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A taste of the pharmaceutical sciences: Development of labs to support student learning

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Abstract

The field of pharmaceutical science requires the integration of key concepts from both chemistry and biology. As part of a new degree program in the Faculty of Pharmaceutical Sciences, I have designed a series of laboratory activities to complement lecture concepts in pharmaceutics, genetics, and nanomedicine that allow students to develop the ability to generate, interpret, and analyze data. Students begin the labs by exploring concepts of solubility, diffusion, and permeability using decalcified eggs, followed by use of a Franz diffusion chamber to see how drugs are able to transit this membrane over time. Building upon the increased familiarity with the lab, we next explore how GST metabolism is affected by both biological sex, and pH, using male and female mouse liver cytosol and the UV detection of substrate metabolites. The third lab introduces concepts from pharmacogenomics with the students having the opportunity to extract their own DNA and see the correlation between their ability to taste bitter compounds and the SNPs present in their TAS2R38 gene. The fourth and fifth labs introduce concepts from nanomedicine with students first preparing liposomes using extrusion techniques and analyzing their liposomes' physical properties and loading efficiencies. The next lab has students assist with the preparation of liposome encapsulated siRNA using a microfluidic chip-based system, and the subsequent use of these particles to knock down expression of GFP expressed in HEK cells. This ties back into lecture content where they have covered the production and use of siRNA drugs like the anti-cancer medication patisiran. These labs help to introduce basic lab techniques while reinforcing lecture concepts, helping to prepare students for more advanced lab courses in their third year.

Keywords: genetics, introductory concepts, metabolism, nanomedicine, pharmaceutics, pharmacogenetics

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INTRODUCTION

Here I describe the five labs I do with second-year students enrolled in PHAR 203, one of the required courses for the Bachelor of Pharmaceutical Sciences degree program at The University of British Columbia in Vancouver, Canada. This course introduces key concepts in drug delivery technologies and personalized medicine, including pharmaceutics, biopharmaceutics, nanomedicine, pharmacogenomics, and precision medicine.

The course consists of 3 hours of lecture per week, and a 4-hour lab held once every two weeks (5 labs total over the term). Coming into the course, most students have little to no lab experience outside introductory chemistry, thus the labs have been designed to be a mixture of demonstrations and hands-on experiences to allow students to make the most of their limited time. Many of these labs could be further simplified, or conversely, made more difficult, to better match the needs of a different population of students. Although the experiences of these labs build upon each other, and familiarity with various concepts assists in the later labs, each the labs stand alone, so an individual exercise could be easily adapted for use with other courses in a variety of disciplines.

Due to the number of exercises presented here, the full student lab handouts and worksheets have been separated from the text and are offered as supplemental information, with lab-specific instructional notes located in the appendices.

Laboratory 1 – Solubility and Transport

This is the first lab of the course and is meant to provide a fun introduction to the lab. This set of experiments reinforces concepts around solubility, diffusion of solutes and solvents, and provides an opportunity for students to practice standard lab calculations to prepare small amounts of solutions. This lab consists of three parts: examination of osmosis using decalcified eggs, measurement of the rate of diffusion of a drug across a biological membrane (i.e. the egg membrane), and an investigation of the solubility of a drug in different solvents. This lab also allows introduces students to common laboratory calculations to determine the amounts of solids and liquids needed to make solutions or set-up reactions.

The students are also able to familiarize themselves with micro pipettors and other equipment that most have not previously encountered in their introductory chemistry courses. The student handout and worksheet are located in SmithPoster_SI_Lab1.pdf, and instructor's notes in Appendix A.

Osmosis: Osmolarity and Osmolality

The use of 'naked eggs', i.e. raw eggs, with the shells removed in a weak acid bath, allows hands-on exploration of the movement of water across a semi-permeable membrane, and is a fun introduction to the lab. I find students really enjoy manipulating the eggs, and being able to see and feel the difference between the various treatments seems to allow a better grasp of the concepts presented in the lab readings and lecture material. This does require approximately one-week of preparation time in order to decalcify and treat the eggs so that students are able to observe any differences during lab.

<u>Experimental design</u>

1. Remove egg from solution and gently blot dry before weighing and recording observations of the exterior and interiors of each egg, comparing against a fresh, non-deshelled egg and the other treatments.
2. Use the information obtained to calculate the % change in weight and write in your observations table.

Semipermeable membranes and drug diffusion

Determining if a compound can pass through a biological barrier, like epithelium, is critical in the development of new drug products. This is done by measuring the amount of flux – the amount of permeant crossing a membrane per unit area into the circulatory system per unit time. Here we use the chicken egg membrane as a crude mimic of an epithelium, and a Franz diffusion chamber to measure the transfer of a drug from the donor chamber through the membrane, and into the receptor chamber. Depending on the choice of drug, it is possible to see even transfer over the time period (e.g. acetaminophen), or no transit through the membrane at all (bromocresol green). Further examples of this type of experiment can be found in Ansari et al. (2006), Tari et al. (2017), and Venkataswamy (2018).

<u>Experimental design</u>

1. Take the membrane from one of the eggs stored in ultra-pure water, and gently rinse in PBS, pH 7.4.
2. Assemble Franz chamber. Fill the receptor chamber with 5 mL of PBS, pH 7.4, overlay with a small (~15 mm diameter) piece of egg membrane. Add the top chamber, and clamp into place.
3. Fill top chamber with 1 mL of drug at 2 mg/mL.
4. Immediately remove 240 µL from the receptor and store in a labelled 1.7 mL microfuge tube as the time 0 sample.
5. Replace the amount taken with 240 µL of fresh PBS, pH 7.4.
6. Collect a sample every five minutes for one hour (13 time points in total).
7. In a UV-transparent 96-well microplate, load 100 µL of each sample in duplicate, as well as an 8-point standard curve (0-100 µg/mL drug) and read at the appropriate wavelength.
8. Using the data collected, determine the average flux for your drug of interest.

Table 1. Compounds for use in drug diffusion and solubility assays

Compound	Max λ (nm)	Flux (µg/cm^{2}min)	Solubility				
			DMSO	100% EtOH	Pure water	0.1M HCl	0.1M NaOH
4-methylumbelliferone	334	5.94 ± 0.23	Yes	Part*	Yes	No	Yes
Acetaminophen	243	7.14 ± 0.84	Yes	Yes	No	No	Yes
Acetylsalicylic acid	230	5.28 ± 0.74	Yes	Yes	No	No	No
Bromocresol green	616	0.00 ± 0.05	Yes	Part*	Yes	Yes	Yes
Bromophenol blue	592	5.73 ± 1.72	Yes	Part	No	No	Yes
Caffeine	273	7.53 ± 0.99	No	No	Part*	Part*	Part*
Chloroquine diphosphate	332	NA**	No	No	Yes	Yes	Yes
Orange G	480	3.67 ± 0.46	Yes	No	Yes	Yes	Yes
Quinidine sulfate	332	NA**	Yes	Yes	No	No	No
Xylene cyanol	616	5.96 ± 0.57	Yes	Part	Yes	Part*	Yes

Solubility at 20 mg/mL in 5 solvents has been indicated for each. *Partially soluble at 20 mg/mL, and fully soluble if diluted to 10 mg/mL. **Not tested.

Solubility: Solvents and pH

Students have been taught that different compounds will have varying levels of solubility in different solvents, and that the pH of an aqueous solvent can affect the ability of a weak acid or weak base to dissolve. Despite it being possible to look up solubility data, in the lab it is often necessary to test this empirically with your particular assay buffers. Here students are provided with several tubes, each containing a small amount of a known compound. They calculate the amount of solvent needed to make a 10 mg/mL solution in each tube, and form hypotheses about which solvent will or will not dissolve the compound before they perform the test. This also serves to provide additional practice in performing the calculations required to make solutions.

Experimental design

1. Calculate the appropriate amount of solvent needed to make a 10 mg/mL solution for each tube of compound.
2. Knowing that your compound is a weak acid/weak base, hypothesize which solvent(s) will work best to dissolve it.
3. Prepare five different 10 mg/mL solutions of your compound using DMSO, 100% ethanol, ultra-pure water, 0.1 M HCl, or 0.1 M NaOH according to your calculations.
4. Mix the tubes well using the vortex and evaluate if all the drug has gone into solution. Let the tube sit in the tube rack for several minutes and look at it again to evaluate if any of the drug has come out of solution.

Laboratory 2 – Drug metabolism

This lab reinforces a number of concepts covered in the lecture material such as drug metabolism, factors affecting metabolism including sex and assay pH, as well as allows students to put common lab calculations to use to prepare the buffers they will use in the experiment. Drug metabolism is known to be significantly different between men and women (Soldin and Mattison 2009), providing an opportunity to reinforce the difference between sex (classification by

reproductive organs) and gender (a non-binary, culturally determined social construct). This experiment looks at the difference in cytosolic GST metabolism in male and female mice at three different pHs (5.5, 6.5, and 7.5). This simple assay uses commercially available mouse liver cytosol, the general cytosolic GST substrate 1-chloro-2,4-dinitro benzene, and a plate reader to directly measure the production of the glutathione-substrate conjugate. The student handout and worksheet are located in SmithPoster_SI_Lab2.pdf, and instructor's notes in Appendix B.

Potassium phosphate buffer preparation

Students apply their knowledge of the Henderson-Hasselbalch equation gained in lecture to calculate the amounts of dibasic and monobasic potassium phosphate to produce a buffer at the desired pH, use the pH meter to first compare their solution's actual pH with the desired pH, then adjust the pH of their solution appropriately. This process is overseen by a TA who uses the opportunity to lead a discussion on why we need to adjust the pH despite their calculations.

Experimental design

1. Use the Henderson-Hasselbalch equation to determine the volumes of 0.5M dibasic (K_2HPO_4) and monobasic (KH_2PO_4) potassium phosphate needed to make 100 mL of 0.1 M potassium phosphate buffer at pH 5.5 or 7.5.
2. Prepare your buffer and check the pH with a pH meter. Under the supervision of a TA, adjust the pH as needed using potassium hydroxide or phosphoric acid.
3. Bring the solution to volume after mixing and transfer to a labeled bottle.

Metabolism of Drugs by the Glutathione S-transferases (GST)

The activity of cytosolic GST enzymes, a superfamily of enzymes that play a critical role in the detoxification of numerous endogenous and xenobiotic compounds (Hayes et al. 2005), can easily be determined by combining liver cytosol, buffer, a general substrate (1-chloro-2,4-dinitro benzene), and glutathione. The production of the glutathione conjugate can be detected by absorption at 340 nm, with the Beer-Lambert law and molar extinction coefficient of 9,600 M^{-1} cm^{-1} used to quantify the amount (Habig et al. 1974; Habig and Jakoby 1981; González et al. 1989). Conversion of raw results to a rate of metabolism can be done by the teaching staff, and students allowed to interpret the data.

Experimental design

1. Place a UV-transparent microplate on ice, and label the side with your section, group, and date.
2. For each reaction at your chosen pH combine the reagents such that there is a final concentration of 0.5 nM 1-chloro-2,4-dinitro benzene, 5 ng/mL mouse liver cytosol, 2 mM Cibacron blue (if needed), and buffer to bring the reaction volume to 90 µL, or 100 µL in the case of the no-GSH negative controls.
3. Incubate the plate for 2 minutes at 37°C in the plate reader.
4. The instructor will very quickly add 10 µL of 10 mM GSH to each well (final concentration of 1 mM) excepting the no-GSH negative control wells.
5. Immediately place back in the plate reader, mix briefly, and read the plate every 10s for 5 minutes at 340 nm.
6. Following lab, use the file uploaded to Canvas containing the analyzed data to complete your lab worksheet.

Laboratory 3 – Pharmacogenetics

This lab is intended to complement a series of lectures on basic genetics, that lead into further discussion of pharmacogenetics, and pharmacogenomics. Here we use the bitter taste receptor *TAS2R38* gene to do a bitter taste phenotype-genotype correlation. The ability to taste bitter compounds is primarily determined by three missense-coding single nucleotide polymorphisms (SNPs) in this gene at positions 145; 785, and 886 of the open reading frame giving rise to the PAV (taster) and AVI (non-taster) diplotypes with the frequency of tasters for PROP and PTC varying by both race and ethnicity (Boxer and Garneau 2015). A student's genotype for the gene can be determined by sequencing (Emerson 2012), however with larger lab classes, this can become expensive rather quickly. Conveniently, each SNP is located at the cut site for either the Fnu4HI or BccI restriction endonucleases and the DNA will cut or not depending on if a person has the taster or non-taster associated genotype at that locus. This allows for same-day determination of genotype without waiting for potentially expensive sequencing data but does lose information about other SNPs or genetic changes that may be present in a given individual. Although the *TAS2R38* gene has not been directly implicated in any particularly negative health outcomes, there are some contradictory data suggesting a weak correlation between certain diplotypes with various

cancers as well as the gut microbiome (Carrai et al. 2011; Schembre et al. 2013; Choi et al. 2016; Giaccherini et al. 2022), however, these links have not been shown to be causal, and small sample sizes and specialized populations make drawing broader conclusions difficult. Students may, for any reason, decline to extract or test their own DNA, and are provided with an alternate DNA sample to use for this laboratory should they not wish to test their own DNA. The student handout and worksheet are located in SmithPoster_SI_Lab3.pdf, and instructor's notes in Appendix C.

Genomic DNA extraction

Students isolate their own genomic DNA from buccal cells using a simple kit-based system to provide enough DNA to perform a subsequent amplification of TAS2R38. This typically yields ~ 2.5 µg of DNA (~50 ng/µL), however yields as low as 5 ng/µL can still provide sufficient DNA for downstream experiments.

Experimental design

1. Swab the entire area of the inside cheek of your mouth using a sterile swab for 15 seconds.
2. Working over a disposable bench pad, place the swab into a 1.7 mL microfuge tube containing 500 µL of Genomic Lysis Buffer and vigorously swirl and rub the swab against the sides of the tube to dislodge any cells.
3. Incubate at room temperature for 10 minutes.
4. Carefully press the cotton swab against the side of the tube to press out as much liquid as possible. Pipette 400 µL of lysis buffer into a labelled Zymo-Spin™ IICR Column in a collection tube. Avoid transferring any of the cotton swab to the column.
5. Centrifuge at 10,000 x g for one minute. Discard the flow through.
6. Add 200 µL of DNA Pre-Wash Buffer to the column and centrifuge at 10,000 x g for one minute. Discard flow-through.
7. Add 500 µL of g-DNA Wash Buffer to the column and centrifuge at 10,000 x g for two minutes. Discard flow-through.
8. Transfer the spin column to a clean, labelled, microfuge tube.
9. Add 50 µL pre-warmed DNA Elution Buffer to the spin column.
10. Incubate 5 minutes at room temperature and then centrifuge at top speed for one minute to elute the DNA.

TAS2R38 amplification

The human *TAS2R38* comprises a single exon producing a 1,143 bp mRNA that encodes a 1,002 bp open reading frame. We will use specific primers to allow us to amply three regions of the gene, each containing a SNP known to be associated with the ability to taste bitter compounds. It is necessary to specifically amplify a small region around each SNP due to the large number of restriction sites present throughout the gene which would make distinguishing between diplotypes exceedingly difficult.

Experimental design

1. You have been supplied with 3 tubes of PCR master mix, one for each of the three TAS2R38 SNPs being tested, with each containing a different primer pair. Record the unique ID code on your tube so that you can identify your results.
2. Add 2 µL of your DNA (or provided sample) to each tube and mix by pipetting.
3. Place your tubes in the thermocycler and perform a two-step cycling reaction: 95°C for 30 sec, (95°C 15 sec, 68°C 30 sec) x 30, 68°C 5 minutes.

Restriction digestion

Each of the three TAS2R38 SNPS associated with bitter taste is located at the site of either the Fnu4HI or BccI restriction endonucleases. Fnu4HI cuts at the recognition site "GC▼N▲GC" and has a cut site at SNP 145 and 785 in taste phenotype, whereas BccI cuts SNP 886 several bases after its recognition site "GGATCNNNN▼N▲" in the non-taster. The uncut PCR amplicon length and the size of the digested fragments of each SNP vary depending on the genotype at that site.

Table 2. TAS2R38 SNP amplicon sizes before and after restriction digestion

SNP	Enzyme	Uncut (bp)	Digested (bp)	
			Non-taster (AVI)	Taster (PAV)
145	Fnu4HI	230	126, 104	104, 99, 27
785	Fnu4HI	169	169	94, 75
886	BccI	175	116, 59	175

Experimental design

1. You have been provided with three tubes of restriction endonuclease master mix, two containing Fnu4HI (SNPs 145, 785), and one containing BccI (SNP 886).
2. Add 17 µL of amplified DNA to the appropriate tube and incubate at 25°C for ~15-20 minutes in a thermocycler.

Polyacrylamide gel electrophoresis

Due to the small size of the fragments generated, they are run out on 10% polyacrylamide instead of agarose. This allows for far better resolution of the bands and increases visibility of the smaller fragments. The smallest fragment (27 bp) is still too small/faint to see, but it is fortunately not required to be able to differentiate between the PAV and AVI diplotypes. Due to time constraints, and hazardous nature of liquid acrylamide, we prepare the gels ahead of time for the students, and load and run them immediately following the lab with images of the stained gels uploaded to Canvas for the students to analyze.

Bitter taster phenotyping

Students evaluate their food preferences and rank a number of bitter and non-bitter foods on a one-to-five scale indicating increasing bitterness, then use compound test strips to evaluate their ability to taste bitter compounds. This is contrasted with their ability to taste the compound sodium benzoate, a common food additive that is not detected by the TAS2R38 receptor, that may be tasteless, sweet, salty, bitter, or some combination of these tastes.

Experimental design

1. Rank the following foods (black coffee*, bitter melon*, brussels sprouts*, broccoli*, cabbage*, carrot, coffee*, corn, dark chocolate*, eggplant, grapefruit juice*, green bean, kale*, matcha*, orange juice, peas, potato, pu'er tea*, red radish, tofu, tonic water*, turnip) on a 1-5 scale of bitterness. Foods marked with an asterisk are commonly considered bitter but may or may not taste bitter to you. Use these results to form a hypothesis of your bitter-tasting phenotype.
2. Taste the provided control, PTC, PROP, thiourea, and sodium benzoate test strips (Precision laboratories), and record your observations about the taste of each.
3. Do these results match your food-preference survey? Why or why not? Based on these results, what do you expect your genotype is most likely to be?

Laboratory 4 – Liposomes

Liposomes, phospholipid vesicles consisting of one or more concentric lipid bilayers enclosing discrete aqueous spaces, are one of the most common nanoscale vehicles for drug delivery, allowing for increased uptake and distribution of compounds by increasing stability and solubility of compounds (Sercombe et al. 2015). Extrusion is a common and simple method for liposome production that can easily be done inexpensively at small scale in a teaching laboratory. Here we make liposomes, encapsulating the fluorescent dye Nile Blue A, from an 80:20 mix of DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and cholesterol, and then measure encapsulation efficiency, particle size, and particle uniformity. The student handout and worksheet are located in SmithPoster_SI_Lab4.pdf, and instructor's notes in Appendix D.

Liposome production by extrusion

We will produce liposomes that encapsulate the fluorescent dye Nile Blue A using passive loading. This allows us to simply mix the compound and liposomes together before production but can lead to low loading efficiencies due to

compound diffusing out of the liposomes. To avoid this, we will try to keep our drug in the ionized form by suspending it in a low pH buffer, and thus unable to pass through the encapsulating lipid shell. Liposomes are typically purified by dialysis against water or buffer overnight. However, due to time constraints, we will perform a quick purification using a 50 nm filter and a cold extrusion apparatus to separate our liposomes away from the ammonium sulfate buffer containing unincorporated free dye (Alves et al. 2013).

Experimental design

1. Add 2 mL of a 100 µg/mL 0.45 µm filtered Nile Blue A in 300 mM ammonium sulfate, pH 5.5 to the lipid thin film.
2. Seal flask with parafilm and place in a sonicating water bath at 60°C for ~20 minutes, shaking every ~2 minutes.
3. Assemble extrusion apparatus, including the filter with the largest pore size (400 nm), place in heating block, warm to 60°C, and test for leaks using ultra-pure water. Once confirmed free of leaks, proceed to the extrusion.
4. Discarding the water, fill the syringe with 1,000 µL of hydrated lipid in the Nile Blue A solution, and place into apparatus. Allow system to equilibrate to 60°C for 2-3 minutes, then carefully depress the plunger containing the solution, forcing the liquid through the extrusion apparatus and into the second syringe.
5. Continue to pass the lipid mixture through the apparatus ~20-30 times. There should be substantial resistance, if the plunger depresses too easily, a leak has formed somewhere, or the membrane shifted such that lipid solution is bypassing the filter.
6. After the final extrusion, collect all the liquid into a syringe and dispense into a labelled 13 mm diameter glass tube. Place this tube in a 60°C temp block to keep the lipids warm during the subsequent step.
7. Carefully disassemble and clean the extrusion apparatus, discarding the membrane.
8. Replace the membrane with the 200 nm filter, reassemble, and repeat steps 3-7.
9. Replace the membrane with the 100 nm filter, reassemble, and repeat steps 3-7.
10. Remove the extrusion apparatus from the heating block and allow it to cool to room temperature.
11. Pipette 200 µL of your extruded liposomes into a microfuge tube labelled with “unpurified liposomes” and your group.
12. Reassemble your extrusion apparatus with a 50 nm filter disk and test it to confirm there are no leaks.
13. Draw your remaining liposomes into a syringe and insert into the extrusion apparatus, with an empty syringe in the other side.
14. Force the majority of the liquid through the filter, being careful not to break the membrane or extrusion apparatus, leaving ~100-150 µL of liquid in the original syringe.
15. Remove the syringe containing the large amount of buffer and discard the liquid. Refill the syringe with an equal volume of 300 mM ammonium sulfate, pH 5.5, and press it through the filter into the original syringe, diluting the lipid with fresh buffer.
16. Repeat steps 14-15 twice more, or until the buffer looks clear and colorless.
17. Remove the syringe containing your lipids and dispense into a microfuge labelled “purified liposomes”.
18. Carefully disassemble and clean the extrusion apparatus, discarding the membrane and filter supports, and rinsing the components in 95% ethanol to remove residual lipids and dye, then again in ultra-pure water before leaving to dry.

Quantification of liposome loading efficacy

The loading efficiency of the liposomes is measured by comparing the amount of Nile Blue A present in lysed versus intact liposomes. Triton X-100 (1%) is used to lyse the liposomes and the amount of Nile Blue A present calculated by comparison with standard curves made in 300 mM ammonium sulfate with or without Trion X-100 added. The standard curves and stock solutions are prepared by the technical staff due to time constraints and to ensure reproducibility between groups.

Experimental design

1. Prepare a dilution of your purified liposomes: mix 50 µL of purified liposomes with 450 µL of 300 mM ammonium sulfate, pH 5.5, and load 100 µL of this into the black 96-well plate in triplicate.
2. Prepare diluted “lysed liposomes” by combining 50 µl of purified liposomes with 450 µl of 1% Triton X-100 made in 300 mM ammonium sulfate, pH 5.5, and load 100 µL of this into the black 96-well plate in triplicate.
3. Technical staff will load in two standard curves of Nile blue (0-400 ng/mL), one made in 300 mM ammonium sulfate, pH 5.5, and one made in % Triton X-100 made in 300 mM ammonium sulfate, pH 5.5.

4. Incubate plate at 37°C for 10 minutes to allow lysis of the liposomes.
5. Read the plate at Excitation: 635 nm, Emission: 680 nm.
6. Using the equation of line for each curve, calculate the amount of Nile Blue A present in your samples.
7. Calculate the entrapment efficiency of the particles:

$$Entrapment\ Efficiency\% = \frac{mass\ of\ dye\ in\ nanoparticles}{initial\ mass\ of\ dye} \times 100$$

8. Calculate the loading efficiency of our particles:

$$Loading\ Efficiency\% = \frac{mass\ of\ dye\ in\ nanoparticles}{mass\ of\ lipid\ +\ dye\ in\ particles} \times 100$$

Measurement of liposome size

Nanoparticle size can be determined using dynamic light scattering. The motion of particles in solution causes light from a monochromatic laser shot into the solution to be scattered at varying intensities. Analysis of the fluctuations in intensity allow measurement of the particles' Brownian motion, and thus particle size using the Stokes-Einstein relationship.

Experimental design

1. Set the Malvern Instruments software measure the particles. Material: lipid polymer, dispersant: water, cell: 4 mL polystyrene cuvette, measurement: 173° backscatter, manual duration: 11 runs, 10 seconds, 3 measurements
2. Add 980 µL of ultra-pure water and 20 µL of your unpurified liposomes to a cuvette. Mix well by pipetting.
3. Place the cuvette into the machine, put the cuvette cover on the cuvette, close the lid, and press start.
4. Repeat steps 2 and 3 using the purified liposomes and record your data.

Laboratory 5 – Lipid nanoparticles (LNPs)

Liposomes made using a mixture of neutral, ionizable cationic lipids, cholesterol, and other modified lipids are commonly used as carriers for nucleic acids, the Pfizer/BioNTech and Moderna COVID-19 vaccines being well known examples of this. These LNPs are typically produced using microfluidics due to the sensitivity of the payload and the difficulties in scaling other methods. Here we use the Precision NanoSystems Spark NanoAssembler and Hepato9 RNA encapsulation kit to produce LNPs encapsulate siRNA that targets green fluorescent protein (GFP), which we will then use to silence GFP expression in a human cell line that stably expresses the GFP gene. The student handout and worksheet are located in SmithPoster_SI_Lab5.pdf, and instructor's notes in Appendix E.

Production of LNPs by microfluidic mixing

LNPs are an effective delivery system for nucleic acids and have been used as delivery vehicles for the mRNA-based COVID vaccines (Spikevax®, Comirnaty®), as well as siRNA drugs such as ONPATTRO® (patisiran). In order to properly encapsulate nucleic acids, a mixture of neutral-, ionizable-, and PEGylated lipids, as well as cholesterol, are complexed with the nucleic acids in a process called microfluidic mixing. Microfluidic mixing is different from the extrusion-based method we used previously in that the particle formation takes place inside a microscale mixing channel that rapidly mixes two liquid streams (an aqueous and organic phase) to promote nanoparticle self-assembly (Zhigaltsev et al. 2012; Leung et al. 2015). We will use the Precision Nanosystems Spark NanoAssembler to encapsulate two different DICER-substrate short interfering RNA (DsiRNA) RNA duplexes into LNPs. One will silence GFP expression, and the other is a negative control RNA that does not target any gene in the human, mouse or rat transcriptome. All steps of nanoparticle creation must be performed in the biological safety cabinet as they will be used to treat cultured cells later.

Experimental design

1. In the biosafety cabinet, diluted DsiRNA is pipetted into a microfluidic chip along with an aqueous buffer and the lipid mix in an alcohol-based solvent in separate wells.
2. The chip is placed into the Spark NanoAssembler, and the machine activated, prompting it to force the various liquids through the microfluidic channels and mix to assemble nanoparticles.
3. Remove the chip from the device and transfer your nanoparticles to a microfuge tube and dilute with buffer.
4. Repeat this process for the second DsiRNA.

Determination of RNA encapsulation efficiency

The amount of RNA encapsulated is determined using RiboGreen, a fluorescent nucleic acid binding dye that only exhibits fluorescence in the presence of DNA and RNA. The amount of RNA present in lysed LNPs will be compared with the amount present in unlysed LNPs. The RNA trapped within the LNPs will not be able to interact with the reagent, thus all signal in the unlysed LNPs will be due to unencapsulated RNA. By comparing these, the amount inside can be calculated.

Experimental design

1. Load 100 µL of each point of the standard curve into a black plate, in duplicate.
2. Load 50 µL of a 3/50 dilution of each LNP sample into 4 wells each.
3. To each of two wells of each LNP sample add 50 µL TE buffer (unlysed samples).
4. To each of two wells of each LNP sample add 50 µL 2% Triton X-100 in TE buffer (lysed samples).
5. To 2 wells, load 50 µL of a 3/50 dilution of PBS in TE buffer plus 50 µL TE buffer (TE blank).
6. To 2 wells, load 50 µL of a 3/50 dilution of PBS in TE buffer plus 50 µL 2% Triton X-100 in TE buffer (Triton blank).
7. Incubate plate at 37°C for 10 minutes to allow for lysis of LNPs.
8. Add 100 µL of diluted Ribogreen working reagent (previously prepared by your TA) to each well and mix gently.
9. Read the plate using a fluorescent endpoint read with excitation at 485 nm, and emission at 528 nm.
10. Calculate the amount of RNA present in each sample and the amount present within the particles.

Measurement of DsiRNA-LNP size

The size of nucleic acid containing nanoparticles is determined in the identical manner as outlined in Laboratory 4.

Gene knockdown mediated by lipid nanoparticle encapsulated DsiRNA

Here we will use our LNPs containing the eGFP-DsiRNA to silence the expression of GFP in a stably transfected Flp In T-REx 293-GFP cell line. This line expresses GFP under the control of the Tetracycline repressor with production stimulated by the addition of tetracycline. The cells are treated simultaneously with our tetracycline induces and the eGFP-DsiRNA, resulting in low gene expression in the treated cells compared to the controls. Unlike lipofectamine, and other similar transfection reagents, which tend to result in high levels of cellular mortality, possibly due to activation of cellular stress response genes (Fiszer-Kierzkowska et al. 2011), treatment with LNPs results in negligible cellular mortality. Due to the specialized skills, limited time, large number of students, and length of time needed for the treatments, cells are plated and treated by technical staff in advance of the lab, and the students make observations on these cells. LNPs produced with the students are used to treat cells for subsequent lab sections.

Experimental design

1. Examine each well of treated and control cells under the inverted microscope and record your observations about the visible levels of fluorescence in each well.
2. Perform a relative quantification of fluorescence in each well by harvesting the cells from each well using 50 µL trypsin per well, rinsing cells with media, and lysing with 100 µL 1% Triton X-100 in TE buffer.
3. Place cell lysate into a black 96-well plate, incubate at 37°C for 10 minutes to lyse cells, and read plate using excitation at 485 nm, and emission at 528 nm.
4. Create a graph illustrating the relative fluorescence of each treatment and discuss why the values may have high variability or imperfectly reflect their observations (cell numbers, loss during harvesting, etc.).

NOTES FOR THE INSTRUCTOR

This manuscript details 5 separate laboratory activities designed to complement an introductory course covering topics in pharmaceutics, drug metabolism, pharmacogenomics, and nanomedicine. Due to the broad nature of the course, our labs needed to be designed in such a way that each would be able to stand alone in terms of content but were still able to develop of basic lab skills that provide a foundation for subsequent courses or work in a research lab.

One of the major challenges that I faced in implementing these exercises in our second-year lab course was the students' lack of previous lab experience combined with the limited amount of time available in the labs. To overcome these challenges, detailed laboratory handouts with background reading were prepared for each lab to provide students with sufficient background for each lab, and whenever possible, lab concepts were repeated in lecture, just as lecture concepts were reinforced during the labs. At the beginning of each lab, students completed a short quiz (primarily multiple-choice, with some short answer) that evaluated how well they had understood the preparatory material. However, I found many students had not read or understood the material, leading to poor grades on these assessments. In future years, the implementation of alternative means of assessment such as the preparation of flow charts or oral presentations may be better ways to assess and encourage appropriate preparation (d'Entremont 2024).

To combat the lack of laboratory experience, we also implemented relatively small lab sections (approximately 14 students in each of the 4 laboratory sections) that were each facilitated by two graduate student teaching assistants and the laboratory instructor. Within each section, students were divided into 2-4 groups, providing ample opportunities for one-on-one and small group interactions with the teaching assistants and instructor, as well as peer-to-peer learning. This allowed the instructional staff to monitor and guide students through the protocols while preventing any accidents that might damage the students or equipment or ruin the experiment. Students were more comfortable speaking in small groups, allowing the instructional staff to better grasp of how well they were understanding the material.

The workload outside of class was also a concern when developing the labs due to the heavy workload in their other courses. I developed a short lab worksheet to accompany each lab, the majority of which could be completed in-lab, with only small portions requiring work after lab. These sheets also had the benefit of being quick and easy for the TAs to mark, reducing the amount of time that they needed to devote to grading.

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About the Author

Alex is an Assistant Professor of Teaching at the University of British Columbia, where he currently teaches all of the laboratory courses for the Bachelor of Pharmaceutical Sciences degree program. He earned his BSc (Hons) in Biological Sciences (Molecular Genetics) in 2005, and PhD in Physiology, Cell and Developmental Biology in 2012, both from the University of Alberta.

APPENDIX A

Laboratory 1 - Solubility and Transport

Reagents

- 4-methylumbelliferone (Sigma, M1508)
- 5% acetic acid (household white vinegar)
- Absolute ethanol
- Acetaminophen (Sigma, A5000)
- Acetylsalicylic acid (Sigma, A5376)
- Bromocresol green (VWR, 97061-890)
- Caffeine (ThermoScientific, A10431-22)
- Chicken eggs, raw
- Chloroquine diphosphate (Sigma, C6628)
- DMSO (Sigma, MX1458-3)
- Hydrochloric acid (VWR, BDH30323.8LP)
- Orange G (ThermoScientific, J62743-22)
- Phosphate-buffered saline, pH 7.4
- Quinidine sulfate (Sigma, Q0875)
- Sodium chloride (Supelco, SX0420)
- Sodium hydroxide (VWR, BDH9292)
- Sucrose (household table sugar)
- Xylene cyanol (TCI, X0027-25G)

Special consumables

- UV-transparent 96-well plates (Greiner Bio-One, 655801)
- Gel-loading micropipette tips (VWR, 76321-828)

Special equipment

- Franz diffusion chamber (PermeGear, 6G-01-00-09-05)
- Microplate reader (Molecular Devices SpectraMax iD3)

Decalcification and treatment of chicken eggs

In order for this lab to proceed smoothly, the decalcified eggs need to be prepared in advance of the lab.

1. Submerge raw eggs in 5% acetic acid, and soak for ~48 hours with gentle agitation.
2. Gently dry and weigh eggs, recording the weights for each egg.
3. Submerge one egg per student group in each 5% acetic acid, ultra-pure water, 3M Sucrose, or 1.5M NaCl for 48-96 hours.

Notes

Decalcification

Regular white vinegar (~3-5%) works well for the decalcification, but lower concentrations may take longer to dissolve the shell. Strong acids like HCl should be avoided as they will rapidly denaturing the albumin, even before all the shell has dissolved. The dissolution of the shell can be sped up by gently rubbing the partially deshelled eggs under a stream of running water every few hours after the first 24-hour soak, but care is needed not to damage the membrane. Table sugar can be substituted for sucrose in this lab as the higher purity of lab-grade sucrose does not really affect the results, only the price. However, table salt should not be used in place of sodium chloride as it contains various other salts ($CaCl_2$, KCl, $MgCl_2$, etc.) and particulates that may not properly dissolve at the desired molarity. Once the eggs have been placed into the treatment solutions, they can be maintained for several days. For more dramatic results, the solutions may be changed out each day to provide a stronger concentration gradient, however you will typically reach a maximum change using these solutions after ~48 hours.

Drug diffusion assay

In order to obtain enough sample at each time point to be able run the samples in duplicate, I need to sample 120 µL twice using a 20-200 µL micropipettor as it is not possible to fit a 1,000 µL tip into the sampling port in my apparatus. Additionally, regular 200 µL tips are too short to take a full sample, so I use long, gel-loading micropipette tips to sample the Franz chamber. A long Pasteur pipette or a 250 µL – 1 mL Hamilton syringe with a long needle could alternatively be used to collect samples and replace the removed volume. I have found that the sampling is very sensitive to any changes in methodology. It is best to have a single person perform all the sampling for a given chamber and they should endeavor to make sure they take the sample and refill the chamber in the exact same manner each time.

I have typically used acetaminophen, acetylsalicylic acid, or caffeine as the drug of choice for the drug diffusion assay, however, these compounds require the use of UV-transparent microplates and a plate reader capable of measuring at UV-wavelengths. If these are not available, it is possible to use alternative compounds that can be seen with visible light, but their concentrations may need to be optimized. I have tested the compounds listed in Table 1, with the exceptions of chloroquine and quinidine diphosphate due to their low maximum absorbances at the stock concentration. This would result in lower sensitivity with low amounts of drug harder to detect in the receptor chamber. If a plate reader is not available, it would be possible to use a NanoDrop, or equivalent spectrophotometer, to read each sample individually at the cost of requiring additional time.

Solubility testing

I typically give students the same compound for the solubility assay as was used in the diffusion assay, but this is not required. Some compounds, like acetaminophen, which are insoluble in water, but soluble in weak base can provide an opportunity for students to link the physiochemical properties of the solute and solvent and how intermolecular interactions can affect solubility. It should be noted that if you are using dark-colored compounds (bromocresol green, bromophenol blue, crystal violet, xylene cyanol, etc) it can be very difficult to determine if the solid has fully dissolved due to the darkness of the solution.

APPENDIX B

Laboratory 2 - Drug metabolism

Reagents

- 1-chloro-2,4-dinitro benzene (Sigma, 138630)
- Cibacron blue (BioVision, 1555R)
- Dibasic potassium phosphate (Sigma, PX1570)
- Female mouse liver cytosol (Xenotech, M1500.C)
- L-glutathione (Thermo Scientific, J62166.14)
- Male mouse liver cytosol (Xenotech, M1000.C)
- Monobasic potassium phosphate (Sigma, PX1565)
- Phosphoric acid (VWR, BDH153155E)
- Potassium hydroxide (VWR, BDH9262)

Special consumables

- UV-transparent 96-well plates (Greiner Bio-One, 655801)

Special equipment

- Microplate reader (Molecular Devices SpectraMax iD3, or any plate reader capable of reading UV-wavelengths)

Notes

GST assay

Although this assay is simple, caution is required as it uses a stock of 1-chloro-2,4-dinitro benzene dissolved in DMSO as the enzyme substrate. This chemical is toxic, corrosive, and a human health and environmental hazard, but is most hazardous in powder form. To reduce risk, the instructional staff prepare the stock solution, and students are provided only the minimum amount needed to perform their assay. Students are warned of the hazard in advance, and told that proper protective equipment (gloves, lab coats, safety glasses) will be required at all times when performing the assay.

The optimal pH for this assay is pH 6.5, however we perform the assay at pH 5.5, 6.5 and 7.5. Typically, the instructor or TAs perform the experiment at pH 6.5, and students perform the assay in small groups, each group getting a particular pH and sex of mouse (i.e. pH 5.5 and female mouse, pH 5.5 and male mouse, etc.). I usually reserve one pH with both sexes for the instructional team because I typically only have four groups of three-to-four students in a single lab section, and also so that we are guaranteed at least one good result for the students, in case they did not pipet their reagents properly.

We use commercially available mouse cytosol for this experiment, as 1 mL of 20 mg/mL cytosol can be purchased for ~$22 USD, and if properly aliquoted and stored at -80°C, this volume will last for many years. Depending on the resources available to you, it may be possible to generate your own liver cytosolic fraction from either waste research organs, or from commercially available liver from a grocery store or butcher. First homogenize the liver tissue in 3x volume buffer (50 mM TRIS-HCl, pH 7.4 containing 150 mM KCl and 2 mM EDTA), centrifuge at 9,000 x g for 20 minutes, and collect the S9 fraction supernatant. The S9 fraction is then centrifuged in an ultracentrifuge at 100,000 x g for 1 hour. The supernatant is the cytosolic fraction, and the pellet is the microsomal fraction (endoplasmic reticulum), which itself is a rich source of other drug metabolizing enzymes.

Students are given the table of reaction amounts below, but are expected to calculate the values of substrate, cytosol, buffer, and inhibitor for themselves during the lab.

Component	Initial concentration	Final concentration	Standard reaction (µL)	Inhibitor control (µL)	No GSH control (µL)
1-chloro-2,4-dinitro benzene	50 mM	0.5 mM	*1*	*1*	*1*
Liver cytosol	0.05 mg/mL	5 ng/µL	*10*	*10*	*10*
Buffer	0.1 M	---	*79*	*69*	*89*
Cibacron blue (GST inhibitor)	20 mM	2 mM	---	*10*	---
Glutathione	10 mM	1 mM	10	10	0
Total volume	---	---	100	100	100

In order to get useable results, it is vital that the glutathione be added to each well very quickly, and immediately before the start of the read. Thus, students can prepare the majority of the reaction, but following the required 2-minute incubation at 37°C, the instructor or a TA adds the glutathione for the students as they do not have the experience needed to pipette the reagent quickly enough as not to affect their results. As we are directly measuring the appearance of the metabolite, the plate reader used must be capable of reading at 340 nm, and plates must be UV-transparent.

The plate reader is a bottle neck for this lab as the assay uses a kinetic read where each plate is read every 5 seconds for 10 minutes. In order to reach these tight timings, we can only load a maximum of 9 wells on the plate (the 3 reaction conditions in triplicate). As such, we try to stagger when the students start preparing their assay plates, but it will not harm the reaction if the plate sits on ice for several minutes. This waiting time is often used by the students to discuss the lab with their TAs, and the instructor is able to talk to each group about the plate reader and how the reaction works while their plate is run.

Data analysis

Due to the complexity of the data analysis, I have performed the data analysis for each lab section and upload a file containing the analyzed data to Canvas (our learning management software) which students then use to complete their lab worksheets. If you have more advanced students, or additional time, it would be possible to have the students complete this analysis or to go through the analysis with them, however, as it requires performing a linear regression for every well of sample, the analysis can be time consuming if you are not familiar with the process. I typically compile the data in excel, export a csv of the formatted data, and do the linear regressions and statistical analysis in R. See SmithPoster_SI_Lab2.pdf for additional details of the analysis procedure and copies of my relevant R scripts.

1. Save and export the absorbance data from the plate reader and import into a spreadsheet program. Sort and clean the data (so that time is in seconds, all samples are clearly labeled, triplicates are collated, etc.).
2. Perform a linear regression to determine the slope for every well of the plate.
3. Using the slope and intercept of each line, calculate the initial and final concentrations of the metabolite and determine the number of nmoles present in the 100 µL well volume.
4. Determine the rate of metabolism in nmol/min/mg for all samples.

Students are able to take this analyzed data to construct a graph comparing the rate of metabolism between the different treatments and suggest reasons as to why pH and sex may have affected their results. Depending on how successful each group was at performing the assay, I sometimes include other hints and details within the analyzed data, as well as statistical comparisons between the different groups.

APPENDIX C

Laboratory 3 – Pharmacogenetics

Reagents

- 6x gel loading dye (NEB, B7025S; or homemade: 20 mM Tris-Cl, pH 7.5; 60 mM EDTA, pH 8.0; 15% Ficoll 400; 0.03% xylene cyanol; 0.03% Bromophenol blue; 0.1% Orange G)
- BccI (NEB, R0704L)
- Acrylamide (Bio-Rad, 1610147)
- Ammonium persulfate (VWR, 97064-592)
- dNTPs (NEB, N0447L)
- FnuHI (NEB, R0178L)
- LabSafe nucleic acid stain (G-biosciences, 786-409)
- Low Molecular Weight DNA ladder (NEB, N3233L)
- Taq DNA polymerase (NEB, M0267X)
- TEMED (TCI, TCT0147-100ML)

Primers

SNP	Direction	Sequence (5'-3')	Length (nt)	%GC	T_m	Product (bp)
145	Forward	GGAGTACATTTCTGTTCATTTCAGTCCTGGAGTTTGCAGTGG	42	45	65	230
145	Reverse	GCTGTGGTTCAGTGGTTCACTCAACTTCTGGAAGTGG	37	51	66	
785	Forward	CCAGAAACTCTCGTGACCCCAGCCTGGAGG	30	63	68	169
785	Reverse	GCCATTATCCCAACACAAACCATCACCCCTATTTTGTCG	39	46	65	
886	Forward	GGTGATGGTTTGTGTTGGGATAATGGCAGCTTGTCC	36	50	66	175
886	Reverse	CCATTCTCAGCACAGTGTCCGGGAATCTGC	30	57	65	

Special consumables

- 0.2 mL PCR tubes
- 10% TBE-Polyacrylamide gels (home-made: 10% acrylamide in 1x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) with 15-wells),
- Control taste test strips (Precision Laboratories, 158-12V-100)
- Gel-loading micropipette tips (VWR, 76321-828)
- Quick DNA extraction kit (Zymo Research, D3025)
- Sterile cotton swabs (Avantor, 89031-270)
- Sodium benzoate taste test strips (Precision Laboratories 166-144V-100)
- PTC taste test strips (Precision Laboratories 165-144V-100)
- PROP taste test strips (Precision Laboratories 125-144V-100)
- TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0)
- Thiourea taste test strips (Precision Laboratories 196-12V-100)

Special equipment

- Benchtop centrifuge (Thermo Scientific, Pico17, 75772411)
- Gel imager (Azure 300, AZI300-01)
- PAGE chambers (Bio-Rad, 1658001FC)
- Thermocycler (Analytik Jena, Biometra Tone 96, 846-4-070-311)

Notes

Genomic extraction

DNA samples were taken by swabbing checks using cotton swabs on wooden sticks. Student groups were provided a pouch of 4 autoclaved swabs (1 per person) to collect buccal cells. Students are repeatedly given the opportunity to opt out of testing their own DNA at any time, for any reason. In this case, they will be provided with another DNA sample provided by the instructor or TA to use to complete the lab.

This kit has given reasonably consistent results, and I have found it unnecessary to measure and adjust the subsequent PCR depending on the concentration of each student sample. Elimination of this step greatly speeds up the experimental protocol, and I have found the PCR relatively resistant to large swings in DNA concentration, I have had success with concentrations ranging from ~6 – 60 ng/µL.

PCR set-up

Due to student inexperience with pipetting and the limited amount of time available in lab, students are provided with tubes of pre-made master mix containing all the reagents needed for the PCR except the template DNA. We prepare our PCR master mix in advance, and have found that the 1x mix, containing everything except the DNA can be made ahead of time, aliquoted into individual PCR tubes, and stored at -20°C for a least a week without any issue, however multiple freeze-thaws of the mix should be avoided. Storing the mix at 4°C should be avoided – this led to poor amplification of the target, non-specific amplification, and substantial primer-dimers.

Component	**Final concentration**	**1 reaction (µL)**
Nuclease-free water	---	39.75
10X ThermoPol Reaction Buffer	1X	5
10 mM dNTPs	200 µM	1
10 µM Forward Primer	0.2 µM (10 pmol)	1
10 µM Reverse Primer	0.2 µM (10 pmol)	1
Template DNA	~ < 20 ng/µL	2
10U/µL Taq DNA polymerase	0.025 U/µL	0.25
Total volume	---	50

All students need to do is add 2 µL of their own extracted DNA to each of their 3 PCR tubes before placing the tubes in the thermocycler. Despite each primer pair having slightly different ideal annealing temperatures in our reaction buffer (as determined by the NEB Tm Calculator, https://tmcalculator.neb.com/#!/main), our testing has found that when using this polymerase and primers, using an annealing temperature of 68°C for all reactions resulted in the lowest amount of non-specific amplification and primer-dimers while giving robust amounts of the desired amplicon.

Bitter taster phenotyping

We typically do the bitter taster phenotyping while the PCR is running. This 'free' time allows for additional discussions on the culturally/socially determined elements of food preferences as well as additional time to discuss the theoretical background of the experiments being performed. Students are made to wipe down their benches with 70% ethanol and wash their hand prior to performing this activity, and the taste-test strips are never allowed to come in contact with any lab surfaces.

Restriction digestion

Due to time constraints, the students are provided with ready-to-use restriction digest mixes to which they add 17 µL of the appropriate PCR. These mixes can also be made ahead of time, aliquoted and stored at -20°C without issue, but multiple freeze-thaws should be avoided. Similarly to the PCR mix, storage at 4°C should be avoided.

Component	Final concentration	Fnu4HI (µL) Tube "F"	BccI (µL) Tube "B"
Nuclease-free water	---	0	0
10X rCutSmart Buffer	1X	2	2
Template DNA	<1,000 ng	17	17
Fnu4HI (10 U/µL)	0.5 U/µL	1	---
BccI (10 U/µL)	0.5 U/µL	---	1
Total volume	---	20	20

Gel Electrophoresis

Students are able to add loading dye to each of their own samples, but the actual loading of the gels is done by the TAs, instructor, and/or technician following the lab due to precision needed, student inexperience with pipettes, equipment limitations, and a lack of time in the lab period.

1. Prepare samples by adding 6.6 µL of 6x gel loading dye to each remaining PCR, and 4 µL of dye to the restriction digests (1x final concentration). Samples are then given to the technical staff to be run on gels.
2. Due to time (and skill) constraints, the instructional staff will load the gel for the students and the results uploaded to the learning management software. Each gel has 15 wells and can hold samples from two students. We load 3 µL (150 µg) NEB Low Molecular Weight DNA Ladder, and 15 µL of each PCR and restriction digest.
3. Gels are run at 80V for ~3 hours.
4. Gels are stained post-run by submerging in ~10 mL of 1X TBE containing 1x LabSafe stain and incubating in the dark with agitation for 20 minutes.
5. Gels are briefly rinsed in pure water and imaged using a gel imager. Photos are labelled using the appropriate anonymous ID code and uploaded to Canvas for students to examine and interpret.

APPENDIX D

Laboratory 4 - Solubility and Transport

Reagents

- Ammonium sulfate (EMD, AX1385-1)
- Cholesterol (Sigma, C8667-5G)
- Chloroform (VWR, BDH1109-4LG)
- DSPC lipid (Avanti Polar Lipids, 850365P-1G)
- Methanol (VWR, BDH1135-4LP)
- Nile Blue A (Sigma, N0766-5G)
- Triton X-100 (VWR, 97063-864)

Special consumables

- 0.05 µm Nuclepore PC 19 mm membranes (Whatman, WHA800308)
- 0.1 µm Nuclepore PC 19 mm membranes (Whatman, WHA110405)
- 0.2 µm Nuclepore PC 19 mm membranes (Whatman, WHA10417004)
- 0.4 µm Nuclepore PC 19 mm membranes (Whatman, WHA10417104)
- Black 96-well microplates (Greiner Bio-One, 655076)
- Cuvette 4 mL (VWR, 97000-584)
- Filter support disks (Avanti Polar Lipids, 230300)
- Parafilm (Bemis, HS234526C)

Special equipment

- 1 mL Syringe (Avanti Polar Lipids, 610017-1EA)
- Analytical balance (VWR, 76446-816)
- Extruder heating block (Avanti Polar Lipids, 610024-1EA)
- Hotplate stirrer (VWR, 76447-044)
- Mini-extruder (Avanti Polar Lipids, 610020-1EA)
- Microplate reader (Molecular Devices SpectraMax iD3)
- Rotary evaporator (Büchi, Rotovapor R-200)
- Rotary evaporator heating bath (Büchi, Heating Bath B-490)
- Round bottom flasks (TLG, CS-F0301425)
- Sonicating water bath (VWR, 97043-988)
- Temperature block (VWR, 75838-286)
- Zetasizer (Malvern Instruments, Nano ZS)

Notes

<u>Thin film preparation</u>

A 40 mg, 55:45 molar ratio, DSPC:cholesterol lipid thin film must be prepared by the technical staff prior to start of lab. These thin films can be pre-made and stored at -20°C for several weeks to months. This thin film allows for faster and more even hydration of the lipids with the aqueous buffer used for production of the liposomes. This molar ratio is equivalent to a weight ratio of 71.41% DSPC to 28.59% cholesterol.

1. Prepare 50 mg/mL stocks of each DSPC and cholesterol in a 5:1 mixture of chloroform:methanol in glass vials.
   - For ~12 flasks of thin films, you will need 350 mg DSPC in 7 mL solvent, and 15 mg cholesterol in 3 mL solvent
2. To a clean round bottom flask, add a total of 40 mg of lipid so that there is a 55:45 molar ratio of DSPC:cholesterol.
   - 571 µL 50 mg/mL DSPC
   - 229 µL 50 mg/mL cholesterol.
3. Mix each flask well by vortex.
4. Using a rotary evaporator with the water bath set to ~40°C, evaporate all the solvent under rotation and vacuum. This may take up to 15 minutes per flask. Do not heat the water too high, or evaporate too quickly, or a poor-quality thin film will result.

<u>Extrusion</u>

Due to limited equipment and personnel, we typically divide the class into two groups of approximately seven students, each of which does their own extrusion experiment. Each group is led by a TA, and the instructor floats between groups assisting as needed. Each group gets their own thin film, and after the students have had a chance to examine it, the TAs add 2 mL of filtered Nile Blue A to the flask before placing it in a sonicating water bath set to 60°C and allowing it to sonicate for at least 20 minutes. The filtration of the Nile Blue A solution is essential, as undissolved particles will clog the extrusion device at later steps. When sonicating the thin film, the longer the sonication period lasts, the better the emulsion of lipid and dye, resulting in a mixture that is easier to pass through the extruder membranes. While we wait for the lipids to emulsify, I hold short quizzes to evaluate student preparedness, and have had the teaching assistants deliver short lectures on the principles and theory behind liposome manufacture to reinforce the material that has been covered in lecture.

The extrusion apparatus can be difficult to consistently assemble without leaking, and I would strongly recommend that TAs be given the chance to practice several times before the start of lab so that they are better able to walk the students through this process. Every time the apparatus is re-assembled (due to a filter change, or to fix a leak), the apparatus should be leak tested using ultra-pure water. If no leaks appear, only then should the lipid mix be added. After adding lipid, the plunger will feel quite stiff to push, but the resistance should not be so great that it cannot be depressed. If the plunger becomes very difficult to press, it is likely the filter has clogged, and it if becomes too easy to press, the apparatus has likely developed a leak. In both cases, the apparatus should be disassembled, checked, and reassembled. If lipid has leaked, it can be aspirated back into the syringe without issue. It is important that the lipid mix be kept hot at all times, or it will become impossible to pass through the filters, thus while filters are swapped, the mix should be dispensed into a glass tube and kept in a temperature block or water/bead bath at ~60°C until you are ready to continue with the extrusion process.

Loading efficacy

Due to time and equipment considerations, as well as to ensure accuracy, the technical staff prepare the standard curves for the students and load them into the plate used for measurement. It is important that the same filtered solution of Nile Blue A that was used in the creation of the liposomes be used for the generation of the standard curves, or at the very least both must have been made in the exact same manner, otherwise the readings will not be correct. Two standard curves are made, one in buffer and one in buffer containing 1% Triton X-100 so that the absorbance of the Triton X-100 can be accounted for when examining the lysed liposome particles.

1. Prepare standard curve (0, 25, 50, 100, 150, 200, 300, 400 ng/mL) of Nile Blue A in 300 mM ammonium sulfate, pH 5.5.
    - Dilute the 0.45 µm filtered 100 µg/mL stock solution to 1 µg/mL with 300 mM ammonium sulfate, pH 5.5.
    - Combine the diluted stock with 300 mM ammonium sulfate, pH 5.5 to prepare 500 µL of each point.
2. Prepare a second identical curve, this time in 300 mM ammonium sulfate, pH 5.5 containing 1% Triton X-100.
    - Using the same diluted stock as above, combine it, 300 mM ammonium sulfate, pH 5.5, and 2% Triton X-100 to make 500 µL of each point of the standard curve.
3. For both curves, load 100 µL of each point into a black 96-well plate in triplicate. Student samples will be added to this plate prior to the 37°C incubation. It should not matter if the curves or the points are loaded first.

Sizing

Accurately determining the size of the nanoparticles requires access to an expensive dynamic light scattering spectrophotometer, which may not be possible for groups outside of chemistry or pharmaceutical sciences departments. Transmission electron microscopy could be used to visualize and size particles; however, this has its own unique challenges (and expenses).

When spectrophotometric methods are employed, I have noticed that it is important that the nanoparticle only be diluted in ultrapure water immediately before the measurement s take place, otherwise the prolonged exposure to ultrapure water results in liposome swelling and a decrease in liposome uniformity, resulting in artificially low quality and high polydispersity index.

Students are taken to see the instrument, the instructor speaks briefly about the features and operation of device, and demos the use to measure the first sample. At this point, the technical staff finish measuring the other sample(s) and the students are returned to the lab room. Final data is collated and uploaded to the learning management software for students to use to complete their worksheets.

APPENDIX E

Laboratory 5 - Solubility and Transport

Reagents

- FBS (Gibco, 12483020)
- DMEM (Lonza, BA1556)
- DPBS (Gibco, 14190136)
- eGFP-S1 DsiRNA (IDT, 51-01-05-07)
- eGFP-expressing HEK293 cells (Invitrogen, Flp-In™ T-REx™ 293, R78007; transfected with eGFP under tetracycline-controlled transcriptional activation)
- Hygromycin B (Enzo Life Sciences, 380-306-G001)
- Negative control DsiRNA (IDT, 51-01-14-04)
- Pen-strep (Gibco, 15140122)
- Ribogreen RNA reagent (Invitrogen, R11491)
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)
- Triton X-100 (VWR, 97063-864)
- Trypan blue (Corning, 25-900-CI)
- Trypsin-EDTA (Gibco, 25200072)

Special consumables

- 48-well cell culture dish (VWR, 10062-898)
- Black 96-well microplates (Greiner Bio-One, 655076)
- Cuvettes (VWR, 97000-584)
- Hepato9 RNA encapsulation kits (Precision NanoSystems, NWS0009)

Special equipment

- Analytical balance (VWR, 76446-816)
- Benchtop centrifuge (Thermo Scientific, Pico17, 75772411)
- Class II A2 Biosafety cabinet (Nuaire, NU-540-400)
- Haemocytometer (Hausser Scientific, 3500)
- Microplate reader (Molecular Devices SpectraMax iD3)
- Spark NanoAssembler (Precision NanoSystems)
- Zetasizer (Malvern Instruments, Nano ZS)

Notes

This experiment builds upon the previous lab on liposomal production via extrusion and showcases the technology that was used to develop and produce the COVID mRNA vaccines. However, it is by far the most expensive experiment we do, and if you do not have access to labs who possess the required instruments already, or you have large lab sections, it may not be feasible. The Spark NanoAssembler (Precision NanoSystems) is approximately $27,000 USD (with educational discount), each single use cartridge (we use 2 per lab section) coasts ~$39 UDS, and the lipid kit I used cost ~$800 USD and was good for ~12 encapsulations. This particular kit (Hepato9 RNA encapsulation kit) has since been discontinued, the in-market kit being the GenVoy-ILM lipid mix. Although the composition of the various formulation buffers and lipid mixes in the kit are proprietary, but it is possible to develop your own formulation based on the literature, however due to time and reagent costs, this may not actually be any more economical.

<u>Gene silencing</u>

GFP-expressing cells must be prepared and treated several days in advance of the lab. I use HEK293 cells containing GFP under tetracycline-controlled transcription activation, due to availability of materials, however a constitutively expressing line would work just as well and be less complicated to prepare. When preparing the plates, I seed low numbers of cells as I need to grow them for several days before the students' lab, and this allows me to forgo additional splitting or media changes over the weekend.

1. Plate cells in a 48-well dish (1.1 cm^2 per well) at ~5,000-10,000 cells per well at least 24 hours prior to treatment. Variable numbers of cells are plated depending on if students will be examining these plates in 4 or 6-days' time.
2. Treat cells in triplicate with 0.1 nM, 1 nM, 10 nM, and 100 nM eGFP-DsiRNA or negative control-DsiRNA, and tetracycline (2 µg/mL) in complete media (High glucose (4.5 g/L) DMEM with 2 mM L-glutamine, 10% FBS, and 200 µg/mL Hygromycin B).

3. Include non-induced controls wells, where cells are treated with media without tetracycline or DsiRNA added
4. Include induced control wells, where cells are treated with media containing tetracycline, but no DsiRNA.
5. Grow cells at 37°C with 5% CO_2 for 3-5 days, then examine under a fluorescent inverted microscope to evaluate silencing.

Production of LNPs by microfluidic mixing

Much of this lab is performed as a demonstration rather than a hands-on activity for the students due to the expense of the equipment and reagents, as well as the precision required to work with very small volumes of reagents in the microfluidic chips. Students are allowed, and encouraged, assist with subsequent steps following the particle production.

1. Each DsiRNA is resuspended to a final concentration of 20 mg/mL in sterile ultra-pure water in the biosafety cabinet, then further diluted to 930 µg/mL using the Hepato9 Formulation Buffer 1.
2. Add 24 µL of Formulation Buffer 2 to the largest well of the Spark Cartridge, 18 µL of the diluted EGFP DsiRNA to the middle well of the cartridge, and 6 µL of Nanoparticle mix to the smallest well of the cartridge.
3. Insert the cartridge into the Spark NanoAssembler, and use setting 3 to produce the particles after purging the air.
4. Transfer your LNPs from the largest well into a microcentrifuge tube containing 48 µL of Formulation Buffer 2 and mix.
5. Repeat steps 2-4 using the diluted Negative control DsiRNA to also produce the negative control LNPs. Particles can be stored at 4°C for several weeks, though the RNA may start to degrade the longer they are stored.

Determination of RNA encapsulation efficiency

Preparation of the working reagents and standard curves is performed by the TAs and/or technical staff immediately before use.

1. Prepare ~30 mL of a 1:100 dilution of Ribogreen reagent in TE is made and stored in the dark.
2. Prepare a five-point standard curve (0.1, 0.25, 0.5, 1.0, and 2.5 µg/mL) of the 930 µg/mL eGFP-DsiRNA stock in TE containing 1% Triton X-100.

The encapsulation efficiency is calculated using the fluorescence readings from this assay. To speed this up in the lab, I prepare an excel sheet ahead of time that will automatically calculate the encapsulation efficiency based on the readings obtained, so all that needs to be done is paste in the values from the plate reader.

1. Subtract the average fluorescence of the blank from each sample.
2. Calculate the average fluorescence of the with and without Triton X-100 samples from the duplicate wells
3. Calculate the encapsulation efficiency:
   - $\%EE = 1 - \frac{without\ Triton}{with\ Triton} \times 100$
4. Create a standard curve of the RNA standards and calculate the slope and intercept
5. Calculate the total siRNA concentration present in each sample:
   - $C_T = (fluorescence \times slope + intercept) \times dilution\ factor$
   - The dilution factor will be 16.67 (RNA was diluted 3/50).
6. Calculate the amount of encapsulated siRNA for each sample:
   - $RNA_{encapsulated} = \frac{\%EE \times C_T}{100}$

Gene knockdown mediated by lipid nanoparticle encapsulated DsiRNA

I typically have approximately half of the students examine the cells under the microscope while the TAs have the remainder assist with the Ribogreen assay, and the groups swap mid-way. As students are not used to examining cells under a microscope, the instructor guides the students through the controls, and tries to get them to hypothesise what the expected results will look like. After all the students have seen the induced (fluorescent) and non-induced (non-fluorescent) controls, they are guided through the observation of the treated cells. Once everyone has seen the cells, they are allowed to freely examine any well.

Students are shown the trypsinization and cell harvest process once everyone has made all their observations. The students assist the TAs with washing, resuspending the cells in 1% Triton X-100 in TE and transferring the cells to a black 96-well plate for a rough quantification of fluorescence.

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