Laboratory 1 – Solubility and Transport

1. Osmosis: Osmolarity and Osmolality

a) Background[1]

Body Water: Without water humans can survive for only a few days as it is essential for cellular homeostasis and life. Water comprises a large portion of the body weight, ranging from ~75% body weight in infants to ~55% in the elderly. The Intracellular Fluid Compartment (ICF) houses 2/3 of the body water volume, and is collectively considered to be the intracellular fluid (aka cytosolic fluid) within the cells of the body. The remaining 1/3 of body water is outside of cells, in the extracellular fluid compartment (ECF). The ECF compartment is divisible into three compartments of unequal proportion: (1) **interstitial fluid** (ISF) ~25% of total body water, the fluid in the spaces between cells; (2) **plasma** ~ 5-8% of total body water, the fluid portion of blood, and (3) **transcellular fluid** ~1-2% of total body water, the fluids forming glomerular filtrate, cerebral spinal, synovial, pleural, pericardial, and intraocular fluids. Fluid balance in the body is regulated by homeostatic mechanisms where there is a controlled balance between fluid, salts, protein, and cellular components in body compartments.

Movement of Water in the Body: The movement of fluids in the body occurs across membranes that are semi-permeable to solutes, but freely permeable to water. Movement of fluids in the body occurs through two fundamental processes (1) **hydrostatic pressure**, the *active* physical pressure exerted by water on the system (e.g. capillary beds), and (2) **osmotic pressure**, the pressure exerted by the small, non-diffusible particles dissolved in a solution or fluid that are unable to pass *passively* through a semipermeable membrane, which causes water to move from areas of high concentration to areas of low concentration. Both **electrolytes**, compounds that easily dissociate into their component ions, such as sodium chloride (NaCl) and **nonelectrolytes**, organic molecules like lipids, sugars, and proteins, contribute to the osmotic (solute) content in the body fluids, although they are not equal in effect. Importantly, electrolytes have much greater osmotic power than nonelectrolytes because each electrolyte molecule disassociates into at least two ions.

Osmolarity is a measure of the mols (or osmoles) of solute per liter of solution. A capital letter M for Molarity is used to abbreviate units of mol/L. Osmolarity is affected by changes in water content, as well as temperature and pressure because the total solution volume (L) *includes* the solute content. This is the most commonly used measure of dissolved solutes used in research labs, and is usually just referred to as molarity.

Common formulas for dealing with molarity in the lab:

$$n = C \cdot V \qquad n = \frac{m}{MW} \qquad C_1 \cdot V_1 = C_2 \cdot V_2$$

Where n = moles, m = mass in grams, MW = molecular weight of the compound in grams/mol, C = concentration in mol/L, and V = volume in litres.

[1]Thanks to Drs. Fong Chan and Tara Klassen who contributed to the background information.

You'll need to remember some of the major SI prefixes – they are essential for lab work:

Prefix	Symbol	Scientific notation	Decimal
kilo	k	10^3	1000
---		10^0	1
milli	m	10^{-3}	0.001
micro	µ	10^{-6}	0.000 001
nano	n	10^{-9}	0.000 000 001
pico	p	10^{-12}	0.000 000 000 001
femto	f	10^{-15}	0.000 000 000 000 001

Although there are other prefixes both larger and smaller, they are very rarely encountered in the lab.

Example calculations:

A) NaCl has a molecular weight of 58.44 g/mol. If you wanted to make 1 litre of a 1.5M NaCl solution, how much solid NaCl would you need?
1. You'll need these two formulas: $n = \frac{m}{MW}$ and $n = C \cdot V$ which can be combined to give the formula $C \cdot V = \frac{m}{MW}$
2. Substitute in the values and solve form: $(1.5M) \cdot (1L) = \frac{m}{58.44g/mol}$
3. $m = 87.66$ g of NaCl

B) Suppose you have a 3 M stock solution of NaCl, how much of this would you need to make 25 mL of a 0.180 M NaCl solution?
1. When dealing exclusively with liquids, you can use the formula $C_1 \cdot V_1 = C_2 \cdot V_2$
2. Next you need to ensure everything is using the same units – either put everything into base units (i.e., M and L), or everything into milli-units (i.e. mM and mL)
   a. 0.025 L = 25 mL
   b. 0.180 M = 180 mM
3. Substitute in the values to find your unknown: $(3M) \cdot V_1 = (0.180M) \cdot (0.025L)$
4. $V_1 = \frac{(0.180M) \cdot (0.025L)}{3M}$
5. $V_1 = 0.0015\ L$
6. It is important that you put your values into units that are useful in the lab – typically mL or µL for small volumes. Similarly, the number of decimal places you need will depend on the precision of your equipment and the units in which you are working. For µL volumes, most pipettes can measure to a maximum of 2 decimal places.
   a. 0.0015 L = 1.5 mL (multiply by 1000 to get to mL)
   b. 0.0015 L = 1500 µL (multiply by 1 000 000 to get from L to µL)

Osmolality is a measure of the moles (or osmoles) of solute per <u>kilogram of solvent</u> expressed as (mol/kg, molal, or Osm). Note that the divisor (kg) *excludes* the solutes and therefore remains constant. Because this remains constant during temperature and pressure changes, two variables that fluctuate in body physiology, osmolality is the preferred in diagnostic applications. Osmolality is commonly used in healthcare where blood solute concentration can be measured with an osmometer, the average plasma osmolality being 280 – 300 mOsm/L.

Using the example above, sodium chloride (NaCl) dissociates into 2 ions, Na^+ and Cl^-, magnesium chloride ($MgCl_2$) dissociates into three ions (Mg^{2+}, $2Cl_2$), but glucose, a nonelectrolyte, contributes a single solute particle despite its relatively large molecular size. Thus, $MgCl_2$ has a higher osmolality than glucose.

Exchanges between the interstitial fluid and intracellular fluid occur across plasma membranes and depend on the **semi-permeable** plasma membrane. Although ion transport is highly regulated via active transport or through ion channels, osmotic flow of water is bi-directional. Three types of environments can exist outside cells that affect the internal environment due to a change in the **osmotic** gradient: (1) **Isotonic**, concentration of solute is the same in ICF and ECF, thus there is no net water movement, (2) **Hypertonic**, concentration of solute is higher in ECF, causing water to leave the cell, (3) **Hypotonic**, concentration of solute is lower in the ECF, so water moves into the cell in an attempt to reach an osmotic equilibrium.

b) Experimental design

The chicken egg is a large self-contained cellular system. Protected by a physically rigid calcium carbonate ($CaCO_3$) eggshell, and two flexible, semi-permeable keratin reinforced shell membranes, the chicken egg is designed to permit the passage of air and moisture while protecting from the external environment and pathogenic bacteria.

Together the membranes are ~70 µm thick, with a thin air chamber between the layers. The outer membrane is ~50 µm thick and consists of 6 layers of fibrous protein that alternately cross each other at various angles, and the inner membrane is ~20µm thick and made up of 3 layers of fibres aligned parallel to the eggshell. There is a fine lamellar structure between the fibres of each layer, reducing open space and providing elasticity to the membrane.

In this experiment, the eggshells have been removed by dissolving the $CaCO_3$ shell in an acid bath to reveal the semi-permeable outer shell membrane. The now *naked eggs* were weighed on a digital scale. Individual eggs were placed into either a **hypertonic** or **hypotonic** solution and left to sit for >48 hours at room temperature.

Decalcification and treatment of chicken eggs

NB: This has already been done for you due to time constraints.

1. Raw eggs are submerged in 5% acetic acid (household vinegar), and allowed to soak for ~48 hours and eggs gently agitated every so often to ensure even dissolution of the shells.
2. Residual egg shell was gently rubbed to remove and deshelled eggs rinsed in mQH_2O.
3. Each egg was gently patted dry using paper towels, and weighed.
4. The deshelled eggs were then submerged in 5% acetic acid, mQH_2O, pH 7.4; 3M Sucrose; or 1.5M NaCl for 48-96 hours.

c) Protocol

At this station you will be measuring the 1) weight of the eggs in each solution and 2) evaluating the viscosity/consistency of the naked egg contents. The procedure is outlined below, but be sure to follow all verbal instructions and suggestions from the teaching team. Treat the eggs gently!

Procedure:

1. Carefully remove a naked egg from each of the container's solutions, and gently blot the eggs dry using paper towels.
2. Place each egg in one of the provided dishes, label the dish with the treatment, and record the egg ID and in which solution the egg had been stored.
3. Record the initial mass of the egg (from the chart provided by the instructors) in the **observation table provided in the worksheet**.
4. Place a weigh boat on the scale and TARE to zero the balance.

5. Remove the first egg from the dish, ensure it is dry, re-blotting if necessary, and place onto scale.
6. Measure the weight of the egg and record in your **observation table**.
7. REPEAT steps 1-6 for all eggs.
8. Obtain a normal shelled egg from your TA.
9. Carefully crack the shelled egg into the dish provided.
10. Break open each of the eggs in their dishes, as well as a non-deshelled egg into a fresh dish.
11. Keep the membrane from your mQH_2O egg and place it in a small beaker of 1x PBS, pH 7.4. PBS stands for Phosphate Buffered Saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).
12. Compare the viscosity and consistency across the eggs and make notes in your **observations table**.
13. Use the information obtained to calculate the % change in weight and write in your **observations table**.

2. Semipermeable membranes

a) Background

Biological membranes are semipermeable, allowing a small number of materials through, while blocking others. Diffusion is the tendency of molecules to move from an area of high concentration to an area with a lower concentration until the two regions have the same concentration. This process of reaching equilibrium is driven by the random motion of molecules.

Passive diffusion is energy independent – it does not require an energy source (chemical or otherwise) for diffusion to take place, rather only a concentration gradient is needed. This is the most common method most drugs make their way through various biological barriers.

Determining if a compound is able to 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 (i.e. the compound being tested) crossing a membrane per unit area into the circulatory system per unit time. Although thickness of the membrane is taken into account in diffusion calculations, the permeability of a particular tissue isn't directly correlated with thickness – other physiochemical (lipid composition, etc) and structural properties of the membrane (s) will affect permeability. Skin taken from different locations on the body will have different structures and the permeability will differ. **Diffusivity** is a measure of how easily a permeant can penetrate a given membrane in units of area/time. The **permeability coefficient (K_p)** describes the rate at which the permeant can penetrate the membrane per unit concentration expressed in distance/time.

Testing of diffusion is typically done *in vitro* using a device known as a Franz diffusion chamber (Fig. 1). This is a device that allows you to test the movement of a compound across a membrane. There are a number of parts:

- **Donor chamber** – the region where the test compound (the donor) is placed at the start of the study (high concentration region)
- **Membrane** – any biological, or synthetic membrane
- **Joint** – the interface between the donor and receptor chambers
- **Orifice** – the opening in the joint surface that will be covered by the membrane
    - **Orifice area** – this is a critical parameter to know for diffusion calculations!
- **Receptor chamber** – the section of the cell where a buffer for the test compound to migrate to is placed (low concentration region)

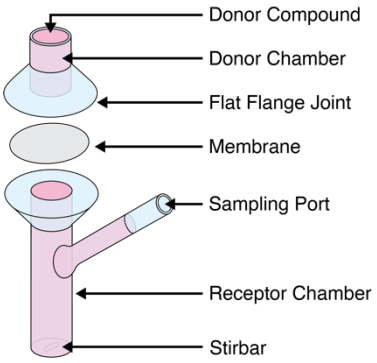
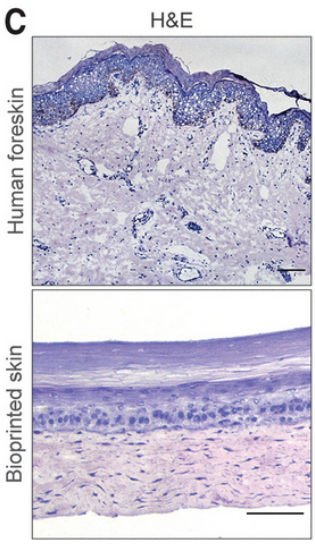


Fig. 1 Unjacketed Franz diffusion chamber.

- **Sampling arm** – a port through which some receptor fluid can be withdrawn for analysis while the experiment runs.

In pharmaceutical testing, the type of membrane used will vary depending on the properties of the drug in development and the formulation being tested. Human tissue, generally obtained from biobanks containing tissues and organs donated by deceased individuals or their families, is the gold standard when developing drugs meant to cross human membranes. However, this can be hard to obtain in sufficient quantities, and if you are looking at a specific condition, you may not be able to test on any samples from individuals that had that condition.

Animals are often used as substitutes for human tissue permeability studies, however small animals such as mice, rats, and rabbits typically have thinner skin with a different underlying structure compared to humans which can lead to higher permeabilities than would be seen in human samples. Tissues from large animals is occasionally used as well, with pig soft tissue being very similar to human soft tissues in both morphology and function, but large animal tissue can be expensive to purchase as they are less commonly used in research compared to mice, rats, and rabbits. Although non-human primates have been used in the past, this has mostly been phased out in favour of other options.



In some cases, synthetic membranes with properties similar to the specific tissue of interest are used. Although these lack the same complexity as the actual tissue, this can provide good preliminary data. Engineered tissues are also becoming more commonly used. It is now possible to use 3D printing and cellular scaffolds to print a human skin equivalent with all the expected layers and vascularization (see Baltazar *et al*., 2020; Javaid & Haleem, 2021). Currently tissue produced in this way typically has higher permeability than natural human skin, however this field is advancing quickly and improvements are being made constantly.

Figure 2. H&E staining of human foreskin and bioprinted constructs 30 days after *in vitro* maturation. Baltazar et al. (2020) Tissue Engineering Part A 26:227–238.

b) Calculating Flux (J)

Flux, as used in permeability studies, is calculated using a simplified version of Fick's law of diffusion, a mathematical expression that states that the rate of passive diffusion (flux, J) is directly proportional to
1) concentration gradient of the solute ($C_1 - C_2$; mg/mL),
2) the surface area (A) of the membrane (cm^2),
3) the diffusion coefficient (aka the permeability constant K_p) of the solute (cm^2/min),
and inversely proportional to the thickness of the membrane (cm).

Flux (J) is the amount of permeant crossing the membrane per time and it is given in units of mass/area/time (μg cm^{-2} min^{-1} in our case).

$$J = \frac{Q}{(A \cdot t)}$$

Here, *Q* is amount transported through membrane at time *t* (in μg), *A* is the surface area of the membrane (cm^2), and *t* is time (min).

Example calculation:

After 15 minutes, a total of 73.0 µg of drug has passed through the membrane. Given that the surface area of the membrane is 0.64 cm^2, what is the flux at this time point?

$$J = \frac{73.0\mu g}{(0.64 cm^2 \cdot 15 min)} \qquad J = 7.6 \mu g cm^{-2} min^{-1}$$

c) Experimental design

In our lab, we'll be using the cheap and easily obtainable egg shell membrane as our membrane for diffusion testing. Depending on the compound being tested, there are a number of ways that it could be detected and quantified. We'll be using UV-Vis spectroscopy to detect and quantify the amount of drug that is able to diffuse through the egg membrane. In order to quantify the amount of drug that passes through the membrane, we have to set up a standard curve.

Due to time constraints, the instructor and TAs have prepared the standard curves in advance, but will run and interpret them with you.

Preparation of Standard Curve

1. Prepare a 2 mg/mL stock solution of drug in PBS, pH 7.4
2. Prepare an 8 point standard curve (0, 1, 2, 5, 10, 20, 50, and 100 µg/mL drug):
    - 200 µg/mL: Add 100 µL of 2000 µg/mL, 900 µL of buffer (for prep use only)
    - **20 µg/mL:** Add 100 µL of 200 µg/mL, 900 µL of buffer
    - **2 µg/mL:** Add 100 µL of 20 µg/mL, 900 µL of buffer
    - **100 µg/mL:** Add 50 µL of 2000 µg/mL, 950 µL of buffer
    - **10 µg/mL:** Add 100 µL of 100 µg/mL, 900 µL of buffer
    - **1 µg/mL:** Add 100 µL of 10 µg/mL, 900 µL of buffer
    - **50 µg/mL:** Add 25 µL of 2000 µg/mL, 975 µL of buffer
    - **5 µg/mL:** Add 100 µL of 50 µg/mL, 900 µL of buffer
    - **0 µg/mL:** 1000 µL of buffer
3. Load 100 µL of each point of the curve, in triplicate, into a 96-well UV-transparent microplate
4. Read the plate at the appropriate wavelength for the drug being tested using the SpectraMax iD3 platereader.

Determine if the drug permeates egg shell membrane using the Franz diffusion cell

1. Carefully slice open an egg that had been stored in PBS, maximizing the amount of intact membrane.
2. Remove and discard interior contents
3. Gently rinse and store membrane in 1X PBS, pH 7.4, until ready to use.
4. Assemble Franz chamber:
    1. Place receptor chamber into a 100 mL beaker containing temperature beads.
    2. Add a micro stir bar to the receptor chamber.
    3. Add 5 mL of 1X PBS, pH7.4 into the receptor chamber.
    4. Take your egg shell membrane and cut a circle approximately 2 cm in diameter.
    5. Place this circle of egg membrane on top of the receptor chamber, completely covering the orifice (9 mm diameter).

6. Gently place the empty donor chamber on top of the membrane, and using the provided clamp, attach it in place.
7. Use a piece of KimWipe to gently blot away any liquid that may have escaped.
8. Place apparatus centred on top of the magnetic stir plate, and set the speed to 200 rpm.

5. Fill top chamber with 1 mL of our 2 mg/mL compound
6. Immediately remove 120 µL from the receptor chamber twice via the sampling tube using a p200 micropipette set to 120 µL and fitted with a long gel-loading tip. Place the samples into labelled 1.7 mL microfuge tube. These are your time = 0 time points (T0) (performed in duplicate).
    1. You **MUST** be consistent when sampling – if you pipette mix 3 times before taking the sample, you must do this every time you sample!
7. Add in 240 µL of fresh buffer via the sampling tube to replace the amount taken (add 120 µL twice).
8. Set a timer for 5 minutes. When it goes off repeat steps 6 and 7 to take your next time point (T5).
9. Continue to sample every five minutes for one hour. You should end up with a total of 13 time points (T0-T60) in duplicate.
10. Load 100 µL of each time point into a micro plate and read at the appropriate wavelength for the drug.

d) References

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3. Solubility: Solvents and pH

a) Background

Ensuring your compound can be put into a liquid form, and ideally into an aqueous buffer, is essential for being able to do a great many of the tests needed to evaluate the drug. There are a number of places you can look for solubility information, including the manufacturer's website, or PubChem (https://pubchem.ncbi.nlm.nih.gov/) if the compound you're dealing with is commercially available. Sometimes you're lucky and can ask the chemist who made it, but usually, it ends up involving a search of the relevant literature then doing trial and error test in the lab when it becomes apparent that the literature disagrees wildly. This can be due to differences in equipment, temperature, the exact solvents, or source compound used. Regardless of the reason, you're left trying to figure out how to dissolve the compound you need to run your assay.

b) Experimental Design

The instructional team have prepared small, pre-weighed amounts of several compounds, and placed them in labelled tubes at your bench. You have 5 tubes each of two different compounds, and will add a different solvent to each tube. Depending on the compound and the amount present in the tube, you will need to add a different amount of solvent to achieve the correct final concentration.

1. Calculate the appropriate amount of solvent needed to make a 10 mg/mL solution for each tube. Add a maximum of 1 mL of solvent.
   a. You are combining a set mass and adding volume to get the ratio 10 mg to 1 mL of solvent
      E.g. If you had 7.6 mg of drug, how much solvent would you need?
      V = 7.6 mg / 10 mg/mL, V = 0.760 mL = 760 µL
      *Confirm: 10 mg/mL * 0.76 mL = 7.6 mg*

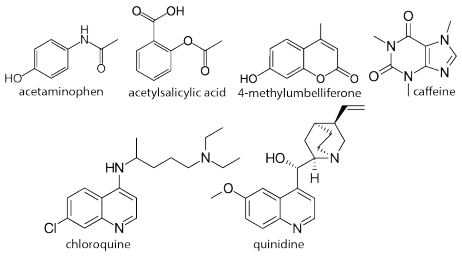
2. To each tube, add the appropriate amount of your calculated solvent:

Solvent	Compound A	Compound B
mQH_2O		
100% Ethanol		
DMSO		
0.1 M acetic acid		
0.1 M NaOH		

3. Tightly close the cap on each tube, and mix well using the vortex.

4. Look at each tube and evaluate if all of the drug has gone into solution. Let the tube sit in the tube rack for several minutes and look at it again – has any of the drug come out of solution?

5. Some physiochemical properties of several drugs are given below. Do any of these properties help predict the solubility of your tested compounds?
   a. Acetaminophen (pKa: 9.46, LogP: 0.46)
   b. Acetylsalicylic acid (pKa: 3.50, LogP: 1.18)
   c. 4-methylumbelliferone (pKa: 7.79, LogP: 1.9)
   d. Caffeine (pKa: 14, LogP: -0.55)
   e. Chloroquine diphosphate (pKa 10.32, LogP: 4.63)
   f. Quinidine sulfate (pKa: 8.56, LogP: 3.44)



Laboratory 1 – Solubility and Transport Worksheet [38 points total]
Due: In lecture, the day after lab.

Osmosis: Osmolarity and Osmolality [16 points]

1. **Observations**

Fill in the chart with your observations and calculations. In the observations column, record what you noted about of the properties of the eggs for each treatment. % Change = ((final-initial)/initial)*100 **[9 points]**

Egg ID	**Initial mass (g)**	**Final mass (g)**	**Observations**
Solution	**% Change**		
Egg ID	**Initial mass (g)**	**Final mass (g)**	**Observations**
Solution	**% Change**		
Egg ID	**Initial mass (g)**	**Final mass (g)**	**Observations**
Solution	**% Change**		
Egg ID	**Initial mass (g)**	**Final mass (g)**	**Observations**
Solution	**% Change**		
Egg ID	**Initial mass (g)**	**Final mass (g)**	**Observations**
Solution	**% Change**		

2. Circle/highlight correct answer for each statement. [4]
    a. The **3M sucrose** solution is **hypertonic | isotonic | hypotonic** compared to the egg.
    b. The **1.5 M NaCl** solution is **hypertonic | isotonic | hypotonic** compared to the egg.
    c. The **PBS** solution is **hypertonic | isotonic | hypotonic** compared to the egg.
    d. The **5% acetic acid** is **hypertonic | isotonic | hypotonic** compared to the egg.

3. What are the **osmolalities** of the 1.5 M NaCl and 3M sucrose solutions? Which solution caused the largest change (% change) in the weight of the naked egg and in which direction (loss or gain of fluid)? [3]

4. What is the purpose of keeping one of the eggs in PBS? Hint: think in the context of the whole lab. [1]

Semipermeable membranes [8 points]

1. What drug did we use to test the permeability of the egg membrane? [1]

2. What wavelength do we need to use to detect our drug of interest? [1]

3. Why is it important to do replicates? [1]

4. In diffusion studies, there is usually an interval between the start of the experiment and the steady state being reached, the lag time. This is the earliest point at which the linear region of the graph of cumulative drug vs time starts when looking at the x-axis.
    a. What is the equation of the line and R^2 for our compound standard curve? [1]

    b. What is the equation of the line and R^2 for the graph of Total permeation over time? [1]

c. What is the equation of the line and R^2 for just the linear region of the graph of Total permeation over time? Hint: You can use the calculation of instant flux at each time to help determine when the system has reached steady state. [1]

d. What is the lag time for this combination of compound and membrane type? [1]

5. What was the average instant flux once the system reached steady state? [1]

Solubility: Solvents and pH [14 points]

1. The compound and the mass provided is written on the top and/or side of each tube. Use this information to calculate how much solvent will be needed to dissolve the mass in the tube to produce a solution with the final concentration of 10 mg/mL. Show your work. [5]

2. Fill out the table below **[5]**

Compound	**Mass of compound (mg)**	**Solvent**	**Solvent volume (µL)**	**Complete dissolution? Yes / No**
		mQH2O		
		100% EtOH		
		DMSO		
		0.1 M Acetic acid		
		0.1 M NaOH		

Write compounds = 1, write masses = 1, solvent volume = 1, dissolve = 1. If >2 values missing for any one category = 0

3. What can you conclude about the solubility of your two compounds? Are they hydrophilic or hydrophobic, weak acids or weak bases? What solvent should you use to best dissolve each compound? [3]

4. Why might you choose one solvent over another if multiple possible solvents would work to dissolve your compound? [1]