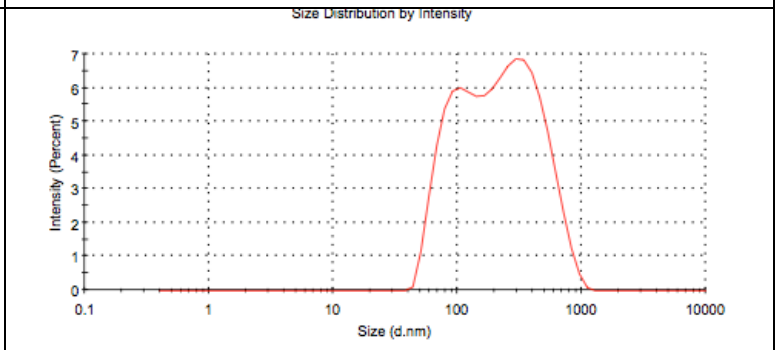
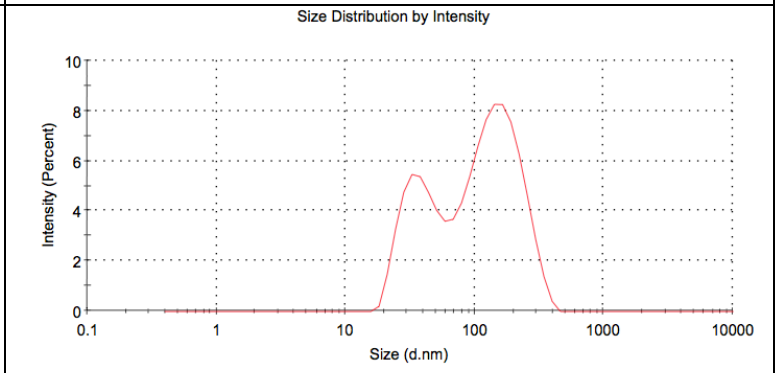
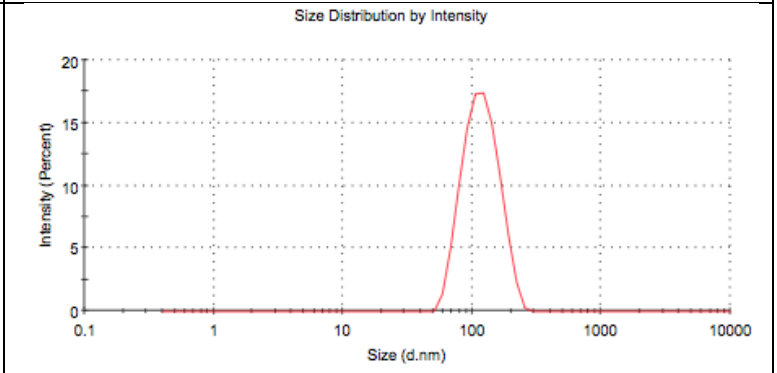
P	928.4	186.1	433.3	
P_A	601.9	115.5	725.9	

Table 9A: DLS results for plain, curcumin and CTAB containing samples

	Peak 1	Peak 2	Z-Aver. (d.nm)	
S	57.82	234.1	75.00	
SC	44.39	218.8	56.74	

SC^{+}	107.9	0	87.60	
SC^{++}	188.2	4937	152.3	
D	341.5	98.02	167.8	
DC	154.4	38.10	74.45	
DC^{+}	121.2	-	110.7	

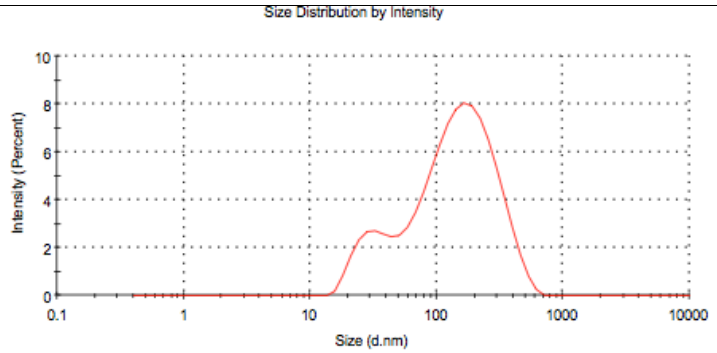
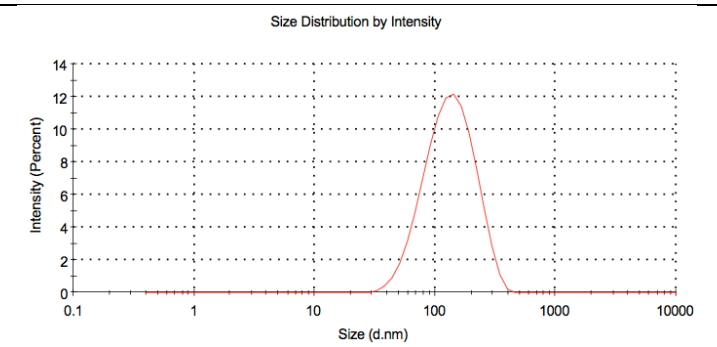
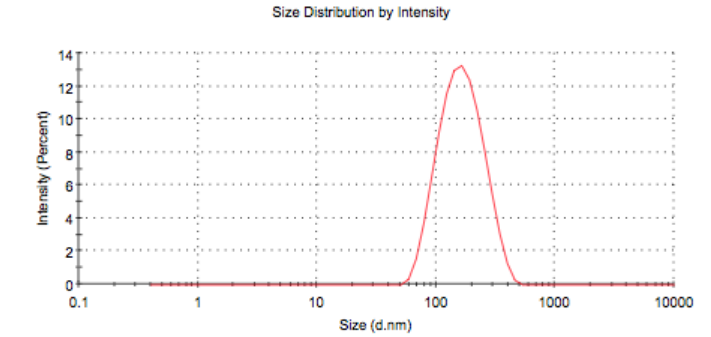
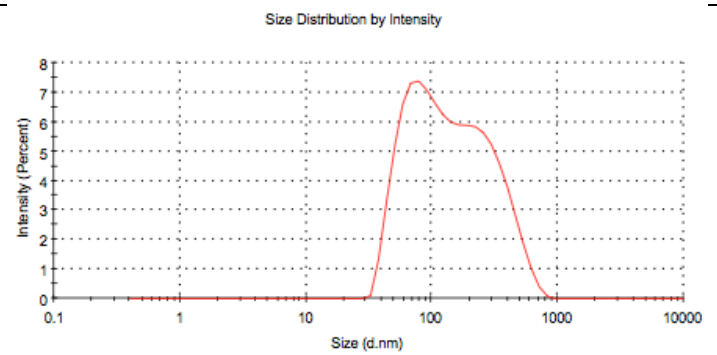
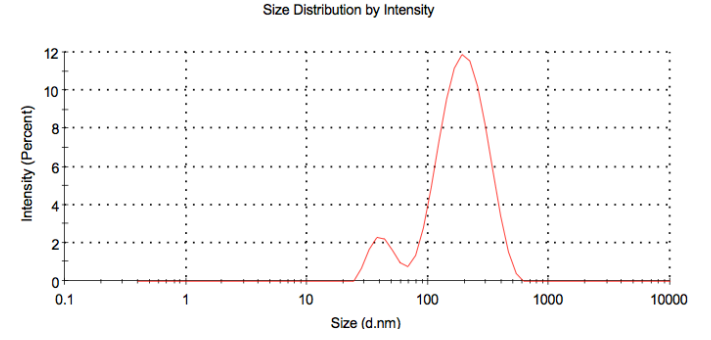
DC^{++}	180.4	31.02	97.49	
P	143.4	-	118.5	
PC	174.2	-	134.8	
PC^{+}	175.6	-	110.0	
PC^{++}	210.1	43.93	144.4	

Table 10A: DLS results for plain conventional liposomes of type L1 and samples loaded with sulphanilamide and ethylaminobenzenesulfonamide

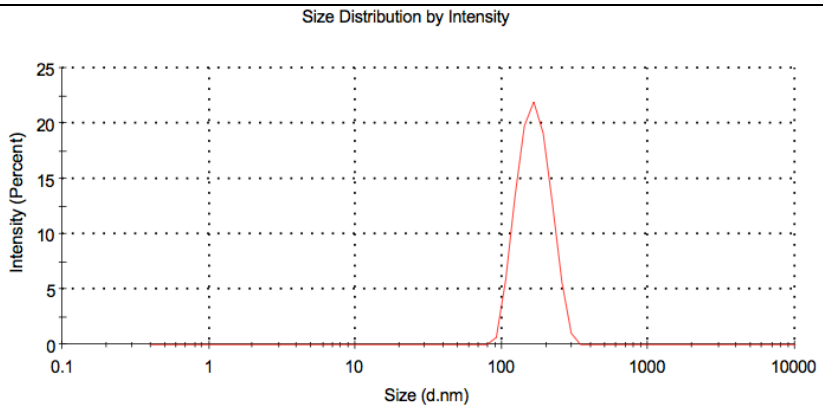
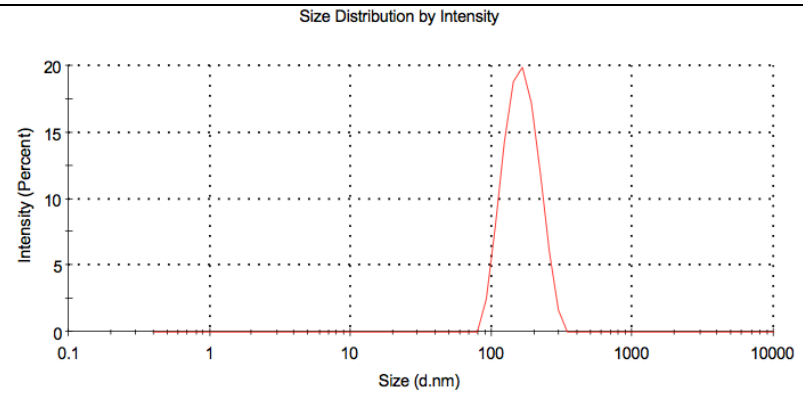
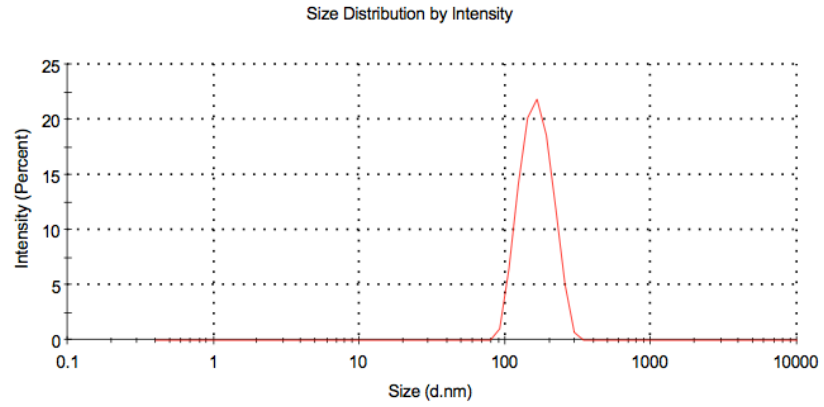
	Peak 1	Z-Aver. (d.nm)	
L1	168.8	160.0	
L1ES	166.3	155.6	
L1S	166.3	157.7	

Table 11A: DLS results for plain conventional liposomes of type L2 and samples loaded with sulphanilamide and ethylaminobenzenesulfonamide

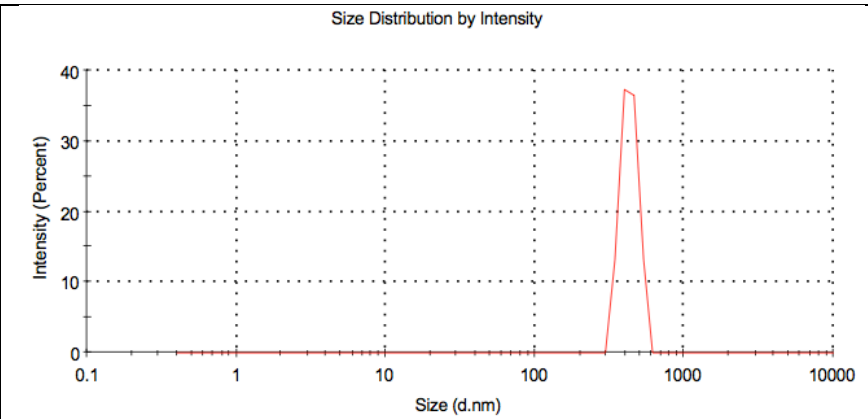
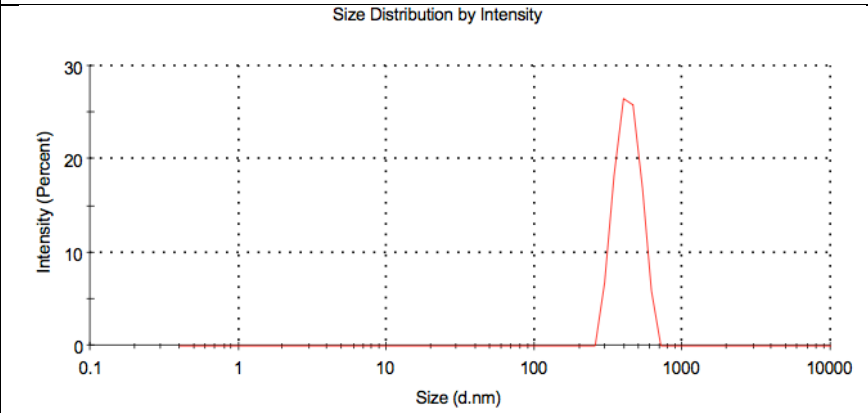
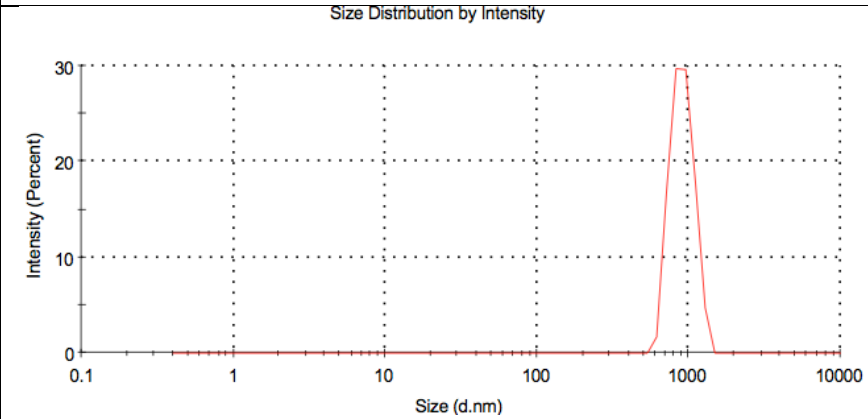
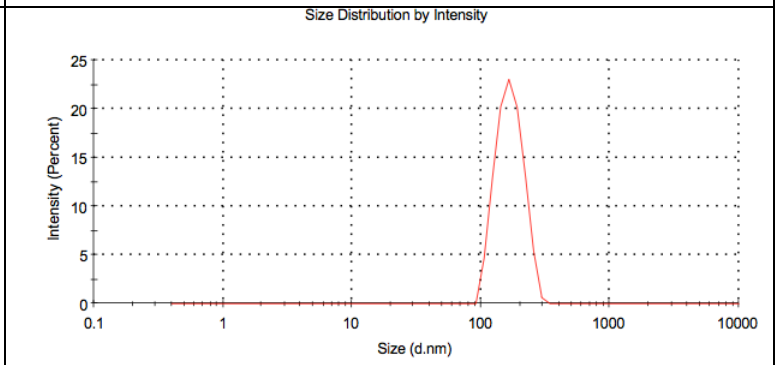
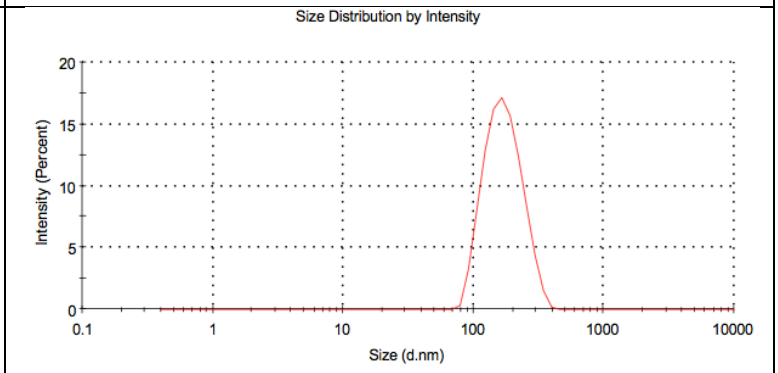
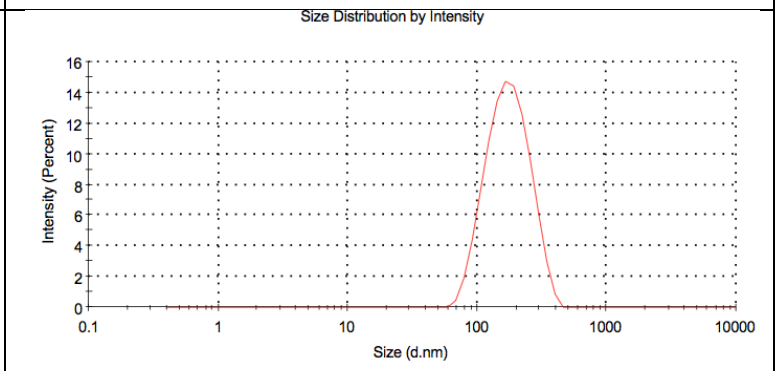
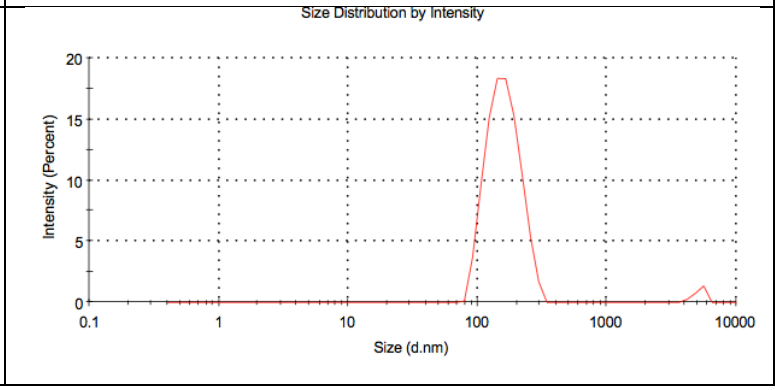
L2	429.5	569.2	
L2ES	431.3	477.9	
L2S	912.7	811.8	

Table 12A: DLS results for plain conventional liposomes of type L1 and samples loaded with penicillin G

	Peak 1	Peak 2	Z-Aver. (d.nm)	
L1	169.8	-	162.0	
L1G	175.2	-	161.0	
L2	181.8	-	159.3	
L2G	162.4	5166	161.5	

Biological Assays

Ampicillin loaded bacterial liposomes

Table 13A: overview from sensitivity assay of ampicillin loaded liposomes of type D (115 µg/mL) against E. coli XL1Blue/pUC18 (Tet^R, Amp^R) and E. coli XL1Blue (Tet^R)

		$OD_{590\ nm}$						
Sample	Conc. (µg/mL)	T_0		T_{24}		Δ OD(T_{24}- T_0)		Growth
XLBlue + D_A	0	0.185	0.185	1.31	1.2	1.125	1.015	+
XLBlue + D_A	7	0.206	0.193	1.21	1.18	1.004	0.987	+
XLBlue + D_A	14	0.217	0.235	1.21	1.32	0.993	1.085	+
XLBlue + D_A	29	0.255	0.262	1.44	1.22	1.185	0.958	+
XLBlue + D_A	58	0.145		0.25		0.105		+
XLBlue + A	0	0.195	0.192	1.29	1.21	1.095	1.018	+
XLBlue + A	**7**	**0.126**	**0.103**	**0.11**	**0.11**	**-0.016**	**0.007**	**-**
XLBlue + A	14	0.088	0.088	0.09	0.1	0.002	0.012	-
XLBlue + A	29	0.082	0.079	0.09	0.09	0.008	0.011	-
XLBlue + A	58	0.078	0.077	0.09	0.09	0.012	0.013	-
XLBlue/pUC18 + D_A	0	0.174	0.175	1.21	1.14	1.036	0.965	+
XLBlue/pUC18 + D_A	7	0.184	0.215	1.22	1.41	1.036	1.195	+
XLBlue/pUC18 + D_A	14	0.212	0.216	1.18	1.19	0.968	0.974	+
XLBlue/pUC18 + D_A	29	0.254	0.243	1.2	1.2	0.946	0.957	+
XLBlue/pUC18 + D_A	58	0.216		1.39		1.174		+
XLBlue/pUC18 + A	0	0.184	0.185	1.19	1.23	1.006	1.045	+
XLBlue/pUC18 + A	7	0.177	0.172	1.14	1.18	0.963	1.008	+
XLBlue/pUC18 + A	14	0.178	0.18	1.16	1.26	0.982	1.08	+
XLBlue/pUC18 + A	29	0.179	0.181	1.17	1.24	0.991	1.059	+
XLBlue/pUC18 + A	58	0.178	0.169	1.14	1.18	0.962	1.011	+
- control	_	0.07	0.07	0.08	0.09	0.01	0.02	-
XLBlue + D	_	0.283	0.267	1.19	1.19	0.907	0.923	+
XLBlue/pUC18 + D	_	0.258	0.267	1.3	1.16	1.042	0.893	+

Table 14A: MIC values based on Table 13A

	XLBlue		XLBlue/pUC18	
Samples	D_A	Amp	D_A	Amp
MIC(µg/ml)	-	≤ 7 µg/mL	-	-

Table 15A: overview from sensitivity assay for D_A(397.3µg/mL), S_A(336.5µg/mL), P_A (399.4 µg/mL) against E. coli XL Blue (pUC 18)

		$OD_{590\ nm}$						
Sample	Concentration (µg/mL)	T_0		T_{24}		Δ OD(T_{24}- T_0)		Growth
Amp	200	0.128	0.126	1.125	1.492	0.997	1.366	+
D_A	198.65	0.133	0.132	1.426	1.509	1.293	1.377	+
P_A	168.25	0.131	0.127	1.448	1.476	1.317	1.349	+
S_A	199.7	0.133	0.133	1.221	1.292	1.088	1.159	+
Control – (LB2x +H_2O)	1:1	0.115	0.118	0.115	0.118	0	0	-
Control + (Inoculate in LB2x + H_2O)	1:1	0.124	0.124	0.765	1.182	0.641	1.058	+

Table 16A: overview from sensitivity assay for D_A(397.3µg/mL), S_A(336.5µg/mL), P_A (399.4 µg/mL) against B. subtilis 168

		$OD_{590\ nm}$			
Sample	Concentration (µg/mL)	T_0	T_{24}	Δ OD(T_{24}- T_0)	Growth
SA	29	0.128	0.2	0.072	+
SA	14.5	0.125	0.258	0.133	+
SA	7.3	0.129	0.347	0.218	+
SA	3.6	0.126	0.348	0.222	+
SA	1.8	0.127	0.286	0.159	+
SA	0.9	0.13	0.276	0.146	+
PA	14.5	0.132	0.363	0.231	+
PA	7.3	0.13	0.367	0.237	+
PA	3.6	0.13	0.3	0.17	+
PA	1.8	0.132	0.384	0.252	+
PA	0.9	0.131	0.267	0.136	+
PA	29	0.13	0.308	0.178	+
+ control		0.136	0.535	0.399	+
- control		0.118	0.119	0.001	-
A	29	0.132	0.255	0.123	+
A	14.5	0.134	0.31	0.176	+
A	7.3	0.134	0.316	0.182	+
A	3.6	0.131	0.303	0.172	+
A	1.8	0.134	0.328	0.194	+
A	0.9	0.132	0.271	0.139	+
DA	29	0.137	0.23	0.093	+
DA	14.5	0.134	0.255	0.121	+
DA	7.3	0.135	0.297	0.162	+
DA	3.6	0.131	0.3	0.169	+
DA	1.8	0.133	0.305	0.172	+
DA	0.9	0.132	0.28	0.148	+
+ control		0.133	0.862	0.729	+
- control		0.113	0.117	0.004	-

Table 17A: MIC values based on Table 15A

Samples	S_A	P_A	D_A	Amp
MIC(µg/ml)	-	-	-	-

Table 18A: overview from sensitivity assay for D_A(397.3µg/mL), S_A(336.5µg/mL), P_A (399.4 µg/mL) against B. subtilis 168

		$OD_{590\ nm}$			
Sample	Concentration (µg/mL)	T_0	T_{24}	Δ OD(T_{24}- T_0)	Growth
DA	199	0.136	0.123	-0.013	-
DA	116	0.138	0.122	-0.016	-
DA	**58**	**0.132**	**0.12**	**-0.012**	**-**
DA	29	0.13	0.221	0.091	+
PA	199	0.141	0.124	-0.017	-
PA	116	0.14	0.123	-0.017	-
PA	**58**	**0.137**	**0.117**	**-0.02**	**-**
PA	29	0.135	0.216	0.081	+
SA	199	0.142	0.128	-0.014	-
SA	116	0.14	0.125	-0.015	-
SA	**58**	**0.135**	**0.12**	**-0.015**	**-**
SA	29	0.134	0.224	0.09	+
AMP	**200**	**0.143**	**0.124**	**-0.019**	**-**
AMP	116	0.137	0.235	0.098	+
AMP	58	0.136	0.261	0.125	+
AMP	29	0.136	0.278	0.142	+
- control		0.124	0.119	-0.005	-
+control		0.133	0.302	0.169	+

Table 19A: MIC values based on Table 18A

Samples	S_A	P_A	D_A	Amp
MIC(µg/ml)	58	58	58	200

Penicillin G loaded bacterial liposomes

Table 20A: overview from sensitivity assay for D_{PG}(272 µg/mL), S_{PG}(29 µg/mL), P_{PG} (17 µg/mL) and PenG against E. coli XL Blue (Tet^R)

		$OD_{590\ nm}$			
Sample	Concentration (µg/mL)	T_0	T_{24}	Δ OD(T_{24}- T_0)	Growth
D_{PG}	58	0.105	0.117	0.012	+/-
D_{PG}	**29**	**0.102**	**0.109**	**0.007**	**+/-**
D_{PG}	14.5	0.105	0.311	0.206	+
D_{PG}	7.3	0.102	1.316	1.214	+
D_{PG}	3.6	0.102	1.2	1.098	+
D_{PG}	1.8	0.102	0.686	0.584	+
P_{PG}	7.3	0.107	0.104	-0.003	-
P_{PG}	3.6	0.106	0.106	0	-
P_{PG}	**1.8**	**0.103**	**0.112**	**0.009**	**-**
S_{PG}	14.5	0.127	0.127	0	-
S_{PG}	**7.3**	**0.113**	**0.112**	**-0.001**	**-**
S_{PG}	**3.6**	**0.105**	**0.124**	**0.019**	**+/-**
S_{PG}	1.8	0.104	0.164	0.06	+
PenG	**58**	**0.1**	**0.119**	**0.019**	**+/-**
PenG	29	0.1	0.127	0.027	+
PenG	14.5	0.102	0.452	0.35	+
PenG	7.3	0.108	1.129	1.021	+
PenG	3.6	0.105	1.414	1.309	+
PenG	1.8	0.114	0.675	0.561	+
LB2x	_	0.091	0.097	0.006	-
Inoculate	_	0.104	0.813	0.709	+

Table 21A: MIC values based on Table 20A

Samples	DPG	SPG	PPG	PenG
MIC(µg/ml)	29	3.6	≤1.8	29-58

Table 22A: overview from sensitivity assay for D_{PG}(272 µg/mL), S_{PG}(29 µg/mL), P_{PG} (17 µg/mL) and PenG against E. coli XL Blue (Tet^R)

		$OD_{590\ nm}$			
Sample	Concentration (µg/mL)	T_0	T_{24}	Δ OD(T_{24}- T_0)	Growth
D_{PG}	**29**	**0.103**	**0.109**	**0.006**	**+/-**
D_{PG}	14.5	0.104	1.068	0.964	+
D_{PG}	7.3	0.104	0.897	0.793	+
D_{PG}	3.6	0.103	1.13	1.027	+
D_{PG}	1.8	0.104	0.818	0.714	+
D_{PG}	0.9	0.113	0.752	0.639	+
P_{PG}	7.3	0.106	0.107	0.001	-
P_{PG}	3.6	0.106	0.107	0.001	-
P_{PG}	**1.8**	**0.111**	**0.124**	**0.013**	**+/-**
P_{PG}	0.9	0.106	0.414	0.308	+
S_{PG}	14.5	0.152	0.137	-0.015	-
S_{PG}	**7.3**	**0.119**	**0.117**	**-0.002**	**-**
S_{PG}	**3.6**	**0.111**	**0.126**	**0.015**	**+/-**
S_{PG}	1.8	0.109	0.384	0.275	+
S_{PG}	0.9	0.108	1.432	1.324	+
PenG	29	0.106	0.234	0.128	+
PenG	14.5	0.105	0.457	0.352	+
PenG	7.3	0.109	0.691	0.582	+
PenG	3.6	0.104	0.642	0.538	+
PenG	1.8	0.103	0.772	0.669	+
PenG	0.9	0.105	0.747	0.642	+
LB2x	_	0.096	0.102	0.006	-
Inoculate	_	0.108	0.789	0.681	+

Table 23A:MIC values based on Table 22A

Samples	DPG	SPG	PPG	PenG
MIC(µg/ml)	29	3.6	1.8	>29

Table 24A: overview from sensitivity assay for D_{PG}(272 µg/mL), S_{PG}(29 µg/mL), P_{PG} (17 µg/mL) and PenG against E. coli XL Blue (Tet^R)

		$OD_{590\ nm}$			
Sample	Concentration (µg/mL)	T_0	T_{24}	Δ OD(T_{24}- T_0)	Growth
D	1:2	0.118	1.162	1.044	+
P	1:2	0.117	1.116	0.999	+
S	1:2	0.147	1.416	1.269	+
D_{PG}	136	0.128	0.104	-0.024	-
D_{PG}	**68**	**0.117**	**0.103**	**-0.014**	**-**
P_{PG}	**8.5**	**0.119**	**0.107**	**-0.012**	**-**
S_{PG}	**14.5**	**0.138**	**0.12**	**-0.018**	**-**
PG	136	0.11	0.1	-0.01	-
PG	**68**	**0.111**	**0.1**	**-0.011**	**-**
LB2x	1:2	0.103	0.097	-0.006	-
LB2x + D	1:2	0.107	0.105	-0.002	-
LB2x + P	1:2	0.114	0.102	-0.012	-
LB2x + S	1:2	0.141	0.129	-0.012	-
LB2x + D_{PG}	1:2	0.111	0.106	-0.005	-
LB2x + P_{PG}	1:2	0.105	0.1	-0.005	-
LB2x + S_{PG}	1:2	0.125	0.122	-0.003	-
Inoculate	1:2	0.11	1.11	1	+

Table 25A: MIC values based on Table 24A

Samples	DPG	SPG	PPG	PenG
MIC(µg/ml)	≤68	≤14,5	≤8,5	≤68

Penicillin G loaded conventional liposomes

Table 26A: overview from sensitivity assay for L1G (176 µg/mL); L2G (220 µg/mL) and PenG against E. coli XL Blue

		$OD_{590\ nm}$						
Sample	Conc. (µg/mL)	T_0		T_{24}		Δ OD(T_{24}- T_0)		Growth
L1	_	1.464	1.458	1.533	1.540	0.069	0.082	+
L2	_	1.315	1.439	1.517	1.439	0.202	0.088	+
L1G	88	1.158	1.12	0.84	0.82	-0.318	-0.3	-
L1G	**44**	**0.527**	**0.595**	**0.465**	**0.567**	**-0.062**	**-0.028**	**-**
L1G	22	0.422	0.415	1.362	1.404	0.94	0.989	+
L1G	11	0.272	0.31	1.435	1.437	1.163	1.127	+
L1G	5.5	0.252	0.27	1.449	1.425	1.197	1.155	+
L1G	2.7	0.183	0.211	1.376	1.444	1.193	1.233	+
L1G	1.3	0.151	0.165	1.4	1.426	1.249	1.261	+
L2G	110	0.602	0.577	0.503	0.522	-0.099	-0.055	-
L2G	**55**	**0.331**	**0.324**	**0.311**	**0.312**	**-0.02**	**-0.012**	**-**
L2G	27.5	0.228	0.227	0.383	0.388	0.155	0.161	+
L2G	13.6	0.178	0.18	0.561	1.354	0.383	1.174	+
L2G	6.7	0.152	0.152	0.866	1.375	0.714	1.223	+
L2G	3.4	0.147	0.143	1.43	1.365	1.283	1.222	+
L2G	1.7	0.141	0.139	1.32	1.38	1.179	1.241	+
PenG	**100**	**0.129**	**0.132**	**0.131**	**0.133**	**0.002**	**0.001**	**+/-**
PenG	50	0.13	0.133	0.143	0.14	0.013	0.007	+
PenG	25	0.128	0.13	0.317	0.424	0.189	0.294	+
PenG	12.5	0.132	0.13	0.577	0.568	0.445	0.438	+
PenG	6.3	0.131	0.131	0.945	1.072	0.814	0.941	+
PenG	3.1	0.136	0.131	0.899	0.789	0.763	0.658	+
PenG	1.6	0.133	0.132	1.172	1.227	1.039	1.095	+
Control + (Inoculate in LB2x + H_2O)	1:1	0.13	0.133	1.164	0.994	1.034	0.861	+
Control – ($LB2x+H_2O$)	1:1	0.122	0.121	0.13	0.128	0.008	0.007	+
Empty cell	_	0.112						

Table 27A: MIC values based on Table 26A

Samples	L1G	L2G	PenG
MIC(µg/ml)	≤44	≤ 55	≥ 100

Table 28A: Optical density of plain liposoms L1 and L2

Sample	Dilution	$OD_{590\ nm}$
L1	1:2	1.570
L1	1:4	1.541
L1	1:8	1.445
L1	1:16	1.105
L1	1:32	0.493
L1	1:64	0.329
L1	1:128	0.248
L2	1:2	1.426
L2	1:4	0.925
L2	1:8	0.459
L2	1:16	0.315
L2	1:32	0.246
L2	1:64	0.241
L2	1:128	0.229

As well, samples of DPG (58, 29, 14.5 µg/mL), SPG (7.2, 3.6, 1.8 µg/mL), PPG (3.6, 1.8, 0.9 µg/mL), PenG (58, 29, 14.5 µg/mL) showed inhibition of growth of E. Coli XL Blue at following MIC: DPG (29 µg/mL), SPG (7.2 µg/mL), PPG (3.6 µg/mL), PenG (58 µg/mL)

Summary of spread plating trials for liposomes containing penicillin G

Table 29A: Initial title at (t_0) for E. coli XL1 Blue

	Dilutions	CFU	CFU/ml	Mean value CFU/ml
Trial 1	_			$5.0x10^6$
Trial 2	10^{-2}	720	$1.4x10^6$	$1.6 x10^6$
	10^{-3}	55	$1.1x10^6$	
	10^{-4}	12	$2.4x10^6$	
Trial 3	10^{-2}	uncountable	-	$5.0 x10^6$
	10^{-3}	170	$3.4x10^6$	
	10^{-4}	33	$6.6x10^6$	

Table 30A: Final title (t_f) for liposomes containing PenG plated with E. coli XL1 Blue

	Sample	Concentration (µg/ml)	CFU	CFU/ml
Trial 1	D_{PG}	136	1	1.0 x10
	D_{PG}	68	60	$6.0 x10^2$
	P_{PG}	8,5	0	_
	S_{PG}	14,5	0	_
	PenG	136	6	6.0 x10
	PenG	68	85	$8.5 x10^2$
Trial 2	D_{PG}	58	0	_
	D_{PG}	29	5	5.0 x10
	P_{PG}	7,3	1	_
	P_{PG}	3,6	0	_
	P_{PG}	1,8	0	_
	S_{PG}	7,3	0	_
	S_{PG}	3,6	16	1.6 x102
	S_{PG}	14,5	0	_
	PenG	58	0	_
Trial 3	PenG	29	uncountable	_
	D_{PG}	29	1656-1832	~1.7x104
	P_{PG}	3,6	uncountable	
	P_{PG}	1,8	2728	~2.7 x104
	S_{PG}	7,3	0-1	
	S_{PG}	3,6	40	4.0 x102

Table 31A: determination of bactericidal effect of penicillin loaded lipomes based on Table 29A and Table 30A

	Sample	Concentration (µg/ml)	Title at t_0	Title at t_f	Ratio t_f/t_0	Bactericidal
Trial 1	D_{PG}	136	$5.0x10^6$	1.0 x10	$2x10^{-6}$	+
	D_{PG}	68		$6.0 x10^2$	$1.2x10^{-4}$	+
	P_{PG}	8,5		0	0	+
	S_{PG}	14,5		0	0	+
	PenG	136		6.0 x10	$1.2x10^{-5}$	+
	PenG	68		$8.5 x10^2$	$1.7x10^{-4}$	+
Trial 2	D_{PG}	58	$1.1x10^6$	0	0	+
	D_{PG}	29		50	$4.5x10^{-5}$	+
	P_{PG}	7,3		10	$0.9x10^{-5}$	+
	P_{PG}	3,6		0	0	+
	P_{PG}	1,8		0	0	+
	S_{PG}	7,3		0	0	+
	S_{PG}	3,6		160	$1.45x10^{-4}$	+
	S_{PG}	14,5		0	0	+
	PenG	58		0	0	+
Trial 3	PenG	29	$5.0x10^6$	uncountable	_	-
	D_{PG}	29		$1.7 x10^4$	$3.4x10^{-3}$	+/-
	P_{PG}	3,6		uncountable	_	-
	P_{PG}	1,8		2.7 x104	$5.4x10^{-3}$	+/-
	S_{PG}	7,3		0-10	$1.0x10^{-6}$	+
	S_{PG}	3,6		400	$1.0x10^{-4}$	+
(+) bactericidal when there is <0,1% ($1 x10^{-3}$) viable cells (-) not bactericidal when there is >0,1% ($1 x10^{-3}$) viable cells						

Table 32A: Lowest bactericidal concentration based on Table 31A

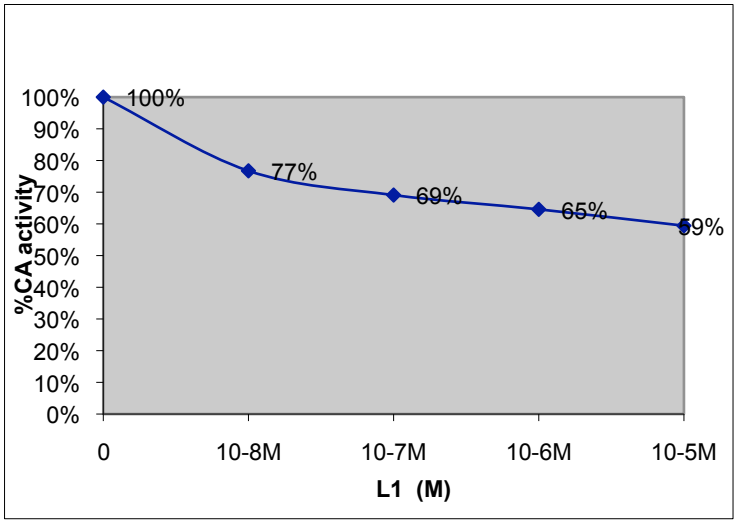
Samples	D_{PG}	S_{PG}	P_{PG}	PenG
Lowest conc. (µg/ml)	≥ 29	3.6	≥ 1.8	58

Enzymatic Assay

Table 33A:Inhibition activity on CAI by conventional liposomes of type L1 loaded with sulfanilamide (S) and ethylaminobenzenesulfonamide (ES)

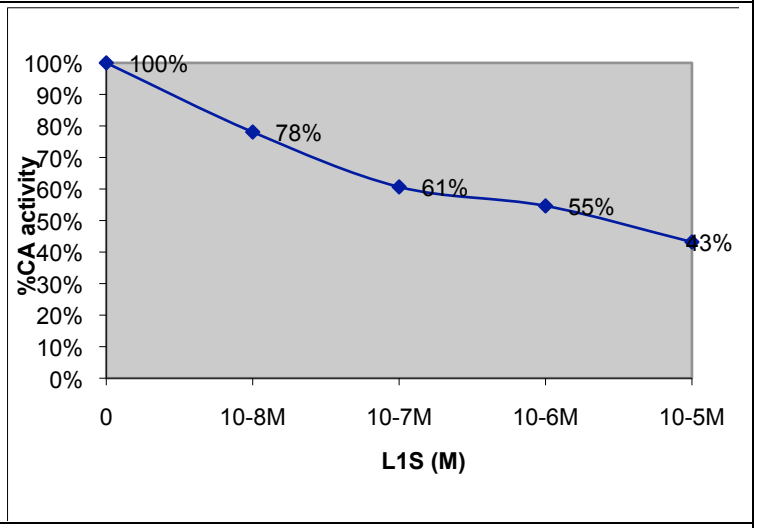
Compound	Inhibitor conc.	%Free Enzyme	% inhibition
CA I 10-6M	0	100%	0%
L1	10-8M	77%	23%
L1	10-7M	69%	31%
L1	10-6M	65%	35%
L1	10-5M	59%	41%

IC50= n.d



CA I 10-6M	0	100%	0%
L1S	10-8M	78%	22%
L1S	10-7M	61%	39%
L1S	10-6M	55%	45%
L1S	10-5M	43%	23%

IC50=4.65111E-06



CA I 10-6M	0	100%	0%
L1ES	10-8M	75%	25%
L1ES	10-7M	59%	41%
L1ES	10-6M	53%	47%
L1ES	10-5M	41%	59%

IC50=3.11602E-06

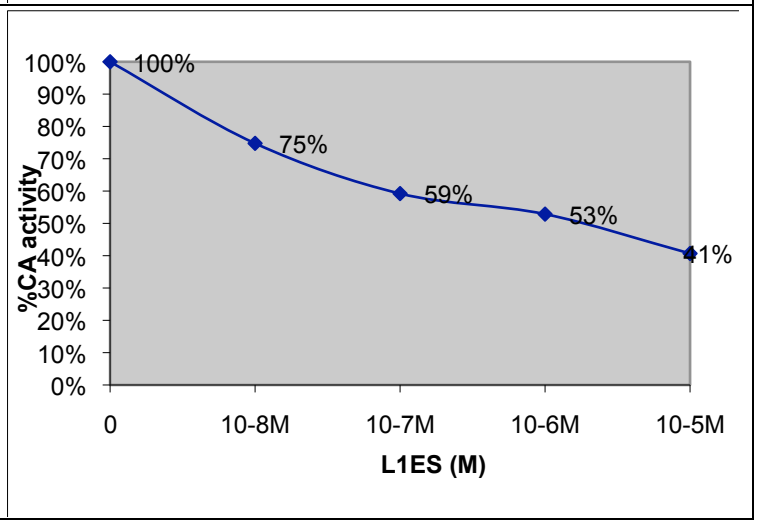
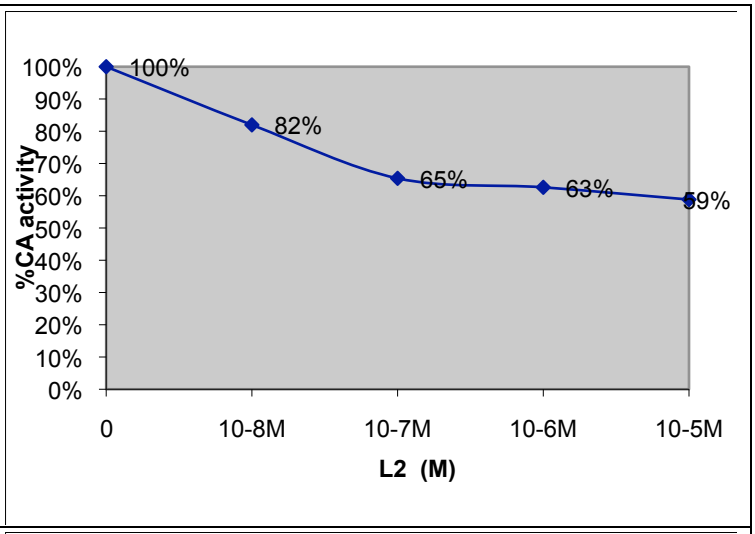


Table 34A:Inhibition activity on CAI by conventional liposomes of type L2 loaded with sulfanilamide (S) and ethylaminobenzenesulfonamide (ES)

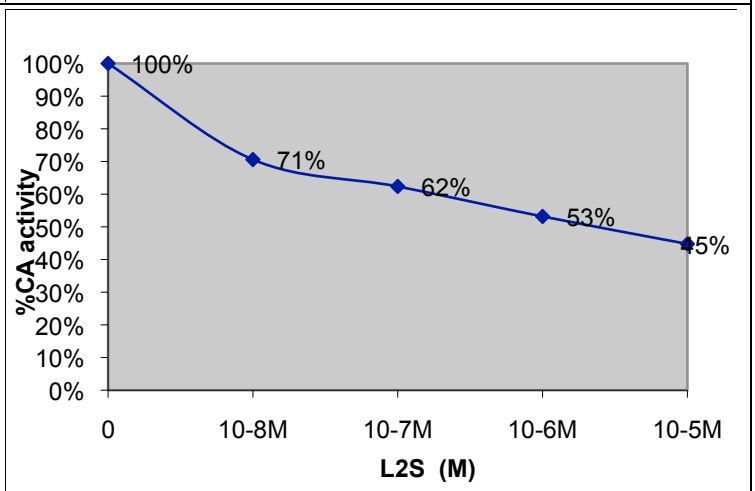
CA I 10-6M	0	100%	0%
L2	10-8M	82%	18%
L2	10-7M	65%	35%
L2	10-6M	63%	37%
L2	10-5M	59%	41%

IC50 = n.d



CA I 10-6M	0	100%	0%
L2S	10-8M	71%	29%
L2S	10-7M	62%	38%
L2S	10-6M	53%	47%
L2S	10-5M	45%	55%

IC50 = 4.65111E-06



CA I 10-6M	0	100%	0%
L2ES	10-8M	78%	22%
L2ES	10-7M	61%	39%
L2ES	10-6M	54%	46%
L2ES	10-5M	39%	61%

IC50 = 3.22208E-06

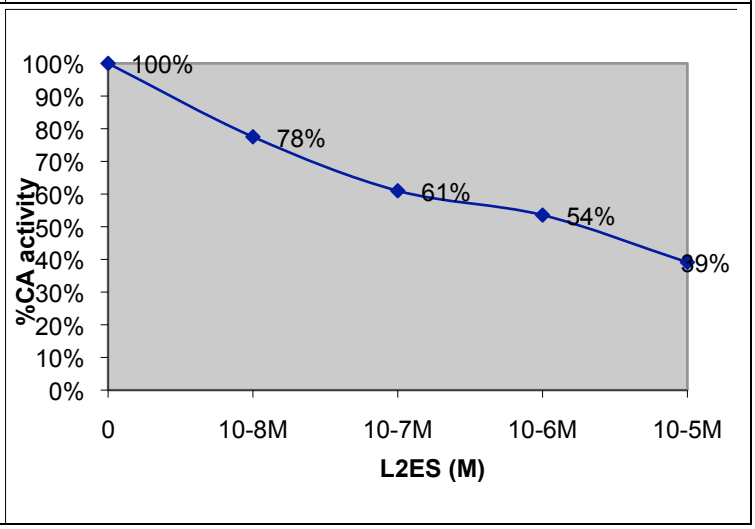
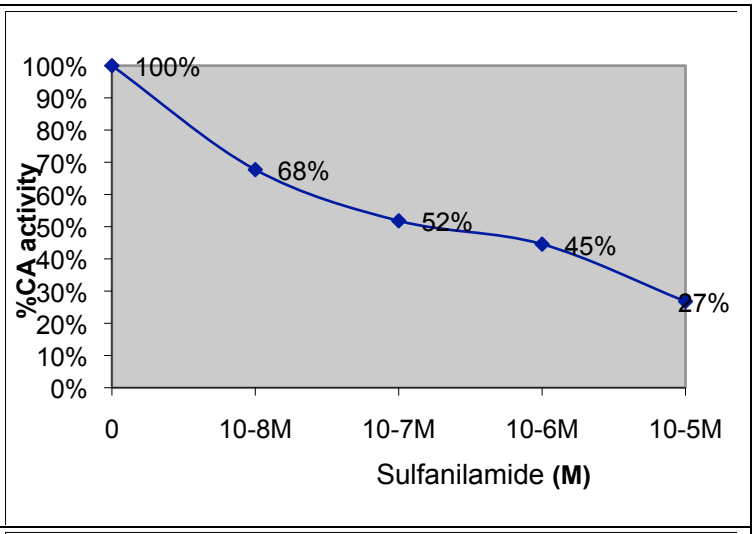


Table 35A:Inhibition activity on CAI by free sulfanilamide (S), ethylaminobenzenesulfonamide (ES) and AAZ

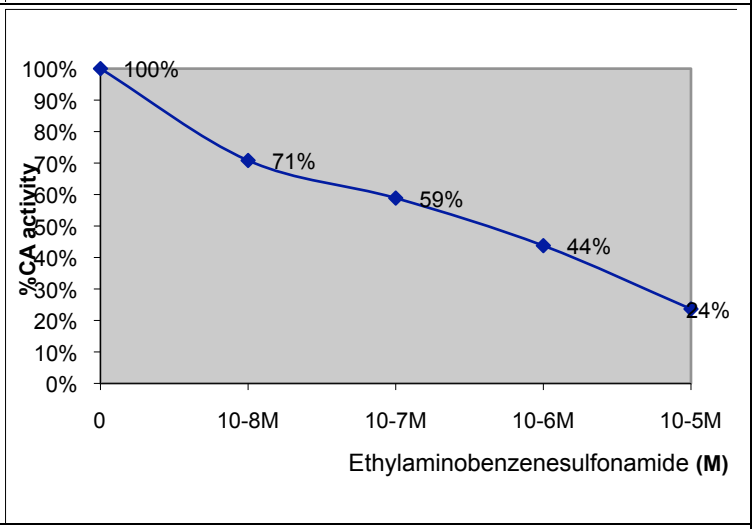
CA I 10-6M	0	100%	0%
SA	10-8M	68%	32%
SA	10-7M	52%	48%
SA	10-6M	45%	55%
SA	10-5M	27%	73%

IC50 = 3.26392E-07



CA I 10-6M	0	100%	0%
EA	10-8M	71%	29%
EA	10-7M	59%	41%
EA	10-6M	44%	56%
EA	10-5M	24%	76%

IC50 = 6.28561E-07



CA I 10-6M	0	100%	0%
AAZ	10-8M	69%	31%
AAZ	10-7M	49%	51%
AAZ	10-6M	27%	73%
AAZ	10-5M	10%	90%

IC50 = 9.49011E-08

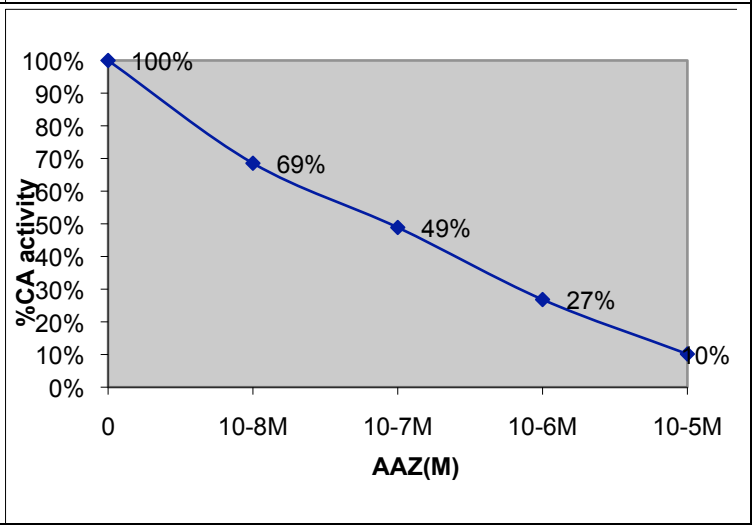
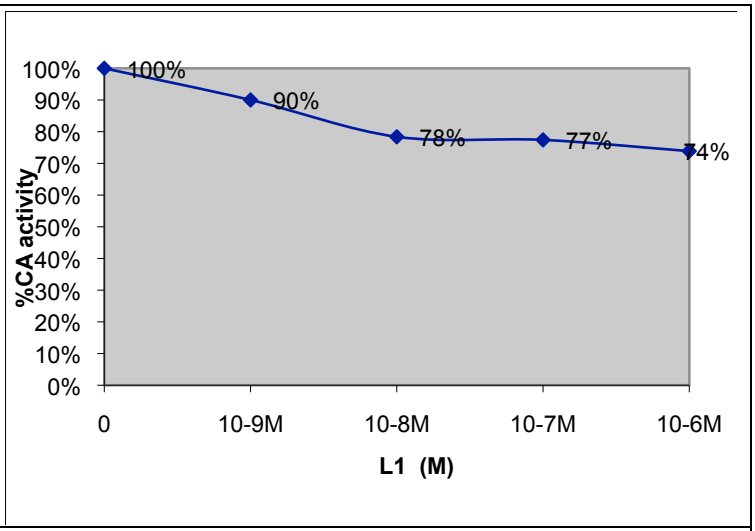


Table 36A:Inhibition activity on CAII by conventional liposomes of type L1 loaded with sulfanilamide (S) and ethylaminobenzenesulfonamide (ES)

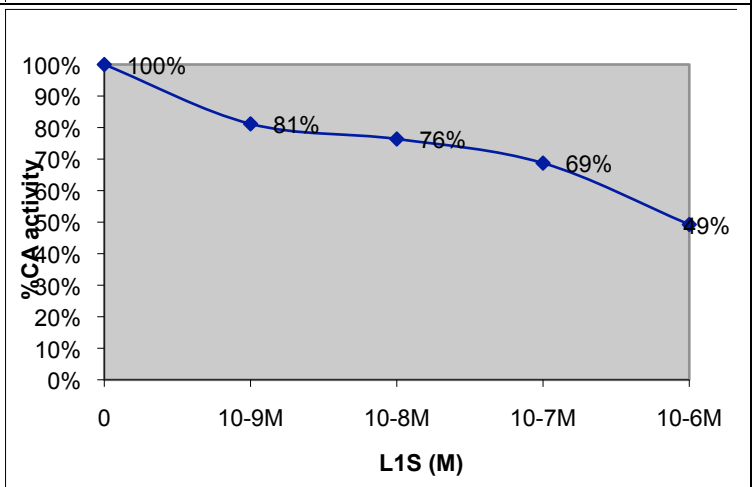
CA II 10-7M	0	100%	0%
L1	10-9M	90%	10%
L1	10-8M	78%	22%
L1	10-7M	77%	23%
L1	10-6M	74%	26%

IC50 = n.d



CA II 10-7M	0	100%	0%
L1S	10-9M	81%	19%
L1S	10-8M	76%	24%
L1S	10-7M	69%	31%
L1S	10-6M	49%	12%

IC50 = 9.65506E-07



CA II 10-7M	0	100%	0%
L1ES	10-9M	78%	22%
L1ES	10-8M	63%	37%
L1ES	10-7M	41%	59%
L1ES	10-6M	34%	66%

IC50 = 6.29905E-08

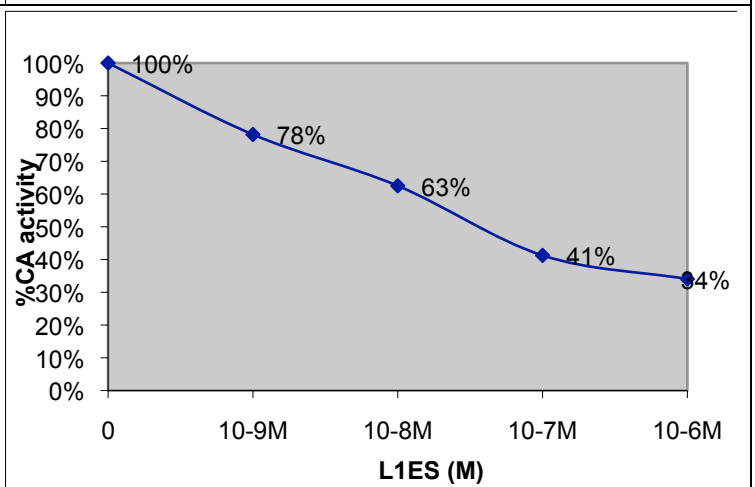
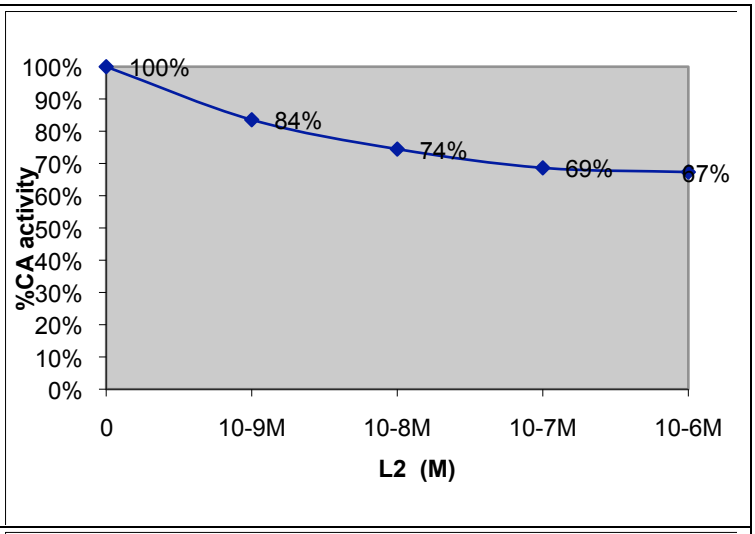


Table 37A:Inhibition activity on CAII by conventional liposomes of type L2 loaded with sulfanilamide (S) and ethylaminobenzenesulfonamide (ES)

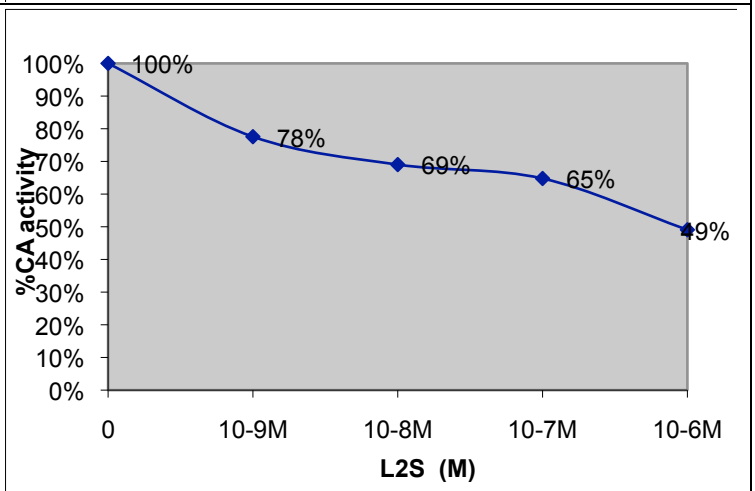
CA II 10-7M	0	100%	0%
L2	10-9M	84%	16%
L2	10-8M	74%	26%
L2	10-7M	69%	31%
L2	10-6M	67%	33%

IC50 = n.d.



CA II 10-7M	0	100%	0%
L2S	10-9M	78%	22%
L2S	10-8M	69%	31%
L2S	10-7M	65%	35%
L2S	10-6M	49%	51%

IC50 = 9.47022E-07



CA II 10-7M	0	100%	0%
L2ES	10-9M	72%	28%
L2ES	10-8M	68%	32%
L2ES	10-7M	58%	42%
L2ES	10-6M	49%	51%

IC50 = 8.93421E-07

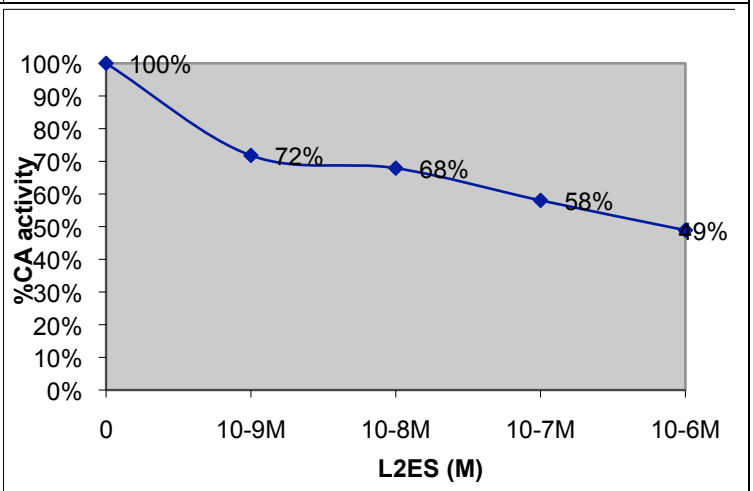
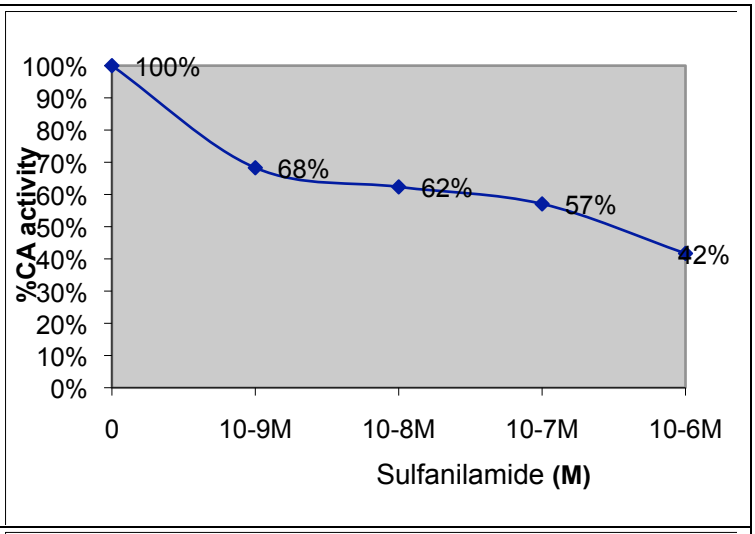


Table 38A:Inhibition activity on CAII by sulfanilamide (S), ethylaminobenzenesulfonamide (ES) and AAZ

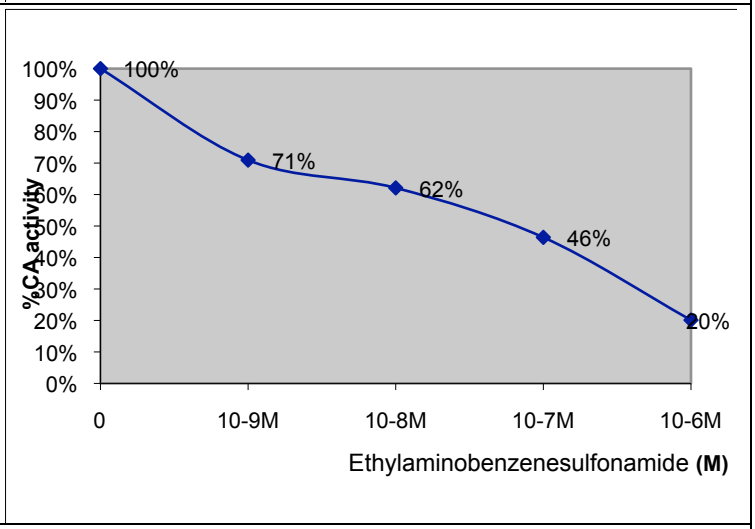
CA II 10-7M	0	100%	0%
SA	10-9M	68%	32%
SA	10-8M	62%	38%
SA	10-7M	57%	43%
SA	10-6M	42%	58%

IC50 = 5.13876E-07



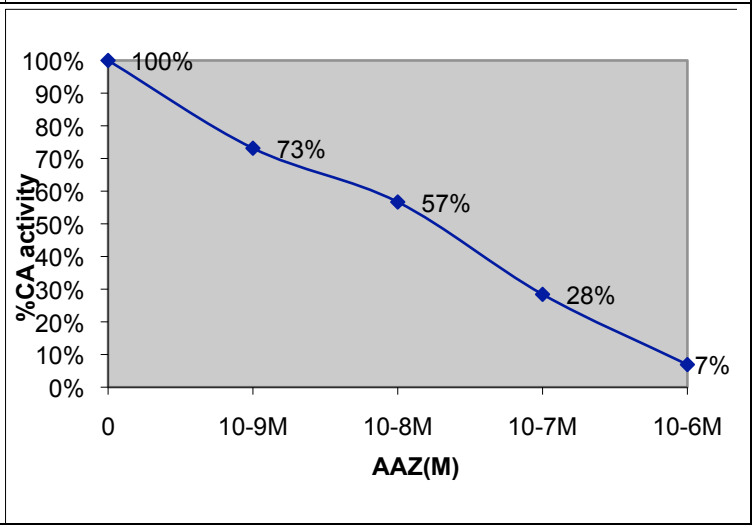
CA II 10-7M	0	100%	0%
ES	10-9M	71%	29%
ES	10-8M	62%	38%
ES	10-7M	46%	54%
ES	10-6M	20%	80%

IC50 = 7.93523E-08



CA II 10-7M	0	100%	0%
AAZ	10-9M	73%	27%
AAZ	10-8M	57%	43%
AAZ	10-7M	28%	72%
AAZ	10-6M	7%	93%

IC50 = 3.12643E-08



Small-angle X-ray scattering (SAXS)

Table 39A:SAXS results for plain liposomes of type D and liposomes loaded with ampicillin and penicillin G

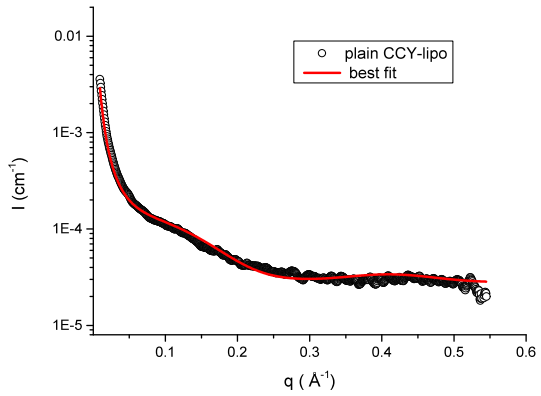
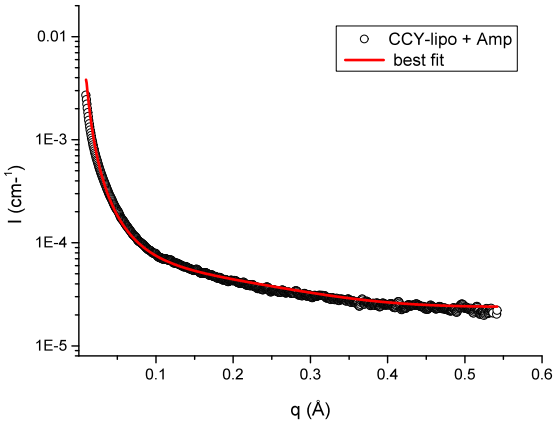
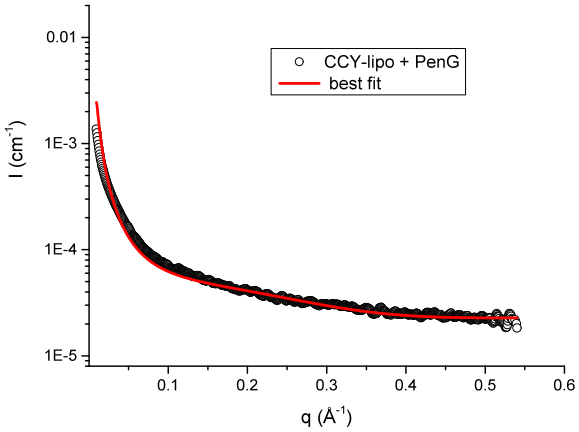
(1) Plain D	
(2) D + Amp	
(3) D + PenG	

Table 40A:SAXS results for plain liposomes of type P and S and liposomes loaded with ampicillin and penicillin G

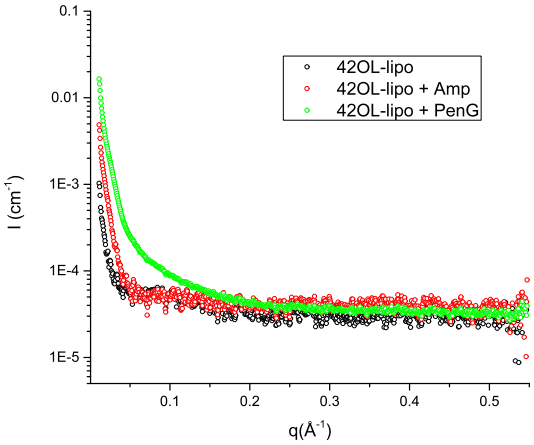
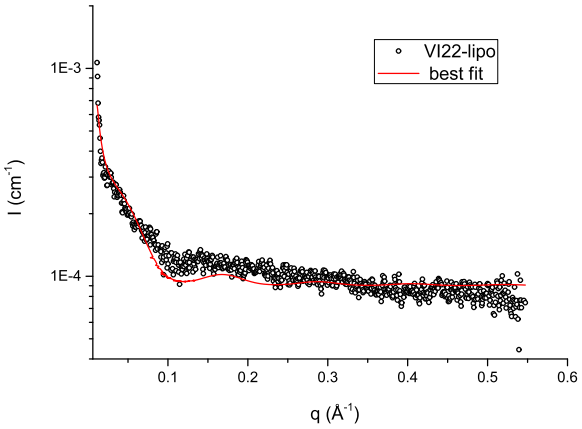
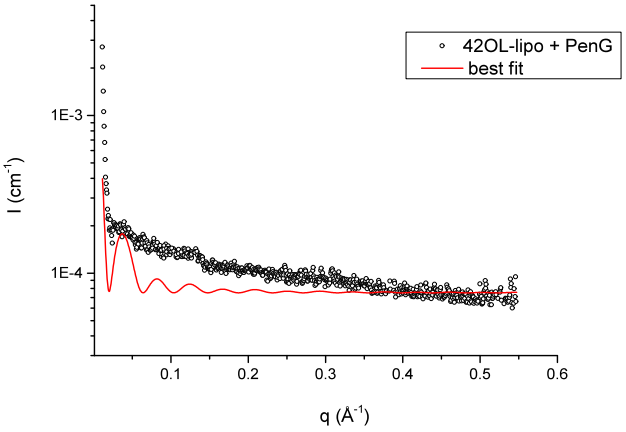
(1) Plain P Plain P + Amp Plain P + PenG	
(2) Plain S	
(3) S +PenG	

Table 41A:SAXT results for plain liposomes of type D, S, P and liposomes loaded with ampicillin and penicillin G

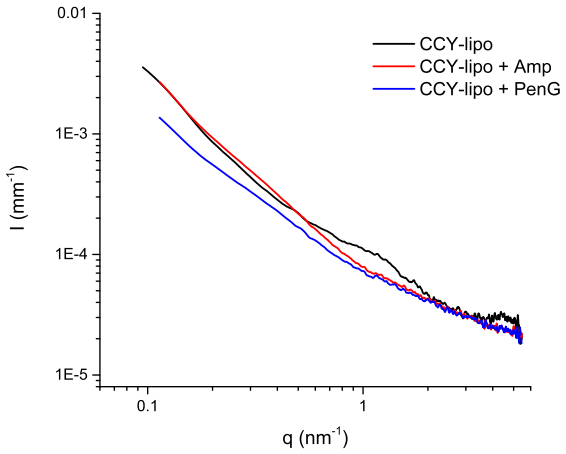
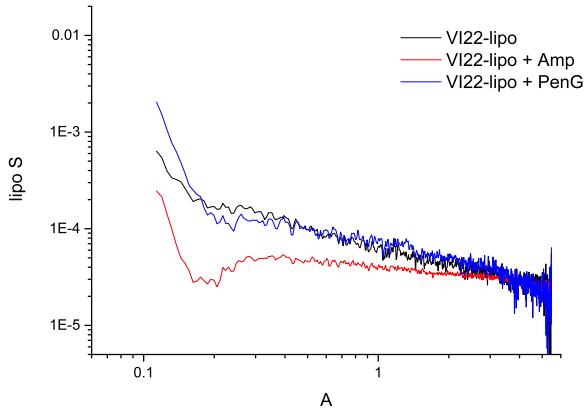
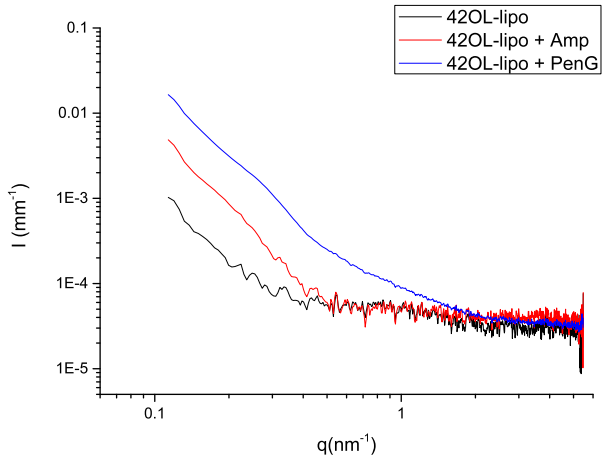
(1) D D_A D_{PG}	
(2) S S_A S_{PG}	
(3) P P_A P_{PG}	

Table 42A: Summary basic information about bacterial strains used in current work

Strain	**CCY0110**	**VI22**	**42OL**
Domain	Bacteria	Bacteria	Bacteria
Phylum	Cyanobacteria	Cyanobacteria	Proteobacteria
Subsection		I	α-2
Class	Oscillatoriophycideae		Alpha Proteobacteria
Order	Chroococcales	Chroococcales	Rhizobiales
Family	Cyanothece	Cyanothece	Bradyrhizobiaceae
Genus	Cyanothece	Cyanothece	Rhodopseudomonas
Species	Cyanothece sp.	Cyanothece sp.	Rhodopseudomonas palustris
Culture Medium	ASN-III	AMA	Modified Ormerod medium (Vincenzini et al. 1981) = RPN medium (Bianchi et al.2010)
Isolated by	U.S. Department of Energy (DOE) – Joint Genome Institute (JGI)	M.C. Margheri < M. Sarrica (1995/96)	Centro di Studio dei Microrganismi Autotrofi, CNR Firenze
References	Rita Mota et al. (2012)	De Philippis et al. 1998; M. Sarrica 1996	Vincenzini et al. 1981; Carlozzi and Sacchi (2001); Adessi et al. 2012
Sampling site	Coast of Zanzibar	Coast of Villasimius, Sardinia, Italy	Castiglion Fiorentino (AR), Italy
Habitat	Marine	Hypersaline	Sugar Refinery Wastewaters
Metabolic Features	photoautotrophic	photoautotrophic	photoautotrophic or photoheterotrophic (anaerobic conditions), chemoheterotrophic or chemoautotrophic (aerobic conditions)
General morphological Features	gram-negative (?)	gram-negative (?)	gram-negative, purple non-sulfur (PNS)
Cell wall layers	contact with cellular membrane: electrontransparent; intermediate: peptidoglycan (murein); outer membrane: lipopolysaccaridic (2 layers)	contact with cellular membrane: electrontransparent; intermediate: peptidoglycan (murein); outer membrane: lipopolysaccaridic (2 layers)	*contact with cellular membrane:electrontransparent; intermediate: peptidoglycan (murein);* outer membrane: lipopolysaccharidic
Glycocalix	glicoproteins	glicoproteins	not present
Exopolysaccaride (EPS) production	mucillage	capsule	not reported
Flagella	_	_	subpolar flagella