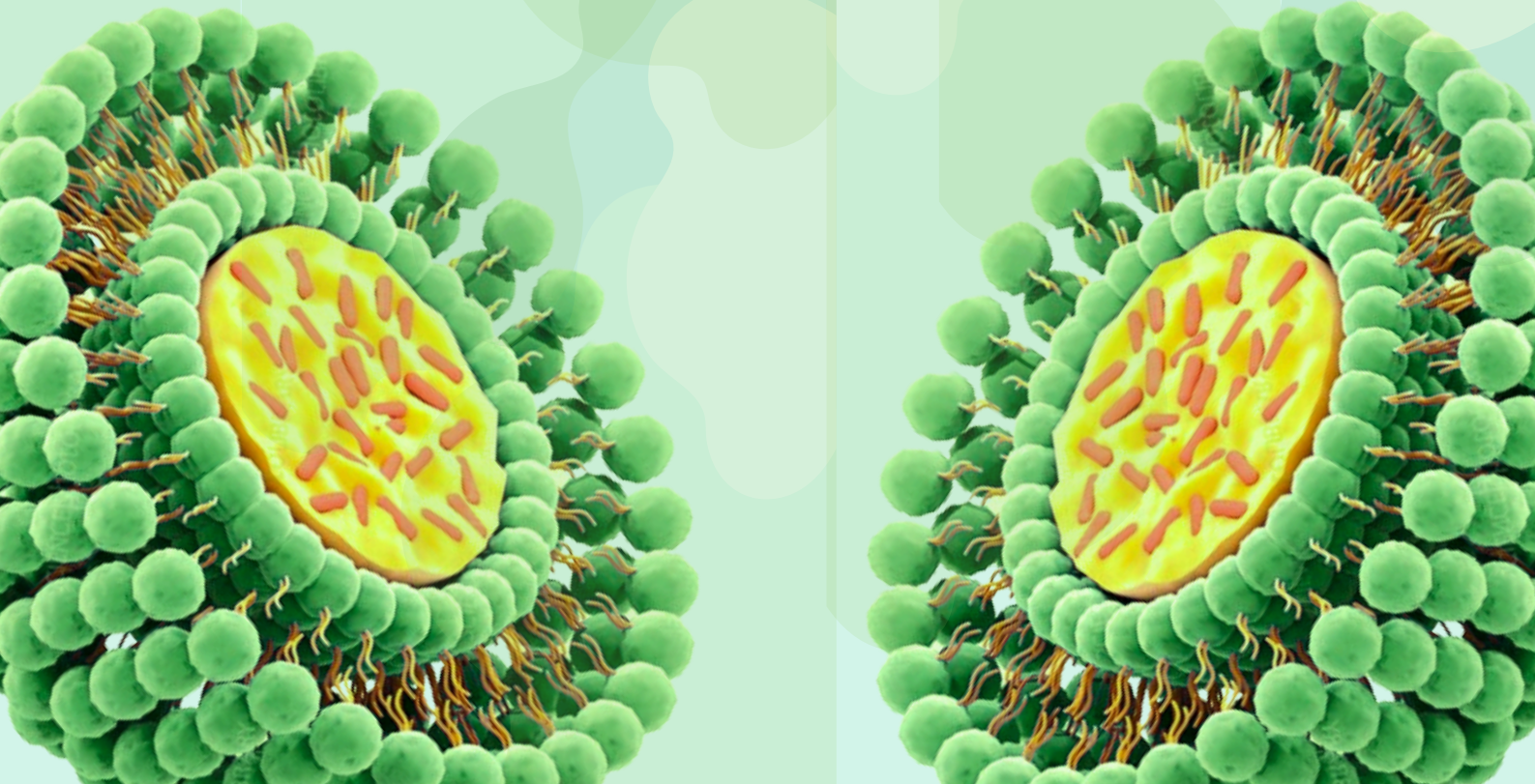
LIPID DELIVERY SYSTEM HANDBOOK

by iGEM teams from

Bielefeld-CeBiTec, Linkoping, Patras, Radboud-University and TERMOSZ-Selye-HUN



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LIPID NANOPARTICES BY BIELEFELD-CEBITEC TEAM

GEM
BIELEFELD
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LIPID NANOPARTICLES (LNPS)

Lipid nanoparticles (LNPs) are an advanced delivery system designed to transport therapeutic molecules like RNA, DNA or proteins into the cells. These nanoparticles are tiny spheres made of lipids that form a protective shell around the cargo. The size of LNPs typically ranges from 50 to 200 nm in diameter, making them incredibly small - about 1,000 times thinner than a human hair [1].

LNPs come in various forms to suit different therapeutic needs. For example, cationic LNPs are ideal for gene delivery due to their positive charge, while neutral and anionic LNPs offer reduced toxicity and unwanted interactions [2].

In many modern therapies, particularly mRNA vaccines, the fragile nature of mRNA requires protection from degradation by enzymes like RNases. LNPs effectively encapsulate and safeguard the mRNA to reach the target cells intact [3]. LNPs are acclaimed for their high drug-loading capacities, which greatly enhance their therapeutic effectiveness [4].

Once inside the body, LNPs are taken up by cells through endocytosis, but the real challenge is ensuring successful endosomal escape (Figure 1). Ideally, the LNPs release their payload into the cytoplasm after escaping from endosomes. If this escape process is inefficient, the mRNA can be degraded by lysosomes, which is a significant challenge for mRNA vaccines and therapies [5].

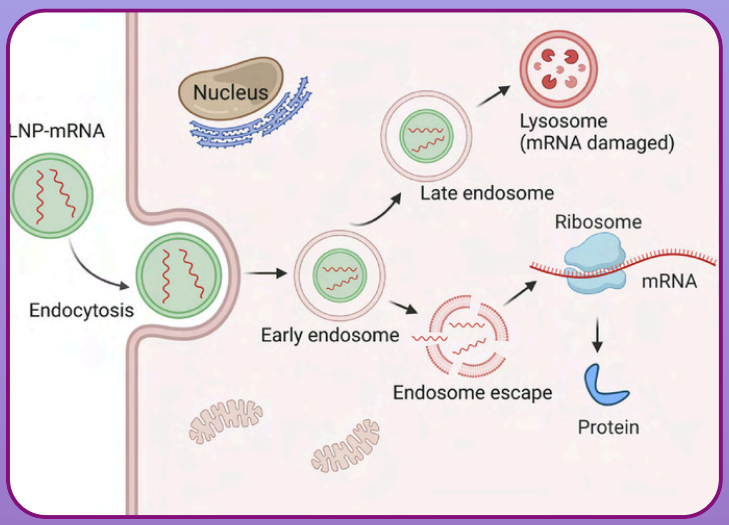


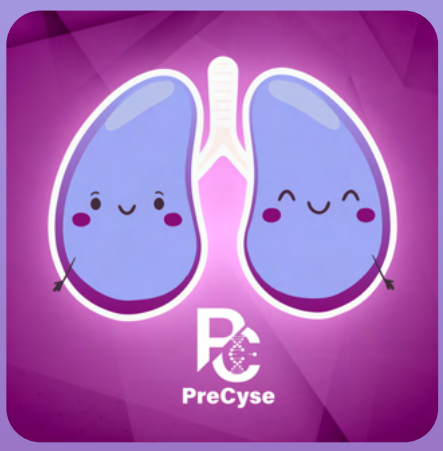
Figure 1. LNP endocytosis and RNA endosomal escape process. Picture taken from reference [5].

A key innovation in LNP technology involves pH-sensitive cationizable lipids, which remain neutral at physiological pH but become positively charged in the acidic environment of endosomes, aiding in membrane disruption and enhancing cargo release into the cytoplasm [5]. Surface modifications further improve LNP targeting. By incorporating specific lipids or modifying the surface with antibodies or charged ligands, LNPs can be engineered to deliver their payload to specific organs like the lungs or spleen [6]. Additionally, LNPs can be engineered with targeting ligands or antibodies to precisely direct their payload to specific cell types, further enhancing their therapeutic efficacy [7]. This versatility in design is essential for optimizing the delivery and effectiveness of LNP-based therapies.

LNPS AS PART OF OUR PROJECT - PRECYSE

Our next-generation prime editing technology, PreCyse, is designed to revolutionize gene therapy for cystic fibrosis by targeting the most common mutation, ΔF508, in the CFTR gene. We are optimizing lipid nanoparticles to ensure efficient and cell-specific delivery of therapeutic mRNA. This approach addresses the limitations of current treatments, which often lack precision, speed, and long-lasting effectiveness, leaving many patients with limited options for sustained improvement. PreCyse aims to offer a groundbreaking solution by providing a treatment that ensures longer-lasting effects, reducing the frequency of application, and making it a more accessible and patient-friendly option.

PreCyse
Precision with every breath



Our lung-specific formulation is based on Selective Organ-Targeting (SORT) LNPs, utilizing a combination of lipids to enhance stability, targeting, and delivery efficiency [8]. With the lung-specific nanoparticle formulation combined with a spray-drying technique to enhance stability and longlivety, we offer a versatile and efficient method for delivering mRNA therapeutics to the lung with our LNP called AirBuddy (Figure 2). The effective delivery of the prime editing complex to lung epithelial cells via inhalation of our spray-dried LNP is a crucial point in our project (Figure 3) [9].

While future enhancements, such as antibody conjugation for targeting CFTR-expressing lung cells, remain possible, our current focus is on achieving precision and safety in delivery. In the context of pulmonary delivery, where the goal is to target the lungs, the size and properties of the LNPs are crucial. Particles smaller than 2 µm are particularly effective for reaching the alveolar regions of the lungs [8]. For instance, chitosan-based nanoparticles have been explored for their ability to adhere to mucus and enhance drug delivery through the respiratory tract. These nanoparticles can penetrate through the mucus layer to reach the lung tissues more effectively [10].

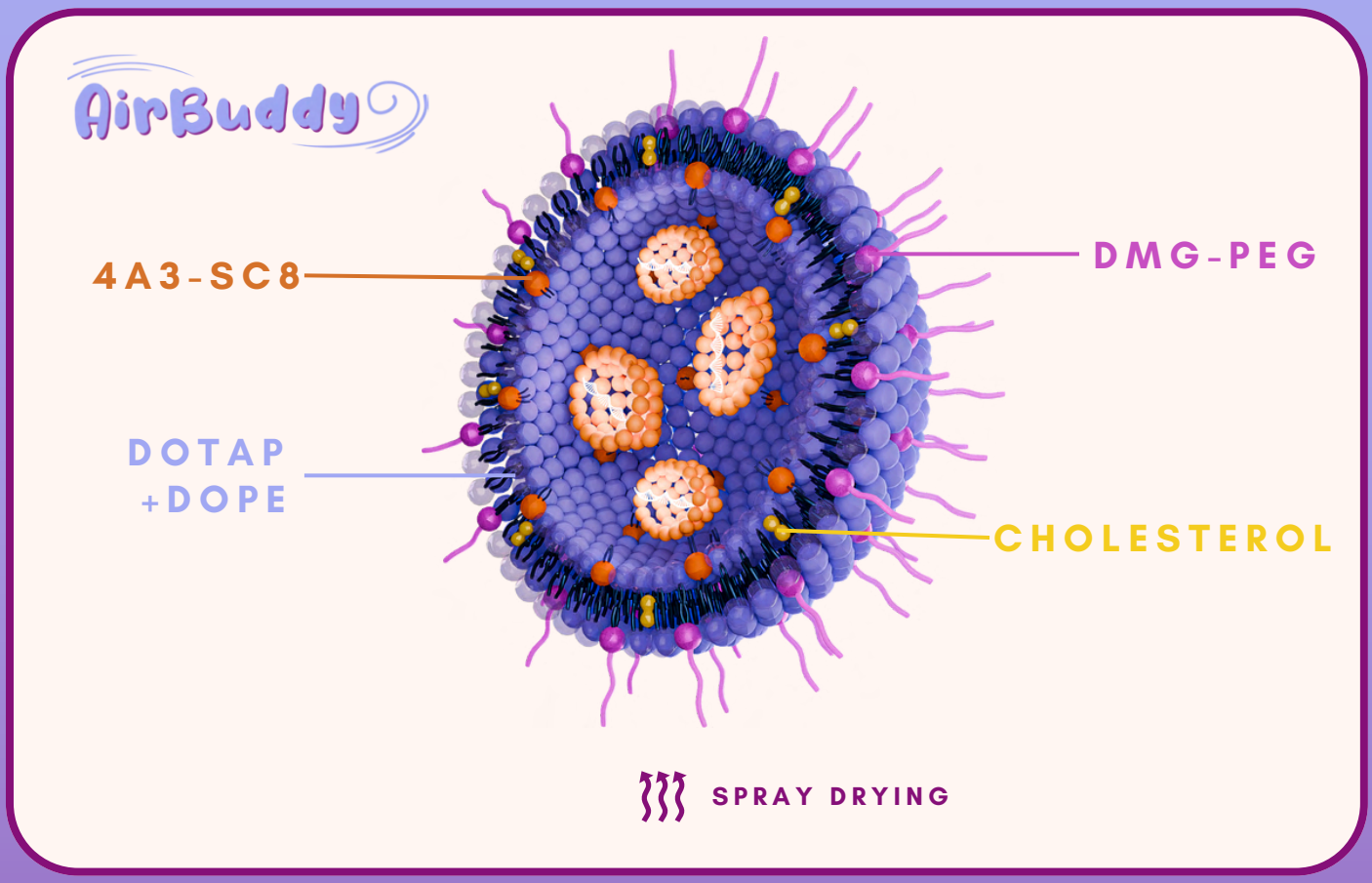


Figure 2. Schematic illustration of AirBuddy.

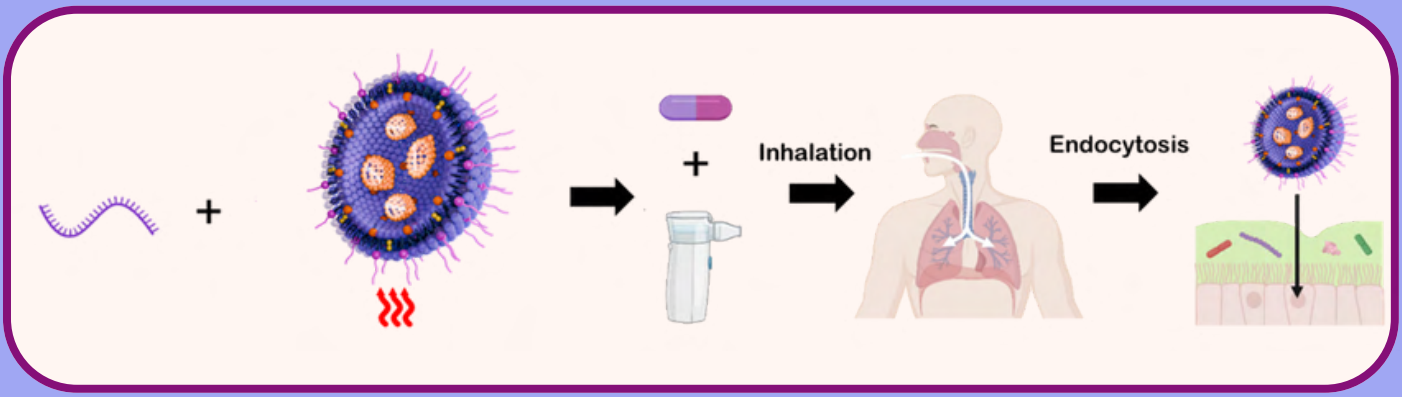


Figure 3. Spray-dried AirBuddy is inhaled, enabling cellular uptake of PrimeGuide via endocytosis.

Components

Ionizable lipid

The primary ingredient, **4A3-SC8**, is an ionizable cationic lipid that forms the core of the LNP. Ionizable cationic lipids become positively charged in acidic environments, such as when a pH change occurs for example in acidic buffers or in the endosome. This allows them to bind to negatively charged nucleic acids and form protective capsules around it. In the endosome these lipids facilitate endosomal escape through electrostatic interactions between the LNPs and the endosomal or cellular membranes.

Helper lipids

DOTAP (Dioleoyltrimethyl-ammonium propane) is a cationic lipid that makes up 50 % of the total molar lipid ratio. It plays a crucial role in binding to the negatively charged surface of lung epithelial cells. This enhances transfection efficiency and helps make the LNP formulation more lung-specific, improving targeted delivery.

The neutral helper lipid **DOPE** (Dioleoylphosphatidylethanolamine) enhances endosomal escape by fusing with the endosomal membrane and improves transfection efficiency.

Sterol

Cholesterol, is an important cationic lipid, providing structural stability, fluidity and permeability to the LNPs, thereby improving their overall transfection efficiency.

PEGylated lipids

DMG-PEG (Dimyristoylglycerin-polyethyleneglycol) is an important component by improving the LNP stabiltity and preventing aggregation of the LNPs.

TIPS AND TRICKS

- To maintain the optimal environment for LNP formation, a citric acid buffer (pH 4) is used, which is particularly important for ionizable lipids like 4A3-SC8 to function properly during mRNA encapsulation.
- When all components are combined, the clear lipid solution should become cloudy and slightly bluish (Figure 4). This is a first indicator that LNPs have formed :) This happens because the LNPs are so small that they scatter light, especially blue light, in a phenomenon called the Tyndall effect - kind of like why the sky looks blue!

Figure 4. LNP formation leading to cloudy and bluish appearance of the solution (the four left tubes, the right one holds water for comparison).

COST ESTIMATION

The following table outlines the required components and quantities for assembling the LNP, along with the suppliers they were ordered from (Table 1). The cost estimate may vary depending on the quantity and manufacturer from which the LNP components are purchased. The quantities in the table are probably more than enough for an igem project, but should be calculated individually for your own project.

Table 1. Cost estimation for Airbuddy.

Compound	Total amount	Manifacturer	Price [€]
4A3-SL8	50 mg	Biozol	319
DOPE	25 mg	Sigma Aldrich	87.70
Cholesterol	25 mg	Biomol	130
DOTAP	25 mg	Sigma Aldrich	203
DMG-PEG 2000	1 g	Sigma Aldrich	295
Citrate Buffer 0.5 M, pH 4.0	250 ml	ThermoFisher	73.35
Pur-A-Lyzer Midi Dialysis Kit	50 midi dialysis tubes	Sigma Aldrich	269

TIME ESTIMATION

Table 2. Time estimation for producing Airbuddy.

Experimental step	Duration
Prepare lipids	1 h
Prepare mRNA	10 min
Assemble LNPs	2 h

FUN FACT

Lipid nanoparticles are so small that you could fit over a billion of them on the head of a pin! Despite their tiny size, they're capable of carrying and delivering powerful genetic therapies, like mRNA, directly into cells, proving that big things really do come in small packages.

OUR PROTOCOL

1.Prepare lipids

1) Weigh 30 mg of 4A3-SC8 into a tared tube. Then add 200 µL of pure ethanol to dissolve 4A3-SC8 to reach a concentration of 150 mg/mL. The solution should appear clear.

2) Weigh 10 mg of DOPE into a tared tube. Add 1.0 mL of pure ethanol to dissolve DOPE and reach a concentration of 10 mg/mL. The solution should appear clear.
Tipp: DOPE has limited solubility in ethanol. Slightly heat the solution to increase solubility.

3) Weigh 10 mg of cholesterol into a tared tube. Add 1.0 mL of pure ethanol to dissolve cholesterol and reach a concentration of 10 mg/mL. The solution should appear clear.

4) Weigh 10 mg of DMG-PEG into a tared tube. Add 1.0 mL of pure ethanol to dissolve DMG-PEG and reach a concentration of 10 mg/mL. The solution should appear clear.

2.Prepare mRNA

1) Combine in a RNase-free 1.5 mL tube 52 µL of citrate buffer (10 mM, pH 4) and 8 µL of mRNA (1.0 mg/mL). Mix well.

Tipp: mRNA is highly susceptible to degradation by ubiquitouse RNases. Use RNase-free reagents, pipette tips and tubes. Clean the working bench properly beforehand and always work fast but careful with RNA.

3.Assemble LNPs

1) To prepare a complete lipid mix solution: Combine 6.7 µL of the 4A3-SC8 solution, 50.7 µL of the DOPE solution, 52.7 µL of the cholesterol solution, 34.2 µL of the DMG-PEG solution and 40.0 µL of the DOTAP solution and mix the solutions well together.

2) Add 19.44 µL of the complete lipid mix solution into a RNase-free 1.5 mL tube.

3) Add 0.56 µL ethanol into the above tube. Mix well.

4) Pipette 60 µL of the mRNA buffer solution quickly into the lipid mix ethanol solution, followed immediately by pipetting up and down rapidly for 20–30 s.
Tipp: Pipette up and down immediately after mixing the two solutions to prepare uniform LNPs. Slow mixing can yield poorly formed LNPs with reduced activity.

5) Incubate the resulting solution at RT for 15 min.

6) Immediately dialyze the LNP solution using the Pur-A-Lyzer Midi 3500 Dialysis tube against 1× PBS for at least 1 h to remove the ethanol and acidic buffers.

7) After dialysis, transfer the solution to an RNase-free 1.5 mL tube and measure the volume.

8) Compensate the solution with 1× PBS to reach a final volume of 800 µL.

Storage

The final LNP solution can be stored at 4 °C for a few days. It is recommended to use the formulated LNPs as soon as possible to maintain consistent results. Storage at RT is not recommended. Storage at freezing temperatures is also not recommended unless optimized cryoprotectants are used.

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LIPOSOMES

By the Linkoping Team

LIPOSOMES

Liposomes are spherical vesicles consisting of phospholipids often arranged in a bilayer. Both sides of the bilayer are hydrophilic, while the middle is hydrophobic [1]. The size of the liposomes usually vary from 50-500 nm in diameter and the liposomes can be both unilamellar (one membrane) or multilamellar (more than one membrane). The liposomes may also contain other lipid chains, such as cholesterol. High biocompatibility, biodegradability, and low immunogenicity are some reasons why liposomes are commonly used nanocarriers. Liposomes are also shown to enhance drug solubility and controlled distribution. Another feature is its capacity for surface modifications making them suitable for targeted, prolonged and sustained release [2].

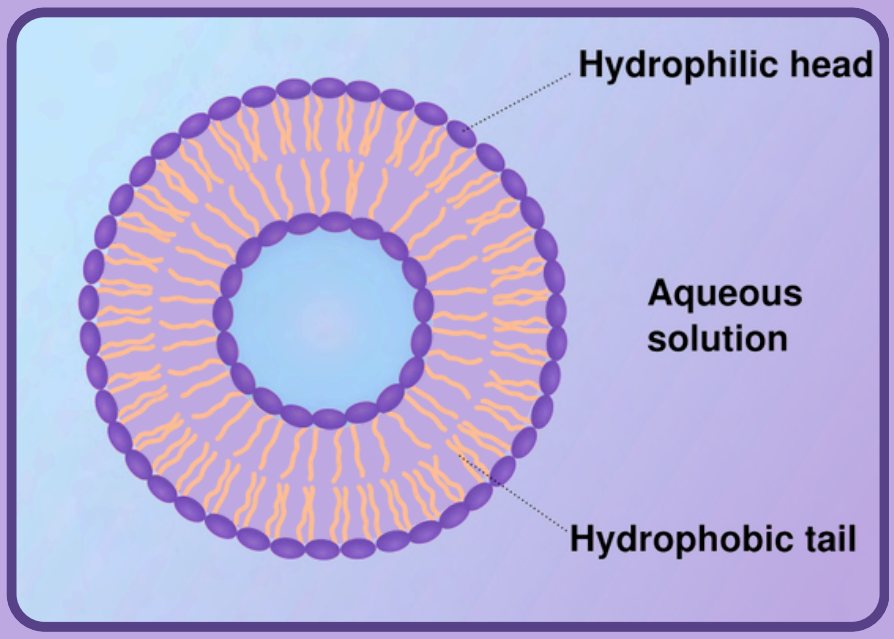
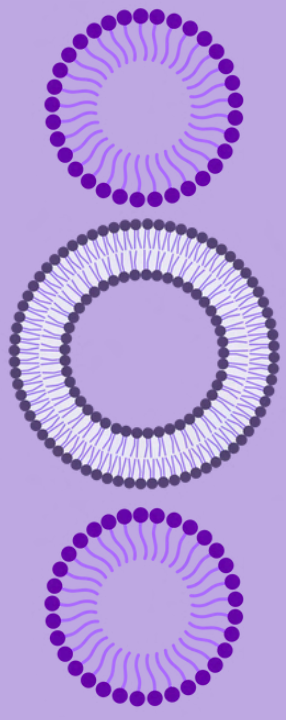


Figure 1: Liposome structure [2]



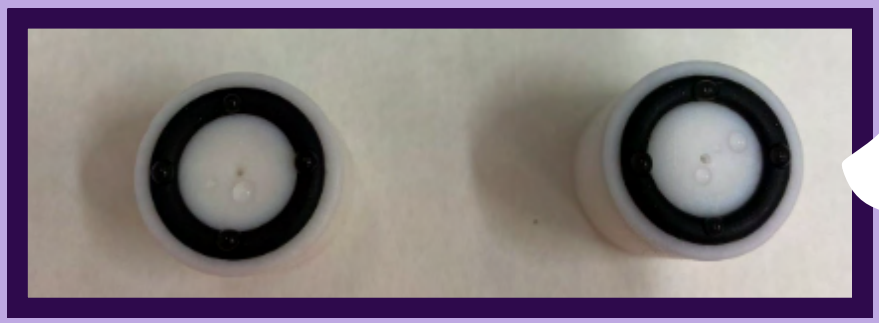
LIPOSOMES AS A PART OF OUR PROJECT

The 2024 LiU iGEM team aimed to develop a treatment for a rare skin disease called Ichthyosis. The disease is caused by mutations that leave certain enzymes important for the skin barrier defective. To solve this problem our team used liposomes to deliver functional enzymes that would replace the damaged ones. In our project we chose to construct large unilamellar liposomes (LUVs) with a diameter of 200 nm. The composition used for the liposomes consisted of SOY lipid, DOPE and cholesterol. The SOY lipid was used as a base, DOPE provided easier fusion with membranes and cholesterol stabilized the liposomes [3][4]. When constructing the liposomes we used different percentage compositions of these three to find the optimum. We tested the different compositions with Dynamic Light Scattering every week to ensure that they remained the same size and had not aggregated. From this we learned that the composition 50 mol% SOY, 25 mol% DOPE and 25 mol% cholesterol was the most stable.

The method used to create our liposomes is called thin film hydration and extrusion, which we describe in our protocol. To get liposomes with the diameter of 200 nm we used membranes with the pore size of 0,2 µm. If you want to learn more about the method you can read the article published by Hongwei Zhang [5]. With our method PBS buffer was used both when suspending the protein and in the DLS measurements. The pH of the buffer was set to 7,4 to imitate the physiological pH.

TIPS AND TRICKS

- Check with your local lab if you can borrow things like the extruder, membranes and filter support. Or if you can get sponsored to buy them.
- Do several batches of liposomes where you vary the amount of the different components to find the optimal composition.
- Be careful when pipetting the lipids since the solutions drip easily from the pipette
- Run a DLS/check the size regularly to test the shelf life of the liposomes.
- Add a drop of Milli-Q water to the center of the internal membrane support before adding the filter support so that it remains sealed (see picture below).



- Add a membrane bound fluorophore such as nile red to be able to study the liposomes through a fluorescence microscope.

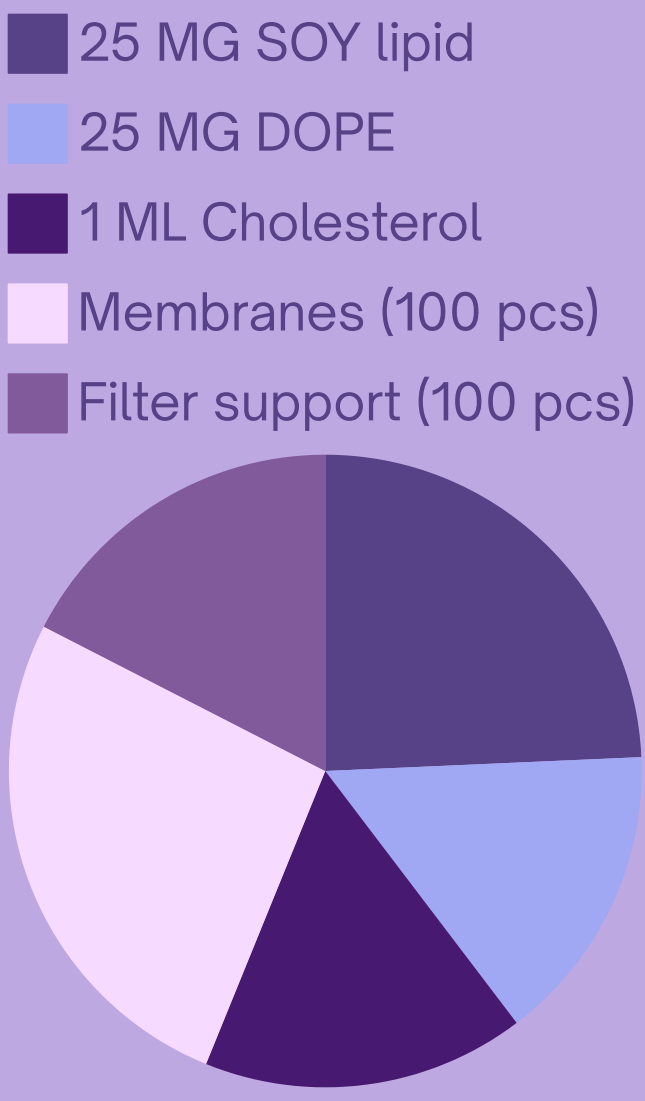
COST ESTIMATION

This is an estimation, and depends on what composition you want to have and what brands you use. Everything is bought in packs and you get much more than needed in a short project like iGEM. Therefore it is wise to consider sharing with some other group at your local lab. Also note that the cost of the extruder is not included.

Cost breakdown:

- 25 MG SOY lipid: 151,16 €
- 25 MG DOPE: 95,56 €
- 1 ML Cholesterol: 102,57 €
- Membranes (100 pcs): 164,19€
- Filter support (100 pcs): 108,59 €

Total cost: 622,07 €



TIME ESTIMATION

The estimated time to assemble liposomes is 2 days and is based on one batch. The time is divided as followed:

- **Day 1**: 3 h working time and drying in a vacuum sealer over night
- **Day 2**: 2 h

FUN FACT

Liposomes were discovered in 1961 by Dr AD Bangham when he and a colleague tested their new electron microscope. They saw that the phospholipids they looked at formed lipid bilayers when in an aqueous solution.

OUR PROTOCOL

MATERIALS

- Erlenmayer flask
- Measuring cylinder
- Solvent safe pipette tips
- Mini extruder
  - Extruder outer casing (silver)
  - Retainer nut (silver)
  - Internal membrane support with channel (white)
  - Internal membrane support (white)
  - Teflon bearing (white)
  - O ring x2 (black)
  - Filter supports x4 (2 on each side of the membrane)
  - Membrane (decides the size of the liposomes)
- 2 Syringes
  - 1 needle
  - 1 silver bolt
  - 1 black rod
- Tweezers
- Vials
- Lid to vials
- Lid to vials that withstands organic materials
- Aluminum foil
- PBS filter

CHEMICALS

- PBS tablet
- Milli-Q water
- 28% Hydrogen peroxide H2O2
- 25% Ammonia NH3
- SOY lipid
- DOPE
- Cholesterol
- Nitrogen gas N2
- Solution that is going to be encapsulated

DAY 1

Preparation of solution that is going to be encapsulated

1. Prepare a PBS buffer by mixing a PBS tablet with 1 L deionized water.
2. Filter the solution with the PBS filter to get rid of any dust.
3. Sonicate in a water bath to remove any air bubbles.
4. Solve the desired molecule to be encapsulated in the buffer. The concentration varies and you might want to consult other researchers and articles.

Calculation of liposome composition

5. Decide the percentage of every component you are going to use and calculate the volume with the excel sheet below. The final volume should be 250 µL.
6. Example: 50 mol% SOY, 25 mol% DOPE and 25 mol% Cholesterol -> 143,2 µL SOY, 70,2 µL DOPE and 36,6 µL Cholesterol.

Washing of Erlenmayer flask

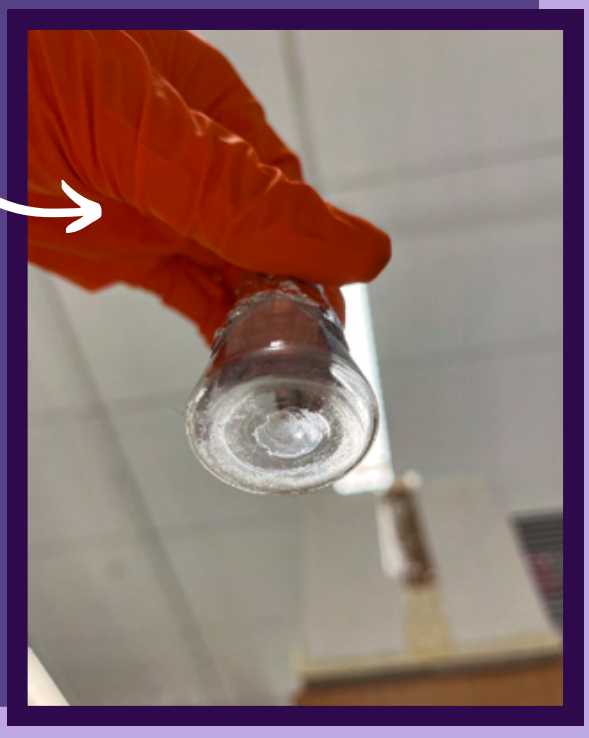
We use TL-1 cleaning when washing the E-flasks to remove any organic contamination. Make sure to use gloves and a lab coat, as well as clean up and handle the waste correctly.

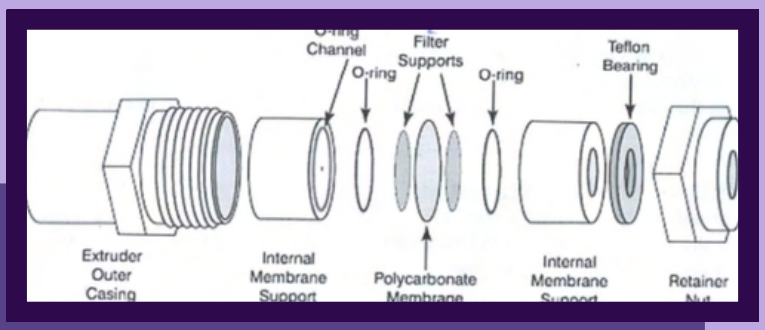
7. Use one 10 mL Erlenmeyer flask per liposome composition

8. With a graduated glassware, measure 5 mL (5 parts) of Milli-Q water and add it to the Erlenmayer flask.
9. In a fume hood, add 1 mL (1 part) of 28% H2O2 and 1 mL (1 part) 25% NH3 to the flask.
10. In a fume hood, put the flask directly on a hotplate with the temperature set to 100 °C. Let it boil for 5 min.
11. Remove the flask from the hot plate, open the water in the sink under the hood, and slowly discard the solution.
12. Wash the flask by filling it up with Milli-Q water and discard it 12 times.
13. Dry the flask thoroughly until completely dry with N2.

Lipid assembly

14. Transfer the lipids and cholesterol into vials with lids that withstands organic material.
15. In a fume hood, pipette the calculated amount of lipids and cholesterol into the bottom of the flask carefully. OBS use chemically resistant pipette tips and make sure that the lipid does not splash on the walls of the flask.
16. Carefully dry the lipids with N2.
    You should see a thin layer on the bottom of the flask.
17. Put aluminum foil on the flask and poke a few holes in it.
18. Put the flask in a vacuum sealer and leave it overnight.
19. Before putting the lipids and cholesterol into the freezer, clean the vials with N2.

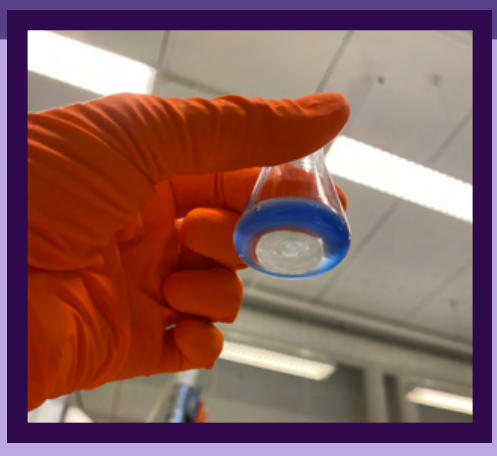




Extrusion

20. Assemble the mini extruder according to the pictures below. Use tweezers to place the membranes and filter supports.

21. Pipette 500 µL of the solution you want to encapsulate in the bottom of the Erlenmayer flask.
22.Put the flask on a shaker table for 5 minutes at low speed.
23.Vortex the flask until the solution is homogeneous and milky as the second picture.



24. Put one empty syringe on one side of the miniextruder and the other one with 0,2 mL Milli-Q water.
25. Slowly push the water through the extruder into the other syringe carefully and discard the water. Repeat 2 times on each side so that there are no air bubbles in the system.
26. When you are certain there are no air bubbles left, fill one of the syringes with 0,2 mL Milli-Q water and slowly push it through the extruder a couple of times. Note that the volume remains the same. This is to ensure that the extruder does not leak.
27. Take up the liposome solution in the Erlenmayer flask and push it through the extruder 21 times. Make sure to note which side you start on.
28. Pour the liposome solution in a dark glass vial, label it, put on a lid and store in the fridge.

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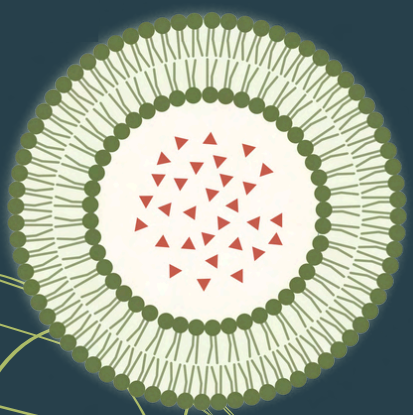
LIPID NANOPARTICLES (LNPS) BY IGEM PATRAS

iGEM PATRAS 2024

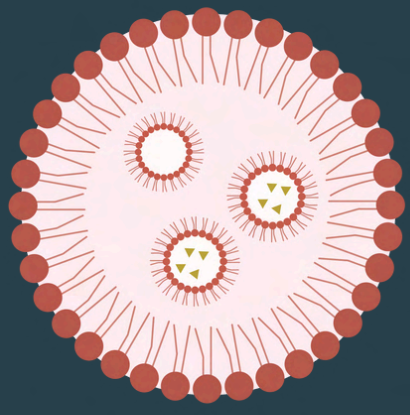
LIPID NANOPARTICLES (LNPS)

Liposomes are spherical vesicles consisting of phospholipids often arranged in a bilayer. Both sides of the bilayer are hydrophilic, while the middle is hydrophobic [1]. The size of the liposomes usually vary from 50-500 nm in diameter and the liposomes can be both unilamellar (one membrane) or multilamellar (more than one membrane). The liposomes may also contain other lipid chains, such as cholesterol. High biocompatibility, biodegradability, and low immunogenicity are some reasons why liposomes are commonly used nanocarriers. Liposomes are also shown to enhance drug solubility and controlled distribution. Another feature is its capacity for surface modifications making them suitable for targeted, prolonged and sustained release [2].

Liposome



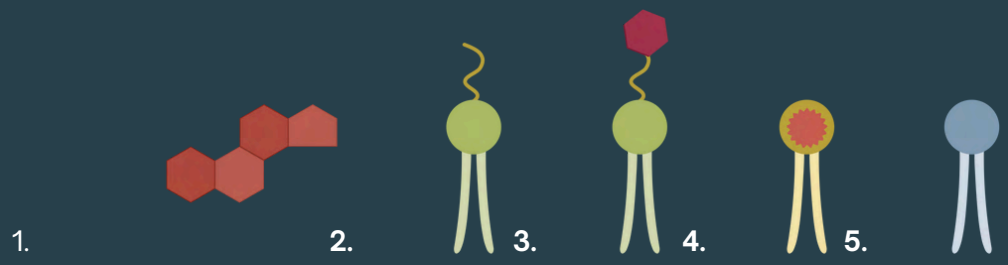
Lipid Nanoparticle



iGEM PATRAS 2024

LIPOSOMES AS A PART OF OUR PROJECT

HER2-positive breast cancer is a subtype of breast cancer characterized by the overexpression of the human epidermal growth factor receptor 2 (HER2) protein. The first-line treatment for HER2-positive breast cancer typically includes a combination of chemotherapy and targeted therapy with trastuzumab (Herceptin). Trastuzumab is a monoclonal antibody that specifically binds to the extracellular domain of the HER2 protein, thereby inhibiting its signaling pathways. Despite the remarkable efficacy of trastuzumab, a major challenge in the management of HER2-positive breast cancer is the development of resistance to this therapy. The development of resistance to trastuzumab poses a significant clinical challenge, as it limits the long-term effectiveness of the therapy and can lead to disease progression [2]. To address the persistent issue of resistance to trastuzumab in HER2-positive breast cancer, we are developing an innovative drug delivery system that utilizes small interfering RNA (siRNA) molecules for silencing genes that participate in resistance mechanisms. To efficiently deliver these siRNA molecules to cancer cells, we employ lipid nanoparticles (LNPs), which have proven to be an excellent vehicle for RNA delivery as mentioned above. LNPs encapsulate the siRNA, protecting it from degradation and facilitating its entry into cells. Our innovative Lipid Nanoparticle integrates multiple lipid components to optimize the delivery and efficacy of siRNA for targeted cancer therapy. The lipid nanoparticle is composed of cholesterol, DSPE-PEG, DSPE-PEG-MALEIMIDE, MC3-DLIN-DMA, and DSPC, each serving a specific role in enhancing the stability, delivery, and targeting capabilities of the system.



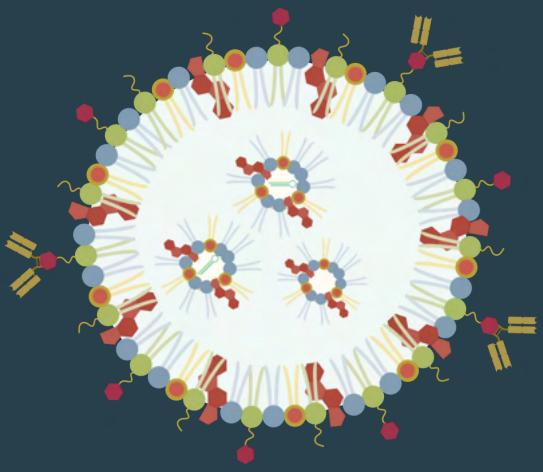
Lipid nanoparticle composition: 1. cholesterol, 2. DSPE-PEG, 3.vDSPE-PEG-MALEIMIDE, 4. MC3-DLIN-DMA, and 5.DSPC, Created with BioRender.com

To specifically target HER2-positive breast cancer cells, we will decorate the surface of these Lipid Nanoparticles with trastuzumab F(ab)'2 fragments [3], using maleimide functional groups that participate in click chemistry reactions with - chemically added - sulfhydryl groups on the antibodies [4]. This modification will not only direct the nanoparticles to HER2-overexpressing cells but also leverage trastuzumab's therapeutic properties. The nanoparticles will engage in receptor-mediated endocytosis, where the binding of trastuzumab to HER2 triggers the internalization of the nanoparticle into the cancer cell [5].

The precise protocols for anchoring antibodies to maleimide-decorated PEG-lipids will be detailed below, highlighting one of the most challenging aspects of our laboratory experiments during our iGEM journey. Targeted lipid formulations present significant opportunities for innovation!

LipOdyssey

~A quest for precision treatment~



Lipid nanoparticle LipOdysseus.

TIPS AND TRICKS

Protocol A

- For decorating the liposome surface, small antibody fragments containing the complementarity-determining regions (CDRs) are considered sufficient and even preferred [4].

Protocol B

- The activated antibody should be stored at 4°C until needed.
- Proceed quickly to subsequent steps after deprotection to avoid unwanted disulfide bond formation between thiol groups.
- Accurate quantification of thiol groups is essential for consistent results in subsequent conjugation steps.

Protocol C

- For the unknown(s), make dilutions so that the 250µL sample applied to the assay reaction has a sulfhydryl concentration less than 1.0mM. Concentrations exceeding 1mM free sulfhydryl will result in high absorbance values and less accurate estimation of the concentration based on the extinction coefficient.

TIPS AND TRICKS

- If sulfhydryl content is lower than expected make sure to minimize time delay between assay of sample and its use in applications that depend on its free sulfhydryl content and make sure to maintain 1-5mM EDTA in sample to chelate divalent metal ions, which can oxidize sulfhydryls [7].

Protocol D

- When using Bradford or ELISA for quantifying the bound antibody, prepare a standard curve for accurate measurement. Ensure all reagents and samples are properly prepared and calibrated.
- Store the lipid nanoparticles appropriately to prevent degradation. Typically, storage at 4°C is recommended.

COST ESTIMATION

Protocol A

- Pepsin enzyme: Ranges between €90 and €270 per gram.
- Trastuzumab: Approximately €1,800 to €5,500 per milligram.
- Isotonic phosphate solution (PBS 10μM): Typically around €27 to €57 for a 500 mL bottle
- Sodium citrate buffer, pH 3.5: Costs around €45 to €90 for 500 mL.
- Na Azide: About €55 to €90 for 25 grams.
- Dialysis tubes: These range from €27 to €135 depending on the size and molecular weight cutoff.

Protocol B

- SATA (N-succinimidyl S-acetylthioacetate): Around €185 for a 50 mg pack.
- DMSO (Dimethylsulfoxide): Price ranging between €35-45 for 100 ml.
- NH2OH (Hydroxylamine): Priced around €50-60 for 100 g.
- G-25 Separation Column: Costing around €75-90 per column.
- Ellman's reagent (DTNB): Costing about €40-50 for a 1g pack.
- Concentration filters (3000 rpm): Generally priced between €80-120 depending on the brand and specifications

COST ESTIMATION

Protocol C

- EDTA (Ethylenediaminetetraacetic acid): This chelating agent is commonly available as a powder. Prices can vary, but it's generally priced around €20 to €40 for a 500g container.
- Isotonic Phosphate Buffer Solution (PBS): Prices are around €20 to €50 per liter.

Protocol D

- siRNA solution: Pricing can vary significantly based on the sequence and modifications. Typically, research-grade siRNA solutions start at around €200-€500 for small quantities (20-50 nmol).

Prices may vary due to differences in purity levels, suppliers, and local distributors.

OUR PROTOCOL

A. Antibody Digestion to F(ab)'2 Fragments

DAY 1-2

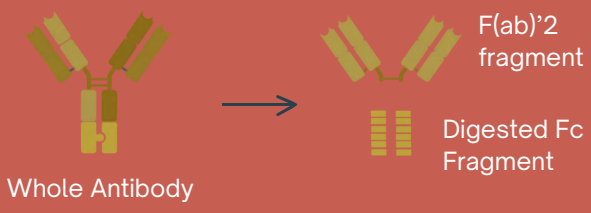
Duration: 1-2 days. It depends on the amount of mixture that will pass through the Dialyses tubes.

Introduction

This protocol outlines the preparation of an antibody solution and the steps for digesting antibodies to produce F(ab)'2 fragments according to the protocol available online by **Sigma Aldrich** titled : **Antibody Fragmentation with Pepsin Digestion.**

Materials

- Pepsin enzyme
- Trastuzumab
- Isotonic phosphate solution PBS 10µM
- Sodium citrate buffer, pH 3.5
- Na Azide
- Dialysis tubes



Procedure

Prepare an antibody solution at 25-35 mg/mL.
Prepare 0.1M sodium citrate buffer, pH 3.5.
Dissolve 10 mg of pepsin (P6887) in 1 mL of 0.1M sodium citrate buffer, pH 3.5.
Add the dissolved pepsin to the antibody solution.
Pepsin should be added at a 3% (w/w) ratio relative to the target antibody. (If processing 10 mg of antibody, add 30 µg of pepsin or 30 µL of the pepsin solution.)
Incubate at 37°C for 1-2 hours.
Stop the reaction by titrating with 2M TRIS-base to reach a final pH of 8.0.
Dialyze the antibody against 0.01M PBS buffer, pH 7.2-7.4 (add 15mM Na Azide for preservation) overnight at 4°C.

B. Conjugation of F(ab)'2 with SATA for the Incorporation of a Thiol Group (Activated Antibody)

DAY 3

Duration: 1 day.

Introduction

This procedure describes the conjugation of F(ab)'2 fragments with SATA (N-succinimidyl S-acetylthioacetate) to introduce thiol groups, resulting in an activated antibody [6], according to the protocol by **Papyrus Bio**, titled: **Antibody conjugation methods, protocols and tips**, Method 2 Step 1.

Materials

- SATA (N-succinimidyl S-acetylthioacetate)
- DMSO (Dimethylsulfoxide)
- NH2OH
- Hydroxylamine
- G-25 separation column
- Isotonic phosphate solution PBS 10μM
- Concentration filters at 3000 rpm
- Ellman's reagent

Procedure

- Immediately before use, dissolve SATA in DMSO at a concentration of 10 mg/mL.
- For each mg of antibody, add 1 mL of SATA to a buffer solution (pH 6.5–7.5).
- Combine the components and incubate for 30 minutes at room temperature.
- For every 10 mL of antibody solution, add 1 mL of hydroxylamine buffer solution to deprotect the thiol groups.
- Mix and allow the reaction to proceed at room temperature for 2 hours. Desalt the solution using a G-25 separation column.
- To avoid disulfide formation, proceed immediately to the next step.
- To remove unconjugated reagent, perform 3 successive washes using deionized phosphate buffer, followed by simple centrifugation with concentration filters at 3000 rpm for 15 minutes per wash.
- The number of introduced thiol groups can be determined using Ellman's reagent (standard curve) or based on the absorption coefficient.

C. Procedure for Quantitating Sulfhydryl Groups Based on Molar Absorptivity

DAY 4

Duration: 1 day.

Introduction

To quantify sulfhydryl groups in unknown samples, an Ellman's Reagent-based assay is performed using a spectrophotometric method. The protocol involves preparing a reaction buffer, mixing the reagent with samples, and measuring absorbance to determine sulfhydryl concentrations based on the molar extinction coefficient of the reaction product. This protocol is provided by **Thermo Fisher** upon ordering Ellman's Reagent.

Materials

- EDTA
- Ethylenediaminetetraacetic acid
- Isotonic phosphate solution PBS 10μM
- Ellman's Reagent

$E = A/b * c$

E → Molar Absorptivity (M-1 cm-1)

A → Absorbance

b → Path Length (cm)

c → Concentration (M)

Procedure

- Prepare a buffer solution containing 0.1M sodium phosphate, pH 8.0, containing 1mM EDTA.
- Dissolve 4 mg Ellman's Reagent in 1mL of Reaction Buffer.
- For each unknown sample to be tested, prepare a tube containing 50μL of Ellman's Reagent Solution and 2.5mL of Reaction Buffer.
- Add 250μL of each unknown to the separate test tubes prepared in step 1. As a blank, add 250μL of Reaction Buffer to a separate test tube prepared in Step 1.
- Mix and incubate at room temperature for 15 minutes.
- With a spectrophotometer set to 412 nm, zero the instrument on the blank and then measure absorbance of each sample.
- Calculate the amount and concentration of sulfhydryls in the sample with a total volume of 2,80mL from the molar extinction coefficient of TNB (14,150M-1 cm-1) using the following equations.

D. Conjugation of Activated Antibodies with Activated Lipids in Lipid Nanoparticles

DAY 5

Duration: 1 day.

Introduction

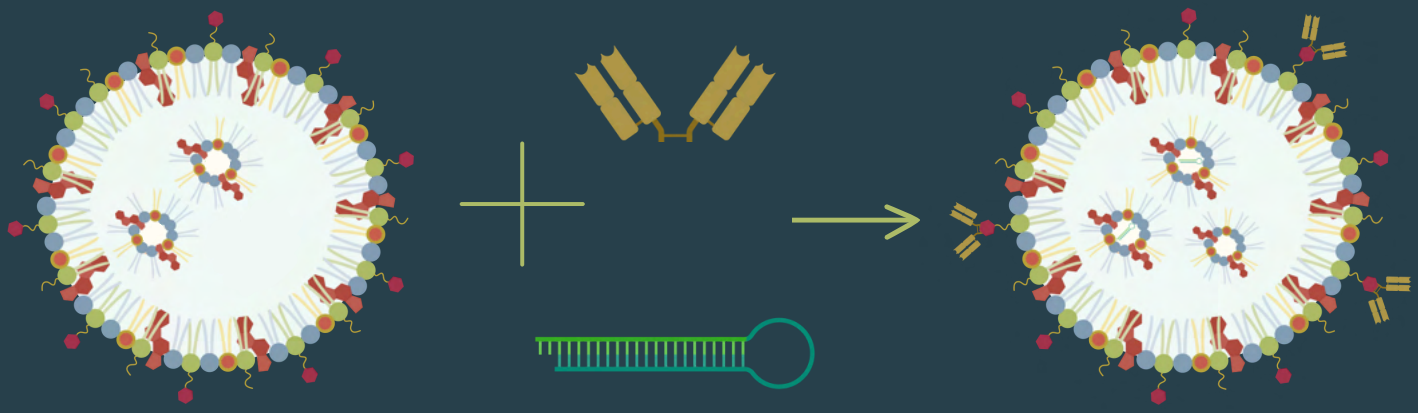
This protocol outlines the procedure for conjugating an activated antibody to Lipid Nanoparticles through maleimide functionalized PEG-Lipids and the subsequent purification and quantification of the conjugated antibody, according to our laboratory standards.

Materials

- siRNA solution
- Ultracentrifuge

Procedure

- Liposomes bearing maleimide or carboxy NHS on their surface are incubated with the activated antibody at 37°C for one hour. The mixture is stirred overnight at room temperature.
- The excess is removed by ultracentrifugation.
- The amount of conjugated antibody can be determined using the Bradford, ELISA or Dot Blot method.



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iGEM PATRAS 2024

LIPID NANOPARTICES BY RADBOUD TEAM

LIPID NANOPARTICLES (LNPS)

The lipid nanoparticles (LNPs) are composed of PEGylated lipids, cholesterol, a type of phospholipid, and ionizable lipids which significantly affect their characteristics.

Lipid components	Functions	Examples
Ionizable lipid	• Nucleic acids complexation • Membrane fusion	• ALC-0315 (Pfizer/BioNTech) • SM-102 (Moderna)
Phospholipid	• Complex support • Provides highly stable structure (saturated lipids) and endosome destabilization (unsaturated lipids)	• DSPC, DPPC (saturated lipid) • DOPE (unsaturated lipid)
Cholesterol	• Integrity • Endosomal release	• Cholesterol
PEGylated lipid	• Hydrophilic surface • Steric hindrance • "Stealth" effect	• ALC-0159 (Pfizer/BioNTech) • PEG-DMG (Moderna)

Table 1. LNP lipid components and their functions. Table taken from reference [1]

Ionizable lipids are crucial for LNP preparation, and they provide effective mRNA encapsulation, as well as safe LNP delivery in the body. Their role is crucial, since they change their charge due to protonation under acidic pH (become positively charged), or deprotonation under the neutral pH (become not charged). This feature makes it possible to encapsulate the mRNA more efficiently, as well as their safe delivery in a blood neutral pH environment. The ionizable lipids are synthesized by an epoxide ring-opening reaction [1] (Figure 1).

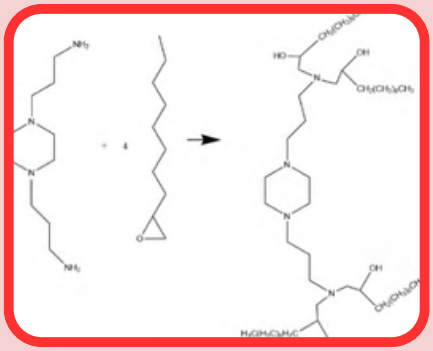


Figure 1. Epoxide ring-opening reaction. In the reference [2], you can see the procedure describing their production.

They increase or decrease the drug encapsulation efficiency; create more or less uniformly-sized particles; and improve the effectiveness of the drug delivery into the target cells [1].

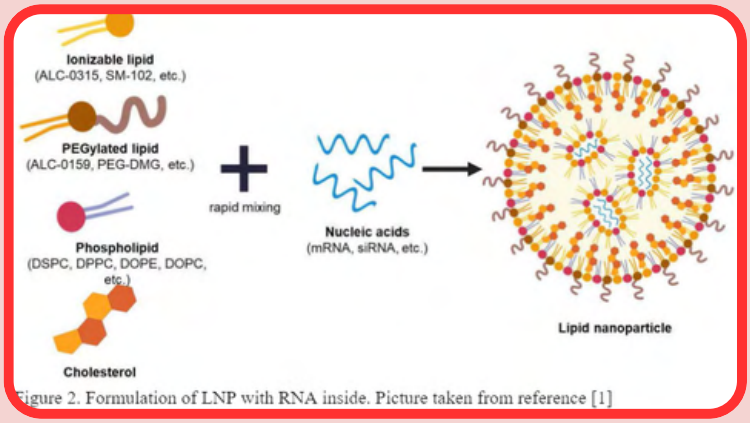


Figure 2. Formulation of LNP with RNA inside. Picture taken from reference [1]

The LNPs can be used for medicine delivery and circulation in the blood. The purpose of LNPs which the Radboud Team is trying to develop in their experiments is the delivery of a FVIII mRNA via LNPs of the following composition: ionizable lipids (synthesized from 1,4-Bis(3-aminopropyl) piperazine and 1,2-epoxydecane), cholesterol, DOPE, and C16-PEG ceramide (of which 2.5% being DSPE PEG-mannose) in the following ratio: 26.5, 52, 20, and 1.5 [2].

The LNPs are then delivered via the nasal spray delivery system by the use of hydrogel, composed of poloxamer 407, poloxamer 188, NA-CMC, and almotriptan (we do not make the hydrogel during our project due to the lack of supplies). This hydrogel provides a biphasic release pattern: the quick release for 30 minutes (this provides an acute release of a medicine upon administration) and a sustained release for a 5 hour period (this maintains a loading dose of the medicine).

LNPS AS A PART OF OUR PROJECT, INFINITYF∞

The 2024 Radboud iGEM Team, the first team of our university, is dedicated to addressing the daily challenges faced by patients with hemophilia A. This condition is caused by a genetic mutation in the FVIII gene on the X-chromosome, which prevents liver sinusoidal endothelial cells (LSECs) from producing coagulation factor VIII, a crucial protein in the blood clotting process. Hemophilia is a widespread condition affecting approximately 1,125,000 people worldwide. In severe cases, hemophilia can lead to fatal blood loss, but even minor inconveniences, like restricted physical activities, significantly impact patients' lives.

To solve this problem our team used LNP containing mRNAs that encode the coagulation factor VIII protein. LNPs administered into the bloodstream travel to the liver and target LSECs with the help of surface proteins. Once inside the cells, the vesicles will release the mRNAs, leading to the production of coagulation factor VIII. This approach enables patients to produce their own coagulation factor VIII, eliminating the need for frequent injections of plasma-derived or recombinant proteins. Due to the lack of financial supplies, a model of a final LNP had to be formulated, as well as HEK293T/17 cell line had to be used instead of LSEC cell line which is quite expensive.

The model LNPs are of diameter of 100 nm, and the composition of the used liposomes consisted of ionizable lipids (synthesized from 1,4-Bis(3-aminopropyl) piperazine and 1,2-epoxydecane), cholesterol, DOPE, and DSPE PEG - 2000 (last two kindly provided by Sadaf Javed, PhD candidate in Coacervates and Soft Interfaces at Radboud university) in the following ratio: 26.5, 52, 20, and 1.5.

2024

iGEM

INFINITY F

COST ESTIMATION

Price for formulation of a single model LNP:

Compound	Total mass of all compounds, mg	Price, Euros
ionizable lipids	85	0.07
1,4-Bis(3-aminopropyl)piperazine	17	0.03
1,2-epoxydecane	68	0.04
DOPE	0.12	0.42
cholesterol	67	1.047
DSPE-PEG 2000	0.03	0.06

Table 2. Price for formulation of a single model LNP

Price for formulation of a single LNP:

Compound	Total mass of all compounds, mg	Price, Euros
ionizable lipids	85	0.07
1,4-Bis(3-aminopropyl)piperazine	17	0.03
1,2-epoxydecane	68	0.04
DOPE	0.12	0.42
cholesterol	67	1.047
C16-PEG ceramide (PEG-lipid)N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]}	0.02925	0.517
DSPE-PEG-mannose	0.00075	0.00405

Table 3. Price for formulation of a single LNP

TIME ESTIMATION

Experiment duration	Minutes
Sonification	5
mRNA solution preparation	30
mixing	10
centrifugation	120
DLS	60
Encapsulation efficiency	240
TEM	180
Total time:	645

Table 4. Time estimation for a single model LNP experiment

OUR PROTOCOL

1. Lipid Preparation

Evaporate Chloroform

(some of our lipids were delivered as compounds dissolved in chloroform, whereas they were required to be suspended in ethanol):

- **Use a gentle stream of nitrogen gas to evaporate the chloroform.**
- **This will form a thin, uniform lipid film on the walls of the flask.**
- **After the chloroform has evaporated, place the flask under a vacuum for 15 and leave it in the desiccator overnight to ensure complete removal of any residual chloroform.**

Rehydrate Lipid Film with Ethanol:

- **Add ethanol to the flask containing the dried lipid film.**

Blow N2 on the new solution - remove O2. Make sure all lipids are completely dissolved in the ethanol before proceeding by sonication 5 min.

Combine the Lipid Solutions:

- Make a mixture of the lipid solutions in molar ratio
- Ionizable lipid, DOPE, cholesterol, PEG-lipid by pipetting: 26.5 : 20 : 52 : 1.5. [2]
- Fill up with ethanol up to 1 ml.

2. Nucleic Acid Preparation

Prepare Citrate Buffer:

- Prepare 10 mL of 10 mM citrate buffer at pH 4.0.

Dissolve Nucleic Acid:

- Dissolve 1 mg of nucleic acid (e.g., mRNA) in 1 mL of the citrate buffer.

3. Lipid Nanoparticle Formation

Ethanol Injection:

- Slowly inject the 1 mL ethanol-lipid solution into 3 mL of the aqueous (1:3 volume ratio, can also try 1:1, or other applicable volume ratio) citrate buffer (10 mM, pH 4.0) containing the nucleic acid, while stirring the buffer rapidly.

The rapid mixing of the ethanol-lipid solution with the aqueous phase leads to the spontaneous formation of lipid nanoparticles.

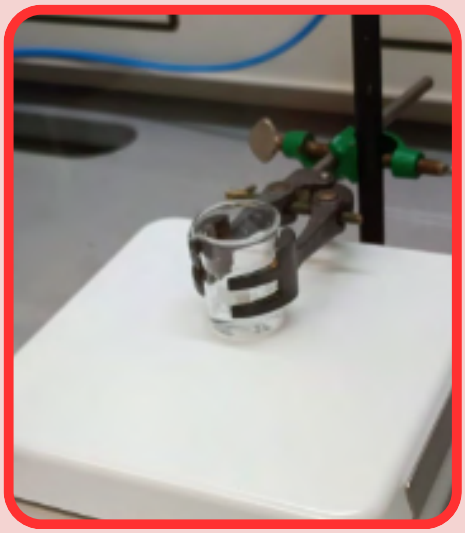


Figure 3. Ethanol Injection, mixing of aqueous and organic phases

Equilibration:

- Continue stirring for an additional 5-10 minutes to allow the system to equilibrate and ensure complete nanoparticle formation.

4. Purification

- Use a dialysis machine to remove the ethanol solvent.

(See video in a reference [5] for the dialysis procedure description).

Figure 4. Dialysis membrane used in our experiment procedure. 10000 kDa molecular cut-off

5. Analysis

- DLS
- Encapsulation efficiency [3]
- TEM

6. Storage

- LNPs without mRNA/ with a placeholder mRNA: 4 C.
- LNPs with a target mRNA: -80 C

DYNAMIC LIGHT SCATTERING (DLS)

The DLS machine in Radboud University uses the ZS XPLORER machine for processing samples with DLS. It is important to get instructions from staff in your university to know how to work with the program.

Otherwise, the principle is to set up a measuring program of your choice, load your sample in a cuvette like in Figure 5, and then insert it in the dialysis machine chamber.

Figure 4. Dialysis membrane used in our experiment procedure. 10000 kDa molecular cut-off

After the analysis, the cuvette can be cleaned by rinsing with MiliQ water, and then drying with N2 flow.

Figure 4. Dialysis membrane used in our experiment procedure. 10000 kDa molecular cut-off

Instructions provided by Dustin van Doeselaar, RU PhD candidate at the Systems Chemistry department

ENCAPSULATION EFFICIENCY

Based on Instructions used from Invitrogen [3]

Dye used:

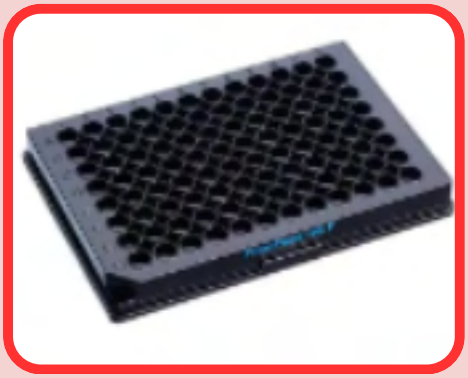
SYBR® Gold Nucleic Acid Gel Stain [4] NOTE: Mutagenic, handle with care!!!

mRNA used: This procedure is done on the Yeast mRNA.

Materials:

1. TE buffer
2. Triton X-100
3. SYBR Gold

- Prepare a standard curve by making 2-fold serial dilutions in 1x PBS of the mRNA stock solution, starting with (change to our concentration) as the highest concentration.
- Prepare the mRNA-LNP sample dilutions. Dilute mRNA-LNP samples with 1x PBS, to achieve an approximate theoretical concentration that lies around mid-point of the standard curve (e.g., Estimated initial concentration = 147.5 µg/mL, after mixing with mRNA citrate solution).
- Prepare the dilution for the theoretical mRNA concentration in LNPs. Prepare 6 replicates of them.



- Add 0.24 ul of LNPs (stock - 147.5 g/ml) to 10 ml of solvent (PBS 1x) -> 0.0036 g/ml LNPs.
- Estimates molecular weight of an average LNP: 590 g/mol weight. [6]
- In a 96-well black fluorescence capable plate, place 50 µL of the standard curve and 50 µL of mRNA-LNP diluted samples. For the samples, prepare at least 6 replicates.
- Add 50 µL of 2% Triton X-100 to each well of standards.

- Triton X-100 (TX100) is one of the most widely used nonionic surfactants for lysing cells to extract protein and other cellular organelles or to permeabilize the living cell membrane for transfection.
- To half of the sample replicates (3) add 50 µL of 2% Triton X-100.
- To the remaining wells of sample replicates (3) place 50 µL of 1XTE.
- Repeat the same for at least 4 reference wells with 50 µL 1x PBS (2 wells with 50 µL of 2% Triton X-100, 2 wells 50 µL 1X TE).
- Note: The wells containing Triton X-100 are used to quantify the total amount of mRNA while the ones containing 1X TE are used to quantify the mRNA not encapsulated inside LNPs.
- Slightly shake the plate to ensure thorough mixing of the reagents. Allow the plate to incubate at 37°C for 10 min. During this time, thaw the SYNC Gold 100x reagent (stored at −20C) and dilute it 1:10000 with 1X TE buffer.
- NOTE: Before opening, each vial should be allowed to warm to room temperature and then briefly centrifuged in a microfuge to deposit the DMSO solution at the bottom of the vial. Be sure the dye solution is fully thawed before removing an aliquot.
- After the 10 min incubation, add 100 µL of diluted SYNC Gold to each well. Avoid creating bubbles when mixing and take precautions to avoid light exposure.
- Measure the fluorescence using a microplate reader with an excitation wavelength of 500 nm, emission wavelength of 525 nm and gain of 50.
- Excitation maxima for dye–nucleic acid complexes are at ~495 nm in the visible and ~300 nm, in the ultraviolet. The emission maximum is ~537 nm. SYBR®. [4]
- After subtracting the fluorescence values of the blanks from each sample well, calculate the concentration of free mRNA (from wells without Triton X-100) using the standard curve and multiplying by the dilution factor used in 25b.
- Repeat the same to calculate the concentration of mRNA both inside and outside the LNP (from wells with Triton X-100).
- The concentration of mRNA in the final formulation is determined by subtracting the concentration of free mRNA from the total concentration of mRNA both inside and outside LNP (calculated from 25k-l).
- The fraction of free mRNA is determined by dividing the fluorescence of the intact particle sample (no Triton X-100) by the fluorescence value of the disrupted particle sample (with Triton X-100).
- Quantify the encapsulation efficiency = 1 – free mRNA fraction (calculated in 25k).

TRANSMISSION ELECTRON MICROSCOPY

For this method, it is useful to address a specialist in your university in charge of a DLS machine. It is quite expensive equipment, and you should consult with the one incharge of TEM on your specific case.
In Radboud, 8 samples can be analyzed in one go on a TEM machine, and the analysis itself lasts 2 h long.

The measurement is provided by Rob Mesman, RU (Cryo) electron microscopy and associated sample preparation specialist

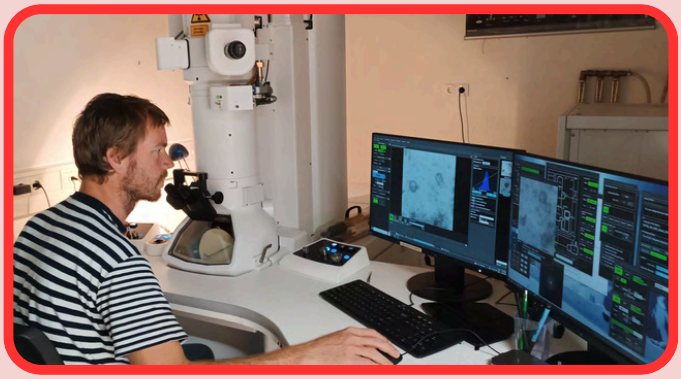


Figure 4. TEM used in our experiment procedure

TIPS AND TRICKS

1. Cleaning of dialysis tubes

The dialysis tubes can be reused for each experiment, and if you do not have enough equipment to perform your experiment, it might be useful info to know! Cleaning of dialysis tubes for LNP purification:

- A 0.5% acetic acid concentration (between 50 and 100 mM) is a good concentration to clean the filters.
- Add 5 mL of prepared solution to the top and centrifuge 10 minutes at 1000 g to wash some acetic acid through it.

- Wash dialysis tubes with around 50 mL MiliQ (just add it to the filter, and then dispose in the sink).
- After that, spin it a few times with around 5 mL MiliQ so some of it goes through the filter.

Instructions provided by Merlijn van Haren, RU Physical Organic Chemistry Department

Figure 5. Our consultation with merlijn van Haaren on dialysis technieque

2. DLS procedure affects LNP mixture

During the experiments it was discovered that during the analysis of LNPs with a DLS machine, they turn purple. It is not clear what is the exact reason - either metallic syringe used upon the transfer, or the DLS rays affecting them. Not only do they change color, but their diameter increased more than 4-fold. Thus, when using DLS technique it is suggested to only use an aliquot of 0.5 ml from your sample.

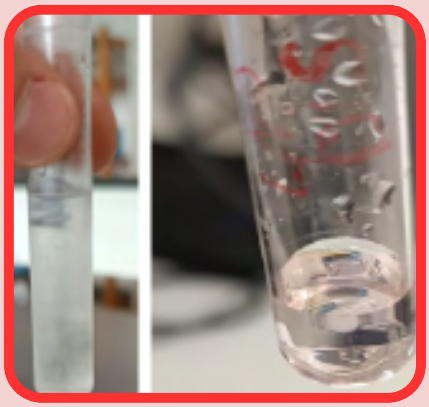


Figure 6. Left to Right: Sample before DLS measurement; Sample after DLS measurement

FUN FACT

Lipids should be stored in glass vials, since the plastic interacts with them, making them stick to it, and reducing the yield of compound significantly. These vials usually have a plastic cap. If lipids are stored in a chloroform solvent, it is important to wrap the vial where they are stored with paraffin wrap to prevent the plastic cap from loosening up open. This happens due to chloroform being a strong solvent. It is also useful not to lean your vial to the side or upside-down for these two reasons listed above!

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Radboud Universiteit

Radboud University 2024

iGEM 2024

LIPOSOMES BY TERMOSZ-SELYE-HUN TEAM

RISING MEDICAL GUYS
HISTAMINA PROJECT

TRANSDERMAL LIPOSOMES

Non-invasive transdermal drug delivery refers to the application of drugs systemically across intact skin without breaking the skin or injection with a needle. Painless administration, lack of needle phobia, and better patient compliance are the most significant advantages of this administration route [1]. However, hydrophilic drugs, proteins, and nucleic acid-based drugs are difficult to deliver into the skin. For that purpose the application of liposomes can be a suitable choice. Liposomes are considered among the most versatile and advanced nanoparticle delivery systems used to target drugs to specific cells and tissues. Structurally, liposomes are sphere-like vesicles of phospholipid molecules that are surrounded by equal number of aqueous compartments. The spherical shell encapsulates an aqueous interior which contains substances such as peptides and proteins, hormones, enzymes, antibiotics, antifungal and anticancer agents. This structural property of liposomes makes it an important nano-carrier for drug delivery [2]. Liposomes possess various surface charges depending on the type of lipid that constitutes the bilayers. Surface-charged liposomes can improve the skin permeation and penetration of active ingredients. Negatively charged liposomes can penetrate faster the upper region of the skin (stratum corneum) through hair follicles and sweat glands, in comparison to positively charged ones [3].

LIPOSOMES AS PART OF OUR PROJECT

Within the project our aim was to design large unilamellar liposomes (LUVs) with the average hydrodynamic diameter between 150-200 nm, which can be applicable for transdermal delivery of HNMT, DAO and selected aptamer. By delivering these substrates into the human cell, we aim decreasing the biological effect of the histamine. The composition used for the liposome formulation consisted of soy L-α-phosphatidylcholine (PC), cholesterol (CH) and DPPE-PEG2000. PC was used to form the phospholipid bilayer, CH to improve membrane permeability and stability of liposome membrane in the presence of biological fluids, while DPPE-PEG2000 to attach polyethylene glycol (PEG) to the surface of liposomes. These pegylated stealth liposomes are more difficult to recognize by the macrophages, therefore their circulation half-life and drug release at the target site is extended. During the formulation of liposomal carrier different molar ratio of the selected lipid components were varied and based on the resulted colloidal parameter (average hydrodynamic diameter, polydispersity index, zeta potential) determined by Dynamic Light Scattering (DLS), the optimal composition was selected. The investigated compositions were the follows:

1. CH:PC:DPPE-PEG2000 20:73:7 mol%
2. CH:PC:DPPE-PEG2000 30:63:7 mol%
3. CH:PC:DPPE-PEG2000 40:53:7 mol%

In each preformulation experiment the amount of DPPE-PEG2000 was constant 7 mol% to ensure adequate packing parameter (PP) for forming vesicle structure and highest biological stability for the LUV [4].

For the preparation of our liposomes the thin film hydration method followed by extrusion was applied, which we describe in our protocol. For the hydration of liposomes PBS pH 7.4 was used. To achieve liposomes with the average hydrodynamic diameter lower than 200 nm a membrane filter with the pore size of 0.2 μm was used.

Dynamic light scattering

The vesicle size, expressed as the average hydrodynamic diameter (DH), the vesicle size distribution (PdI, polydispersity index) and the zeta potential were measured via the Malvern Nano ZS Zetasizer (Malvern Instruments, Worcestershire, UK) based on dynamic light scattering. The samples were placed in folded capillary cells, and the measure-ments were carried out at 25 °C with a refractive index of 1.450. All measurement were carried out in triplicate with individual batches (n =3), and the results are ex-pressed as average ± SD.

Based on the results of the dynamic light scattering (DLS) measurements (Table 1.) the optimal blank liposomal carrier, which consisted of CH:PC:DPPE-PEG2000 30:63:7 mol%, had a vesicle size lower than 200 nm indicating suitable dermal permeability property. The PdI values were below 0.300, which indicates uniform absorption behaviour in the monodisperse formulations. The zeta potential value shows the surface charge of liposomes, the negative value improves the penetration of liposomes to the upper region of the skin (stratum corneum) through hair follicles and sweat glands.

Table 1. Average hydrodynamic diameter (DH), polydispersity index (PdI) and zeta potential (ζ) values of blank liposomes after dispersion in pH 7.4 PBS . Measurements took place via dynamic lights scattering and zeta potential measurements. All data are presented as means ± SD from three individual batches.

	Blank lipo
DH (nm)	178.5 ± 4.8
PdI	0.124 ± 0.016
ζ (mV)	-8.37 ± 2.16

Size Distribution Report by Intensity

v2.2

Sample Details

Sample Name: CH_PC_DPPE_PEG2000

SOP Name: mansettings.nano

General Notes:

File Name: LP.dts

Record Number: 3

Material RI: 1,45

Material Absorbtion: 0,010

Dispersant Name: Water

Dispersant RI: 1,330

Viscosity (cP): 0,8872

Measurement Date and Time: 2024. september 20.

System

Temperature (°C): 25,0

Count Rate (kcps): 289,0

Cell Description: Clear disposable zeta cell

Duration Used (s): 10

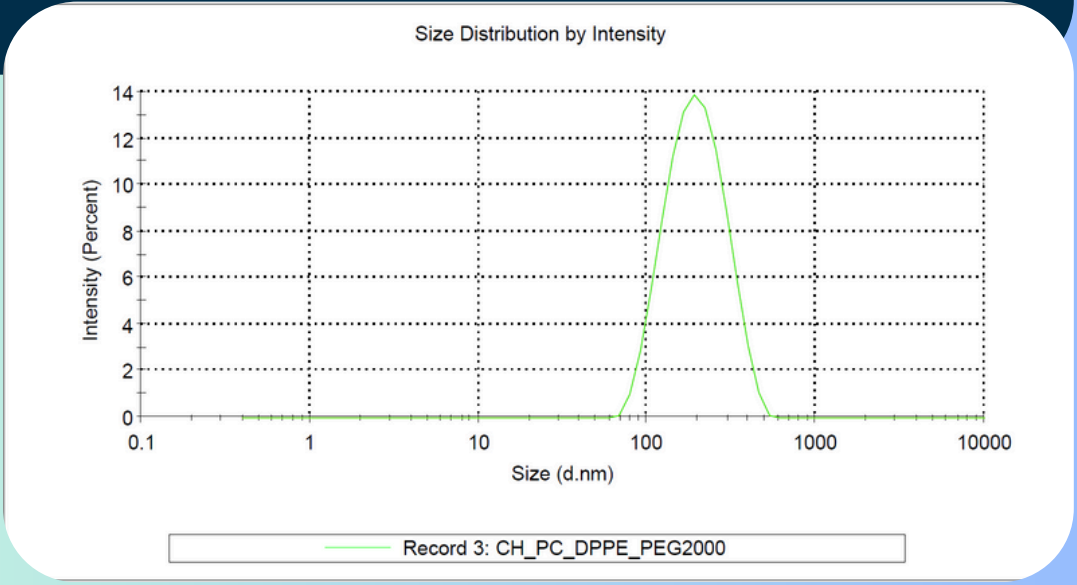
Measurement Position (mm): 5,50

Attenuator: 5

Results

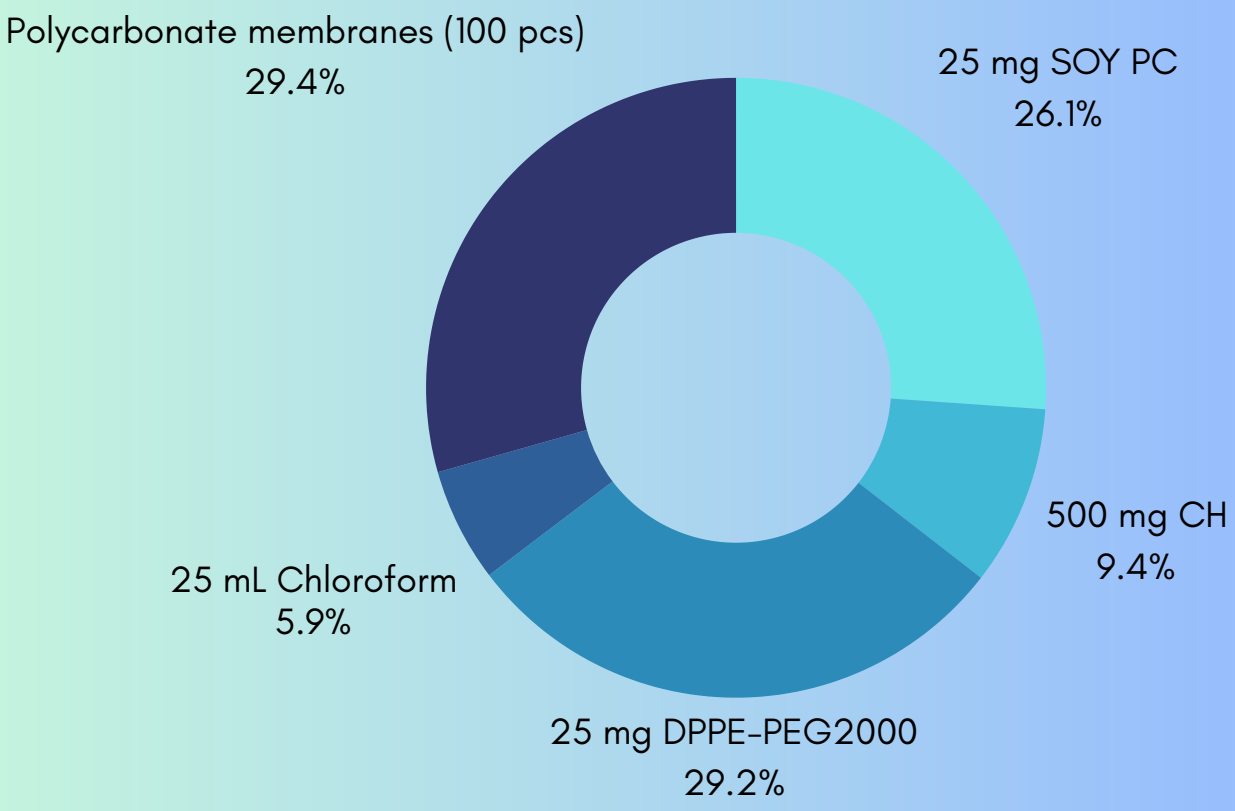
		Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 178,5	Peak 1:	206,1	100,0	80,41
PdI: 0,124	Peak 2:	0,000	0,0	0,000
Intercept: 0,949	Peak 3:	0,000	0,0	0,000

Result quality: Good



COST ESTIMATION

25 mg SOY PC: 114.82 € [1]
500 mg CH: 41.32 € [2]
25 mg DPPE-PEG2000: 128.26 € [3]
25 mL Chloroform: 26.11 € [4]
Polycarbonate membranes (100 pcs): 129.27 € [5]
Total cost: 439.78 €



[1] https://www.sigmaaldrich.com/HU/en/substance/soypc1234597281475
[2] https://www.sigmaaldrich.com/HU/en/search/cholesterol?focus=products&page=1&perpage=30&sort=relevance&term=cholesterol&type=product
[3] https://www.sigmaaldrich.com/HU/en/product/avanti/880160p
[4] https://www.sigmaaldrich.com/HU/en/search/chloroform?focus=products&page=1&perpage=30&sort=relevance&term=chloroform&type=product
[5] https://www.sigmaaldrich.com/HU/en/product/mm/gttp02500

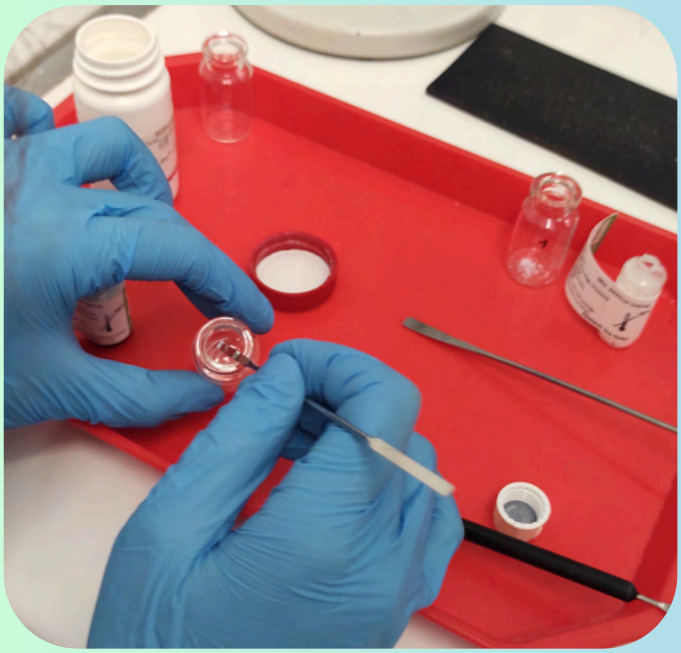
OUR PROTOCOLS

Materials

- Scale
- spoon
- Round bottom flask
- rubber cone
- Pipette
- Solvent safe pipette tips
- Rotary vacuum evaporator
- Syringes
- Polycarbonate membrane
- Tweezers
- Parafilm foil
- Vials
- Lid to vials that withstands organic materials
- PBS filter
- Ultrasound bath

Chemicals

- PBS pH 7.4
- Milli-Q water
- PC
- CH
- DPPE-PEG2000
- Solution that is going to be encapsulated



DAY 1

Calculation of liposome composition

Washing of round bottom flask

We use TL-1 cleaning when washing the E-flasks to remove any organic contamination. Make sure to use gloves and a lab coat, as well as clean up and handle the waste correctly.

1. Use one 100 mL round bottom flask per liposome composition.
2. With a graduated glassware, measure 5 mL (5 parts) of Milli-Q water and add it to the round bottom flask.
3. In a fume hood, add 1 mL (1 part) of 28% H2O2 and 1 mL (1 part) 25% NH3 to the flask.
4. In a fume hood, put the flask directly on a hotplate with the temperature set to 100 °C. Let it boil for 5 min.
5. Remove the flask from the hot plate, open the water in the sink under the hood, and slowly discard the solution.
6. Wash the flask by filling it up with Milli-Q water and discard it 10 times.
7. Dry the flask in a drying cabinet at 60°C for 1 hour.

Lipid assembly

8. Measure the lipids and cholesterol into a vial with lids that withstands organic material.
9. In a fume hood, pipette 1 mL of Chloroform to the lipids and cholesterol carefully. OBS use chemically resistant pipette tips.
10. Transfer the lipid solution into a 100 mL round bottom flask and cover with parafilm foil.
11. Connect the lipid solution containing round bottom flask the rotary vacuum evaporator, set the water bath to 60°C, the pressure to 400 mbar, the rotational speed to 20/min and operate it until the solvent evaporates. At the end of the process you should see a thin layer on the bottom of the flask.
12. Switch off the equipment, set the pressure to ambient and remove the round bottom flask.
13. Pipette the required amount of PBS 7.4 and rehydrate the lipid film under ultrasonication for 30 seconds.
14. Filter the liposomes through the 0.2 µm pore size polycarbonate filter using a syringe.
15. Check the average hydrodynamic diameter and polydispersity (PdI) with DLS. If the PdI is >0.3 repeat step 14.

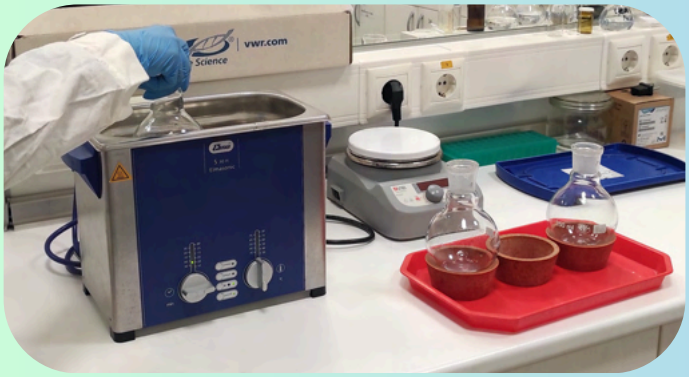
DAY 2

Encapsulating solutions

Washing of round bottom flask

Lipid assembly

1. Measure the lipids and cholesterol into a vial with lids that withstands organic material.
2. In a fume hood, pipette 1 mL of Chloroform to the lipids and cholesterol carefully. OBS use chemically resistant pipette tips.
3. Transfer the lipid solution into a 100 mL round bottom flask and cover with parafilm foil.
4. Connect the lipid solution containing round bottom flask the rotary vacuum evaporator, set the water bath to 60°C, the pressure to 400 mbar, the rotational speed to 20/min and operate it until the solvent evaporates. At the end of the process you should see a thin layer on the bottom of the flask.
5. Switch off the equipment, set the pressure to ambient and remove the round bottom flask.
6. Pipette the required amount of solution and rehydrate the lipid film under ultrasonication for 30 seconds.
7. Repeat the steps for the other solutions.



DAY 3

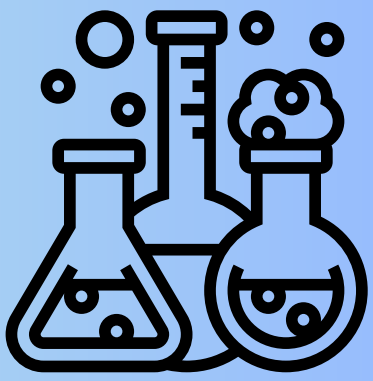
IN VITRO PERMEATION TEST

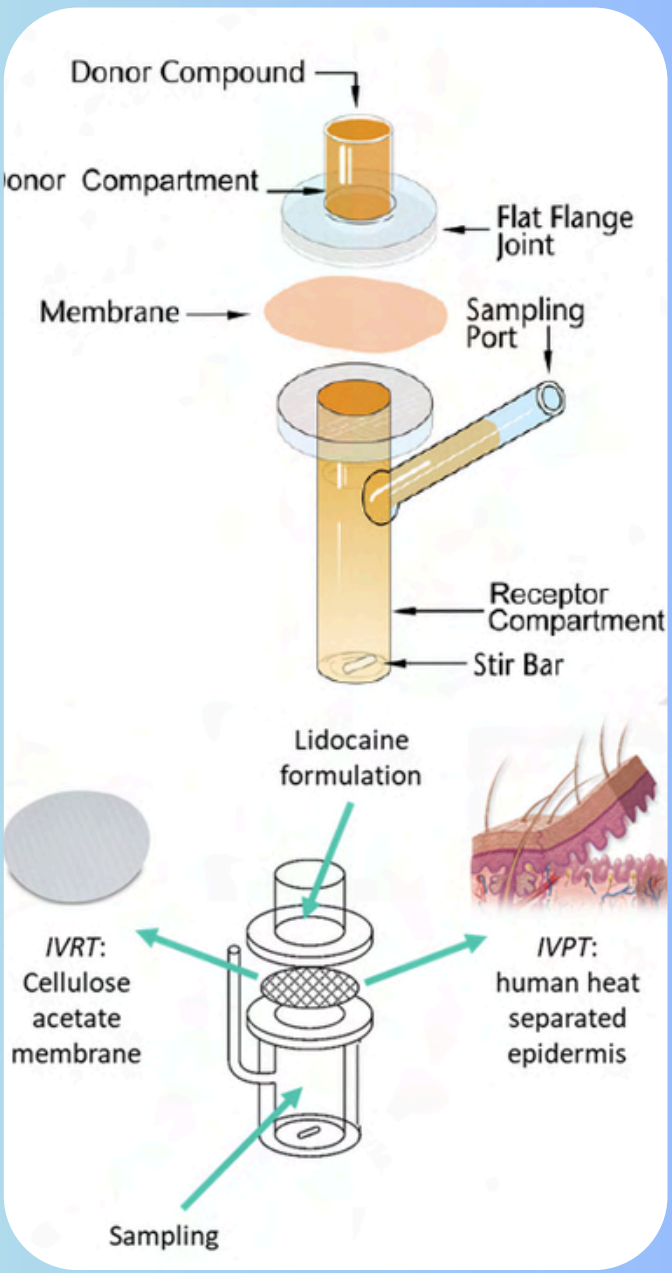
Method of skin permeation

In vitro skin permeation test (IVPT) were evaluated with a Franz diffusion cell (Phoenix RDS automatic diffusion system, Teledyne LABS, Thousand Oaks, CA, USA). It was designed with a donor and an acceptor phase, which were separated by heat-separated human epidermis (HSE).

Human skin was acquired from a Caucasian female patient who underwent an abdominal plastic surgery procedure at the University of Szeged, Department of Dermatology and Allergology. The investigations were performed with the approval of the Hungarian Medical Research Council (ETT-TUKEB, registration number: BMEÜ/2339–3/2022/EKU).

The HSE, which was supported by a synthetic membrane on mounted of cells, was prepared after it was placed in a water bath (60 ± 0.5 °C) and then the epidermis was separated from the dermis. A volume of approximately 0.3 g of the semisolid formulations was placed on the membrane. The acceptor chamber, containing 10 mL of PBS, was kept at 32.5 °C with a stirring speed of 400 rpm. In the case of IVPT, the experiment ran over 24 h in the three parallel cells (sampling times: 1; 2; 4; 6; 12; and 24 h. The analysis of the samples.....

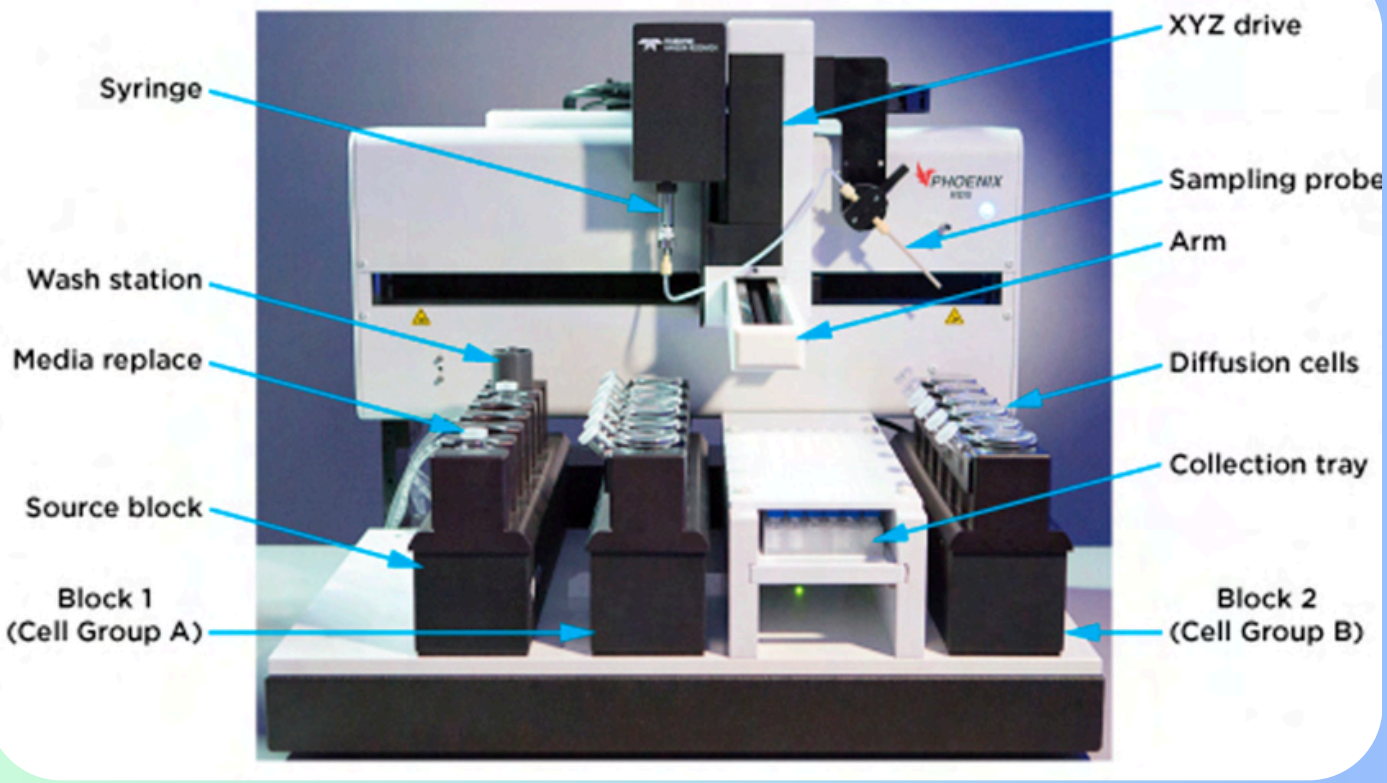




IN VITRO PERMEATION TEST (IVPT)

The in vitro permeation test (IVPT) is intended to characterize the rate andextent to which a drug applied on the surface of a biologicalmembrane permeates intoandthrough it, using method parameters aimed to simulate in vivo conditions. IVPT methodscanutilize a variety of biological membranes, including excised human skin, which canexhibitnatural variations in permeability that are reflective of the variabilityobservedin vivo.This variability can be substantial; it is not uncommon to observe a 10-fold differenceinskin permeability for a givencompound (in the same formulation) between individualsin thepopulation or between different anatomical regions on the same individual.Experimentalvariability may also be a result of the physicochemical properties of a molecule.For allproducts or treatments compared in anexperiment, the replicate skin sections usedshould besourced from the same donor (or the same set of donors), the same anatomical site(e.g.,abdomen, back, etc.), the same source (e.g., elective surgery or cadavers) and mannerofpreparation (e.g., dermatoming, freezing, etc.) tominimize variability.

An IVPT study can be sensitive and discriminating to differences in the rateand extent to which compounds applied on the skin from differentformulations becomeavailable in and through it. Notably, unlike an IVRT study, differences in permeationthatare observed in an IVPT studycomparing a test versus reference product, if any, maycorrelate with and/or be predictive of differences in bioavailability in vivo. TheIVPT isroutinely carried out to guide semi-solid topical formulation development.



TIME ESTIMATION

The estimated time to assemble liposomes is 2 days.
The time is divided as follows:
Day 1: 1,5 hours to create the thin lipid layer on the bottom of the flask
Day 2: 1 hours

TIPS AND TRICKS

Keep your lipids and hydration buffer at the appropriate temperatures based on the lipid's phase transition temperature (Tm). Above this temperature, liposomes form more easily and are more stable. This ensures the lipids are in a fluid state for optimal liposome formation.

TIPS

FUN FACT

A fun and lesser-known fact about liposomes is that they can act as "stealth" drug carriers by evading the immune system! When polyethylene glycol (PEG) is attached to the surface of liposomes (a process called PEGylation), it creates a protective shield that makes the liposomes less recognizable to the body's immune defenses. This allows the liposomes to circulate in the bloodstream for longer periods, improving drug delivery to target tissues like tumors or infected areas. This "invisibility cloak" has been crucial for improving the efficacy of certain chemotherapy treatments and other drug therapies!

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