

Droplet Interface Bilayers for Drug Discovery

Robert Strutt

A thesis presented for partial fulfilment for the degree of Doctor of Philosophy
March 2022

Department of Chemistry
Imperial College London

I, Robert Strutt, declare that all work contained within this thesis is my own unless otherwise stated.

‘Looking at biology from a chemical design point of view is like staring up at the Great Pyramid
on your first day of an engineering degree’ – *Nessa Carson*

For Nerissa.

Copyright Statement

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-NonCommercial 4.0 International Licence (CC BY-NC). Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose. When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes. Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Abstract

Droplet interface bilayers (DIB) formed at the contact of two aqueous in oil emulsion droplets, show huge potential in a range of fields. This work is concerned with the application of DIB technology in drug discovery and industrially relevant processes as a model system for measuring passive, biological membrane diffusion. A current limitation of the field has been an over reliance on fluorescence as a read out for permeant concentration. This thesis presents two separate methods for label-free DIB translocation experiments engineered with a suite of fabrication techniques, *in situ* data analysis pipelines and semi automation to perform UV spectroscopy in nL volume droplets. In one method, one of the DIB droplets is quantitatively measured and in the other, full translocation dynamics are obtained through mobilisation of a beam path, across multiple DIB droplet pairs. These methods unlock highly relevant permeant structure - permeability relationships decoupled in a DIB environment.

With these developed methods, studies were performed across a series of distinct chemical and biophysical spaces. Systematic functional group changes afforded a correlation between DIB permeability and drug-likeness. Within a biological dataset it was found that native compounds behaved in a biomimetic fashion within DIBs, highlighting the role of compound labelling. Over 30 compounds were assayed across wide chemical space, broadly representative of drug design with a MW range of 150 to 1130 Da. Permeant structure and physicochemical metrics were linked to the DIB permeability. It was found that oil partition as a model for bilayer permeability is inherently flawed, opening DIBs as a replacement of current, industrially applied technologies. Finally, efforts were placed on a more mature synthetic membrane. A bacterial mechanosensitive transmembrane protein was gated in a concentration dependent manner through lipid composition of the membrane. Upon activation, a membrane spanning, non-selective pore was opened, independent of the permeant concentration gradient across the membrane. This thesis concludes with an outlook of the potential of DIB technology in achieving fully biomimetic lipid interfaces with the view to provide bespoke models of biological membranes which barrier protein targets. These results, enabled by the novel methods designed herein, suggest DIBs have the potential to provide a step change in *in vitro* drug translocation assays.

Acknowledgements

This research would not have been possible without support. Since starting this programme in 2018, much of my development can be attributed to my interactions with a fantastic range of people. As part of my appreciation, I wish to address each individually.

I must start by acknowledging the major contributor to this work. To Oscar Ces, I will always be pleased that in official OC canon, I was the Kingston University student with the pen, paper and thoughts on your research. I am thankful on the first day we met, you interpreted my questions positively. Thank you for taking a chance on me. It speaks to your talents as a mentor that I can identify in many ways how your guidance will continue to aid me. I hope that one day, to even half your capacity, I can nurture talent within people just as you have done for me.

Working within the Membrane Biophysics Group has been a fulfilling process and I have been lucky to have learnt from other academics. To Yuval Elani, it has been all parts inspiring, insightful and fulfilling to follow the growth and development of your research team in conjunction with my PhD. Thank you for your support, time and I hope I am not misplaced in saying, friendship. To Nick Brooks, thank you for always having the time to discuss ideas with me and to point me toward the next thing to tinker around with. To Rob Law, for putting ideas through the ringer and for knowing when to take decisive action. To John Harling for always providing insight and discussing my results from the industrial viewpoint. To David Miller, for your commitment to our collaboration and for all you have taught me – working together at the Hackspace during tumultuous times was a highlight of these last few years.

I have been lucky to also have worked alongside some of the finest people I have met. This has fostered close friendships and I hope that these will extend beyond these years. To Callum Hay, I will always be indebted to you incorporating me into your closest circle of friends and for taking me under your wing when I first arrived at Imperial. I hope that your unwaveringly calm outlook has rubbed off on me, even in the slightest. It has been an absolute pleasure to share the lab with you. Experiments within this thesis were often conducted during, what my good friend Nathaniel Gallop would refer to as; a 'stinker' (noun) - 'to stay unsocial hours (within H&S guidelines) in the laboratory'. To Neath, thank you for taking on the role of friend and mentor. Your wise insight and support was ever present. To Mohit Devgan, one of the kindest people I have met, an all-round good friend, colleague and role model. To Nacho Gispert, for being a fine source of clarity and wisdom. For finding the time and for being sensitive and in tune. To Jenny Liu, for being a constant source of cheeky humour and friendship. I should insert a pun here but I don't think it would fit the tone. To Greta Zubaite, for effortlessly upholding a skillset in science and art. For being a great friend, a confidant and a source of good humour. To Felix Sheffield, fellow Kingston alumnus, thank you for being a fantastic colleague, collaborator and friend. To Victor Chang, for supporting me with my recovery from knee surgery. Our days playing football together were some of my favourites. To Divya Malia, thank you for being a great colleague and friend to myself and Nerissa. To James Hindley, thank you for being a role model and friend. I will always be thankful for your ability to find time, make a good recommendation (science or media) and for your sage like advice. I hope we can continue to work together in the future. To Hisham Abdel Aty, thank you for being a constant source of coding inspiration and for your

collaboration. To Matt Allen, thank you for your advice and collaboration. I eagerly await your development as a scientist and your burgeoning career, but most of all the continuation of our great friendship.

Whilst I have shared the lab with brilliant colleagues, I have also shared it with some equally brilliant students. To Ziyang (Sherry) Wu, Dat Doan, Yingting Jia, Ignacy Bartnik, Jordan Gregg and Niall McIntyre, thank you all for your patience with my supervision. I hope my involvement in your research was as rewarding and insightful as the experience of supporting yours has been. I wish you all the very best in your next career steps.

And finally, to all other lab mates and colleagues; Susie Molisso, Norman Chan, Miguel Paez Perez, Lucy Rowlands, Nate Barlow, Divesh Baxani, Claudia Contini, Yu Chen, Shaobin Zhang and Mark Friddin. An emerging theme of these acknowledgements is that I may have difficulty accounting for the contribution of a multitude of people and my thanks for all of your involvements is hard to quantify. For any I have left out, it is completely inadvertent.

There are those within my private life who, without, I would not have been able to pursue this research. I thank my dearest friends, Asith Rajapakse, Scott Montague Murdoch, Moe Ali, Tay Devenny, Rob Lefebour, Hugo DeJonge, Jamie Livens, Alexandra Stevens, Ben Stafford, Kaleb Markham, Ben Hughes, Charlie Cobb, and Martha Fillery. Your support, patience and good humour has enabled me throughout these years. I also recognise all those who, independent of intention, assured my pursuit of a youthful hunch. I hope that this thesis stands as testament to such faith placed in me.

To Nerissa Schuit, you have been core to my personal and psychological maintenance. Your kindness, gentleness and love for me has supported this work. Thank you.

And finally, to my family, for your patience, unwavering support, and for always being a phone dial away. I know that I have made you proud, independent of the potential success of this work. I will always know that I pursued this doctorate through my own intention and that this pursuit was carved out by my own will. Thank you for encouraging this within me.

Publications

Journals

- 1st – January 2022 – Strutt et al. - UV-DIB: Label – Free Permeability Determination Using Droplet Interface Bilayers –**RSC Lab on a Chip**
- 1st - January 2021 – Strutt et al. - Activating Mechanosensitive Channels Embedded in Droplet Interface Bilayers using Membrane Asymmetry – **RSC Chemical Science**
- 1st – April 2022 – Strutt et al. - A Multiplexed High Throughput Platform for Label Free Permeation Studies in Droplet Interface Bilayers – (In preparation)

Conference

- 1st - July 2021- The Design and Application of Label Free Detection in Droplet Interface Bilayers for Drug Discovery Relevant Permeation Studies – Talk – **nanoGE Emerging Investigators in Microfluidics Conference**
- 1st – June 2021 - A Novel Droplet Interface Bilayer Platform for Drug Discovery Relevant Permeation Studies – Talk – **SoftComp EUSMI Conference**
- 1st - July 2019 - The Design and Application of Novel Permeability Systems to Probe Analyte Transport Across Droplet Interface Bilayers – Poster - **European Biophysical Societies' Association and The International Union of Pure and Applied Physics conference**

Code and CAD

All code and CAD files / drawings used throughout this thesis are available via Github [link](#).

All CAD drawings are included in the appendix:

- Data processing from single cannula device (Chapters 3 - 4)
- Fitting methods and analysis pipelines (Chapters 3 – 6)
- Controlling spectroscopic capturing through Arduino (Chapters 5 and 6)
- Pipeline for analysing electrophysiology data (Chapter 7)
- Single cannula CAD
- Parts for fabricating a spectroscopic platform

Table of Contents

1	<i>Droplet Interface Bilayers for Drug Discovery – An Introduction</i>	22
1.1.	Literature introduction – overview	22
1.2.	The plasma membrane and transport phenomena – understanding a highly complex and dynamic organelle	23
1.2.1.	Cell membrane structure – the organisation of lipids in membranes	23
1.2.2.	Permeability mechanisms of simple diffusion at the plasma membrane	27
1.3.	Modelling the plasma membrane in a drug discovery pipeline	29
1.3.1.	Oral bioavailability	29
1.3.2.	The Parallel artificial membrane permeability assay (PAMPA)	30
1.3.3.	Cell based assays	31
1.3.4.	Modelling bilayer permeability within a drug discovery perspective	31
1.3.5.	The Lipinski rules have informed small molecule drug discovery	34
1.4.	Droplet interface bilayers (DIBs) – A platform for <i>in vitro</i> transport modelling?	35
1.4.1.	Droplet interface bilayers (DIBs)	35
1.4.2.	Reconstitution of membrane proteins into DIBs	38
1.4.3.	Droplet interface bilayers for permeability	38
1.4.4.	Label free measurements in DIBs – a review of the literature so far	40
1.5.	Thesis aims	42
2	<i>Materials and Methods</i>	43
2.1.	Chapter overview	43
2.2.	Fabrication	43
2.2.1.	Laser cutting	43
2.2.2.	Bonding materials	43
2.3.	DIB formation	44
2.3.1.	Lipid film preparation	44
2.3.2.	Lipid out	45
2.3.3.	Lipid in and extrusion	45
2.3.4.	Sample preparations	46

2.4.	Theory and application of analytical methods.....	47
2.4.1.	UV-vis spectroscopy	47
2.4.2.	Electrophysiology.....	48
2.4.3.	Deployed python libraries and analysis	49
2.5.	Chapter specific methods.....	50
2.5.1.	Chapter 3.....	50
2.5.2.	Chapter 5.....	50
2.5.3.	Chapter 7	51
2.6.	Purchased chemicals and materials.....	51
2.7.	Permeability model	52
2.7.1.	Model for single beam path chip	52
2.7.2.	Model for permeability and partition measurement – chapter 6	54
3	<i>The Build and Design of a Simple Label Free Permeability System – Laser cut milli fluidic systems</i>	57
3.1.	Chapter overview	57
3.2.	Introduction to laser cut fluidic devices	57
3.3.	Using PMMA chip design to enable label free measurements.....	59
3.3.1.	Fiber implementation	59
3.3.2.	A heat and pressure block for sealing PMMA chips and arranging fibers	61
3.4.	A simple label free DIB permeability chip	62
3.4.1.	Single beam path chip design	63
3.5.	An experimental workflow for permeability assessment in DIBs with a single beam path chip.....	64
3.5.1.	Theophylline and caffeine as model permeants	65
3.5.2.	Spectral collection and data processing	65
3.5.3.	Converting to concentration	68
3.5.4.	Testing for oil partition.....	69
3.5.5.	Extracting a permeability value	70
3.5.6.	Data analysis pipeline	72
3.6.	A millifluidic build out of a single beam path chip.....	74

3.7.	A millifluidic chip for studying reactions under flow	77
3.8.	Chapter conclusions	79
4	<i>Single Droplet Label Free DIB Permeability Studies – DIB biophysical features, systematic chemical space and small molecule drugs</i>	80
4.1.	Chapter overview	80
4.2.	Introduction to <i>in vitro</i> structure – permeability relationships	80
4.3.	DIB biophysical experiments	81
4.3.1.	DMSO % in buffer	81
4.3.2.	Bulk oil phase	84
4.3.3.	Lipid location and DIB formation mechanism	86
4.3.4.	Forming bilayers of higher biomimicry and complexity with lipid in	88
4.3.5.	Zip up time – all conditions.....	90
4.3.6.	Section conclusion	91
4.4.	Systematic permeant modifications – establishing permeability – structure relationships with commercial compounds.....	92
4.4.1.	The xanthine series.....	92
4.4.2.	DOPC vs DPhPC permeability reduction across the xanthine series.....	97
4.4.3.	Colchicine and chloramphenicol – oral drugs	99
4.4.4.	Iodipamide and derivative – Radiolabelling molecules	101
4.5.	GSK compounds – industrial compounds.....	102
4.5.1.	Supplied compounds	102
4.5.2.	Oil partition GSK compounds.....	102
4.5.3.	Permeable GSK compounds.....	103
4.5.4.	Section conclusion	105
4.6.	Chapter conclusions	106
5	<i>The Build and Design of a High Throughput Label Free Method – A custom spectroscopic platform</i>	107
5.1.	Chapter overview	107
5.2.	Further development of label free concentration assessment.....	107
5.3.	Recording in both droplets - Dual beam path chip.....	108

5.3.1.	Commercial multiplexer	108
5.3.2.	Dual beam path chip	109
5.4.	Custom fabricated general fiber multiplexer	113
5.5.	Bespoke fabricated fiber multiplexer	114
5.5.1.	System overview.....	114
5.5.2.	A bespoke, linearly actuated spectroscopic platform.....	115
5.5.3.	Fiber support alignment testing	118
5.5.4.	UV transparent removable chips	120
5.5.5.	DIB formation in spectroscopic platform chips.....	122
5.5.6.	Custom software for controlling the spectroscopic platform with example theophylline DIB permeability system.....	123
5.5.7.	Droplet locating.....	126
5.6.	Chapter conclusions	127
6	<i>Permeability Studies with a Multiplex Label Free Method – Complex drug oil partition, permeant chirality and DIB networks</i>	129
6.1.	Chapter overview	129
6.2.	Introduction	129
6.3.	Multi - droplet permeation assessment.....	131
6.3.1.	Theophylline DIB permeability	131
6.3.2.	Theophylline DOPC networks	133
6.3.3.	Bromocaffeine – assessed with all methods.....	135
6.4.	Quantifying translocation behaviour – multi beam experiments.....	138
6.4.1.	Drug array.....	138
6.4.2.	Comparing permeability derived between methods.....	140
6.5.	The role of permeant chirality on translocation	144
6.5.1.	Amino acid permeability – systematic assessment of tagging and charge ...	145
6.5.2.	Chiral permeability – enantioselective diffusion of a tagged amino acid	146
6.6.	Oil partition – quantitative assessment.....	148
6.7.	Chapter conclusions	150

7	<i>Mechanosensitive Channel Gating – A tool for controlling membrane permeability</i>	151
7.1.	Chapter overview	151
7.2.	Mature synthetic bilayers with embedded flux control	152
7.2.1.	The mechanosensitive channel of large conductance (MscL)	152
7.2.2.	Overview – controlling membrane porosity with bilayer composition	153
7.2.3.	Protein expression	154
7.3.	Assessment of LysoPC mediated MscL activation - a vesicle assay	154
7.4.	Mechanosensitive channel sensing – a DIB assay	156
7.4.1.	System controls	156
7.4.2.	Concentration dependent LysoPC mediated MscL activation in DIBs	158
7.4.3.	Coding pipeline to assess gating events – example of gating analysis.....	160
7.4.4.	Partial activation	161
7.4.5.	Full activation.....	163
7.4.6.	DSA findings.....	164
7.4.7.	The potential utility of LysoPC mediated MscL activation in DIBs.....	166
7.5.	Chapter conclusions	167
8	<i>Conclusions and outlook – Toward improving biomimicry in DIB systems.....</i>	168
8.1.	Conclusion - Major findings of this research.....	168
8.1.1.	Two novel methods for label free DIB permeability measurement.....	168
8.1.2.	Structure – permeability relationships derived from DIB interfaces.....	168
8.1.3.	Controlling permeability with membrane composition.....	168
8.2.	Conclusion - Correlating DIB permeability to structural signatures	169
8.2.1.	Summary of measured permeability response	169
8.2.2.	PCA	170
8.3.	Outlook - The use of DIBs as an alternative to PAMPA.....	171
8.3.1.	Application in novel small molecule drug development space – the case for PROTAC permeability.....	171
8.4.	Outlook - The relationship between biomimetic bilayer composition and permeability response	173

8.4.1.	A method for heating droplets	173
8.4.2.	Permeability response of a range of small molecule drugs to membranes of high compositional complexity and biomimicry – an outlook for the future of translational DIB permeability assays.....	174
8.5.	Final remarks	176
9	<i>References</i>	177
10	<i>Appendix</i>	197
11	<i>Appendix 2 - CAD drawings</i>	201

Table of Figures

Figure 1.1 An overview of transport at the plasma membrane.	23
Figure 1.2: The molecular self-assembly of lipids depends on their chemical structure	25
Figure 1.3: A summary of physical properties of the PM originating from the lipids.	27
Figure 1.4: Molecular mechanisms of passive lipid – drug interactions at the PM.	29
Figure 1.5: Industrial in vitro assays for permeability and absorption.	30
Figure 1.6: Summary of logP determination..	31
Figure 1.7: Droplet interface bilayers.....	35
Figure 1.8: An overview of the surface energy of a DIB.	37
Figure 1.9: An overview of the bilayer method presented by the Choi group.....	41
Figure 2.1: Bonding techniques.....	44
Figure 2.2: Schematic for forming a DIB from a lipid film..	46
Figure 2.3: An overview of the light path taken from light source to the production of a spectrum.	47
Figure 3.1: A brief comparison of the workflows employed in traditional PDMS as compared to PMMA based chip generation.	58
Figure 3.2: Overview of spectroscopy equipment employed in this chapter and throughout..	60
Figure 3.3: Embedding fiber optics within PMMA chips.	61
Figure 3.4: Custom aluminium heating block.....	61
Figure 3.5: Single beam path DIB chips for full broadband spectral collection.....	63
Figure 3.6: The structure of the methylxanthines theophylline and caffeine, with a brief overview of the physicochemical and spectral properties of each.....	65
Figure 3.7: Spectra standardisation enabled smooth intensity time curves..	67
Figure 3.8: Theophylline calibration curve.	69
Figure 3.9: Different partition droplet morphologies within the single beam chip have no impact on the absolute intensity value or intensity distribution.	70
Figure 3.10: Concentration time distribution of theophylline DOPC DIB acceptor droplet.	72
Figure 3.11: Overview of python script to automate the determination of permeability from label free measurement in DIBs..	73
Figure 3.12: A millifluidic approach to DIB formation.	74

Figure 3.13: Results from millifluidic single beam path chip.	75
Figure 3.14: A millifluidic chip for studying reaction kinetics.....	77
Figure 3.15: Results from millifluidic chemiluminescent reaction chip.	78
Figure 4.1: The biophysical features of a DIB permeability system explored in chapter 4.....	81
Figure 4.2: Adjusting DMSO content in the aqueous phase.....	83
Figure 4.3: Adjusting the bulk oil phase to squalene.....	85
Figure 4.4: Adjusting the monolayer formation mechanism.....	87
Figure 4.5: Adjusting SM content in DIBs using the lipid in approach.....	89
Figure 4.6: DIB zip up times.....	91
Figure 4.7: Summary of studied methyl xanthine derivative compounds.	93
Figure 4.8: Measured distributions of xanthine series.	94
Figure 4.9: Measured permeability of xanthine series.....	96
Figure 4.10: DOPC and DPhPC permeability compared across the xanthine series	97
Figure 4.11: Measured distributions of commercial drugs.....	99
Figure 4.12: Measured distributions of radiolabelling compounds..	101
Figure 4.13: Summary of studied GSK compound set..	102
Figure 4.14: Compounds that demonstrated high oil partition.	103
Figure 4.15: Permeable GSK compounds	104
Figure 5.1: Overview of different measurement methods.....	107
Figure 5.2: Image and cartoon of purchased Ocean Optics UV fiber multiplexer MPM-2000.....	109
Figure 5.3: Dual beam path chip overview	110
Figure 5.4: Caffeine translocation distributions collected with the dual beam path chip	112
Figure 5.5: A bespoke design for a custom general fiber multiplexer.	113
Figure 5.6: Overview of a bespoke method to achieve label free, high throughput, multi droplet DIB permeability assessment..	115
Figure 5.7: Cartoon side view of a linearly actuated platform.....	115
Figure 5.8: Custom fiber supports	116
Figure 5.9: The assembled spectroscopic platform with fitted fiber optics..	118
Figure 5.10: Fiber support alignment experiments.....	120
Figure 5.11: Chip formation for spectroscopic platform.....	121

Figure 5.12: DIBs formed in spectroscopic platform chips.	122
Figure 5.13: Operation of spectroscopic platform with custom software.....	123
Figure 5.14: An illustration of the utility of custom software for permeation experiments..	125
Figure 5.15: Droplet scanning revealed a suitable measurement band in DIBs.....	127
Figure 6.1: Dual droplet monitoring with different lipids.....	131
Figure 6.2: DIB networks with theophylline.....	133
Figure 6.3: Experiments with 8-bromocaffeine – a compound with significant oil partition.	136
Figure 6.5: Partition behaviour across a range of drug structures	139
Figure 6.6: Permeability values compared across methods..	141
Figure 6.7: Uncovered trends across permeability systems and fitting approaches.....	143
Figure 6.8: Tryptophan and tyrosine are DOPC bilayer impermeable..	145
Figure 6.9: The permeability and oil partition for each enantiomer of two chiral permeants..	146
Figure 6.10: Summary of findings in this section, highlighting how subtle changes to the chemical structure can yield large differences in the measured permeability.	148
Figure 6.11: Oil partition across a wide physiochemical space.	149
Figure 7.1: Structure of MscL.....	152
Figure 7.2: Activation of MscL with asymmetric DIBs – an electrophysiology approach.....	153
Figure 7.3: Overview of vesicular system for LysoPC mediated MscL activation.....	154
Figure 7.4: Results from vesicle assays of MscL	155
Figure 7.5: Control systems for the role of LysoPC in bilayer response.....	156
Figure 7.6: Control experiments for baseline MscL activation in symmetric membranes.	157
Figure 7.7: Increasing activation of MscL with increasing asymmetric LysoPC content..	159
Figure 7.8: Analysing MscL gating activity.....	160
Figure 7.9: Flickering activity of MscL between the closed and smallest gating state at a consistent resolution.....	161
Figure 7.10: Gating of MscL from partial to fully open states.....	163
Figure 7.11: DSA results of LysoPC compositions.....	165
Figure 7.12: All recorded MscL gatings for 10 and 15 % LysoPC asymmetric activation in DIBs.	166
Figure 8.1: Summary of permeability values measured within this thesis	169

Figure 8.2: PCA analysis of Lipinski descriptors (HBA, HBD, MW, AlogP, TPSA and RBN) of all measured compounds	170
Figure 8.3: A cartoon overview of the novel therapeutic mechanism of PROTAC compounds.....	172
Figure 8.4: Custom heating block for temperature-control in label free DIB permeability measurements.....	174
Figure 8.5: An outlook for building biological complexity in DIBs – toward an improved understanding of membrane permeability.	175

Abbreviations

BBB	- Blood brain barrier
BSA	- Bovine serum albumin
CAD	- Computer aided design
CNC	- Computer numerical control
DHB	- Droplet hydrogel bilayer
DI	- Deionised (water)
DIB	- Droplet interface bilayer
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
DSA	- Droplet shape analysis
Fa	- Fraction of dose absorbed
FRR	- Flow rate ratio
GSK	- GlaxoSmithKline
HBD/A	- Hydrogen bond donor / acceptor respectively
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	- High-performance liquid chromatography
ICAH	- Imperial college advanced hackspace
IFT	- Interfacial tension
IMHB	- Intramolecular hydrogen bonding
logP	- octanol / water partition coefficient
(A)logP	- atomistic logP
(c)logP	- calculated logP
logP_{alk}	- alkane / water partition coefficient
MB	- Megabyte
MDCK	- Madin-Darby canine kidney (cells)
MLV	- Multilamellar vesicles
MS	- Mass spectrometry
MscL	- Mechanosensitive channel of large conductance
MTSET	- [2-(Trimethylammonium)ethyl]methanethiosulfonate bromide
MW	- Molecular weight
MWCO	- Molecular weight cut off
ODE	- Ordinary differential equation

PAMPA - Parallel artificial membrane permeability assay

PBS - Phosphate buffered saline

PCA - Principal component analysis

PDMS - Polydimethylsiloxane

p-GP - p-glycoprotein

PID - Proportional integral derivative

PM - Plasma membrane

PMMA - Polymethyl methacrylate

PROTAC - Proteolysis targeting chimera

PTFE - Polytetrafluoroethylene

QSAR - Quantitative structure activity relationship

RBC - Red blood cell

RBN - Rotatable bond number

S/L/GUV - Small / Large / Giant unilamellar vesicle

SDOM - Standard deviation of the mean

SLB - Supported lipid bilayer

SLC - Solute carrier protein

SMA - Sub miniature assembly version A (connector)

TPSA - Topological polar surface area

UV - Ultraviolet (wavelength light)

UWL - Unstirred water layer

Vis - Visible (wavelength light)

αHL - Alpha hemolysin

Lipids

PA - Phosphatidic acid

PC - Phosphatidylcholine

PE - Phosphatidylethanolamine

PEp – Phosphatidylethanolamine plasmalogen

PI - Phosphatidylinositol

PS - Phosphatidylserine

DOPC - 1,2-dioleoyl-sn-glycero-3-phosphocholine

DOPE - 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

DOPG - 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)

DPhPC - 1,2-diphytanoyl-sn-glycero-3-phosphocholine
LPC - Lyso-phosphatidylcholine
LysoPC - 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine
POPC - 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
SPLE - Soy polar lipid extract

Permeants

17D - 1,7 dimethyluric acid
3MX - 3-methylxanthine
8BR - 8-bromocaffeine
CFN - Caffeine
PPF - Propentofylline
PTX - Pentoxifylline
TBR - Theobromine
TPH - Theophylline
XNE - Xanthine

Symbols

A - Bilayer area

C_{A/D(t=0)} - Initial concentration of acceptor / donor respectively

C_{EQ} - Equilibrium concentration

I_{UV/VIS} - Intensity of UV and Vis standardisation regions respectively

k₂ - Oil partition rate constant

L_c - Lamellar crystalline phase

L_α - Lamellar fluid phase

L_β - Lamellar gel phase

o/w - Oil in water

P - Permeability

T_g - Glass transition temperature

T_m - Melting temperature

V_{A/D} - Volume of acceptor / donor droplets respectively

w/o - Water in oil

λ_{max} - Absorption maxima

γ_{m/b} - Surface tension of a lipid monolayer / bilayer respectively

θ - Contact angle

1 Droplet Interface Bilayers for Drug Discovery – An Introduction

1.1. Literature introduction – overview

The broad aim of this thesis was to develop approaches for the screening of small molecule drugs across droplet interface bilayers (DIBs). This chapter is not intended as an introduction to every aspect of this thesis, but rather to establish a foothold in the literature. Across this thesis, each chapter opens with a targeted introduction. The results chapters (3 – 7) are split into method development (chapters 3 and 5) and application of the developed methods (chapters 4 and 6), ending with an approach for controlling the permeability profile of synthetic membranes (chapter 7).

This thesis begins by illustrating the transport of small molecules across biological membranes. A review is presented on current industrially applied methods for modelling transport phenomena *in vitro* and critically reviews historical trends within drug discovery literature from the perspective of membrane complexity. Next, the field of synthetic biology is introduced, from which DIBs emerged. The biophysical properties and behaviours of model bilayers are described, with a focus on their role in permeability. A critical review of the literature is presented from a methodological perspective, for which the field requires novel direction.

1.2. The plasma membrane and transport phenomena – understanding a highly complex and dynamic organelle

This section covers the fundamental biology of membrane organisation from the perspective of lipid compositional and behavioural complexity at the plasma membrane.

1.2.1. Cell membrane structure – the organisation of lipids in membranes

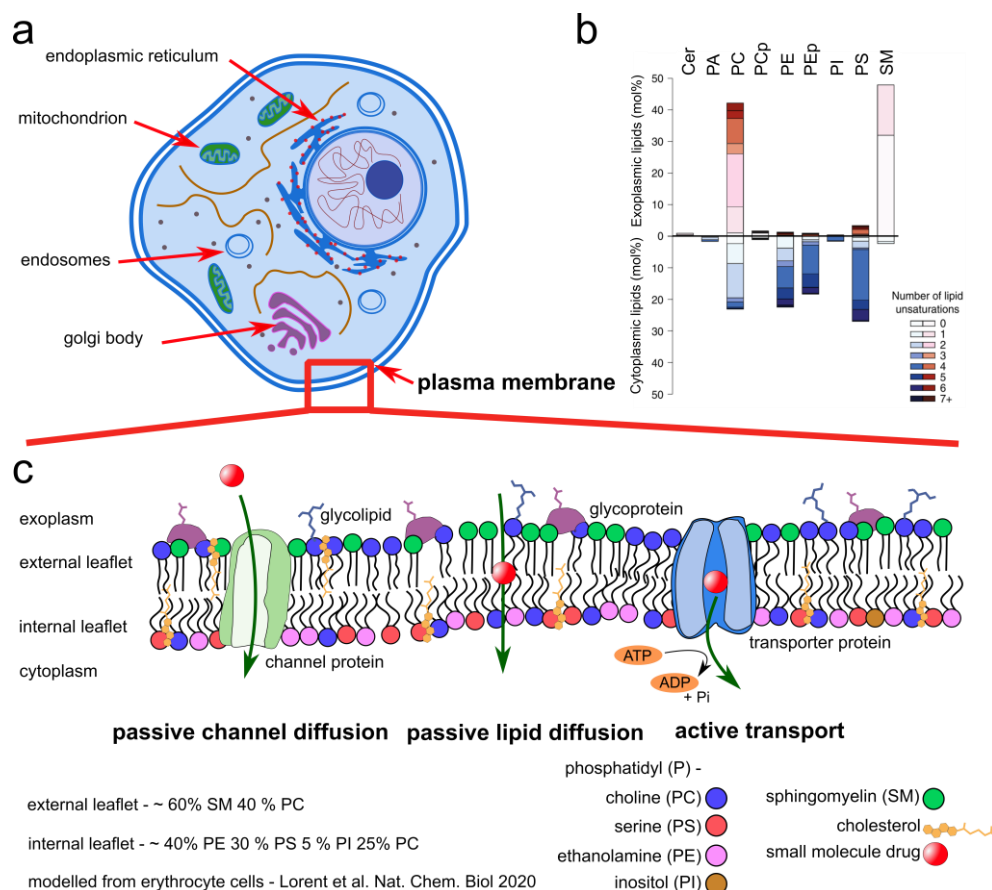


Figure 1.1 An overview of transport at the plasma membrane **a** Depiction of membranes within eukaryotic cells **b** The lipid composition of the plasma membrane – data adapted from Lorent et al, a lipidomic assessment of red blood cells (RBC).¹ Bar graph arranged by lipid head group; ceramides (Cer), phosphatidic acid (PA), phosphatidylcholine (PC) plasmalogen (PCp), phosphatidylethanolamine (PE) plasmalogen (PEp), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM). **c** Transport processes at the plasma membrane. Depiction of lipid head groups modelled from the distribution in b. Note that PEp has been interpreted as PE.

A cell is generally defined as a self-replicating unit, where the milieu of biological processes can be allocated to and conducted by internal structures known as organelles. The exterior and the organelles of eukaryotic cells are defined by membranes - semi permeable barriers which outline three-dimensional space within an aqueous volume. The plasma membrane (PM) is ~3 nm thick and defines all eukaryotic cells (figure 1.1 a - c).² The PM is built from concentrations of lipids, proteins and saccharides. Phospholipids are the major lipidic species of biological membranes³ and these molecules consist of two identifiable portions, a

polar lipid headgroup and at least one apolar lipid tail group. In an aqueous environment, the hydrophobic effect dictates that phospholipids undergo molecular self-assembly to form phospholipid bilayers, minimising the water contact of tail groups.⁴ The entropic cost of phospholipid organisation, is less than individual solvation. Membrane proteins can attach into membranes due to the maintenance of this enclosed hydrophobic volume. The incorporation of biological macromolecules generally distinguishes a 'membrane' from a 'lipid bilayer'. Therefore, lipids act simultaneously as the core building block of the PM as well as the facilitators of its immense functional complexity.

Phospholipids exhibit structural diversity in head and tail group composition which has led to estimations of ~400 PM species. Lipid head groups can be charged and glycosylated. Lipid tail groups can have double bonds or methylations and differ by total length, which effects the net membrane thickness (figure 1.1 b).⁵ Furthermore, an asymmetry exists between the composition of each leaflet of most biological membranes.^{1,6} Lorent et al. (2020) presents the most up to date PM lipidome, which can be seen in figure 1.1 b. The inner PM leaflet is more compositionally complex than the outer leaflet, which is dominated by SM and PC. The concentration of each lipid species at a given time can be affected by internal and external factors.⁷ Lipids can laterally diffuse within the membrane and flip across it, thus affecting the preservation of asymmetry.^{8,6} A transmembrane potential is established through charged lipids almost exclusively occupying the internal, cytoplasmic leaflet as well as ionic concentration differences across the membrane.⁹ Cellular physiology therefore demands that the membrane composition is maintained and controlled by a variety of mechanisms.¹⁰ Such compositional diversity has a functional consequence; the structure of lipids define the physical properties of membranes.

In the simplest terms, lipids behave dependent upon their cross-sectional area (figure 1.2 a, b). Bilayer forming lipids are known as type 0 and non-bilayer forming as type 1 or 2. The lateral pressure profile of a membrane results from the close packing of lipids arranged into self-organised structures. The pressure is highest between the head and tail group due to repulsion between head groups and an attraction between tail groups due to the hydrophobic effect. The lateral pressure profile is affected on a global and local level by the inclusion of type 1 or type 2 lipids which exert a deformation stress on the bilayer.¹¹ Type 2 lipids (such as many phosphatidylethanolamine (PE) species), are conserved in the inner PM leaflet, excellently reviewed by McMahon et al.¹²

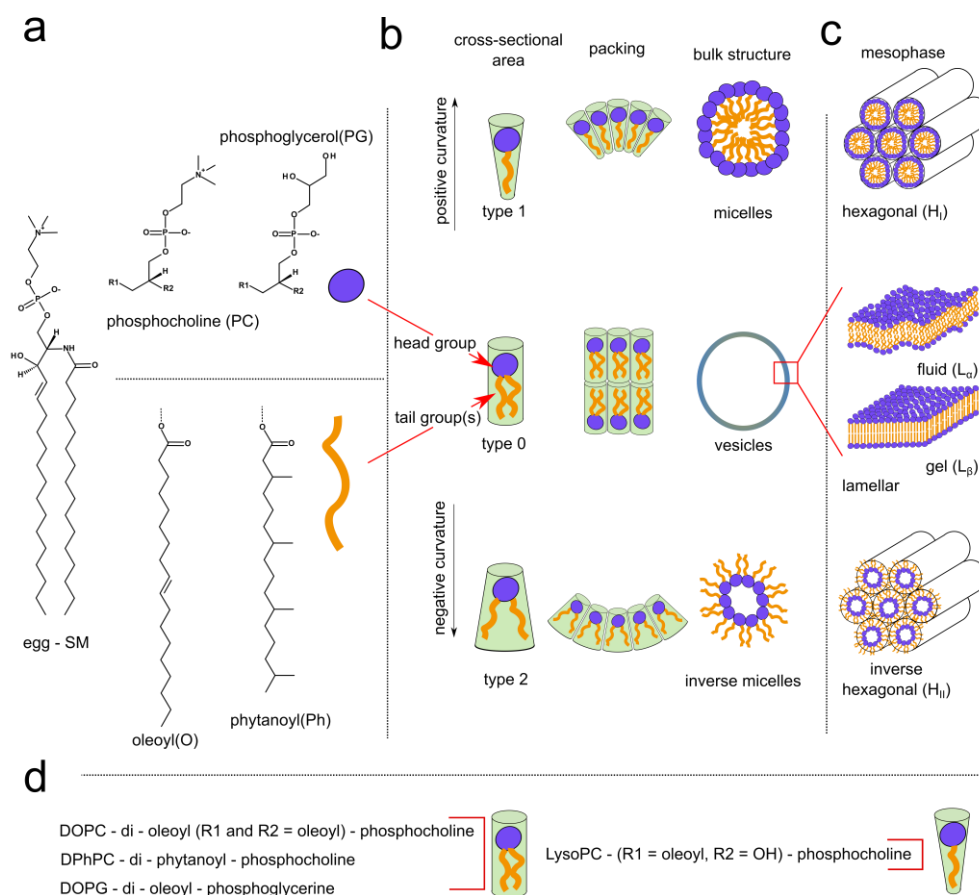


Figure 1.2: The molecular self-assembly of lipids depends on their chemical structure **a** Diagram of the chemical structure of lipids. **b** Dependent upon lipid cross-sectional area, the hydrophobic effect determines different bulk structures. **c** Example phases of macromolecular lipid assembly. For detail on the cubic phases, please see the following reference.¹³ **d** The naming convention of lipids. Here shown with the lipids used throughout this thesis. Figure adapted in part from multiple works.^{14,13}

The mesophase refers to a macromolecular assembly, suspended between the isotropic and crystalline liquid state.¹³ With respect to lipids, the mesophase is dictated by type as illustrated in figure 1.2, as well as the charge, tail group saturation and hydration. The lamellar mesophase describes essentially flat bilayers which can be assigned a phase based on fluidity and packing (figure 1.2 c). The typical lamellar phases from most packed to least are liquid disordered / fluid (L_α), liquid ordered (L_o) $\approx$ liquid gel (L_β) and liquid crystalline (L_c). Lipids which are medially organised in the plane of a bilayer by other lipids, result in the L_o phase.¹⁵ This phase maintains the fluidity of L_β however has reduced hydration and higher lateral diffusion.¹⁶ Overall, biological membranes exhibit the lamellar mesophase however within the membrane a coexistence of distinct phases can occur. L_o domains can be suspended within an L_α phase which is rich in unsaturated lipids.¹⁷ Phase boundaries outline the area of domains. The fluid mosaic model introduced by Singer and Nicholson (1972) was updated in 2013 to recognise highly packed L_o domains as ‘rafts’.^{11,18,19} Moreover, recent work suggests yet further complexity with a separation between the physical properties of

leaflets. The bulk PM exoplasmic leaflet is packed almost to the degree of the L_o phase and the cytoplasmic leaflet packed between that of the L_o and L_α phases.²⁰ Regulation of lipid composition at the PM, thus maintains asymmetry in lateral and medial ordering.¹

Lipid bilayers can undergo thermal, chemical or pressure induced phase transitions. In such a context, the melting temperature (T_m) refers to the thermal phase transition from L_α to L_β . Cholesterol acts as a chemical phase modifier within biological membranes where it increases the fluidity of L_β and decreases the fluidity of L_α . Cholesterol therefore has a propensity to arrange either leaflet toward L_o .²¹ This phenomena has been the focus of model systems, which have aimed to understand the biological consequence of cholesterol induced L_o domains. A ternary, DOPC, SM and cholesterol mixture is a classical model for the exoplasmic leaflet.²² In model systems, cholesterol is ~four times more soluble in bilayers of the L_α phase compared to the L_β phase. A positive linear correlation has been attributed to the amount of cholesterol in a leaflet and difference in inter-leaflet order.²³ The asymmetric distribution of cholesterol at the PM is contentious,¹ with debate surrounding an apparent high flip flop rate as compared to phospholipids.²⁴ Regardless, it seems plausible that biology exploits the ability of cholesterol to relieve mechanical stress through phase modulation.

Much of the observations made in pure lipid systems carry physiological relevance, however care must be taken when attributing to biological membranes.²⁵ Particularly, in how the properties of pure lipid bilayers account for protein dense membranes. A charitable interpretation of model systems is that lipid patches within biological membranes operate independent from membrane proteins apart from in the immediate protein vicinity. Many additional poorly understood factors exist such as phase organisation of membrane proteins,^{26,27} and the role of glycolipids in the exoplasmic leaflet.²⁸ The lateral pressure profile of the PM (figure 1.3) is mostly informed from model systems. Since leaflet order asymmetry is likely to affect this, an averaged lateral pressure profile may not adequately describe biological membranes. Whilst much work has uncovered fundamental physical forces obviously at play, in many ways biological membranes remain poorly understood, yet ubiquitous organelles.

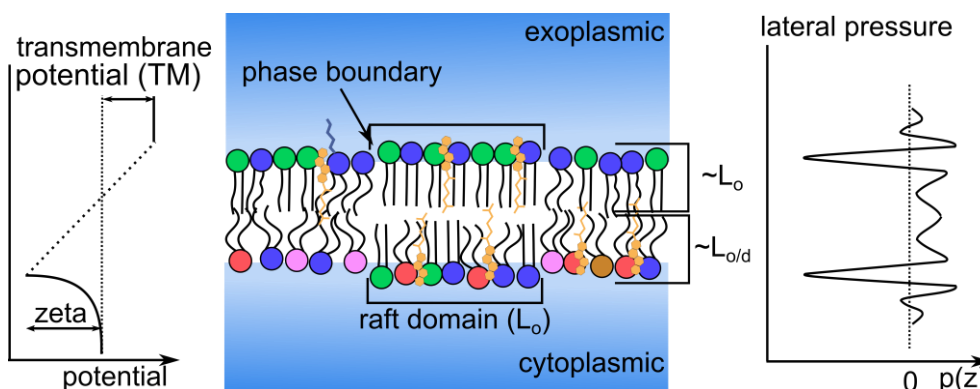


Figure 1.3: A summary of physical properties of the PM originating from the lipids. A simplified cartoon of the lipids is shown to indicate the emergence of raft domains, note the role of cholesterol in organising both leaflets. Lipids same as in figure 1, adapted from Lorent et al.¹ The transmembrane potential is maintained through two contributions; a difference in ion concentration between intra and extracellular volumes and a more local, zeta contribution from negatively charged cytoplasmic lipids. The lateral pressure profile is generated by the molecular composition and the interactions of, the lipids in the membrane. Both graphs have been adapted from reference publications.^{9,29}

1.2.2. Permeability mechanisms of simple diffusion at the plasma membrane

Molecular interaction with and translocation across biological membranes is a dynamic process. A concentration gradient across a membrane incurs a chemical potential gradient.³⁰ Additional mathematical detail is provided in chapter 2. Molecules in the exoplasmic volume are driven by diffusion across the PM to reduce the Gibbs function. From the perspective of a particular chemical structure distributed in the exoplasm, there are multiple transport routes at the PM (an overview is shown in figure 1.1 c). Passive and active mechanisms are distinguished by a dependence upon energy. Passive processes at the PM include passive lipid diffusion, and passive channel diffusion.^{31,32} Active processes at the PM include carrier mediated influx, carrier mediated efflux and transcytosis.³³ P-glycoprotein (p-GP) is a global regulator of non-native compound efflux, and represents a barrier that must be overcome for maximal drug efficacy.³⁴

As appropriate for this work, a focus is placed here on molecular mechanisms which describe passive diffusion of permeants through the lipids of biological membranes. The lipophilicity of a chemical structure is classically measured by its solubilisation into model polar and apolar solvents through the octanol-water partition coefficient (logP):

$$\log P = \log \frac{[\text{Solute}]_{\text{octanol}}}{[\text{Solute}]_{\text{aqueous}}} \quad \text{Eq. 1}$$

Where, the partition coefficient is defined as the natural logarithm of the ratio of equilibrium solute concentration ([Solute]) in octanol and water. A partition coefficient (logP) can be ascertained from the interface presented by any two immiscible fluids however literature generally assumes logP in reference to an octanol – water system specifically. The

membrane permeability as predicted by an o/w partition coefficient was first described in 1896 and is known as the solubility – diffusion model or Meyer-Overton rule.³⁵

$$P = \frac{\log P \cdot D_{oil}}{h} \quad \text{Eq. 2}$$

Where P is the intrinsic membrane permeability coefficient (cm s⁻¹), D_{oil} is the diffusion coefficient through the partition oil phase and h is the thickness of the biological membrane. During passive lipid diffusion, a permeant undergoes temporary desolvation from the aqueous phase and partitions into the hydrophobic core of a membrane. The Meyer-Overton rule postulates that the membrane can be modelled simply as a hydrophobic slab of a given thickness. For a partition to be a suitable predictor, it must reflect the rank order amount of permeant dissolved in a biological membrane during diffusive flux. The thermodynamic penalty of losing a hydration sphere, is thus key within the diffusion solvation model of passive lipid diffusion. This relatively simple model has historically been viewed as a standard model for drug uptake.^{35,36,37,38} Due to the influence of logP in drug discovery, further review is presented in a subsequent section. The inhomogeneous solubility-diffusion model has been proposed to account for conformational freedom during translocation.³⁹ Here, the mechanism is broken into interaction with the exoplasmic leaflet lipid head group, partition into tail region, diffusion across and interaction with the cytoplasmic leaflet lipid head groups.

There are further mechanistic considerations beyond permeant desolvation in passive lipid diffusion. Membranes can become porous under various naturally occurring mechanisms, reviewed by Marrink et al.⁴⁰ The retention of hydrophobic molecules within membranes can also drive porosity, a mechanistic contribution to bilayer translocation often ignored in favour of solubility-diffusion models.⁴¹ Moreover, porosity is exploited in the therapeutic mechanism of some peptides (figure 1.4).^{42,43} Tighter L_β lipid packing results in a higher thermodynamic penalty for desolvation.⁴⁴ Thus, passive transport across the PM is dependent upon the local lipid phase. The phase boundary is also known to be more leaky than the rest of the membrane.⁴⁵ Finally, evidence from model and simulated membranes challenges the notion that molecules must lose their entire hydration sphere within the tail region.^{46,47}

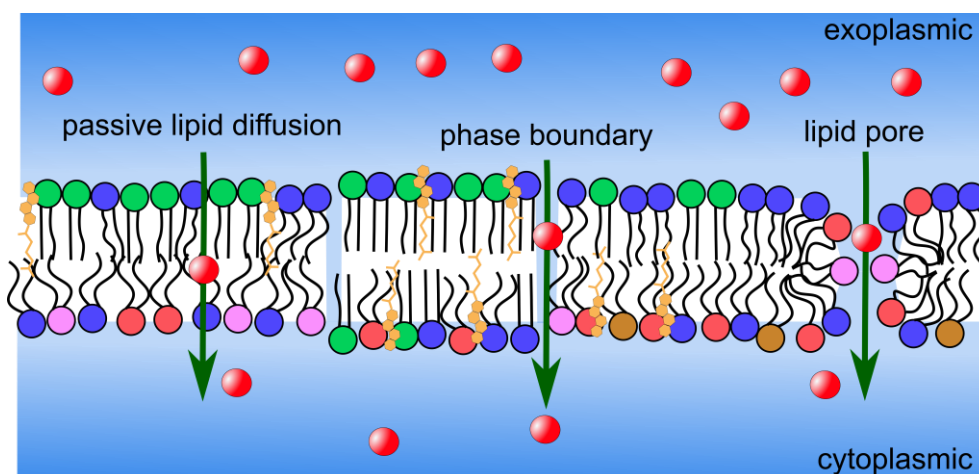


Figure 1.4: Molecular mechanisms of passive lipid – drug interactions at the PM. Figure adapted from references.^{46,48}

1.3. Modelling the plasma membrane in a drug discovery pipeline

Biological membrane transport is critical to drug discovery. Here, a review is presented on current approaches to measure transport phenomena.

1.3.1. Oral bioavailability

Drugs can be ingested orally, intravenously and topically. In short, oral drug metabolism requires a drug to pass through the oesophagus, into the stomach and through the intestinal tract, where during this passage it must cross the outer cell lining, into the blood stream.^{49,50} Oral bioavailability refers to the amount of drug which, after oral ingestion, reaches the target cell to execute a therapeutic mechanism (fraction of a dose absorbed - F_a %). Poor oral bioavailability generally indicates potential biological attrition or that a compound minimally enters systemic blood circulation.⁵¹ Due to patient compliance, oral bioavailability is widely viewed as an optimal method of drug delivery in the clinic. Metabolomic research places focus on quantitative structure activity relationships (QSAR) – where a drugs oral bioavailability may be predicted from its structure alone. Permeation across biological membranes is one of many processes from delivery to therapeutic action which can affect QSAR models.⁵² Dependent upon the drug target, other biological barriers may also be present including the blood brain barrier (BBB), a layer of endothelial cells arranged in a tightly packed structure.⁵³

Regardless of permeation mechanism at the PM of a cell, the chemical structure of the permeant and membrane affect the translocation.⁵⁴ Lightly amphiphilic drugs are attractive prospects due to their observed favourable bioavailability, permeability, and protein binding. Hydrophobic residues within protein targets encourage drug desolvation to increase binding

site residence times as well as off target binding to blood plasma to improve distribution.⁵⁵ Hydrophobicity is considered a key early determinant of success in the drug discovery process and due to its connection with passive lipid diffusion it is sometimes assumed that passive lipid diffusion is the dominant permeability mechanism and that only small, apolar compounds are PM permeable.^{56,57} This assumption is also common to *in silico* experiments, where the complexities of bilayer diffusion can be compared to *in vivo* data.^{58,59} Despite such advances, reliable QSAR approaches for permeability mechanisms are yet to emerge.

1.3.2. The Parallel artificial membrane permeability assay (PAMPA)

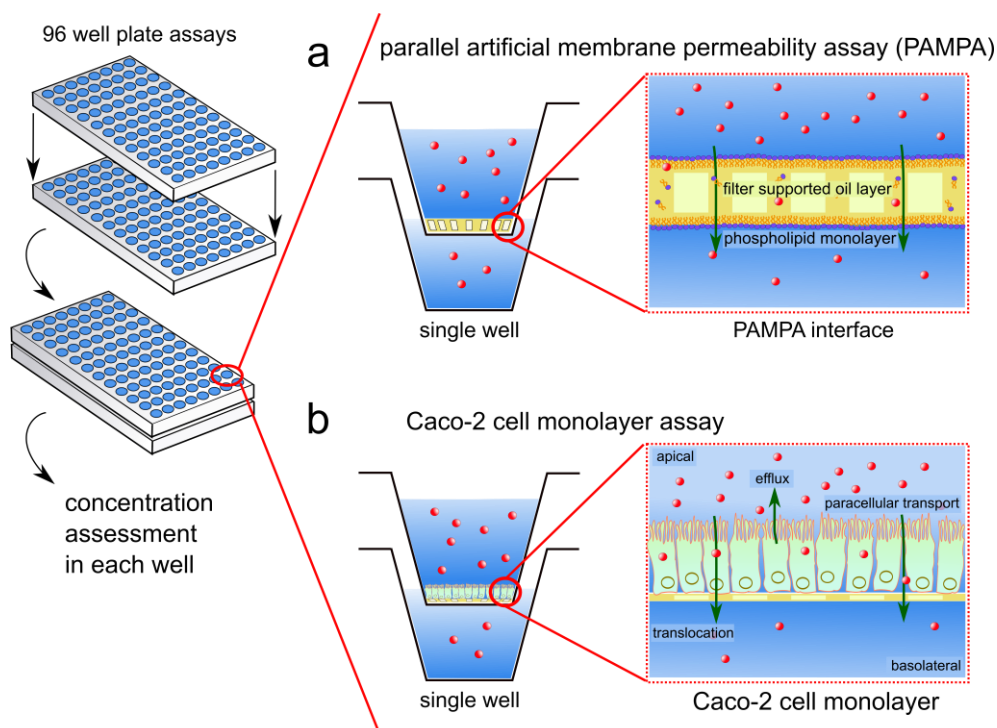


Figure 1.5: Industrial *in vitro* assays for permeability and absorption **a** A schematic of PAMPA, a widely utilised assay for passive lipid diffusion. **b** A schematic of the Caco-2 cell monolayer assay for intestinal absorption.

The parallel artificial membrane permeability assay (PAMPA) was first introduced by Kansy et al. (1998) (figure 1.5 a) as a means to decouple passive lipid diffusion from active transport processes.⁶⁰ This high throughput, low cost and non-cell based assay separates two volumes of water with an oil partition supported upon a hydrophobic filter support layer (~10 μm thick).⁶¹ Lipid dissolved in the oil phase can be used to form monolayers on either side of the oil however, no discrete bilayer structure is formed. Despite this, PAMPA is widely interpreted as an optimal model for passive lipid diffusion at the PM.^{62,63} Due to the nature of the interface compared to the heterogeneity of lipid bilayers, the utility of PAMPA permeability values has been critiqued.^{64,65}

1.3.3. Cell based assays

Translocation of the PM of the intestinal cell layer is an absorption barrier for orally administered compounds. Caco-2 cells are the most widely used cell line as a model for the intestinal epithelium (figure 1.5 b).⁶⁶ Similar to PAMPA, the assay is based in a 96 well format with a measure of the concentration made in both wells. A permeant is typically added in the apical side and driven down the concentration gradient, toward the basolateral chamber, which is separated by a monolayer of Caco-2 cells attached to a carbonate membrane. In this assay, the heterogeneity of the PM is a drawback as it is difficult to decouple active vs passive permeability mechanisms. Moreover, paracellular transport is accounted for, a passive diffusional route distinct from membrane permeability between cellular junctions.⁶⁷ Genetic engineering can be used to change the proteome, however this can affect cell integrity.

1.3.4. Modelling bilayer permeability within a drug discovery perspective

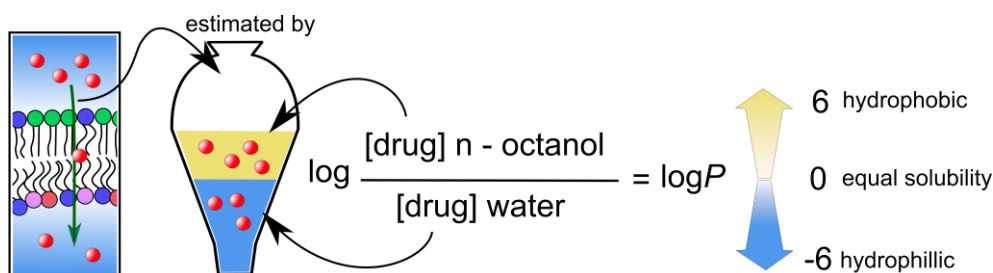


Figure 1.6: Summary of logP determination. Comparing cartoons of the permeability problem next to a traditional shake flask method illustrates the conceptual oversimplification of logP as a measure of actual bilayer permeability. The logarithmic scale (here indicated as the typical space occupied by small molecule drugs) can be characterised as either hydrophilic (negative) or hydrophobic (positive) where a value of 6 indicates a 1×10^6 -fold concentration of drug in octanol over water.

LogP can be determined experimentally through the shake flask method (figure 1.6),^{38,68} or more recently, via high-performance liquid chromatography (HPLC).⁶⁹ Algorithmic approaches allow determination of 'calculated logP'. However, the first calculated logP approach introduced by Leo et al., is referred to as 'clogP'.³⁸ This approach uses a representative experimental sample for the relative polarity contribution of each functional group of the molecule and weights each individually and in tandem. AlogP (atomic logP) was developed for computational integration in QSAR) assessment and considers each atom individually.⁷⁰ Variations of the Leo (clogP) or Ghose-Crippen (AlogP) algorithms are now typically embedded in standard analytical programmes such as ChemDraw. Medicinal chemists rely on calculated logP due to the tedious nature of experimentally determining logP (time to conduct experiments, amount of available material etc.).

Some example linear regression R^2 values between $\log P$ and $\text{clog} P$ are: for a group of steroids (1997) 0.88,⁷¹ a group of ketones (1998) 0.74 and a group of enols (1998) 0.425.⁷² An 893-compound analysis of the Ghose-Crippen algorithm (with no correction factors) yielded a $\log P - \text{clog} P$ R^2 of 0.86, thoroughly reviewed in 2009 by Mannhold et al.⁷³ Correlations in proprietary algorithms are likely much improved due to available training data. Therefore, the accuracy of calculated $\log P$ (as a stand in for $\log P$) varies from software to software in line with developments which improve the accuracy of the available algorithm. This variation of $\log P$ was also observed for compounds studied in this work (supplementary table 1). The correlation between early $\text{clog} P$ and $\text{Alog} P$ was reasonable ($R^2 = 0.91$),⁷⁰ however larger discrepancies in contained chemical spaces have been shown.^{73,74} Another atomistic method referred as $\text{Mlog} P$ (Moriguchi $\log P$ - 1992), showed a comparative R^2 of 0.95 with a 1230 compound data set however the method featured 11 correction factors.⁷⁵ It is therefore pertinent, when comparing calculated $\log P$, either in literature or within a laboratory to ensure that the method used to determine the coefficient is consistent. Significant consideration should also be placed on the choice of method to calculate $\log P$ depending on structural considerations of the molecule (such as structural complexity, electronic structure or rarity of functional groups). There appears to be no consensus with regard to open source approach for the determination of calculated $\log P$ despite public availability of $\log P$ data.⁷³

The validity of $\log P$ within a lipid system and indeed its application in further drug discovery is worth consideration. Octanol can form hydrogen bonds with water, known as the water dragging effect. Octanol compound solubility is increased by the presence of water solubilised within octanol itself, a potential mechanism for false hydrophobic positives.⁷⁶ Such observations thus conflict the standard of utilising n-octanol as a model hydrophobic solvent for the inner lipid membrane. A partition coefficient for alkane – water interfaces ($\log P_{\text{alk}}$)⁷⁶ has emerged as an alternative approach. $\log P_{\text{alk}}$ is generally considered as the partition coefficient determined from any alkane and water, thus independent of the alkane interface.^{77,78} A more recent reporting (Kenny et al. 2013) suggests there may actually be minor variance.⁷⁹ Regardless, $\log P_{\text{alk}}$ is reliably lower than $\log P$ attributable to attenuation of the water dragging effect. The so called ‘wettability’ of hexadecane is 3 orders of magnitude lower than octanol.^{79,80} Water can translocate model bilayers down a water potential gradient, however to what end the solubilisation model accounts for this remains unclear.^{81,82,83} $\log P$ and indeed many other partition coefficients also fail at modelling charged compounds, when compared to human fraction absorbed (F_a (%)) for negatively, positively and partially ionized drugs producing correlations (R^2) of 0.12, 0.21 and 0.21 respectively.⁸⁴ These correlations were found to drastically improve when comparing the

liposomal partition coefficient (0.58, 0.52, 0.47 respectively). Note, that neutral drugs from logP 0 – 4 (liposomal or octanol) all showed strong absorption (Fa).

ρ (logP, P) - R ²	logP range (no. compounds)	model membrane	notes	reference
0.43	0.2 – 5.1 (49)	Madin-Darby canine kidney (MDCK) cells	0.63 R ² for ρ (P, POPC H - bonding (MD)). <0.42 for other Lipinski metrics. clogP used. 2018.	85
0.85	-3 – 4.8 (15)	S49 – lymphoma cells	Radiolabelling used for concentration. logP used. 1984	86
0.44-0.75	n/a (18)	MD simulated PAMPA	Compared SlogP, milogP (0.75), (c)logP, clogP.	87
0.0023	1 - 9.5 (712)	Caco-2 / MDCK cells	332 compounds with a P value >4*10 ⁻⁶ cm s ⁻¹ . Caco2 only used for non-polarized transport. clogP (Pomona)	59
Minimal correlation	-1 – 5 (19)	Caco-2 cells	Permeability showed no correlation with activity. All P values <1*10 ⁻⁴ cm s ⁻¹ .	88
0.0043	5.7 – 7.4 (9)	PAMPA	Highly lipophilic <700 MW macrocycles. PAMPA purports to model bilayer transport. clogD used.	89
0.66	5.7 – 7.4 (9)	MDCK cells	Improved correlation in MDCK cells over PAMPA. Intramolecular H bonding correlated with permeability.	89

Table 1.1: Summary of literature showcasing the correlation between various model membranes and a traditional octanol – water partition coefficient. Independent of model membrane and logP space, poor correlation is observed. This illustrates the limitation of logP as a direct predictor of drug permeability, this is compounded even further by unclear relationships between model membrane permeability and Fa.

Despite the heterogenous environment of the PM and the dynamics of transport at it, logP is still often used as a predictor of bilayer permeability. Due to the infancy of reliable, pure bilayer permeability systems, no study has investigated correlations between any relevant partition coefficient (octanol / alkane / lipid monolayer) with P. The bilayer permeability may correlate with a lipid-based partition coefficient. When sampling diverse chemical space, such as that presented during a typical drug discovery process, the predictive capacity of logP on membrane permeability has been shown to be highly limited^{37,85} as demonstrated in Table 1.1.

1.3.5. The Lipinski rules have informed small molecule drug discovery

The seemingly false perception of strong correlations between physicochemical metrics and permeability measures has shaped drug discovery through the Lipinski 'rules'.⁹⁰ The rules were based off the observation that lead oral compounds prior to 1997 occupied a definable physicochemical space. The principle was that for optimal absorption properties, a compound should therefore satisfy molecular weight (MW) >500 Da, >5 hydrogen bond donors (HBD), >10 hydrogen bond acceptors (HBA) and clogP >5. To date, these parameters have not been linked to a physical process in the mechanism of biological membrane permeability. Despite this, interpretation of Lipinski principles as 'rules' may have affected drug development physicochemical space.^{62,91} Through assessment of cell based assays, the Kihlberg group have presented a convincing account that cellular permeation is not limited by Lipinski principles.^{92,93,94} Further metrics linked to favourable properties include rotatable bond number (RBN) and the topological polar surface area (TPSA).⁹⁵

In the age of high throughput screening, metrics are used more in concert with additional cell assays to mitigate permeability from the efficacy profile of a drug. Based on current non – cell based *in vitro* assays, it appears that physicochemical metrics may define the limits of PM permeability but not the kinetics. The goal of PAMPA was to provide an *in vitro* assay which could decouple the rate of passive diffusion processes from active ones.⁶⁰ Perhaps, this goal still remains, and an improved approach is thus required which can account for the highly dynamic lipid bilayer that supports the PM.

1.4. Droplet interface bilayers (DIBs) – A platform for *in vitro* transport modelling?

The engineering of biomimetic membranes has enabled novel biophysical and technological applications. This section presents a literature review on DIBs - a construct which can act as a mimic of biological membranes.

1.4.1. Droplet interface bilayers (DIBs).

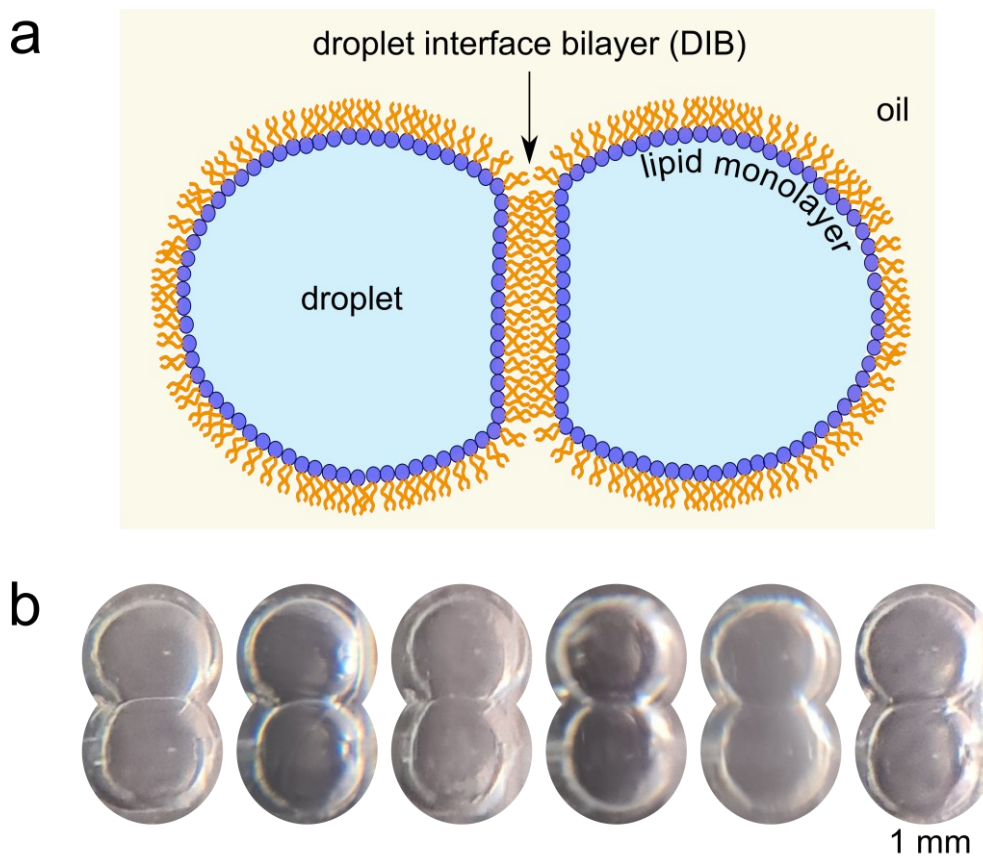


Figure 1.7: Droplet interface bilayers **a** A cartoon showing an assembled DIB. **b** Photographs of DIBs formed within this work with either DPhPC or DOPC. All images from within a chip introduced in chapter 3.

The field of bottom up synthetic – biology is concerned with novel technologies built from biological components.⁹⁶ As with the cell, compartmentalisation through membranes is key, and one of the foundations of the field is to engineer biomimetic structures through self-assembly. These structures can deliver enhanced *in vitro* models of real systems,^{97,98} and enable *in vivo* applications.^{99,100,101} One promising architectural modality that has emerged from bottom-up synthetic biology is the droplet interface bilayer (DIB) (figure 1.7). DIBs are formed at the contact of two water in oil (w/o) droplets stabilised by lipid monolayers. Upon droplet introduction, a lipid bilayer forms after oil is displaced between them. A lipid

monolayer can be formed via the ‘lipid out’ method, from lipids dissolved in the oil phase,¹⁰² and ‘lipid in’, from the deposition of aqueous vesicles at the w/o interface.¹⁰³ Further detail on monolayer formation is provided in chapters 2 and 4.

DIBs were established in the literature by two independent papers, Funakoshi et al. in 2006 from the Takeuchi group,¹⁰² and Holden et al. in 2007 from the Bayley group.¹⁰⁴ A follow up review in 2008 (Bayley et al), is often cited as the seminal work.¹⁰⁵ Since all chemical aspects of the system are user-defined, DIBs are an excellent platform for the study of, and interactions with, bilayers. Since their introduction, many groups have utilised DIB methodologies in a range of biomimetic and de novo *in vitro* applications.

To form a DIB, spherical droplets denature at their contact to present a membrane that occupies a spherical cap defined by the bilayer radius.¹⁰⁶ Equilibrium geometry is underpinned by a balance of monolayer tensions (γ_m) which dictate the surface energy (γ_b) of the bilayer,¹⁰⁷ as described by Young’s equation:

$$\gamma_b = 2\gamma_m \cos\theta \quad \text{Eq. 3}$$

A contact angle (θ) can be calculated from the DIB geometry as outlined in figure 1.8. It is important to note that such equations are upheld for interfaces which approach minimal thickness. For a DOPC DIB a thickness of 3.7 nm closely reflects that of the PM.¹⁰⁸ The ‘zip-up’ is the time taken for an equilibrium geometry, (where the surface energy is matched in the monolayers and bilayer) to be reached from the point of droplet contact during oil preclusion.¹⁰⁹ Zip up has been identified and measured by multiple groups.^{64,65,110} Depending on the adhesive strength of monolayers, the free energy of adhesion (ΔF) can be calculated through the Young-Dupre equation:

$$\Delta F = 2\gamma_m(1 - \cos\theta) \quad \text{Eq. 4}$$

Additionally, these equations consider the bilayer isolated from external conditions, however deviations with lipid and oil type are expected.¹¹¹ Moreover, γ_m is considered here with symmetric volume, osmotic and monolayer properties, however curvature can evolve in the bilayer as a function of unbalanced droplet compositions. Methods for calculating γ_b in such instances have been proposed.¹¹⁰

The experimental utility of DIBs, is in part due to their attractive length scales (μm – mm), as they can be formed with pL to μL volumes.¹¹² Guiselin et al. suggest that, dependent upon system size, the contact angle can predict DIB stability.¹¹³ DIB stability is affected by droplet size, incubation time, oil type, lipid location and lipid complexity. Droplets for DIBs can be

formed by pipetting,¹¹⁴ 3D printing and microfluidics.^{115,116} Within microfluidics DIBs can be formed on guided rails or within larger oil reservoirs.¹¹⁷ The versatility of DIBs as mimics for bilayer stacks, such as those found in the skin or at the blood brain barrier,¹¹⁸ has been realised by Allen et al.¹¹⁹ Stacked bilayer DIBs were formed by passing droplets through arranged w/o interfaces separated by density differences and lipid monolayers.

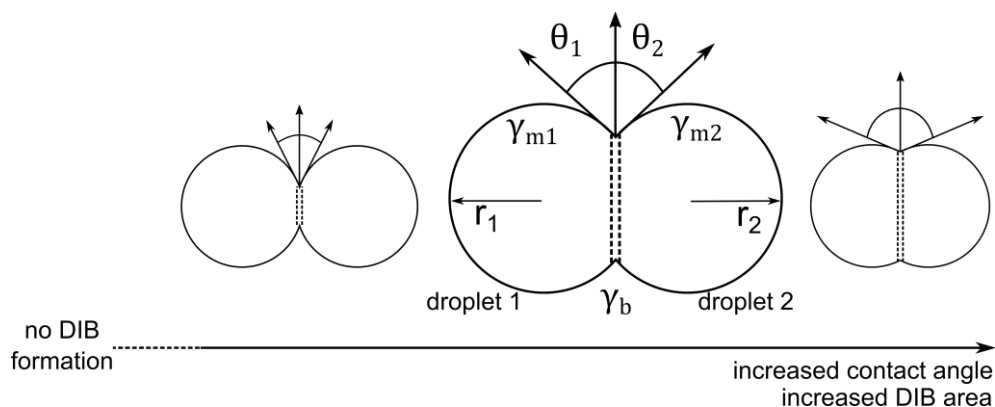


Figure 1.8: An overview of the surface energy of a DIB. Figure adapted from multiple references.^{110,120}

Larger DIBs (nL to $\sim 1 \mu\text{L}$) allow for engineering of local measurement probes and instruments. Within limits, DIBs are stable under mechanical stress, and can be manipulated to control position and arrangement.¹²¹ Barlow et al. show multiplexed DIB formation with sliding layers to introduce the droplets to one another in wells.¹¹⁴ A support around the DIB enabled control of the interfacial area. By compressing or separating droplets, Najem et al. show that the mechanical stress across the membrane can be influenced.¹²² If droplets can be separated without incurring coalescence, downstream content analysis can be performed with mass spectrometry (MS) or HPLC.^{109,123}

Macrostructures of DIB interlinked droplets are known as DIB networks.¹⁰⁴ These structures can be built in 2 and 3 dimensions.¹²⁴ Owing to the modularity of each droplet compartment, this allows for complex patterning within the network. The diffusive pathways within the network can be designed by controlling the permeability profile of each junction.¹²⁵ The Bayley lab have pioneered the development of biomimetic tissue-like materials based on 3D DIB networks.¹²⁶ Villar et al. introduced an approach to print a network consisting of tens of thousands of droplets.¹¹⁵ DIB networks can also act as the chassis for *de novo* technologies based on compartmentalisation. Through leveraging an initial demarcation of fluid space within the network, and by loading each droplet with a different monomeric quantity, a polymerised hydrogel can be formed with the same fluid patterning. Downs et al. show that such an approach can enable magnetic, temperature and light responsiveness within

hydrogels.¹²⁷ Through wrapping the perimeter of DIB networks with additional monolayers, the entire assembly can be transposed from the bulk oil into an aqueous environment, with long term stability.^{128,129} Baxani et al. also show that DIB networks are stable outside of a laboratory through encapsulation within a so called triple emulsion – an oil volume suspended in hydrogel.¹³⁰

1.4.2. Reconstitution of membrane proteins into DIBs

The DIB platform is highly scalable and modular, and its interfacial architecture is close to that of biological membranes. Whilst the relative physiological relevance of vesicle bilayers is rarely questioned, the process of oil deposition from between two droplets remains a contentious matter. Perhaps the clearest evidence of the biomimetic nature of DIBs is that, as with vesicles, membrane proteins can be reconstituted *in vitro*.^{131,132} A growing library of membrane proteins have been incorporated into DIBs, excellently reviewed by Allen-Benton et al.¹³² Capturing non-soluble proteins for *in vitro* study is a considerable challenge and the DIB environment carries central advantages as compared to vesicles as measurements of content either side of the membrane can be made and protein insertional direction can be controlled. Proteins can be grown in bacteria and reconstituted into vesicles or synthesised *in situ*. α -Hemolysin (α -HL) is a water soluble toxin which oligomerizes into a pore structure at lipid bilayers.¹³³ α -HL is commonly deployed to functionalise DIB networks through control of the bilayer permeability profile, allowing passive diffusion of larger solutes.¹³⁴ Functionality studies of membrane protein behaviour have also been carried out with the DIB platform. Findlay et al. used a cell free expression kit to perform *in situ* synthesis of an active transporter protein to enable simultaneous passive and active transport in DIBs.¹³⁵ Since many proteins affect bilayer conductance, the protein gating behaviour can easily be assessed.¹³⁶ Further discussion on reconstituted membrane proteins can be found in chapter 7.

1.4.3. Droplet interface bilayers for permeability

One of the most promising applications that has evolved within the literature is DIBs for permeability assessment. It is therefore common within the literature to reaffirm the advantages of DIBs compared to PAMPA.^{117,65,137,64} Thermodynamically considering a DIB permeability system, a chemical energy gradient is separated by a bilayer in which to lower total energy, a temporary energy penalty needs to occur (desolvation). DIBs formed with fluid lipids such as DOPC or DPhPC are highly popular within the literature due to the resultant bilayer stability. The DIB architecture may be viewed as an *in vitro* complement to *in silico* bilayer models since multiple lipids can be incorporated. Extending from the notion that

DIBs capture true bilayers, the effect on permeability of PM lipid features have been successfully studied in DIBs including: lipid tail groups,^{65,138} lipid head groups,⁶⁵ lipid asymmetry,^{139,140,123} and the role of lipid phase in biomimetic lipid compositions.^{141,142,143} Furthermore, since the environment is intrinsically a bilayer, more complex passive mechanistic interactions representative of the heterogeneity of the PM have been studied such as membrane pore formation and oil trapping.^{144,145}

Faugeras et al. show that the net bilayer permeability is correlated to the bilayer lateral pressure profile which can be modulated by type 1 and type 2 lipids.⁶⁵ Charged lipids increased permeability which was hypothesised to be due to packing deficiencies. Moreover, it was shown that PE lipids retain oil in the interleaflet space more than PC lipids. Stephenson et al. show that bilayer fluidity asymmetry can lead to increased permeability relative to a compositional blend of each leaflet.¹¹⁷ Bachler et al. show that with charged lipid asymmetry, if a positively charged permeant first encountered a negatively charged leaflet this lead to an increase in permeability relative to a symmetrically charged bilayer. The opposite was true when the permeant first encountered a neutral leaflet, leading to a roughly comparative decrease in permeability.¹²³ Korner et al. show that the bilayer permeability reduced as the mammalian derived lipid complexity increased – in general agreement with improved packing efficiency.¹⁴³ This trend agrees in part with a similar study in Barlow et al. which utilised plant lipid extracts.¹⁴² The magnitude of the permeability ranges between these studies do not agree, which suggests intrinsic permeability variation across kingdoms. Each of these studies utilised DIBs, and when taken with biological evidence suggest that to neutrally charged permeants, the external leaflet of the human PM ($\sim L_o$) is less permeable than the internal leaflet (between L_o / L_d).¹ This establishes the notion that a compounds propensity for passive diffusion is affected by interaction with the external PM leaflet.

At the contact between lipid head groups and the surrounding aqueous volume, a long-range ordering of water can occur known as the unstirred water layer (UWL).

In Barlow et al. (2017), recirculation flow was generated in DIB droplets with a spinning disk apparatus.¹⁴⁶ The mixing intensity positively correlated to the permeability, indicating that the UWL plays a role in stagnant DIB permeability interfaces. This adds an additional consideration with respect to the thermodynamics of bilayer translocation. Indeed, there is also substantial biological significance of the UWL. A UWL thickness of 40 – 100 μm has been reported in mammalian gut epithelial cells.¹⁴⁷ The UWL thickness is proportional to the length scale of the probed interface which leads to PAMPA and DIB intrinsic UWL thicknesses of ~ 3800 μm and 137 μm respectively.^{148,146} DIBs, even under stagnant

conditions, thus provide an intrinsically biomimetic interface with respect to bilayer architecture and UWL thickness.

1.4.4. Label free measurements in DIBs – a review of the literature so far

Biologically active compounds are not typically fluorescent. One aspect that has stymied the translational potential of DIB permeability measurements, is a reliance on fluorescence approaches. The principles of fluorescence are introduced in chapter 2. DIBs have been limited to the assessment of a handful of fluorescent permeants such as fluorescein,^{143,65,149} calcein (impermeable),^{144,123} resorufin,^{142,146,112} and rhodamine.¹²³ Stephenson et al. looked to address this limitation with doxorubicin, an example fluorescent chemotherapy drug, thus yielding intrinsically relevant permeation values.¹¹⁷ Additionally, some groups have turned to tagging strategies.¹⁵⁰ A popular method to achieving fluorescence is through click chemistry - a technique from the field of chemical biology.^{151,152} By covalently bonding additional functional groups to a molecule, this can enable the production of fluorescent dyes which act as a measure of concentration. One of the assumptions of tagging is that the physicochemical properties of the tagged molecule are consistent with the untagged molecule. A further common assumption is that the diffusive behaviour of a single dye across bilayers is representative of wide drug chemical space. This has major implications for the impact of the studies described in section 1.4.3 which attempt to link biophysical bilayer properties from experiments carried out with a single permeant chemical structure.

A label free measurement herein, refers to a method with access to wide chemical space without the need of tagging. Furthermore, 'label free' thus also defines a measurement strategy which exploits the core structure of the measured permeant. Within the literature, some label free DIB approaches have been engineered, to limited success. Braziel et al. showcased a Raman microspectroscopy approach for the determination of D₂O bilayer transport.⁸³ Whilst highly promising, Raman is limited by equipment costs and the inclusion of functional groups with measurable stretching modes, such as nitrile groups or deuterated compounds. Nisisako et al. introduced a ultraviolet (UV) microspectroscopy, microfluidic strategy which is again stymied by equipment costs.¹³⁷ Mass spectrometry (MS) was performed in sequestered DIB droplets by the Dittrich group.¹²³ The measurement range of MS means it is perhaps the most powerful approach for detecting molecules. One of the drawbacks, is that the measurement is not *in situ* and requires individual droplets to be captured. The measurement resolution is therefore inherently low unless coupled with massive throughput or the removal of small volumes.

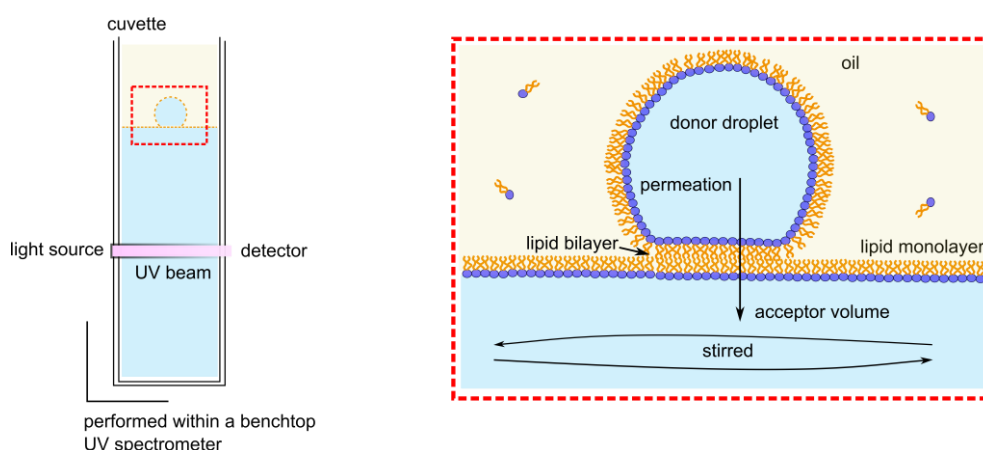


Figure 1.9: An overview of the bilayer method presented by the Choi group.^{153,64} A bilayer is formed within a UV cuvette, housed in a benchtop UV-Vis spectrometer to collect permeation data in a large (1 mL) acceptor volume. This measurement approach is the closest literature compliment to the work performed herein and is compared to in chapter 4.

Two papers from the Choi group are perhaps the best example of a label-free measurement in a reconstituted lipid bilayer system (figure 1.9).^{64,153} In these works, an w/o water partition is formed in a UV cuvette. By introducing a droplet at the interface, a DIB is formed at this partition. The bilayer is not presented between two droplets and thus represents somewhat of a hybrid between DIB and supported lipid bilayer (SLB) and is close in architecture to a droplet hydrogel bilayer (DHB).¹⁵⁴ The translocation of UV absorbent drugs across the bilayer were studied using traditional UV-Vis absorbance spectroscopy. PAMPA and Caco-2 utilise UV-Vis or HPLC to facilitate the measurement of wide chemical space. The method presented by the Choi group successfully enabled study of comparative chemical space across a more relevant interface. Major limitations remained, including limitation of measurement to a single compartment, a large discrepancy in volume between compartments, the requirement of stirring to mitigate the UWL and diffusional distance, and an apparent increased amount of oil trapping - potentially attributed to vertical droplet arrangement.

1.5. Thesis aims

The central goal of this thesis was to unlock DIBs as a platform technology for drug discovery relevant permeation studies. The work required considered design of the fabrication strategy, experimental workflow and analytical pipeline, which can be expanded into the core aims of this thesis:

- **Enable a label free and non-invasive measurement in DIBs such that small molecule drugs can be detected and studied.**
- **Build such a label free system into a methodology which is robust, easy to use and amenable to higher throughput strategies.**
- **Conduct assessment of wide permeant chemical space and assess the response of DIBs as permeability screens.**
- **Delineate permeant structure–permeability relationships to showcase comparative utility in DIBs to industrial assays.**
- **Build a tool for controlling the membrane permeability to large molecules as a function of membrane composition.**

2 Materials and Methods

2.1. Chapter overview

In this chapter, specifics are supplied for replication of the experiments throughout this work. Two permeability models are derived from Fick's first law of diffusion. The two main analytical techniques are described, and their theory detailed. Further experimental details are discussed at the relevant section of each chapter. The source of materials used throughout this work are detailed.

2.2. Fabrication

The novelty of this work was driven through engineering bespoke measurement approaches.

This section covers laser cutting techniques for building plastic architectures to house droplets.

2.2.1. Laser cutting

Laser cutting is an advanced machining process for marking, engraving and cutting materials.¹⁵⁵ An infra-red (IR) light beam is generated by electrically exciting gas molecules within a tube. This beam passes through a mirror into a focusing lens to produce a circular spot of intense light. Depending on power and the focus, this spot can melt a surface.¹⁵⁵ This beam can be controlled in the x and y axis whilst the laser bed controls the z axis. In this work, a bench top CO₂ laser source VLS 2.30 Universal Systems laser cutter was utilised. This laser cutter was held in the central laboratory. Schematics for cutting were produced using Inkscape, CorelDraw or Autodesk Fusion 360. Laser settings were optimised for each material thickness and composition. All laser cut sheets were washed with 70% ethanol post cutting.

2.2.2. Bonding materials

Bonding refers to the process of joining layers of material together. Bonding was pertinent in this work to ensure that oil did not leak from any reservoirs (summarised in figure 2.1). In chapter 3, layers of polymethyl methacrylate (PMMA) were sealed through arrangement on a heating block, since this formed a core part of the result, specifics are extensively discussed in the chapter. For the chips in chapter 5, heat bonding resulted in warping and fracturing of the thin layers of PMMA in contact with the heating block so instead, layers were solvent bonded. Since polytetrafluoroethylene (PTFE) has excellent chloroform resistance, it could act as a support for chloroform assisted PMMA bonding. PTFE supports with holes (3.2 mm) suitable for M3 screws were laser cut. Chip layers were designed with holes in the same

place, allowing for alignment onto the PTFE supports. A syringe with a thin needle was then used to deposit chloroform between the layers. The supports were screwed tightly together and left in a fume hood for around 5 minutes. Care was taken to minimise chloroform contact at the DIB well, sealing layers around the window. Additional chloroform was added at poorly sealed junctions utilising capillary action. This produced a seal between the inner and outer PMMA layers which allowed for DIB formation at each well.

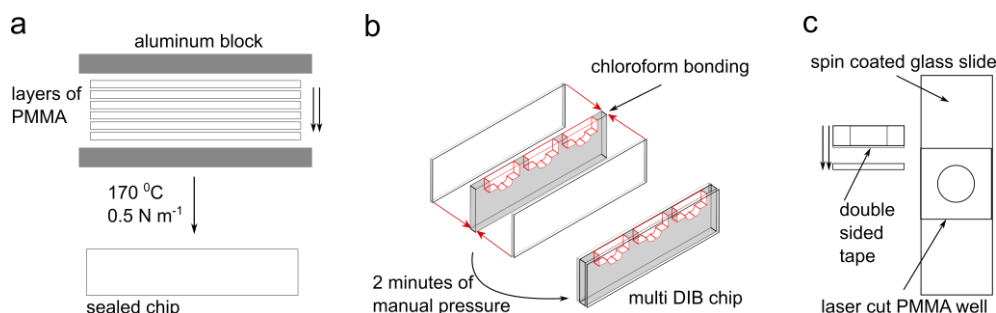


Figure 2.1: Bonding techniques. **a** Cartoon of heat and pressure bonding PMMA layers used to make devices in chapter 3. **b** Cartoon of chloroform bonding of PMMA layers, used to make chips in chapter 5. **c** Tape bonding to seal PMMA to glass. Used in chapter 7 to make a reservoir for DIB generation with a hydrophobic bottom.

In chapter 7, DIB wells were formed by laser cutting a 10 mm diameter hole into a 5 mm thick PMMA layer. Prior to cutting, this sheet was coated with 260 μm thick double sided adhesive tape (3M VHB Adhesive transfer tape F9473PC – RS components). The wells were sealed onto PDMS spin coated glass slides. This produced an oil tight reservoir with a hydrophobic base for droplet electrophysiology.

2.3. DIB formation

Droplet interface bilayers (DIBs) were core to all experiments across this thesis. The following covers the generation of DIBs starting from a lipid film.

2.3.1. Lipid film preparation

All lipids were kept at a known concentration in a chloroform stock. To produce a lipid film, a desired volume of lipid stock was pipetted into an 8 mL glass vial. In the case of mixtures, such as in chapter 4 and chapter 7, mixtures of stocks were formed at this stage to the desired molar ratio. A stream of N_2 gas was applied 130° from the sample vial which was gently rotated. Within minutes, this caused evaporation of the chloroform, which left behind a thin lipid film which covered a significant proportion of the vial walls. To remove residual chloroform, films were dried overnight in a desiccator. All samples were used within 1 day of preparation and typically 10 mg films were prepared.

2.3.2. Lipid out

For lipid out measurements, oil was pipetted into the prepared lipid film to a desired lipid oil concentration (typically 5 mg / ml). The vial was wrapped with parafilm, vortexed for 5 minutes and sonicated at 40 °C for 1 hour to give a fully dissolved, lipid in oil solution. Lipid oil was kept within a concentration range of 1 – 5 mg / ml. For the wells in the single beam path chip this volume was 70 µL (chapters 3 and 4) and for the multi droplet method (chapters 5 and 6) this was 20 µL. To facilitate monolayer assembly, droplets were incubated at the lipid oil / air interface for around 1 minute prior to pipetting into the well.¹⁵⁶ In the multi droplet method, the droplets were smaller which appeared to drive rapid monolayer assembly in conjunction with interfacial evaporation. In the single beam path chip, droplets were further incubated on a platform above the DIB well for at least an additional 5 minutes. After this, droplets were carefully manipulated with a pipette tip and pushed into the DIB well. In some experiments, the acceptor droplet was pipetted into the DIB well whilst the donor droplet incubated above. In general, DIBs were formed with the lipid out method and were highly stable.

2.3.3. Lipid in and extrusion

For lipid in measurements, buffer was pipetted into the prepared lipid film to a desired multilamellar vesicle (MLV) solution concentration (10 mg / ml). Phosphate buffered saline (PBS) buffer (1X Corning) pH 7.4 was used throughout this work. A standard electrophysiology buffer was used in chapter 7 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20 mM, KCl 100 mM pH 7.4). For lipid mixtures, at least 5 freeze thaw cycles were performed by freezing the solution with liquid nitrogen before gradually heating above the T_m . This process is known to aid encapsulation and content exchange between vesicle populations.¹⁵⁷ The sample was extruded at least 15 times through a polycarbonate, porous (100 nm) filter to produce small unilamellar vesicles (SUVs) with a specific size distribution that matched the pore size of the filter. To produce DIBs in the single beam path chip, droplets (900 nL) were placed into a pure hexadecane well and left to incubate. For lipid in, the optimal incubation time was found to be 10 minutes. After this period, droplets were pushed together as described prior. A full overview of both lipid in and lipid out methods for forming DIBs is shown in figure 2.2.

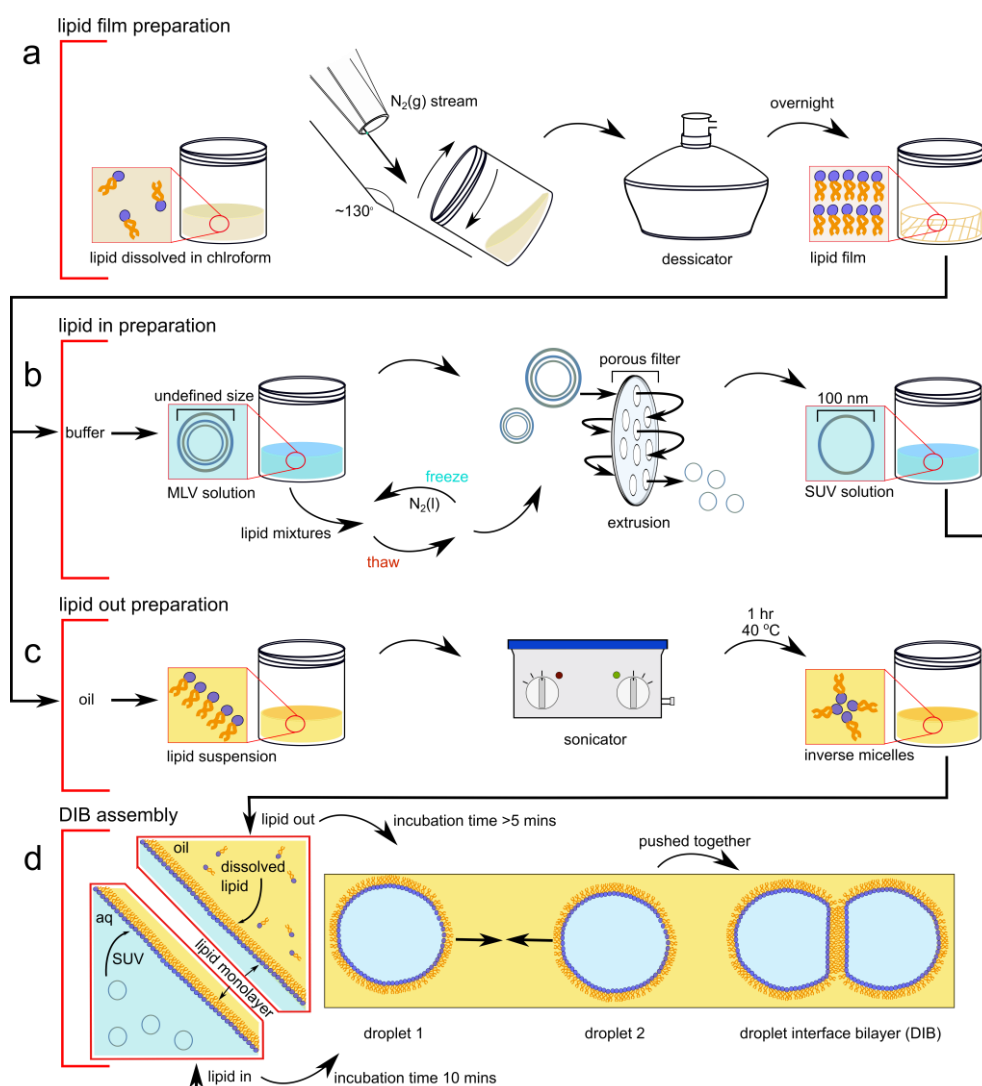


Figure 2.2: Schematic for forming a DIB from a lipid film. **a** Lipid film preparation detailed in 2.3.1. **b** Lipid in sample preparation detailed in section 2.3.3 **c** Lipid out oil preparation, detailed in 2.3.2 **d** DIB assembly, detailed in both section 2.3.2 and section 2.3.3.

2.3.4. Sample preparations

Permeant stock solutions (20 mL, unless otherwise stated 1 mM) were calibrated to pH 7.4 through addition of concentrated HCl or NaOH. All permeant stock solutions were kept in the fridge ($6^\circ C$) and used within 2 months. Permeant samples were prepared via serial dilution from the stock. Samples were used for calibration and to form concentration gradient DIBs in chapters 3,4,5 and 6. Dimethyl sulfoxide (DMSO) was not included in solutions unless otherwise stated and where applicable was added by volume %. In the case of lipid in, equal volumes of the serial dilution with the dissolved permeant and SUV solution were mixed to yield a desired permeant concentration and vesicle concentration of 5 mg / ml. Samples were vortexed for 3 minutes.

2.4. Theory and application of analytical methods

UV-vis spectroscopy facilitated a label free measurement. The porosity of membranes was assessed with electrophysiology in chapter 7. This section details the integration of these technologies into DIB droplets.

2.4.1. UV-vis spectroscopy

UV-vis spectroscopy is a technique that measures the characteristic light absorption of a particular chemical species (figure 2.3). Electrons within ground state bonding orbitals (S_0) can undergo an energy dependent electronic transition into the excited state non-bonding orbital (S_1). If the energy of exposed light irradiation matches the energy of this transition, the molecule will absorb light of the same wavelength. It is well observed that during irradiation, a molecules absorption remains constant through time. This is because molecules lose the absorbed energy as kinetic energy through molecular collisions.¹⁵⁸ By measuring a samples spectrum in relation to a reference, this allows quantification of the sample's concentration.

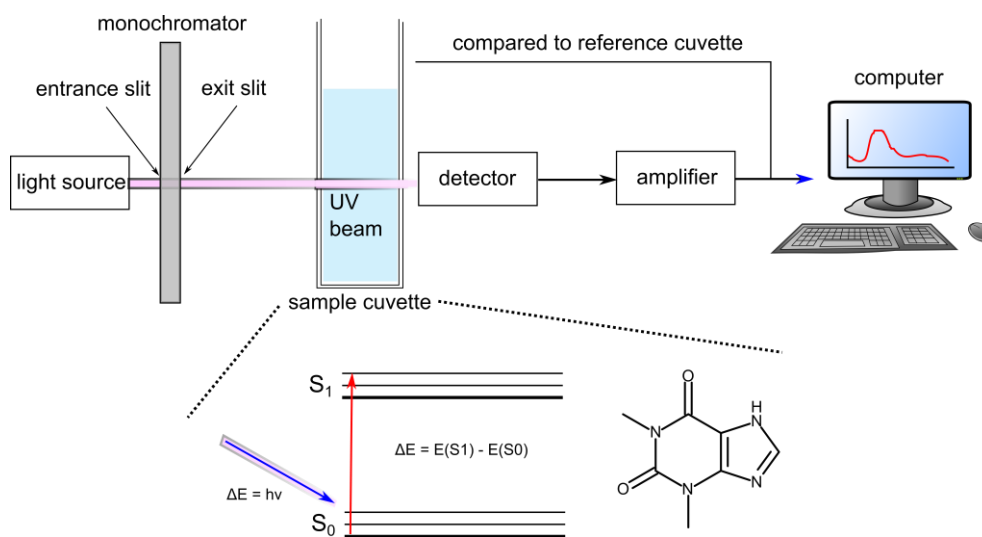


Figure 2.3: An overview of the light path taken from light source to the production of a spectrum. Light can be passed through a monochromator with a diffraction gating to select for a wavelength range to irradiate a sample. A molecule can undergo transition from ground (S_0) to excited state (S_1). Signal amplification can be performed either on computer or at the detector to allow for reduction in the signal to noise ratio of the measured spectrum.¹⁵⁸

Considering a broad wavelength spectrum with intensity of each measured wavelength, the transmittance (T) can be written as:

$$T = \frac{I_0}{I} = 10^{-\epsilon cl} \quad \text{Eq. 5}$$

Where, I_0 = incident irradiation and I = transmitted intensity, c = concentration, l = path length and ϵ = molar absorption coefficient. The intensity of transmitted light is reduced in the wavelength range at which the compound absorbs light. Across large concentration ranges, transmittance follows an exponential relationship with the concentration and so to linearise this relationship, Beer Lambert law defines the absorbance (A) as:

$$A = \log\left(\frac{I_0}{I}\right) = \epsilon cl \quad \text{Eq. 6}$$

The absorbance spectrum for a measured compound depends upon its molecular structure. In general, aromaticity (pi bonding) confers strong absorption in the UV range detectable by most spectrometers from around 200 nm. Delocalisation of the pi bonding orbitals reduces the energy required for the $S_0 - S_1$ transition and shifts the absorption maxima to higher wavelengths. Because of this, most fluorescent compounds consist of highly conjugated interlinked aromatic ring structures. Many biologically active compounds contain aromatic groups and so can be studied using UV-vis spectroscopic approaches.

In this work, UV-vis spectroscopy was conducted within DIB droplets. This was enabled by novel methods engineered in chapters 3 and 5. The methods are referred throughout as the single beam path and multi-droplet method respectively. Both methods used a DH – mini tungsten and deuterium bulb light source and a HR 2000+ spectrometer (OceanOptics). In chapters 3 and 4 data collection was conducted with OceanView software and in chapters 5 and 6, with custom python software. The integration time refers to the time required to collect light at the spectrometer to record a spectrum with a suitable signal to noise ratio. The integration time was optimised for each experiment according to the path length. A larger path length dictated a higher integration time. Further experimental details are provided in the relevant sections of each chapter.

2.4.2. Electrophysiology

Electrophysiology is a technique for the study of ion flow within biological samples. As applied in DIBs, this allows for the determination of Cl^- ion flux across membranes, where ion movement acts as an equivalent of electrical current. In such a system, an ion impermeable lipid bilayer thus acts as a capacitor, where a voltage gradient across the bilayer allows recording of the transmembrane current.¹⁵⁹ When integrated into the measured bilayer, non-specific membrane proteins act as resistors, which allows for the relative increase in ion permeability to be measured as a function of the protein state. Blockades to this flux, either

from molecular interactions within, or conformational changes of the protein thus affect the measured current. The channel conductance (nS) is yielded by the current (Δ pA) divided by the voltage (V).¹⁵⁹ Such a relationship has driven the industrial application of single-channel nanopore recordings with huge potential in sequencing.¹⁶⁰

Droplet electrophysiology is performed with Ag/AgCl electrodes arranged in both droplets to measure current across the membrane (see chapter 7 figure 7.2). In this work, Ag/AgCl electrodes were formed by soldering two equally cut pieces of silver wire (Goodfellows – 0.5 mm) onto a resistor circuit consisting of a battery strap and a Pt wire. The circuit was suspended in concentrated HCl solution and attached to a 9V battery for around 10 s. Bubbles formed around the Pt wire and a browning of the electrodes indicated successful electroplating. Electrodes were attached to gold plated wires which were fitted to a headstage. One aspect of this method to consider, is the significant spatial control required to facilitate the contact of electrode probed droplets. To achieve this, each wire was also fitted to a micromanipulator with fine 3-dimensional resolution. A 5% agar in de-ionised water (DI) water solution was heated and gently stirred for 1 hr at 170 °C, and then kept stirred at 70 °C. To support droplets, the ends of each electrode were carefully coated with the agar. These were washed with buffer to displace the DI water. Depending on experiment, 1 μ L of vesicle solution was pipetted onto each electrode and lowered into a reservoir of pure hexadecane. Droplets were incubated for 5 – 10 minutes before they were manipulated into alignment to form a DIB. The headstage was connected to an Axopatch 200B amplifier and a Digidata 1440A digitiser. The system was controlled with Axon pCLAMP software. Current recordings were performed with a holding potential of -100 mV, a sample rate of 50 kHz and a 5 kHz low pass Bessel filter. Further data handling is discussed in chapter 7.

2.4.3. Deployed python libraries and analysis

The analysis performed in this thesis was enabled by the following python libraries; numpy, matplotlib, scipy, pandas, RDKit, seabreeze, pyABF, sk-learn, skimage, re, serial, random, itertools, glob, os, time, datetime, IPython and astropy. Specifics regarding analysis are provided at the relevant sections of each chapter and all code can be accessed via github link. All analysis was performed with python unless otherwise stated. All calculated logP values were calculated with BIOVIA Draw software, employing a variation on AlogP (The Ghose – Crippen algorithm)⁷³. A specific R^2 for this variation of the algorithm (correlated to logP) could not be ascertained.

2.5. Chapter specific methods

Further methods were deployed within certain chapters of this work. To aid readability they have been detailed here.

2.5.1. Chapter 3

Operation of millifluidic devices – Millifluidic PMMA devices were engineered in sections 3.7 and 3.8 by following the chip fabrication procedure described in section 2.2 and 3.3. At each outlet, PTFE tubing (internal diameter 0.8 mm, outer diameter 1.58 mm) was fitted into circular apertures on the chip surface with epoxy glue. The systems were operated with Fusion 200 model syringe pumps. For the system in section 3.7, droplets were formed at t-junctions manually. All experiments were performed under a stereoscope microscope equipped with a Ximea camera. Fiber optic cannula were aligned either side of the DIB well as described in chapter 3 with the same spectrometer and light source. Spectra were collected as described above, upon a droplet entering the DIB well. For the system in section 3.8, a fiber was aligned at the channel interface. Spectra were collected with a Flame Spectrometer (Ocean Optics) and constantly monitored with an in-house LabView programme. Further details are provided in the relevant sections.

2.5.2. Chapter 5

3D printing – 3D printing allows for the rapid production of computer-aided design (CAD) parts. These parts can be made from a variety of materials and, dependent upon multiple variables, can be of a very high resolution. 3D printing has found multiple applications in chemistry laboratories,¹⁶¹ particularly in microfluidics.¹⁶² All 3D printing was performed at the Imperial College Advanced Hackspace (ICAH) with a FormLabs form stereolithography (SLA) resin 3D printer with Tough 2000 resin (FormLabs). All CAD was designed with Autodesk Fusion 360.

CNC milling – Computer numerical control (CNC) milling uses a drill bit and an automated stage to engrave material. Laser cutting is generally limited with regard to z axis resolution and is optimal for cutting material with a thin, highly precise kerf. CNC milling allows engraving across any dimension of a material piece. All CNC milling was performed at the ICAH with a AXYZ CNC Router. All CAD was designed with Autodesk Fusion 360.

2.5.3. Chapter 7

Fluorescence spectroscopy – Fluorescence spectroscopy is underpinned by a relationship between light excitation of and emission from a molecule. Relative to the maxima of each, the excitation wavelength has a different energy than the emitted wavelength, a difference known as the Stokes shift. In most instances across biology, the Stokes shift is toward lower energies, yielded by vibrational relaxation of the excited state. The fluorescence of calcein ($\lambda_{\text{ex/em}} = 494/514 \text{ nm}$) was studied in chapter 7 with a Cary Eclipse Fluorescence Spectrophotometer (Agilent USA).¹⁶³ The excitation slit was 5/5 nm. All experiments were assessed in a 96 well plate, with reagent aliquots added to vesicle solutions to a total volume of 200 μL . At the end of each experiment, Triton X-100 (3 v/v%) was added to each well and the maximal fluorescence recorded after an equilibration period (15 mins). An addition of buffer sample was used as a negative control. For each distribution, a min-max normalisation was performed with the following equation:

$$\text{Calcein release (\%)} = \frac{F_t - F_0}{F_{\text{END}} - F_0} * 100 \quad \text{Eq. 7}$$

Where F_0 represents the initial value of the distribution, F_t the fluorescence at a point in the distribution and F_{END} the maxima yielded post vesicle rupture.

Droplet shape analysis (DSA) – DSA is a means to assess the interfacial tension (IFT) of aqueous phase boundaries. As lipids pack at an w/o interface, this results in a reduction in the IFT which changes the shape profile of the droplet.¹⁰⁶ IFT can be measured through image analysis of droplet volume, contact angle and radius. DSA measurements were conducted with a Krüss EasyDrop pendant tensiometer. Following a previously published method, a syringe was filled with varying lipid in solutions. Droplets were carefully pipetted from a 0.52 mm flat needle within a cuvette filled with hexadecane. Droplets were left to incubate for ~8 minutes before images were captured and analysed with OpenDrop software.¹⁶⁴ To calculate the IFT, a density of 1000 kg m^{-3} and 770 kg m^{-3} for water and hexadecane respectively and an acceleration of gravity of 9.8 m s^{-2} were utilised.¹¹⁰

2.6. Purchased chemicals and materials

All lipids were purchased from Avanti Polar Lipids. Xanthine, 3-methylxanthine and theobromine were purchased from Tokyo Chemical Industries. Propentofylline was purchased from Toronto Research Chemicals. All compounds introduced in section 4.5 were supplied by GalxoSmithKline (GSK – collaborators during this work) for commercially available compounds within this set, they were also sourced from Sigma Aldrich. PMMA

sheets of varying thickness (Clarex 001 precision or Clarex UV transmission filter) were purchased from Weatherall Equipment and Instruments Ltd. All electronic and fabrication components were purchased from RS components. Fiber optic cabling and optical tools were purchased from Thorlabs. Sub miniature assembly (SMA) ports were purchased from Farnell. All other reagents were purchased from Sigma Aldrich. Additional detail is supplied throughout.

2.7. Permeability model

To interpret the results of this thesis, a permeability model was derived from Ficks 1st law of diffusion. To account for the results of chapter 6, a system of ODES was derived to account for simultaneous permeant partition and permeability.

2.7.1. Model for single beam path chip

Ficks 1st law of diffusion (1855), is an ordinary differential equation (ODE) that describes the movement of solutes through aqueous media.³⁰

$$J = -D_{aqueous} \left(\frac{dC}{dx} \right) \quad \text{Eq. 8}$$

Analogous to Ohms and Fourier's laws of electrical / heat conductivity respectively, Ficks 1st law describes diffusive flux (J [$\text{mol m}^{-2} \text{s}^{-1}$]) as being proportional to the diffusivity of a molecule (D [$\text{m}^2 \text{s}^{-1}$]) and the difference in concentration between two arbitrary points in space ($\frac{dC}{dx}$ [mol m^{-4}]). Ficks 1st law is widely used to interpret solutes moving down a concentration gradient and across lipid bilayers.^{165,166,146} As the utilised DIB permeating system is on the μm scale, an acceptor and donor model is applied where, $D_{\text{membrane}} \gg D_{\text{aqueous}}$ (this assumption is experimentally evidenced in section 4.3.5). Donor and acceptor correspond to droplets of high and low concentrations at t_0 respectively. Fixing the concentration thus moves away from the interpretation of concentration as points in space and instead toward a continuous concentration across each droplets volume. Adjustment through the units of J allows the change in concentration with time across a membrane from a donor to an acceptor compartment to be interpreted.

$$\frac{dC_{A(t)}}{dt} = -D_{\text{membrane}} \frac{A}{V_A} \frac{(C_{D(t)} - C_{A(t)})}{h} \quad \text{Eq. 9}$$

Where $C_{A/D}$ is the concentration of permeant in the acceptor / donor volume respectively (mol m^{-3}), note, that donor / acceptor can be equally referred as source / sink where a sink is typically described as $C_{\text{sink}}(t=0) = 0 \text{ mol m}^{-3}$, A = membrane area (m^2), V_A = volume of acceptor compartment (m^3), h = membrane thickness (m). Within a DPhPC DIB formed in hexadecane the membrane thickness was found to be 3 nm.¹⁰⁹ Note that as $A \gg$ membrane

thickness, the membrane permeability constant is simply derived through a 1 - dimensional approach as:

$$P = \frac{D_{\text{membrane}}}{h} \quad \text{Eq. 10}$$

Where P is the membrane permeability constant (m s^{-1} interpreted throughout as cm s^{-1}), and:

$$k = P \frac{A}{V_A} \quad \text{Eq. 11}$$

Where k is a rate constant for permeant diffusion through the membrane (s^{-1}). Note the similarity here to equation 2 - the Overton-Meyer permeability rule. A permeating DIB system is considered closed when all available permeant remains within the aqueous compartments or solubilised within the membrane, this formed a significant part of investigation in chapter 4 and 6. If it is further indicated that the membrane solubility of the permeant is minimal then a suitable mass balance is:

$$C_{D(t=0)}V_{D(t=0)} + C_{A(t=0)}V_{A(t=0)} = C_{D(t)}V_{D(t)} + C_{A(t)}V_{A(t)} \quad \text{Eq. 12}$$

Where, $V_{D/A}$ is the volume of the donor / acceptor droplets (m^3), respectively. An excellent review of the physical forces governing DIB stability is covered in Guiselin et al., in applicability here, osmotic and Laplace pressures within the DIB control the rate of droplet evaporation.¹¹³ Due to the use of a buffering solution (salt content $>100 \text{ mM}$) which greatly exceeded permeant concentration ($500 \text{ }\mu\text{M}$), all DIBs were effectively osmotically balanced. Moreover, at typical lab conditions droplet evaporation for w/o hexadecane emulsions has been shown to be negligible for systems in which the hexadecane volume greatly exceeds the water volume.¹⁶⁷ In this work, droplet volume was assumed as constant with time. A permeating system in this work uses equal volume DIBs where $V_D = V_A$, this allows concentration to determine the mass balance simply as:

$$C_{D(t=0)} + C_{A(t=0)} = C_{D(t)} + C_{A(t)} \quad \text{Eq. 13}$$

In a closed system with the above assumptions, this allows the equilibrium concentration to simply be defined as:

$$C_{EQ} = \frac{(C_{D(t=0)} + C_{A(t=0)})}{2} \quad \text{Eq. 14}$$

Where, C_{EQ} is the equilibrium concentration (mol m^{-3}) in either droplet. The permeability coefficient of the system can be calculated from the concentration time distribution of a single droplet, and a series of experimentally determined constants with:

$$P_{(t)} = \frac{V_A}{2A(C_{EQ} - C_{A(t)})} \frac{dC_{A(t)}}{dt} \quad \text{Eq. 15}$$

Note, the above equations are adjusted for monitoring of an acceptor compartment, which is the standard for this work, although are equally applicable in the study of donor compartments. This approach is similar to one utilised by Yohan Lee et al.^{64,153} The units of P show how concentration cancels out, and thus concentration can be considered in any unit, which is linearly correlated across the experimental concentration window. The permeability constant can be estimated from a single point in time through linear approximation. Note how A , C_{EQ} and V_A are expressed as constants, where the assumption is made these remain unchanged following DIB zip up. In considering equations 15 and 11, a general solution for an exponential concentration time plot is next derived. Swapping P with the differential term flips the fraction, bringing P into the right-hand side:

$$\frac{dC_{A(t)}}{dt} = P_{(t)} \frac{2A(C_{EQ} - C_{A(t)})}{V_A} \quad \text{Eq. 16}$$

This can be further simplified through the following equation, driving out the 2 term as well:

$$k = P \frac{2A}{V_A} \quad \text{Eq. 17}$$

This yields the following:

$$\frac{dC_{A(t)}}{dt} = k(C_{EQ} - C_{A(t)}) \quad \text{Eq. 18}$$

Fitting could be performed with either the numerical solution to the above equation solved with `scipy.odeint` or the analytical solution which was yielded by integrating the equation:

$$C_{A(t)} = c_1 e^{-kt} + C_{EQ} \quad \text{Eq. 19}$$

Where, the curve is constrained between the starting and end acceptor concentration with an initial value condition as the pre-exponential factor:

$$c_1 = C_{A(t=0)} - C_{EQ} \quad \text{Eq. 20}$$

And so:

$$C_{A(t)} = (C_{A(t=0)} - C_{EQ}) e^{-kt} + C_{EQ} \quad \text{Eq. 21}$$

The above general solution can fit either acceptor or donor curve for $C_{A(t=0)}$, C_{EQ} and k with the permeability yielded with equation 17.

2.7.2. Model for permeability and partition measurement – chapter 6

The above model assumes that the system is closed with respect to the oil phase and so the fitted permeability k value is representative of both acceptor and donor droplets. The surface tension at w/o interface for octanol and water is 8.5 mN m^{-1} ¹⁶⁸ and for hexadecane and

water is 55.2 mN m^{-1} .¹⁶⁹ The most relevant partition value for an estimation of mass loss in a DIB system is one between the concentrations of the permeant in water and in the oil across a lipid monolayer stabilised w/o interface. Such a partition value is here termed, $\text{clogP}_{\text{mon-16}}$. By comparison of $\text{clogP}_{\text{mon-16}}$ with known $\log P_{16}$ (hexadecane – water partition coefficient)^{78,76} or more broadly $\log P_{\text{alk}}$, an estimation of the molecules monolayer interaction may be determined. Across the course of this work, compound partition into the oil phase was observed, yielding an asymmetry in the acceptor and donor distributions due to dynamic permeant hexadecane partition. It was assumed that the oil would act as an infinite sink for partition, due to the 25-fold greater oil compared to aqueous volume. This behaviour is thus not described by a typical exponential change in concentration in either droplet.

In chapter 6, a measurement was made of both droplet distributions by linear actuation of a beam path across a chip. There was a transmittance difference across each chip position. In the described iteration of the method, each measurement position could not be calibrated to yield a typical concentration time curve as in chapters 3 and 4. Instead, intensity time curves were normalised on a pair wise basis such that the oil partition curve was represented through the following:

$$I_{O(t)} = 1 - (I_{A(t)} + I_{D(t)}) \quad \text{Eq. 22}$$

And in the case of DIB networks:

$$I_{O(t)} = 1 - (I_{A(t)} + I_{A2(t)} + I_{A3(t)} + I_{D(t)}) \quad \text{Eq. 23}$$

Where $I_{O(t)}$ represents the lost intensity to surroundings. Acceptor and donor curves were min – max normalised such that $I_{O(t)} \leq 1$ (± 0.05). Experiments were measured for sufficient time such that a measure of the oil partition rate could be made after the equilibration of permeant bilayer flux. As is seen in the model, membrane retention was not accounted for and so the concentration was assumed equal in each droplet. As was seen in chapter 6, a strong agreement was observed for non-partitioning compounds when permeation was characterised quantitatively or with this method. To fit such data, a system of ODEs was determined through:

$$\frac{dI_D}{dt} = -(k_1 + k_2)I_D + k_1I_A \quad \text{Eq. 24}$$

$$\frac{dI_A}{dt} = k_1I_D - (k_1 + k_2)I_A \quad \text{Eq. 25}$$

$$\frac{dI_O}{dt} = k_2I_D + k_2I_A \quad \text{Eq. 26}$$

Where k_1 and k_2 refer to the rate constants of bilayer permeability and partition respectively.

This system can be represented in matrix form with additional dummy values.

$$\begin{pmatrix} I_D' \\ I_A' \\ I_O' \end{pmatrix} = \begin{bmatrix} -(k_1 + k_2) & k_1 & 0 \\ k_1 & -(k_1 + k_2) & 0 \\ k_2 & k_2 & 0 \end{bmatrix} \cdot \begin{bmatrix} I_D \\ I_A \\ I_O \end{bmatrix} = A\vec{v} \quad \text{Eq. 27}$$

The matrix was solved through computational algebra embedded within wolfram alpha to obtain eigen vectors and eigen values. Through the general solution to a homogenous system of coupled ODEs, the following was yielded:

$$I_{D(t)} = -C_2 \cdot e^{-2k_1 t} \cdot e^{-k_2 t} - \left(\frac{C_3}{2} \cdot e^{-k_2 t} \right) \quad \text{Eq. 28}$$

$$I_{A(t)} = C_2 \cdot e^{-2k_1 t} \cdot e^{-k_2 t} - \left(\frac{C_3}{2} \cdot e^{-k_2 t} \right) \quad \text{Eq. 29}$$

$$I_{O(t)} = C_1 + (C_3 \cdot e^{-k_2 t}) \quad \text{Eq. 30}$$

The following additional boundary conditions were included, which assume that the equilibrium concentration is half the starting donor concentration (due to min max normalisation), and the oil partition will eventually tend to total solubilisation, as seen in C_3 :

$$C_1 = I_D(t=0) \quad \text{Eq. 31}$$

$$C_2 = -\frac{I_D(t=0)}{2} \quad \text{Eq. 32}$$

$$C_3 = -I_D(t=0) \quad \text{Eq. 33}$$

Which finally gives a particular solution for the donor, acceptor and oil compartments:

Donor:
$$I_{D(t)} = \frac{I_{D(t=0)}}{2} \cdot e^{-2k_1 t} \cdot e^{-k_2 t} + \left(\frac{I_{D(t=0)}}{2} \cdot e^{-k_2 t} \right) \quad \text{Eq. 34}$$

Acceptor:
$$I_{A(t)} = \frac{I_{D(t=0)}}{2} \cdot e^{-k_2 t} - \left(\frac{I_{D(t=0)}}{2} \cdot e^{-2k_1 t} \cdot e^{-k_2 t} \right) \quad \text{Eq. 35}$$

Oil:
$$I_{O(t)} = I_{D(t=0)} - (I_{D(t=0)} \cdot e^{-k_2 t}) \quad \text{Eq. 36}$$

3 The Build and Design of a Simple Label Free Permeability System – Laser cut milli fluidic systems

3.1. Chapter overview

Enabling label free experimentation is a challenge that has emerged within bottom-up synthetic biology. An ideal DIB permeability platform is one able to screen relevant chemical structures with an *in situ*, non-invasive measurement. This chapter addresses this challenge, with a PMMA based chip design strategy. Uniquely, PMMA allows for the integration of analytical equipment embedded within chips. By heat pressing layers of acrylic, label free systems can be developed for enabling UV spectroscopy across 200 μm apertures. This system of engineering chips yielded a simple and easily deployable chip which was used for DIB permeability assessment. The system, experimental procedure and analysis procedure for measuring label free permeability in a single droplet is detailed. It was found that the engineering principles could be readily transferred into pump controlled fluidic systems. A series of alternative devices for enabling novel experimentation with fluidic chips and devices is presented. Work from this chapter was published in Lab on a Chip in 2022.

3.2. Introduction to laser cut fluidic devices

The process of engineering (on the scale of micro to milli) fluidic devices typically follows a series of key stages. In the simplest terms, these stages can be thought of as chip conception, CAD design, printing and testing. Failure at the chip testing stage can be due to issues in any of the stages along this process. Success can broadly be defined as a chip that facilitates a measurement, often yielded from iterating the engineering pipeline multiple times. Soft lithography with polydimethylsiloxane (PDMS) requires access to a cleanroom (figure 3.1 a), a highly specialised and expensive suite.¹⁷⁰ For soft lithography, the process of engineering a chip can take on the order of a day without factoring in mask lead time.

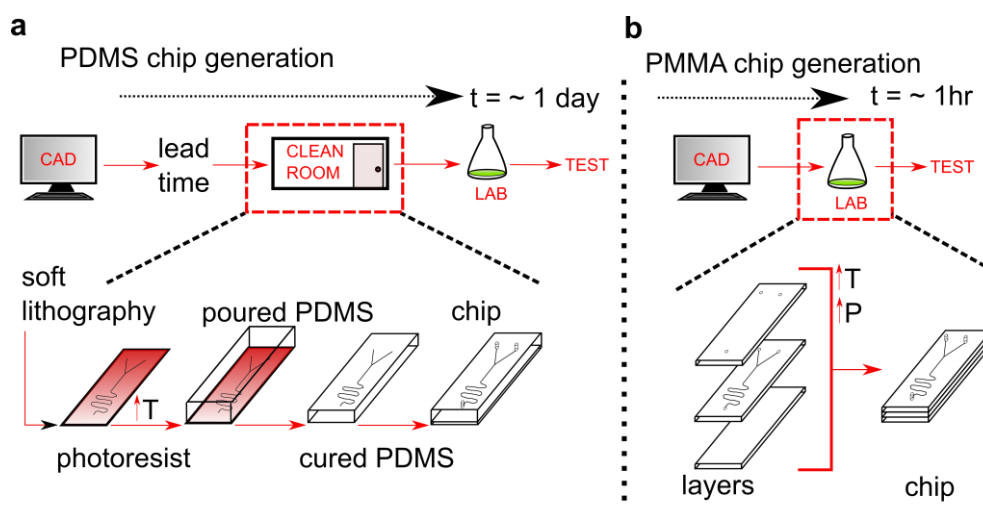


Figure 3.1: A brief comparison of the workflows employed in traditional PDMS. **a** As compared to PMMA based chip generation. **b** PDMS fluidic chips can take around one day to generate from conception to chip due to the more complex procedure of chip generation, whereas PMMA based chips typically take around an hour and can be formulated entirely within a standard laboratory. Note, this is a non-exhaustive protocol for either procedure, for PDMS, please see the following references,^{171,172,173} PMMA chip generation is further covered in this chapter, and chapters 2 and 5.

Polymethylmethacrylate (PMMA) is a thermoplastic that is cheap, readily available and safe. PMMA can be laser cut, etched and milled (figure 3.1 b). PMMA typically has a glass transition temperature (T_g) of 70 – 170 °C (dependent on polymerisation).^{174,175} If heated above the heat distortion temperature PMMA can also be bent into shape. PMMA can be glued, heat and solvent bonded. PMMA is widely utilised in fabrication as a material for designing three dimensional structures built from laser cut two dimensional sheets. A well-documented issue is that small hydrophobic molecules can partition from the aqueous phase into PDMS.¹⁷⁶ Considering the typical physicochemical profile of small molecule drugs and the drive toward industrial application of fluidic technologies, this factor may present significant challenges for drug screening with PDMS based chips. For further reading, see the excellent perspective by Mukhopadhyay.¹⁷⁷ PMMA is starting to see widespread use as an alternative material to PDMS for the generation of fluidic devices, particularly in settings where small molecular adsorption may be a significant problem.¹⁷⁸

Perhaps the most apparent advantage of building fluidic devices with laser cut PMMA over PDMS, is that the process of engineering a successful chip is faster. As the throughput of chip generation is high, issues within the engineering pipeline can be immediately remedied. Laser cutting PMMA can be performed by commercial laser cutters, where all aspects of this chapter were performed with a VLS 2.3 Universal Systems Laser Cutter held within the central laboratory. After cutting layers of PMMA, the layers can be sealed together through heat bonding above the T_g . All devices within this chapter were fabricated with Weatherall

Clarex Precision Acrylic, which was found to exhibit favourable properties for chip engineering when compared to other commercially available acrylics.¹⁷⁹ The work within this thesis contributes to a growing field of novel technologies which exploit the unique aspects of PMMA fluidic chip engineering.

3.3. Using PMMA chip design to enable label free measurements

3.3.1. Fiber implementation

The field of PMMA based fluidic devices has rapidly expanded in recent years. To date, droplet microfluidics has been performed with laminated PMMA layers,¹⁷² multiplexed DIB formation with sliding PMMA layers, and PMMA chips with embedded electrodes.^{114,42} An excellent review from 2016 further covers the maturation of the field.¹⁷⁹ A unique aspect of the engineering process of PMMA based devices is that three-dimensional features are easily facilitated through assembly in a layer-by-layer fashion. Integration of analytical equipment is possible with PDMS chips but requires significant skill and resources.¹⁷³ Within this chapter, layer by layer PMMA chip assembly was exploited to align and integrate analytical equipment within fluidic chips.

As introduced previously, UV spectroscopy is a powerful technique which can be used to determine analyte concentration in solution. In recent years there has been a drive toward portable spectrometers and light sources for enabling custom spectroscopic applications. Ocean Insight (formerly known as Ocean Optics) is a leading company for the supply of benchtop spectroscopy equipment. All fiber optic cabling was purchased from Thorlabs, a leading supplier of fiber optics (figure 3.2 a). The light source and spectrometer used throughout this thesis were purchased from Ocean Insight (figure 3.2 b). Fiber optic cannulae, attach to patch cables through a ceramic support and the patch cables can be attached to spectroscopy equipment through SMA connectors. Fibers allowed transmission of light from 240 – 840 nm.

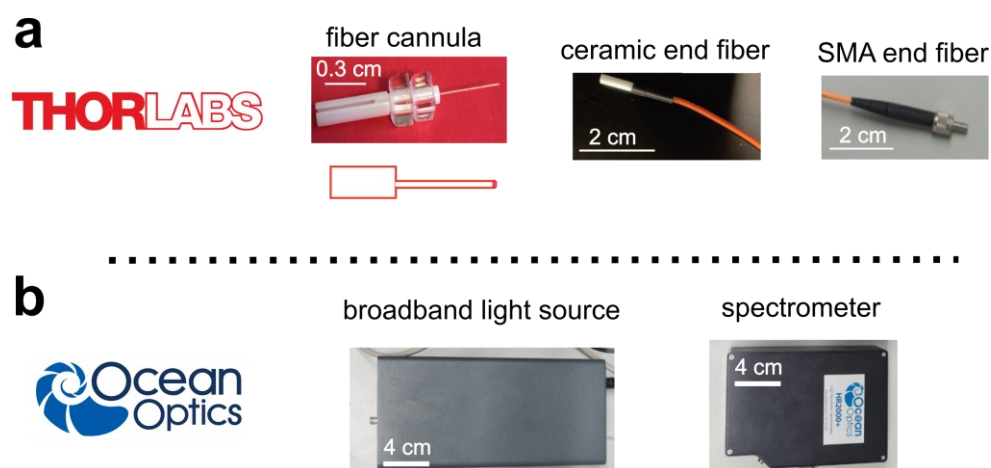


Figure 3.2: Overview of spectroscopy equipment employed in this chapter and throughout. **a** Optical fibers for the transmittance of broadband light (200 – 1250 nm), all purchased from ThorLabs. **b** Spectroscopic devices for generating (light source) and detecting (spectrometer) broadband light.

The resolution for laser cut PMMA based devices is constrained in the z -axis by the thickness of the purchased PMMA layer, and in the x and y axis by the resolution of the laser cutter. For work within this chapter, the thinnest layer was 0.5 mm and the smallest feature size for a cut was 0.2 mm, yielded by a straight etch of the laser cutter. Fiber implementation was initially introduced in 2009 in a glass / siloxane chip.¹⁸⁰ Schafer et al. utilised a custom laser to ablate and align a fiber in grooved glass. Without access to specialist equipment, this ablation strategy is difficult to reproduce. In this work, the surface of PMMA was etched to controllably create a groove with an estimated depth of 0.1 mm. Prior to heat sealing, fibers (diameter 0.2 mm) were aligned along this lasered etch on one of the layers. Fibers were sealed into the chip with a layer of PMMA above and below which melted around the fiber, sealing it in place and alignment (figure 3.3 a).

Figure 3.3 b shows how fiber embedding was coupled with a chip design which aligned the light path across a single droplet of a DIB, discussed later in section 3.4. To ensure access to the fiber in the chip, the ceramic connection port was exposed. A small PMMA support was used to hold the fiber cannula in place, where the support would then melt into the surrounding PMMA.

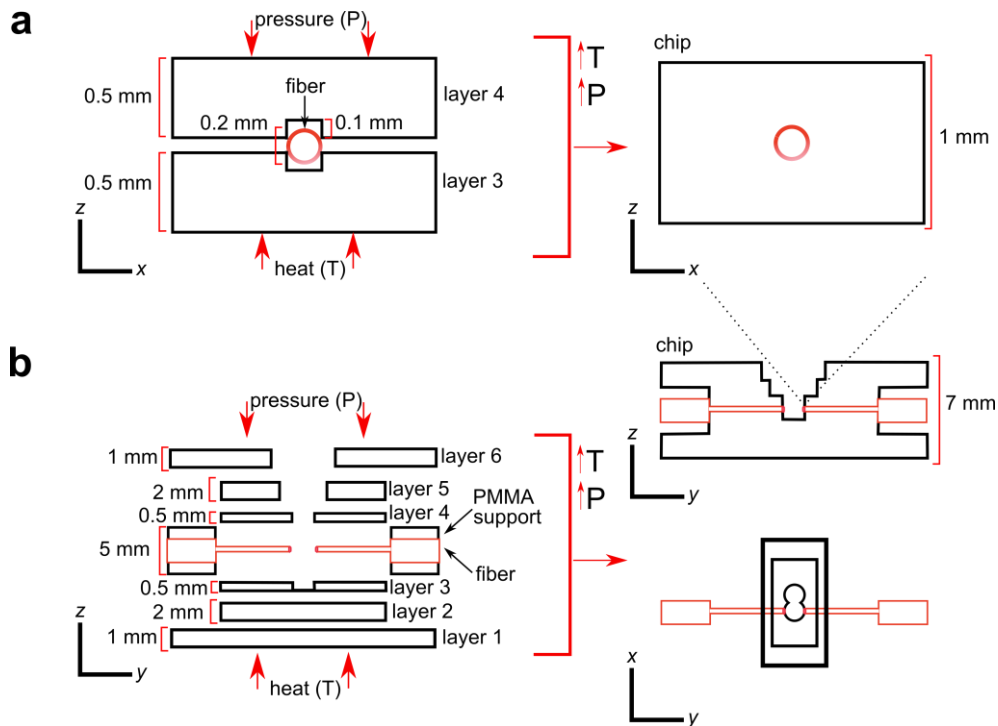


Figure 3.3: Embedding fiber optics within PMMA chips. **a** A generic overview of fiber embedding viewed down the axis of the embedded fiber. Here shown with dimensions of specific layers in optimised single beam path system. **b** Fiber embedding within the optimised single beam path system, here viewed across and above the DIB well space. After heat and pressure bonding the layers of PMMA, the fiber is embedded within the chip, ready for plugging into spectroscopy equipment. Fibers are aligned across a bespoke DIB shaped well. Note that features within schematics are not to absolute scale.

3.3.2. A heat and pressure block for sealing PMMA chips and arranging fibers

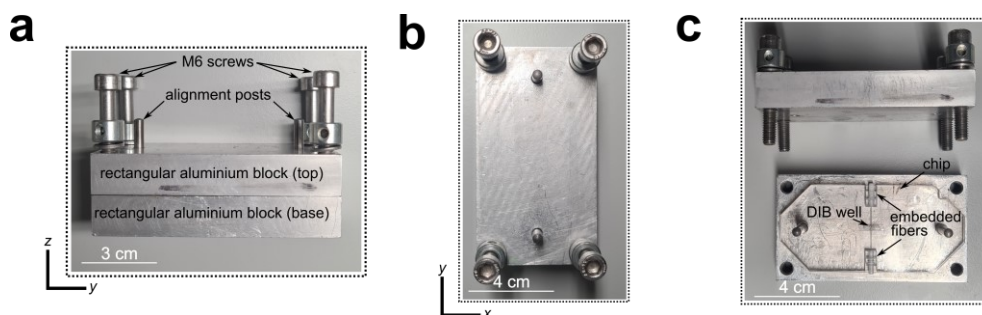


Figure 3.4: Custom aluminium heating block **a** Side on view of heating block. **b** Top-down view of heating block, screws are tightened to apply downward pressure. **c** Image of heating block with single beam path permeability chip post heating. Chip were arranged along alignment posts, ensuring features were aligned during the heat bonding process.

To heat bond chips, a simple, custom aluminium heating block was made in the department by the metal working engineer (Lee Tooley). The heating block consisted of two rectangular blocks which could be sealed by M6 screws. The base rectangular block featured alignment posts (diameter = 0.4 cm). The principles behind the operation of the block were as follows.

All PMMA layers were arranged using the alignment posts on the base rectangular block sequentially up until the layer which would form the base of the embedded fiber (e.g., figure 3.3 b layer 3). The fibers were then carefully arranged across the layer with the supports slotting into the lower layers, care was taken such that the fibers were not exposed into the fluidic well ensuring the fibers sat flush against the well surface (e.g. figure 3.3 b). Layers above the fiber were then added ensuring the position of the fibers was not disturbed (e.g., figure 3.3 b layers 3 – 6), the upper rectangular block was then added on top of the arranged layers. The heating block was sealed using a torque wrench and placed on a hot plate to heat bond the PMMA layers. After a period of time, the heating block was removed from the hot plate, allowed to reach room temperature and the sealed chip would then be taken off the alignment posts.

Chips were optimised following the above procedure. A series of initial experiments aimed to characterise the sealing efficiency of the layers at given temperature, length of seal and pressure conditions. If chips were heated too high, or sealed with too great a pressure, chips would bend and melt, destroying the fine features within the chip. If chips were heated with inadequate temperature or without pressure, then the layers would not seal properly and peeled away from each other. All chips were cooled to room temperature, to avoid heat shock. If PMMA layers were not washed and cleaned prior to sealing, this led to defects at the interface of the layers, such as bubbles, non – uniform layer sealing and highly foggy or blurry chips (an example of minor chip defects can be seen in figure 3.5 b). Depending on application, optical clarity could be controlled through a slower heat bonding process. To ensure that each layer of the PMMA reached the T_g , the heating block was heated well above the literature T_g of 90 °C.^{175,179} Based on visual assessment of failed chips with a chip height of 5 mm, the ideal conditions were found to be 45 minutes, 0.5 mN m⁻¹ pressure at 170 °C.

Having established fiber embedding, the following sections of this chapter will focus on experimental systems built using PMMA chip formation with embedded fiber optics. Major developments in this thesis were enabled by a single beam path device, on which this chapter centres. This label free DIB permeation system was used to perform a range of experiments in chapters 4 and 6.

3.4. A simple label free DIB permeability chip

The central goal of this work was to design a simple, reusable platform for label free DIB permeability assessment through engineering a PMMA chip with embedded fiber optics.

3.4.1. Single beam path chip design

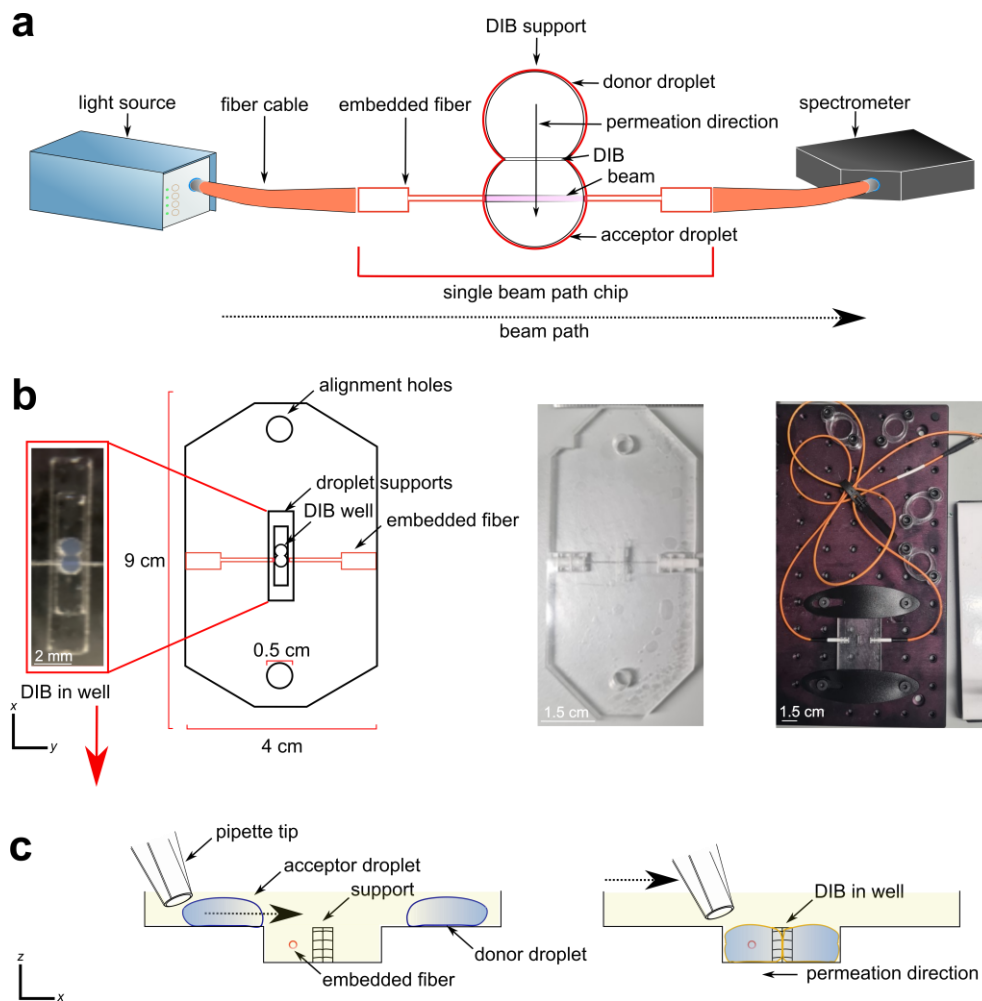


Figure 3.5: Single beam path DIB chips for full broadband spectral collection. **a** Cartoon schematic of single beam path system. Light was passed from a light source to a spectrometer across a single droplet of the DIB. Features not drawn to scale. **b** From left to right - zoom in of a DIB formed in the DIB well of the single beam path DIB chip, cartoon schematic of single beam path DIB chip features not drawn to scale, actual single beam path DIB chip formed post heat press, single beam path DIB chip arranged on optics table with fiber cables attached at exposed ceramic ends of chip. **c** DIB formation mechanism utilising DIB well support. DIBs were guided utilising a small support, also seen in first image in b, figure 3.3 b and DIBs formed in chip in figure 1.7 b. Droplets were incubated in a step above the DIB well, before they were pushed into the beam path of the embedded fiber.

To perform a label free measurement with optical fibers, a schematic of the system is presented in figure 3.5 a. Here it can be seen how a broadband light source was passed across a single droplet of a DIB, facilitating read out of the broadband spectra which could be captured through time. Therefore, a time dependent concentration read out in either the acceptor or donor droplet could be achieved. By controlling the concentration gradient relative to the fiber position, the direction of permeation could be controlled toward or away from the beam.

To arrange the light path, the DIB geometry within the chip had to be controlled. Free standing DIBs formed in oil reservoirs can zip up incompletely, depending on how the droplets are pipetted and manipulated within the reservoir. Due to this, it is common within the literature to control DIB geometry with supports or rails, either in PMMA or PDMS fluidics.^{114,181,182,143} The DIB well was designed such that droplets were guided into arrangement through cut PMMA which would surround the perimeter of the DIB (figures 3.5 b and 1.7 b). This part of the DIB well is referred throughout as the 'DIB support'. This design feature was implemented so as to reduce error within a permeability measurement, repeat to repeat. This feature ensured that both droplets would sit in a uniform position, that (repeat to repeat) the fibers would pass across the same relative section of the droplet volume, and that the bilayer area was controlled, as the droplets could not freely extend beyond this support. Due to the thickness of the lower most layer, as well as defects, the PMMA chips were difficult to study with inverted microscopy as the focal plane became too clouded when focused at the DIB well. To circumvent this, stereoscopic approaches were employed for measurement. Multiple DIBs were formed within the chip with each bilayer area measured with microscopy (first image figure 3.5 b and figure 1.7 b). It was found that the mean DIB area within the chip was $2.9 \times 10^{-3} \text{ cm}^2$ with 1 standard deviation of $0.28 \times 10^{-3} \text{ cm}^2$. A 9.4 % variation in DIB area repeat to repeat suggests that the PMMA supports successfully controlled the DIB area. This successful controlling of the DIB area is evidenced throughout this thesis (chapter 1 figure 1.7 and is also discussed in section 3.5.5 and section 6.4.2).

Utilising layer by layer assembly also allowed for an incubation area to be added into the DIB well. Droplets could be pipetted into this incubation area, allowed to sit and reach a monolayer coating of lipid and then moved into the supported part of the DIB well with a pipette tip (figure 3.5 c). After incubation, the droplets were guided into arrangement as described above by the DIB support feature.

3.5. An experimental workflow for permeability assessment in

DIBs with a single beam path chip

An analysis and experimental procedure was developed for conducting a permeability assessment across wide molecular space with a single beam bath chip.

3.5.1. Theophylline and caffeine as model permeants

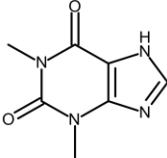
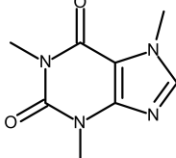
a	theophylline	b	caffeine
			
MW	180 Da		194 Da
AlogP	-0.31		-0.01
absorption range	247 - 302 nm		247 - 302 nm
absorption maxima	273 nm		273 nm

Figure 3.6: The structure of the methylxanthines theophylline and caffeine, with a brief overview of the physicochemical and spectral properties of each. Both of these compounds are used as model permeants within this thesis. AlogP calculated with Biovia Draw software.

Theophylline and caffeine are commercially available small molecule drugs belonging to the methylxanthine series of molecules. The core xanthine ring is a purine derivative with carbonyls at positions 2 and 6 on the heterocycle. Purine based compounds are some of the most abundant metabolites in mammalian cells as they are a core building block of the nitrogenous bases adenine and guanine.¹⁸³ Theophylline and caffeine are found naturally in plants and are rapidly absorbed in the human body. Theophylline causes bronchodilation through increased contraction of diaphragm muscle and so has application in asthma and chronic lung disease.¹⁸⁴ A full picture of the biological mechanism of theophylline remains unknown, despite phosphodiesterase being implicated.^{185,186} Caffeine has an identical structure to theophylline apart from an N-methylation (position 7 in the heterocycle). Caffeine is the most widely used stimulant of the central nervous system.¹⁸⁷ Caffeine is an antagonist of adenosine receptors and inhibits phosphodiesterase. Due to widespread use and interest, both compounds are well studied within the literature. Spectroscopically, caffeine and theophylline are identical however their physiochemical properties differ (figure 3.6). In consideration of the many favourable factors discussed above; theophylline and caffeine were used as model permeants within label free systems throughout this thesis. The methylxanthine series of compounds was further studied in chapters 4 and 6.

3.5.2. Spectral collection and data processing

It was desired that the single beam path chip could be used in an open laboratory without the need for a dark cloth. This would allow the experimenter to easily access the DIB well

and inspect the DIB during the recording of a permeation experiment. Any spectra from the chip therefore required further processing to account for: fluctuation in light source power (assuming variations in wavelength intensity are proportional), electrical fluctuations at the spectrometer detector, background intensity changes (such as laboratory light) and minor changes in droplet arrangement across the fiber.

In consideration, transmittance spectra (180 – 1100 nm, resolution 0.5 nm) were captured within the single beam chip with Oceanview software, an example is shown in figure 3.7 a. Concentration gradient DIBs were formed as discussed in chapter 2, with an acceptor and donor starting concentration of 50 and 500 μM respectively (permitted as a linear concentration – standardised intensity region – see next section). The integration time for each spectrum was between 20 – 40 ms and each spectrum was taken as the average of 3 sequential captures. A cut off was observed in spectra between 240 nm and 850 nm due to the wavelength range of the fiber optic cabling (figure 3.7 a). Some visible regions were occasionally saturated to maximise counts in the UV regions (seen in figure 3.7 a ~575 nm). An example of the difference in spectra between two points in the permeation process was seen in the theophylline absorption region (figure 3.6 a – theophylline and caffeine) where the rest of the non-absorptive regions of the spectra remained constant. In further consideration of the elements discussed in the first paragraph of this section, figure 3.7 b highlights how spectra were standardised by taking the ratio between an absorptive band (in the UV wavelength) and a non-absorptive band (near visible - visible wavelength) utilising the following equations:

$$I_{uv} = \frac{\sum_{uv1}^{uv2} I}{uv2 - uv1} \quad \text{Eq. 37}$$

$$I_{vis} = \frac{\sum_{vis1}^{vis2} I}{vis2 - vis1} \quad \text{Eq. 38}$$

Where $I_{UV / vis}$ are sums of the intensities in the UV and visible regions of the spectra, $uv1/2$ indicate a wavelength range around the permeant absorption maxima (λ_{maxima}) and $vis1/2$ represent a standardisation range around a non-absorbing region of the spectra. Note that in general spectra standardisation was constrained to $uv2 - uv1 = vis2 - vis1$, i.e. the size of the windows were equal. The two regions were ratiometrically represented via:

$$\text{standardised intensity} = \frac{I_{uv}}{I_{vis}} * 100 \quad \text{Eq. 39}$$

The standardised intensity converted a spectrum into a single value which accounted for dynamic fluctuations in spectral intensity.

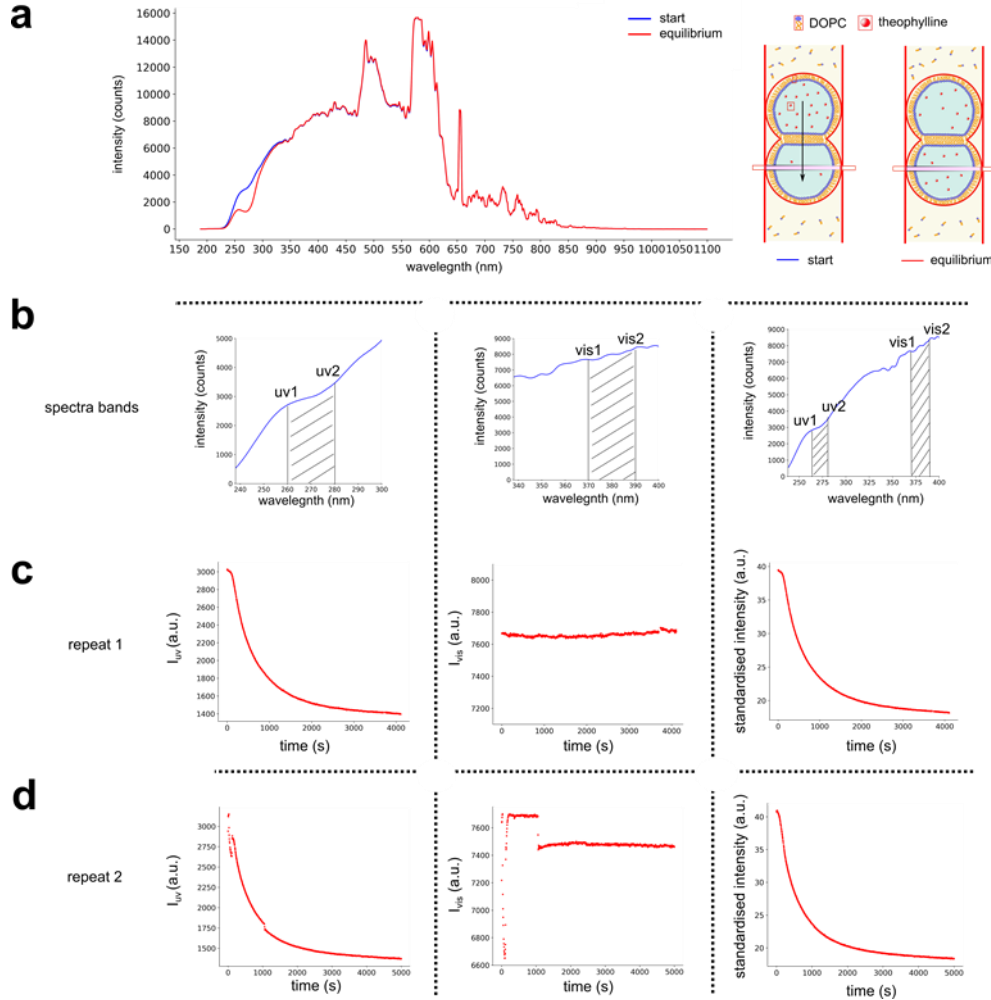


Figure 3.7: Spectra standardisation enabled smooth intensity time curves. **a** Spectra obtained across a DOPC concentration gradient DIB in the single beam path chip. Spectra of the acceptor droplet at the start of the experiment and at the equilibrium point at which diffusion across the bilayer had plateaued. **b** Zoom in of spectra highlights the experiments integration bands and standardisation process. From left to right; a band accounting for UV absorption (I_{uv}), a band representing non-absorption (I_{vis}), the standardisation achieved with both prior bands and equations 37 - 39. **c** Typical distributions of each integration band and standardisation in a permeation experiment with minimal noise in either band. **d** As in c, but in this repeat of the permeation experiment there was significant noise in the measurement in both the I_{uv} and I_{vis} bands. Here it can be seen how the standardisation yields a smooth curve of near identical shape to the one in c. The noise in this run was proportional across the spectrum, suggesting that the source for the noise was likely related to the spectroscopy equipment.

A 5 s time interval was used between each record, to conserve computer memory without compromising data quality. Depending on integration time, the data interval could be as low as 0.02 s. The results of monitoring the standardised intensity through time across an acceptor droplet are shown in figure 3.7 c and d. It can clearly be seen that the change in

standardised intensity followed a classic exponential profile, with an initial modest lag. The exponential character reflected an increase in the concentration of theophylline passing across a DIB from the donor droplet into the acceptor droplet. The lag in the distribution was attributed to DIB zip – a phenomena in which there is a lag time between droplet contact and Fickian diffusion.^{64,110,188} This phenomena was easily observed for fast moving permeants and is compared and discussed in chapter 4 section 4.3.5. These distributions make clear that the method was successful in recording a label free assessment in a droplet interface bilayer and that DOPC bilayers are permeable to theophylline.

3.5.3. Converting to concentration

Due to the spectra standardisation process the relationship between concentration and the standardised intensity was determined. For all permeants studied with the single beam path chip, a rigorous calibration procedure was performed as follows: 1.8 μ L droplets of varying concentration were pipetted into the chip and recorded for at least a minute. The mean standardised intensity was then determined from each distribution with this experiment repeated in triplicate and for each concentration. This yielded a concentration – standardised intensity calibration curve. In almost all studied compounds, a strong linear region ($R^2 > 0.99$) was observed between 50 and 500 μ M indicating that this procedure of spectra normalisation supported a quantification of concentration in the measured droplet (figure 3.8).

Typical UV absorbance spectroscopy could be performed with the method if it was assumed that the standardised intensity value acted as I with an I_0 value derived from a PBS droplet. This approach was decided against due to: the requirement of the standardisation procedure (figure 3.7), linearity was strongly observed without the need for further processing and that a nonlinear region was typically observed between 0 and 50 μ M. For all permeants studied within chapter 4, the permeation distribution is presented as a concentration time curve with the calibration R^2 value between the acceptor and donor concentrations which were kept within a small range (10:1).

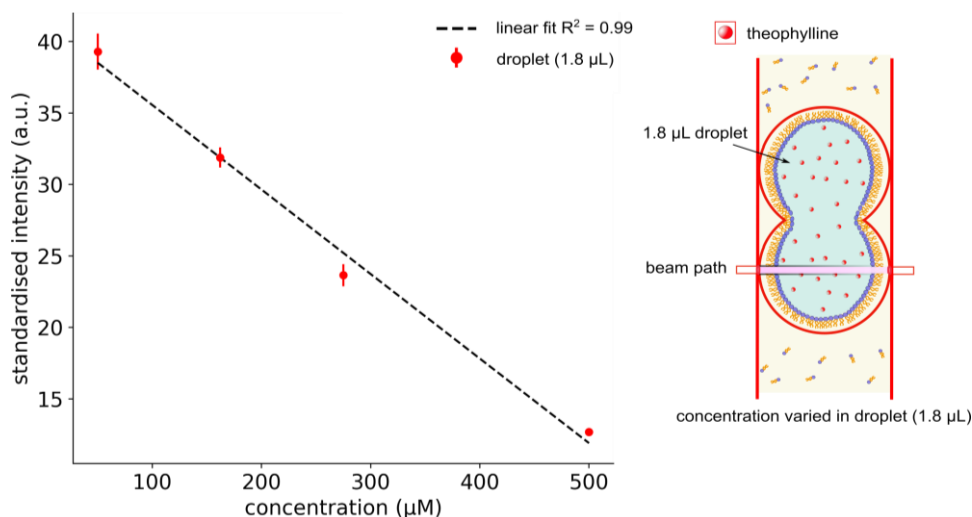


Figure 3.8: Theophylline calibration curve. A linear relationship between standardised intensity and 50 and 500 μM concentration was observed ($n = 3$, error bars represent the standard deviation).

3.5.4. Testing for oil partition

As discussed in chapter 2, it is pertinent to address the role of mass retention within the aqueous phase in a two-phase system such as a DIB. Mass lost from the permeant partitioning into the oil phase may otherwise complicate the kinetics of the permeation profile. The aim of the work was to develop a flexible method to allow study in wide physicochemical space in DIBs. Therefore, it was anticipated that oil partition would be encountered whilst studying a wide hydrophobicity range. To test for this, 1.8 μL droplets of the donor concentration were measured for all permeants. The resultant distribution would thus indicate if the compound was partitioning into the oil phase from the aqueous phase. It is important to note that this additional control is not typically performed within the literature, and this formed the basis of work in chapter 6 and is discussed extensively in chapter 8.

Figure 3.9 shows partition distributions for different w/o emulsion droplets within the chip. Partition of theophylline from the aqueous phase to a DOPC in hexadecane phase (3 mg / ml) was recorded within a single droplet volume (0.9 μL), in a DIB (2*0.9 μL) or in the fused droplet volume (1.8 μL). The distributions in figure 3.9 a are all linear, which clearly indicated that theophylline did not partition into hexadecane at a rate comparable to its DOPC bilayer permeation (quantified in chapter 6). Statistical testing evidenced significant differences between linear regressions of the partition and permeation distributions (figure 3.9 c). Further, the distributions showed that independent of the droplet morphology within the well, there was no difference in absolute intensity value. In tandem, this indicated that when a DIB split, returning to a morphology similar to a 1.8 μL droplet, the measured intensity value remained representative of the aqueous concentration in a DIB droplet. This aspect of the

measurement was exploited throughout chapter 4, to investigate bilayer retention in DIBs. After reaching equilibrium, DIBs were occasionally split to assess the concentration change in the single 1.8 μL droplet following DIB rupture. Any concentration retained within the bilayer would thus diffuse into the droplet bulk as the bilayer lipids redissolved into the aqueous phase. The direction of this distribution was used to indicate if any concentration was retained within the bilayer or the UWL.

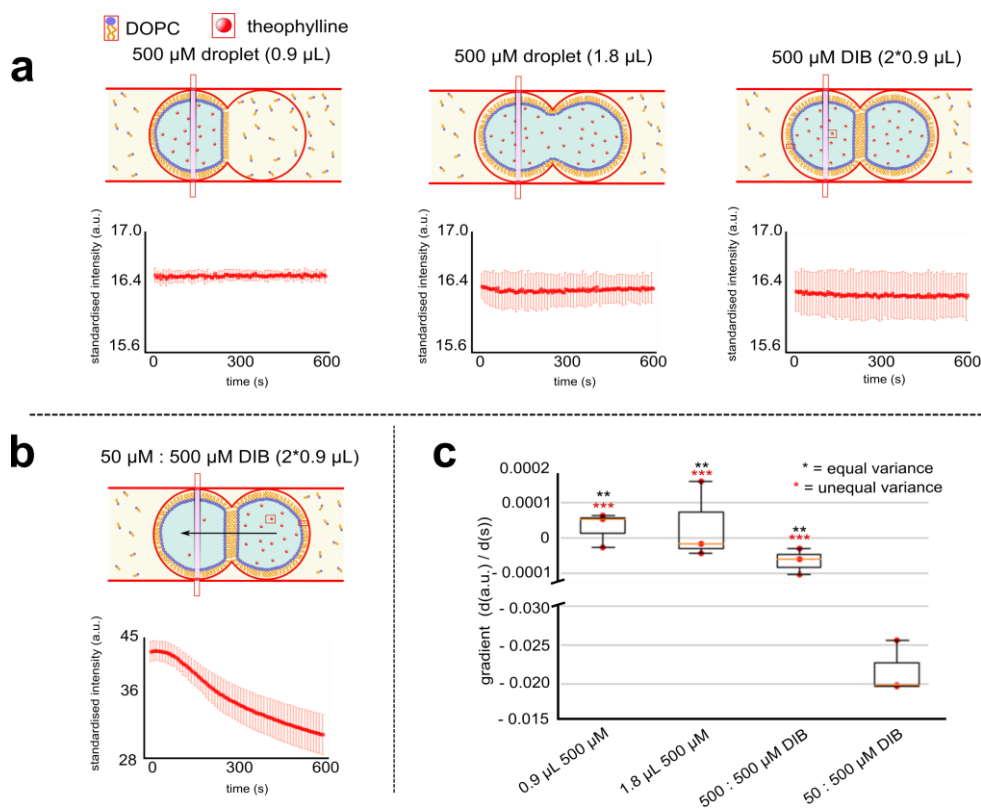


Figure 3.9: Different partition droplet morphologies within the single beam chip have no impact on the absolute intensity value or intensity distribution. **a** Cartoon and intensity time plot for various droplet configurations formed with theophylline 500 μM lipid out DOPC ($n = 3$ for each, error bars represent the standard deviation). Note, these experiments were performed with a different sample from figure 3.8 (calibration). **b** Cartoon and intensity time plot of a concentration gradient DIB where the acceptor droplet was monitored ($n = 4$, error bars represent the standard deviation). The permeation of theophylline can clearly be seen. **c** The gradients of a linear fit through each of the distributions in a and b with statistical testing between the gradient calculated in b and the gradient from each distribution in a. Comparing a permeation system to a partition system with equal or unequal variance showcased the strong statistical significance between each set of distributions (* = $p < 0.05$ two tailed independent t test).

3.5.5. Extracting a permeability value

Figure 3.10 shows the full exponential growth in concentration of the acceptor droplet, indicating the rapid bilayer permeability of theophylline. It was predicted that a compound demonstrating minimal hexadecane partition, would arrive at the expected equilibrium

concentration (C_{EQ}) in both droplets. This assumption informed the mass balance equation 14 and assumptions described above. C_{EQ} was experimentally confirmed for fast moving permeants where possible by monitoring of the full translocation profile to equilibrium. For theophylline, the C_{EQ} value was $\sim 273 \mu\text{M}$ (figure 3.10) ratifying these assumptions and the calibration method. As is described in chapter 4, for slow moving permeants, confirmation of C_{EQ} was not always possible. It is worth noting that a system reaching equilibrium would thus provide the most accurate C_{EQ} value and pervaded fitting with the exponential equation 19 (see chapter 2). Furthermore, in the case of minor discrepancies in volume, C_{EQ} measured per DIB reflected the permeant mass distribution of the individual experiment. In the case of a compound which does not partition into hexadecane, deviation between the expected C_{EQ} and the measured, may be due to the effects of permeant adhesion toward any vesicles within solution (in the case of lipid in), droplet volume differences or bilayer partitioning. This aspect is discussed in chapter 6 section 6.4.

For compounds not reaching equilibrium, a suitably accurate method for determining the permeability was a linear fit applied in the early region of a concentration time plot with an otherwise arbitrary R^2 value >0.95 (δm , m and R^2 were calculated inside the employed least squares regression method - `scipy.stats.linregress`). In further consideration of this, the permeability value was calculated for each concentration point within this linear regression with the mean reported per analysis (plateau region supplementary figure 1). For calibration curves, the error per point was negligible and so was not considered, an assumption expected to minimally affect the reported final variance of the measured P value. This linear fitting approach was also investigated with a designed algorithm which performed a linear fit across sections of a permeation concentration time curve (supplementary figure 1). It was found that so long as the R^2 value remained high, the permeability value plateaued, indicating that the fitting method is viable within the initial part of the curve. The permeability value determined through linear fitting, was near identical to the one extracted from fitting the full distribution with equation 19.

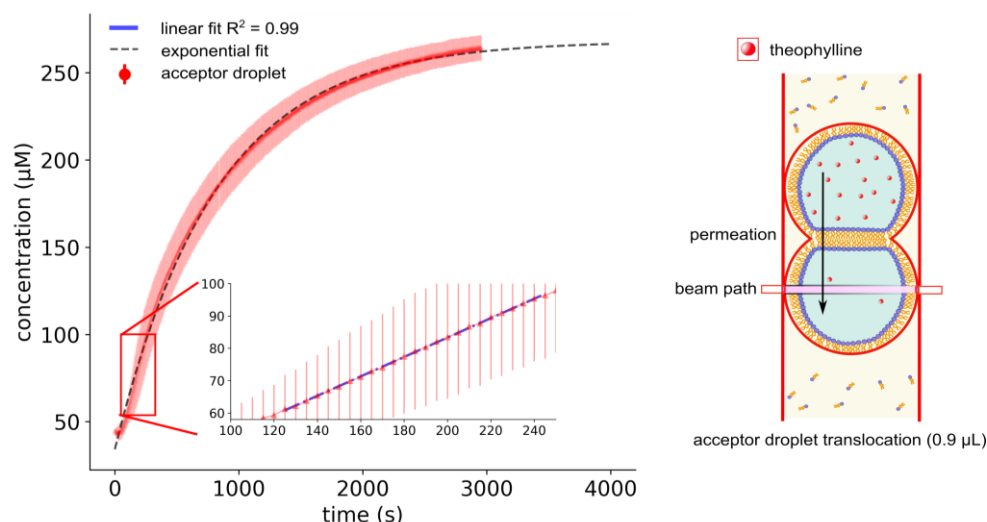


Figure 3.10: Concentration time distribution of acceptor droplet ($n = 3$, error bars represent the standard deviation). Measured with theophylline across DOPC DIBs formed with the acceptor ($50 \mu\text{M}$) and donor ($500 \mu\text{M}$) concentrations. The two methods of fitting are shown, with the insert showcasing an example of a linear fit approach, post DIB zip up for when the full distribution could not be measured.

3.5.6. Data analysis pipeline

The above experimental and analytical procedure was employed extensively throughout this thesis, thus a method was desired which would allow assessment of the bilayer translocation during an experiment. As an example, the theophylline translocation profile shown in figure 3.7 c was captured with a 5 s interval, constructed from 816 spectra which occupied 28.6 MB of disk space. An analysis pipeline written in python processed spectra in a high throughput manner. Depending on computational power, the translocation distribution could be extracted in around 3 s. This pipeline (figure 3.11) was also employed by colleagues who collaborated on aspects of this work within the core research group as well as the Barter lab. In future sections of this work, similar pipelines were also developed, the code of which are included in the appendix (via Github link) and are briefly discussed in each relevant section. An overview of the permeability pipeline is provided:

The Build and Design of a Simple Label Free Permeability System – Laser cut milli fluidic systems

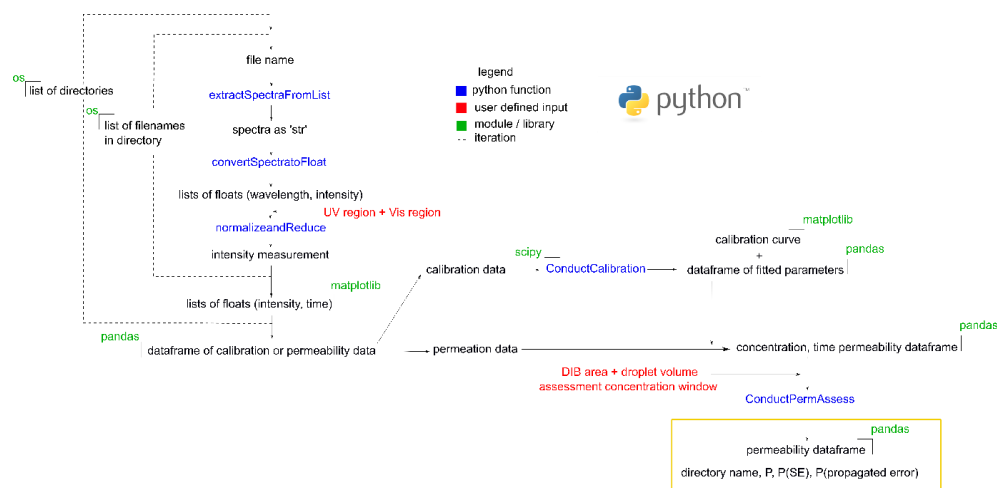


Figure 3.11: Overview of python script to automate the determination of permeability from label free measurement in DIBs. Relevant modules and libraries imported for each stage highlighted in green.

Having established the above system, the final sections of this chapter cover two millifluidic systems utilising methods and tools developed in this section. One system demonstrated potential for label free permeability assessment automation, the other with application in chemical reaction optimisation.

3.6. A millifluidic build out of a single beam path chip

Droplets were manually pipetted into the single beam path chip between each repeat. A means to increase experimental throughput could be achieved through a millifluidic strategy. By switching droplet generation from pipette to syringe pump, the role of the experimenter could be reduced. Complimenting this with *in situ* image analysis the system becomes amenable to automation - an attractive approach toward enabling high throughput data collection. As a proof of concept, this approach for permeability assessment under flow was investigated.

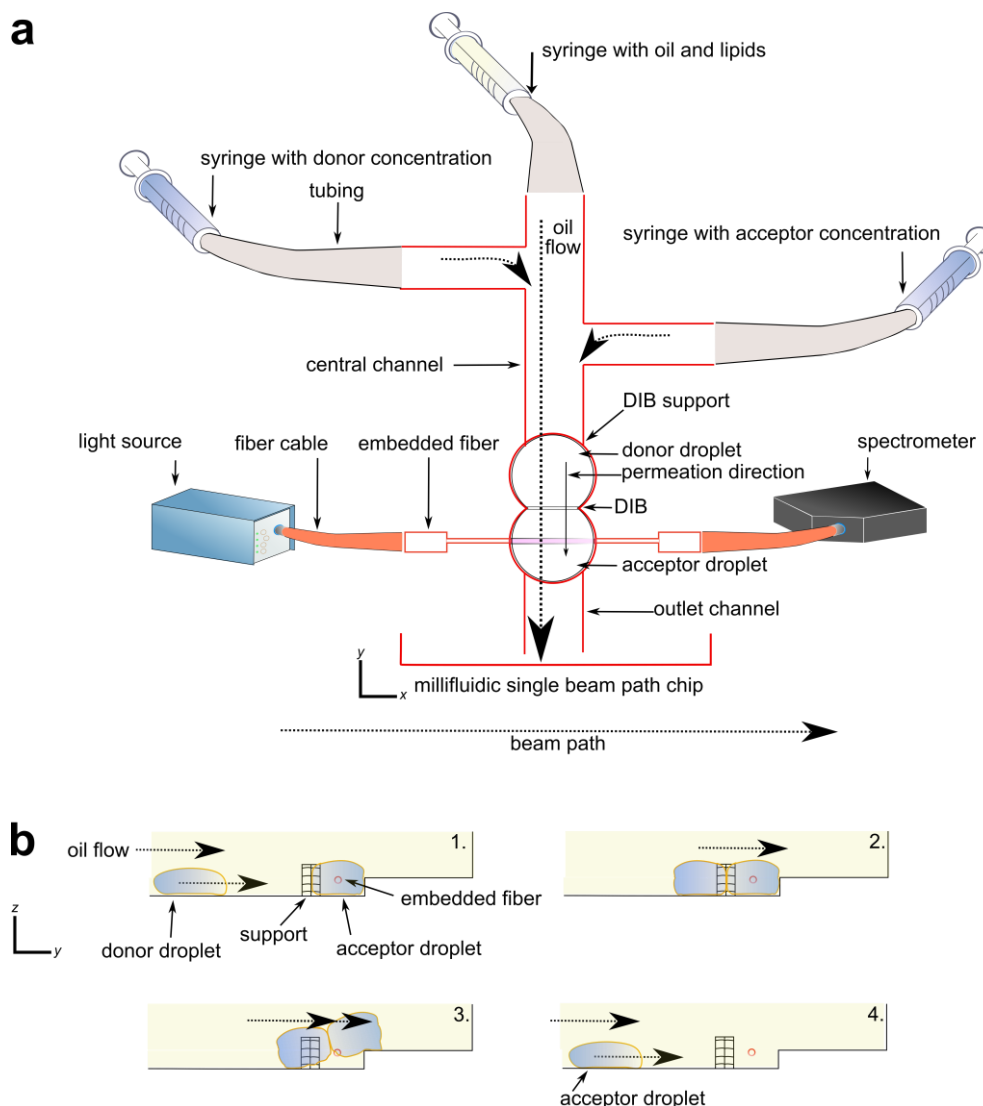


Figure 3.12: A millifluidic approach to DIB formation **a** Cartoon schematic of millifluidic single beam path chip system. A light path was passed from a light source to a spectrometer across a single droplet of the DIB where w/o droplets are generated using T-junctions. Features not drawn to scale. **b** DIB formation mechanism utilising 3D architecture millifluidics and droplet supports to arrange droplets across a fiber. Droplets arrived at the DIB well pre-coated with a lipid monolayer.

A PMMA milli-fluidic chip was conceptualised as in figure 3.12 a; the DIB well from the single beam path chip was placed at the end of a central flow channel. The outlet channel collected waste from the chip and was arranged above the DIB well in line with the central channel. The chip consisted of two T – junctions for w/o droplet generation into the central channel. These junctions were filled with either the donor or acceptor concentration. Droplets were introduced down the central channel into the DIB well (figure 3.12 b 1.). Droplets could then be left within the chip until concentration equilibrium was reached as monitored *in situ* by the analysis pipeline (figure 3.12 b 2.). If significant oil flow was applied, this would push the droplets from the DIB well out of the chip into the outlet channel (figure 3.12 b 3.), leaving the DIB well open for a newly generated acceptor droplet (figure 3.12 b 4.). By controlling droplet generation at either T- junction, the permeation direction could be controlled. An assessment in the device was performed with azolectin in hexadecane (5 mg / ml) with caffeine as the permeant (donor 500 μ M and acceptor 50 μ M). Azolectin was used at a high concentration to aid monolayer assembly. Free standing syringes were used for droplet generation with the oil flow controlled by syringe pump.

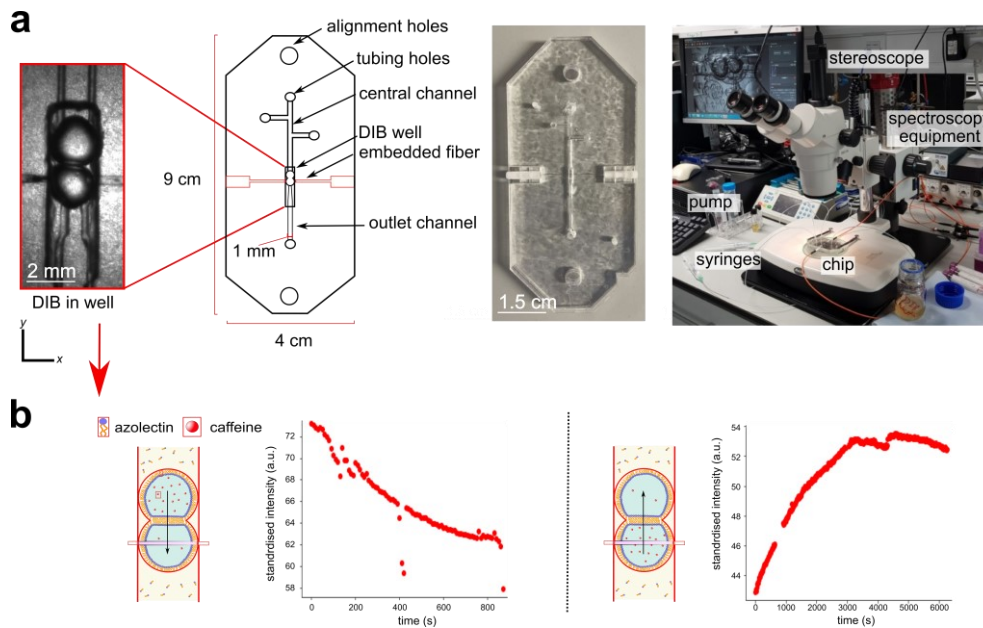


Figure 3.13: Results from millifluidic single beam path chip. **a** From left to right, a DIB formed in the DIB well, a cartoon of the chip features (features not drawn to scale), an image of the actual millifluidic single beam path chip and finally, an image of the system on a stereoscope for image capture. **b** Intensity time distributions captured from the chip, here showing two experiments where the permeation direction was controlled in each.

Figure 3.13 shows the results from DIB production inside the millifluidic chip. It can clearly be seen that DIB geometry was successfully constrained by the supports within the DIB well, as in the prior method. The direction of the distributions in figure 3.13 b, reflect the order of

droplet formation. If forming an acceptor droplet first, a transmittance decrease was observed as caffeine was driven in from the donor droplet, if forming a donor droplet first, the transmittance increased as a result of caffeine leaving to the acceptor. In tandem, this platform shows that fiber embedding in PMMA chips can be implemented into more complex DIB permeability systems. These results indicate that a fully automated label free DIB permeability system may be achievable with this approach. A high throughput system such as this, could have great utility in automating data collection across broad molecular space.

Ultimately, this iteration of the method was limited by a number of factors. As the syringes were controlled by hand, there was significant variation in droplet size, this could be remedied through use of further syringe pumps and fine droplet formation control. Considerable back flow along the central channel was observed, likely due to improper sealing of the tubing onto the PMMA surface. This pulled the droplets out of the beam path and caused DIB instability. This led to the noise seen in the distributions in figure 3.13 b, this could be remedied through use of traditional microfluidic fittings and fine control over the oil flow profile. Due to the contact velocity and the small length of the central channel, droplets frequently merged, a common problem with droplet microfluidics.¹⁸⁹ A more tightly packed monolayer could be achieved through addition of a serpentine in the central channel, after each T-junction. In this chip, deposits in the flow, either small droplets or undissolved lipid also occasionally blocked the beam path, requiring significant oil flow to clear the DIB well.

3.7. A millifluidic chip for studying reactions under flow

One area of emerging interest is fluidics applied to reaction optimisation.^{190,191} Reaction yield can be maximised by determining a reactions optimal parameter space with respect to reactant concentration, reaction equilibria, temperature, pressure and mixing. Fluidic systems have a great deal of potential for streamlining such data intensive tasks as they are amenable to automation. A reactions parameter space can thus be determined in a feedback loop if reaction conditions can be controlled as a function of product formation.¹⁹⁰ As a proof of concept, a millifluidic PMMA chip and flow system was developed which employed embedded fiber optics for the study of a chemiluminescent reaction (figure 3.14).

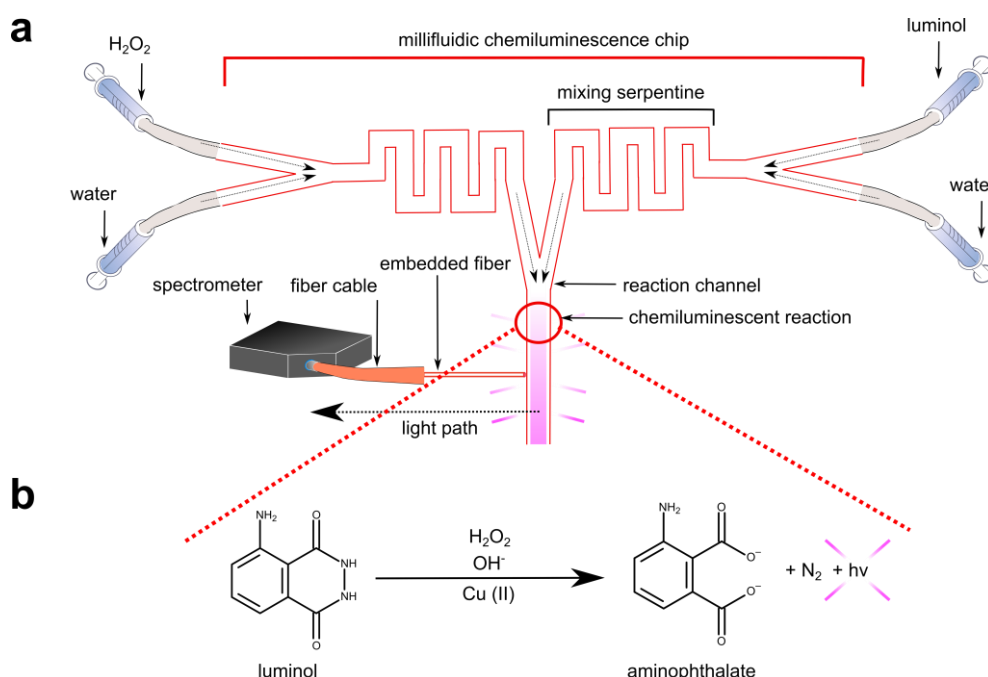


Figure 3.14: A millifluidic chip for studying reaction kinetics **a** Cartoon schematic of millifluidic chip for the study of a chemiluminescent reaction. Light emitted from the reaction was passed to a spectrometer. Features not drawn to scale. **b** Chemiluminescent luminol oxidation reaction.

Luminol is oxidised to aminophthalate via Cu (II) catalysis in an acidic environment (figure 3.14 b). Excited aminophthalate emits violet light between 452 – 489 nm depending on metal catalyst.^{192,193} A successful chip was yielded through rapid prototyping. The design of the experimental system is shown in figure 3.14 a. Operation of the chip was as follows: the reaction concentration was controlled on chip through two aqueous inlets fed into a mixing serpentine, one inlet containing solvent (distilled water), the other containing reactant. One of the serpentine's diluted reactant 1 (luminol (4.52 mM), sodium bicarbonate (570 mM), sodium carbonate (75 mM) and copper(II) sulfate (5 mM)) whilst the other diluted reactant 2 (hydrogen peroxide (30 v%)). By controlling the flow rate ratio (FRR) between the two inlets,

the reactant concentration could be controlled. The two serpentine channels converged at a reaction channel with an embedded fiber. A cleaved fiber optic patch cable was used to save costs during chip development (figure 3.15 a), this method of fiber insertion is further discussed in section 5.3.2. When the reactants mixed in the reaction chamber, a chemiluminescent reaction would begin (figure 3.15 b), in which emitted light intensity is proportional to the amount of product (aminophthalate).

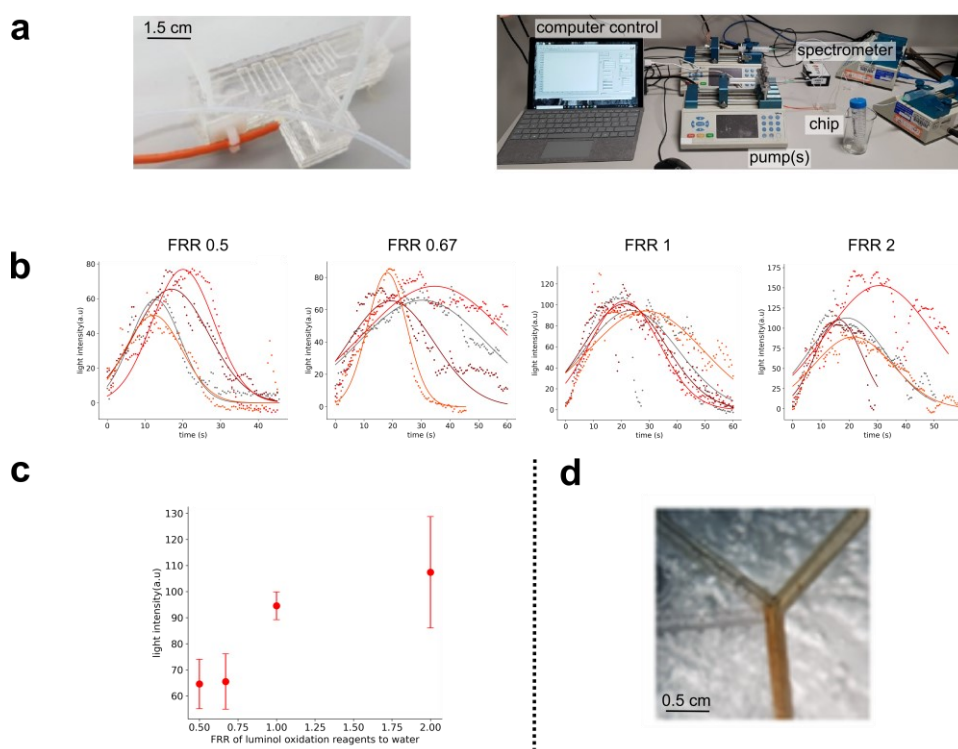


Figure 3.15: Results from millifluidic chemiluminescent reaction chip. **a** From left to right, images of the millifluidic chip with an embedded stripped fiber and the full experimental system. **b** Four chemiluminescence intensity distributions at various FRRs. **c** Positive correlation between Gaussian fit maxima and FRR ($n = 8 - 15$, error bars represent the standard deviation). **d** Residue built up at reaction channel attenuated measurement.

For this system, four syringe pumps were employed as each inlet required continuous flow. Each inlet was pumped at a given FRR for 5 s before shutting off the flow, allowing for the reaction to take place at the fiber. A custom LabView script recorded chemiluminescence through monitoring a spectral band between 440 – 510 nm. A spectral band outside of the chemiluminescent band was also monitored to ensure the intensity was due to the reaction. Shortly after the reactant streams met in the chip, a purple light was emitted from the reaction channel indicating that luminol had been oxidised whilst the non – chemiluminescent band remained flat. The intensity of chemiluminescence was fit to a Gaussian distribution. When changing the reactant FRR, increasing reactant concentration,

the maxima of this Gaussian distribution increased whilst the full width half maxima appeared to remain roughly consistent (figure 3.15 b). In plotting FRR against this fitted maxima, a clear positive correlation was observed (figure 3.15 c).

The results from this system clearly showcase that chemiluminescence can be recorded on chip. As expected, the product concentration (indicated by light intensity) appeared to follow a positive correlation with the reactant concentration. Full automation of this system would thus allow for determination of the parameter space with respect to FRR and maximum product yield. From these results alone, it is difficult to decouple the reaction kinetics from reactant diffusion within the reaction channel. The nature of the distribution could be investigated by adjusting the employed 200 μm channel width and controlling the absolute flow rate, where the degree of mixing in the serpentine can be accounted for. Finally, it was found that oxidised luminol built up in the chip in the form of a brown residue likely including Cu(II) ions (figure 3.15 d). As this aggregated around the fiber, the chip was used for a single sweep of experiments and a new chip was generated, all data in figure 3.15 was recorded from a single chip. This system supports the idea that fiber embedded PMMA chips can have significant utility in fields outside of DIB permeability studies.

3.8. Chapter conclusions

In this chapter, a series of experimental systems enabled by PMMA fluidic chips with embedded fiber optics were engineered. A focus was placed on a single beam path DIB permeability chip, and an experimental system for characterising label free permeability was determined. Since aromaticity is a near ubiquitous structural feature of drug molecules, this method engineered a UV absorption-based measurement to improve upon fluorescence-based approaches employed extensively within the field. The developments of this chapter thus unlock label free experimentation in DIBs. By employing the outlined experimental procedure, a small molecule drugs bilayer transport in DIB can be determined. The flexibility of this fabrication strategy was showcased through implementation of fiber optics into more traditional flow-based systems, demonstrating the potential role of this measurement strategy in automated pipelines.

4 Single Droplet Label Free DIB Permeability Studies – DIB biophysical features, systematic chemical space and small molecule drugs

4.1. Chapter overview

Understanding structure – permeability relationships is of critical importance to *in vitro* biological transport modelling where DIBs can be used to uncover biophysical aspects of this process. In this chapter, the developments in chapter 3 were employed to uncover structure – permeability relationships across a wide chemical space. The DIB environment was first studied, understanding how experimental considerations, such as lipid location, oil phase and dimethyl sulfoxide (DMSO) content can affect the measured permeability. As the developed method was label – free, fine permeant structural changes were then studied, rationalising a structure – permeability relationship in a contained chemical space. From here, commercially available biologically active compounds were tested across DOPC bilayers showcasing the facileness of the developed method. The potential of this approach in an industrial context was successfully demonstrated through the assay of a range of small molecule drugs supplied by GSK. In this chapter, the potential for the label free DIB platform in wide chemical space was demonstrated. Aspects of this chapter were published in Lab on a Chip in 2022.

4.2. Introduction to *in vitro* structure – permeability relationships

Subtle structural changes to permeant structure can drastically affect a permeants bilayer and biological membrane translocation.^{98,37} An outlook in the field of synthetic biology is the engineering of biomimetic constructs which provide physiologically relevant *in vitro* models. As was introduced in chapter 1 DIB interfaces are inherently more biomimetic than the PAMPA interface.^{65,117,64} PAMPA applications, which utilise UV spectroscopy for permeant measurement have produced simple models correlating PAMPA permeability to the physicochemical metrics of the permeant.^{194,63,36} This modelling is an example of a data intensive structure – permeability relationship (QSAR) which attempts to reduce the need for case by case experimentation. A central assumption is that physicochemical metrics capture the biophysical properties of a permeant structure which dictate its translocation behaviour. As PAMPA is easy to use and can study wide chemical space, this has driven its industrial

uptake.¹⁹⁵ PAMPA is therefore readily integrated into drug development pipelines where small structural modifications, which otherwise do not affect the potentiality of measurement, can be screened. DIBs, whilst exhibiting massive potential as an alternative model interface, have been constrained to fluorescent permeants. This aspect has prevented structure - permeability models derived from DIBs. It was thought that the novel DIB bilayer permeation method from chapter 3 could be used to address this major limitation of DIB permeability systems. This chapter aimed to yield a better understanding of the experimental considerations of a DIB permeability system and a series of structure – permeability relationships in DIBs.

4.3. DIB biophysical experiments

In chapter 1, the biophysical considerations of DIB permeability systems were introduced. By understanding how experimental factors influence DIB permeability (figure 4.1) within the single beam path chip, the relative changes in permeability could be determined ahead of the change in permeability afforded across wider chemical space.

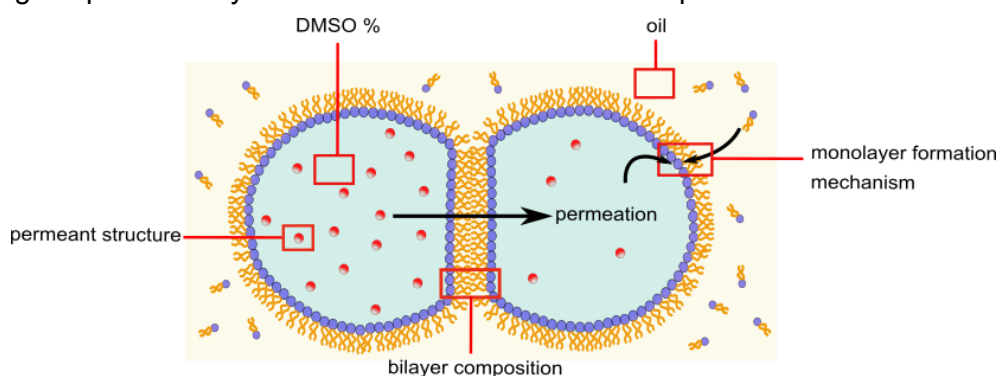


Figure 4.1: The biophysical features of a DIB permeability system explored in this chapter.

4.3.1. DMSO % in buffer

Dimethyl sulfoxide (DMSO) is a solvent extensively used to co-solvate small molecule drugs into aqueous solutions. Co-solvation can achieve aqueous concentration (usually >nM) above the limit of detection of a particular measurement. DMSO's role in biological measurements can be contentious due to its interaction with membranes as well as the bulk aqueous solution.¹⁹⁶ It is well known that DMSO can act as a permeability enhancer of the PM, a significant utility in topical applications.^{197,198} Perhaps most pertinently, Gironi et al. found that red blood cells were permeable to ATP above 1% DMSO, suggesting a modulation of the intrinsic PM permeability to biological permeants.¹⁹⁶ Multiple studies have reported nonmonotonic relationships between membrane properties and bulk DMSO concentration.^{196,199} The behaviour of DMSO is further complicated by membrane

composition and bulk system properties which has led to conflicting results in the literature, particularly in comparing between model and biological systems.²⁰⁰ Multiple studies have reported that DMSO can increase the packing density of membranes through dehydration of the head groups.^{196,199,201} Such is the conventional application of DMSO, it has also been used in DIBs to achieve co – solvation of fluorescent permeants and to attenuate oil partition.^{202,153} Careful control is therefore required to delineate the impact of DMSO in experiments where measurement is based on the system bulk.

In anticipating study across wide chemical space, co-solvation may be required to facilitate sufficient permeant aqueous concentration for measurement. To address this, the effect of DMSO volume in the aqueous phase on the measured bilayer permeability was characterised (figure 4.2 a). Lipid out DOPC DIBs were formed with an equal volume % of DMSO in either droplet, each stock solution was pH corrected to 7.4 (figure 4.2 a). It was observed that DMSO played a minor role in the measured bilayer permeability.

In comparing the concentration time distributions, a clear difference was observed between 0 and 5 % DMSO (figure 4.2 b). The 5% distribution followed a clear and simple exponential fitting well to the model. 0,1, 3 and 5 vol % DMSO were characterised finding a negative correlation with the lipid out DOPC bilayer permeability. This trend appeared to follow a linear dependence (figure 4.2 c). Interestingly, there was no statistical significance between 0% DMSO and any of the DMSO experiments (figure 4.2 c). Of particular importance was the lack of measured difference between 0 and 1 % DMSO, as 1% DMSO is typically used as a standard within *in vitro* experiments. This trend suggested that DMSO minimally increases the packing density of DOPC bilayers between 0 – 5% DMSO, agreeing with literature.¹⁹⁹

DPhPC forms lamellar fluid bilayers at room temperature however its alterations to permeability are attributed to tail methylations. These methylations increase the hydrophobicity and reduce diffusion within the bilayers tail region.²⁰³ DPhPC bilayers are of a similar thickness to DOPC bilayers despite a larger area per lipid. The potentiality of lipid specific DMSO interactions were investigated through assessment of DMSO with DPhPC. A clear and obvious separation was observed in the concentration time distributions for 0 % DMSO lipid out DOPC and DPhPC. As expected, bilayers comprised of DPhPC as opposed to DOPC were less permeable to theophylline. The DPhPC curve equilibrated in around 5000 s as compared to 3000 s for DOPC (figure 4.2 b). This difference carried into the permeability value, which showed a highly statistically significant separation (figure 4.2 c) and was lower for DPhPC compared to DOPC. This factor is further discussed in section

4.4.2 of this chapter. No statistical significance was observed between the measured permeability of DPhPC bilayers formed with 0 and 1 % DMSO in the aqueous phase, mirroring the same experiment with DOPC. A similar absolute difference in the mean permeability was recorded between 0 and 1 % DMSO conditions between DOPC and DPhPC lipids. Statistical significance also carried between 1 % DMSO DOPC compared with 1 % DMSO DPhPC experiments. In tandem, these results suggest that slight permeability reduction at higher DMSO % may carry for DPhPC bilayers.

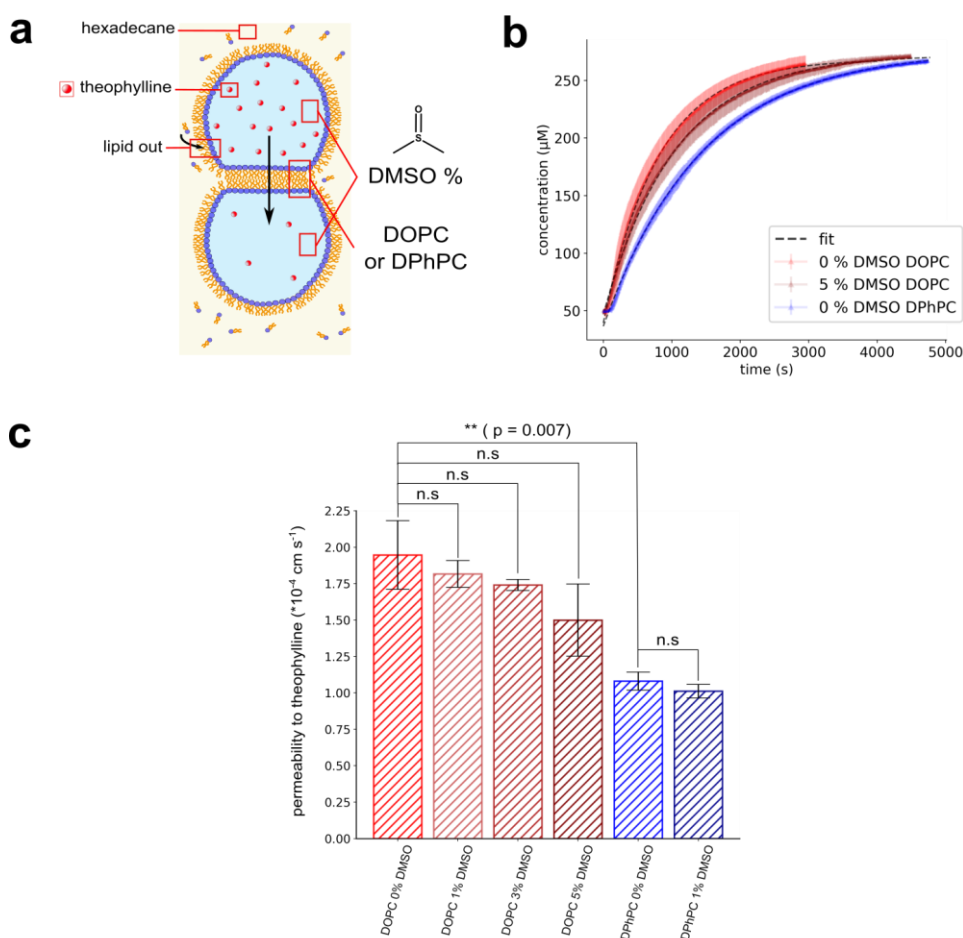


Figure 4.2: Adjusting DMSO content in the aqueous phase. **a** Cartoon showcasing adjusted parameters - DMSO content and lipid (DOPC or DPhPC). Experiments were all performed with theophylline droplets in hexadecane using the lipid out method. **b** Concentration time distributions. For clarity, select distributions with significant separation have been included ($n = 3$, error bars represent the standard deviation) **c** Permeability values determined with adjusted parameters (all measurements $n = 3$, fitted to equilibrium distributions, error bars represent the standard deviation, statistical testing carried out with two tailed t test, $** = p < 0.01$, n.s = non-significant ($p > 0.05$)).

These results require consideration with respect to measuring the DIB permeability from the bulk aqueous concentration. The UWL increases local viscosity at the bilayer reducing permeability through adjustments to the hydrogen bond network.^{146,204,205} The role of DMSO on the UWL remains poorly understood. The bulk aqueous diffusion coefficient can be

increased by the presence of DMSO.¹⁹⁹ This seems unlikely to have substantial impact in the case of theophylline due to its low MW (180 Da), however delineating a general trend for permeants with larger MWs requires further study. The negative correlation observed between permeability and increasing DMSO% (figure 4.2) suggest a bilayer packing increase through dehydration, however the trend likely resulted from modulation of any of these biophysical aspects in combination. Perhaps the most significant finding of these experiments, is the lack of statistical significance between the derived permeability value and low aqueous DMSO%. This therefore suggests, that low DMSO% minimally affects the intrinsic rate of DIB translocation.

4.3.2. Bulk oil phase

The role of the bulk oil phase in DIB biophysics is an important consideration and one that has been readdressed as DIB technology matures. DIBs have successfully been formed in oil reservoirs containing, decane,¹³¹ hexadecane,^{124,141,146,140} squalene,¹⁸² silicone oil,¹¹⁹ mineral oil,¹¹⁹ and glycerol trioctanoate.⁶⁵ In most instances the oil is pure, however mixtures have also been used.¹³⁰ Perhaps most pertinent to a DIB permeation experiment is the notion that residue oil deposits between bilayer leaflets can affect tail contact and thus the biomimetic nature of the bilayer interface.²⁰⁶ This hypothesis is in part based on observed relationships between oil viscosity, membrane thickness and membrane formation time.^{141,106,109} Hexadecane is the most popular oil used throughout the literature with controls showcasing negligible bilayer retention.¹⁰⁹

Faugeras et al. investigated the role of reservoir oil on the measured DIB permeability.⁶⁵ Lipid out DIBs formed in squalene were less permeable than those formed in trioctanoate. When formed in squalene, DOPE bilayers were more permeable than DPhPC ones however no permeability difference between the lipids was reported for the trioctanoate system. This is particularly interesting when noting the difference in aqueous phase behaviour between DOPE (H_{II}) and DPhPC (L_{α}) at room temperature. It has been suggested that a greater concentration of squalene is retained in the bilayer compared to hexadecane and that the lateral diffusion of these deposits can also differ.²⁰⁷ These results point towards a measurable difference in the measured permeability as a function of oil content where lipid specific oil interactions may be significant. In light of this, the permeability of DIBs formed in reservoirs of hexadecane or squalene were interrogated.

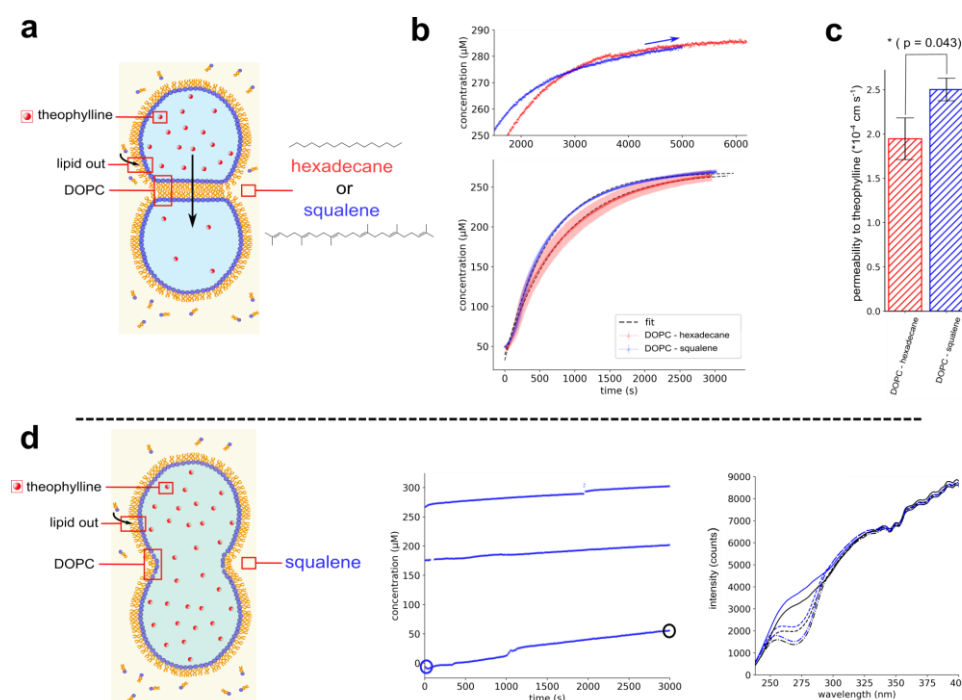


Figure 4.3: Adjusting the bulk oil phase to squalene **a** Cartoon indicating experimental parameters. Experiments were all performed with theophylline droplets in hexadecane or squalene using the lipid out method. **b** Concentration time distributions for squalene and hexadecane experiments ($n=3$, error bars represent the standard deviation). Zoom in shows $n=1$ distribution for each experiment monitored for an extended duration – a significant upward trend for the squalene distribution was noted post apparent equilibrium. **c** DOPC permeability determined as a function of surrounding oil phase (all measurements $n=3$, fitted to equilibrium distributions, error bars represent the standard deviation, statistical testing carried out with two tailed t test, $* = p < 0.05$). **d** Overview of squalene oil partition experiments. Distributions with 3 separate starting concentrations of theophylline (0 (pure PBS), 162 and 275 μM , $n=1$). As with b, an upward trend was observed in each distribution. Spectra at 0 and 3000 s for each distribution.

The results in figure 4.3 a – c indicated that the permeability of DOPC, lipid out DIB bilayers to theophylline was faster when squalene was used as the reservoir oil phase compared to hexadecane (two tailed t test $p = 0.043$). There was also minimal overlap in the standard deviation in the permeation distributions of these two oils. Both distributions followed exponential increases (figure 4.3 b). This result broadly replicated the findings of Faugeras et al.; the reservoir oil can affect the measured permeability value.

One issue encountered with squalene experiments was a significant upward drift toward the end of the permeability distribution (zoom in figure 4.3 b). Theophylline did not partition into hexadecane (chapter 3 section 3.5.4), and the resultant permeability distribution in hexadecane thus represented an exponential increase to equilibrium. This drift was also observed in squalene partition experiments (figure 4.3 d), indicative of an apparent increase in the concentration in the aqueous phase, the opposite of permeant partition. There was also an absolute difference in the standardised intensity in droplets of the same

concentration of theophylline formed in squalene as compared to hexadecane, for which a separate calibration was conducted ($R^2 = 0.99$). Upon close inspection of the spectra, there was a clear transmittance decrease between 245 – 325 nm. This indicated that this phenomenon was likely attributable to a chemical process rather than squalene affecting light scattering or droplet morphology.

As the oil phase was replaced between measurements, this drift could not be attributed to theophylline entering the aqueous phase if it had partitioned into squalene in prior experiments. Interestingly, the distributions in figure 4.3 appeared to share similar rates of increase for both PBS droplets and those with varied theophylline concentration. This indicated a process far from equilibrium and one that is not dependent upon starting concentration. Therefore, it was thought that this phenomenon may be accounted for by solvent exchange at the w/o interface, potentially assisted by DOPC.^{80,79} This hypothesis may explain the reported differences in lipid permeability between trioctanoate and squalene systems.⁶⁵ Alternatively, squalene can photo-oxidise under UV-A irradiation (320 – 400 nm) to form monohydroperoxides.²⁰⁸ Such a UV induced chemical reaction of squalene, may thus have affected the bulk absorption properties, as the monohydroperoxides may have partitioned into the aqueous media.

If the results in figure 4.3 c are to be taken as an absolute representation of the bilayer permeability, then this does not agree with the notion that oil viscosity correlates with the measured bilayer permeability.¹⁰⁶ Therefore, whilst a measurable difference in the DIB permeability between oils was apparent, understanding the reliability and thus the significance of this comparison requires further experimentation. In particular, whether the measured difference was a result of the partition behaviour of squalene at the w/o interface or due to UV irradiation in the measurement. In light of this, hexadecane was employed exclusively as the reservoir oil phase throughout this work.

4.3.3. Lipid location and DIB formation mechanism

A central advantage of the lipid in method, is that asymmetric bilayers can be readily achieved. This was exploited in chapter 7. Asymmetric bilayers have been formed using lipid out, however this typically requires a microfluidic approach, and the assumption of minimal monolayer – oil exchange.¹²³ It is known that monolayer formation time varies with lipid location due to the different mechanism of interface stabilisation.^{209,156} To the best of my knowledge, there exists no experiment within the literature for how these different formation mechanisms may affect the integrity of the resultant bilayer in DIBs. In understanding how

each experimental aspect affected the measured DIB permeability, it thus became necessary to understand this factor.

DIBs were formed with the same experimental conditions, varying the location of the lipid (figure 4.4 a). When assessing droplets containing vesicles with the same concentration of theophylline, lower absolute standardised intensity values were observed. This is evidenced through comparison of figure 3.8 and figure 4.4 b, this observation was due to the turbidity of small unilamellar vesicle (SUV) solutions and the size of vesicles used (100 nm). A separate concentration – standardised intensity calibration curve was established for lipid in droplets. As with the lipid out calibration, a linear region ($R^2 = 0.99$) was observed between 50 and 500 μM .

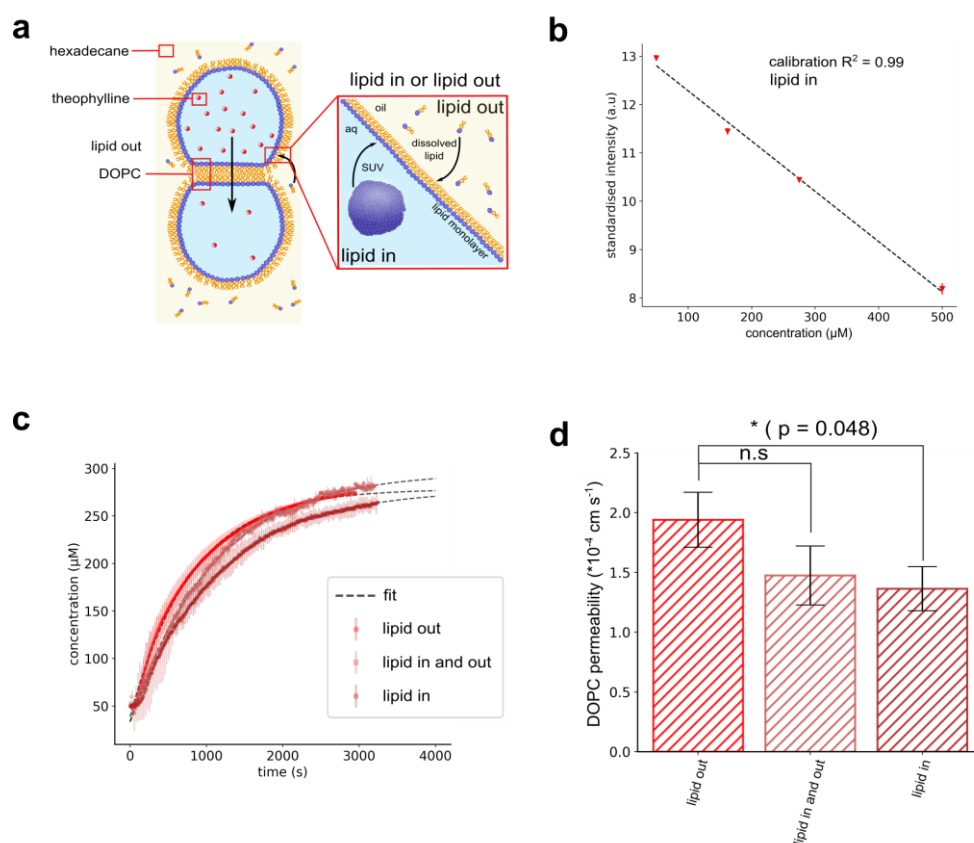


Figure 4.4: Adjusting the monolayer formation mechanism **a** Cartoon indicating experimental parameters. Experiments were all performed with theophylline droplets across DOPC bilayers in hexadecane using the lipid in, lipid in and out or lipid out methods. **b** Additional calibration for lipid in experiments. **c** Concentration time distributions for each formation method ($n=3$, error bars represent the standard deviation). **d** Permeability derived from the distributions ($n=3$, error bars represent the standard deviation, statistical testing carried out with two tailed t test, $* = p < 0.05$)).

A difference in permeability was found between lipid in and lipid out formation methods, evidenced in figure 4.4 c and d. Due to the reduced absolute difference in standardised intensity between acceptor and donor droplets for lipid in measurements, there was considerably higher noise present within the measured concentration time distribution. The

extracted permeability values showcased a statistically significant difference between the permeability of lipid in and lipid out bilayers.

Lipid in and out, where lipidic oil and SUV solution were used to form DIBs, was also tested. Interestingly, the permeability was measured between that of lipid in and lipid out systems, closer to lipid out (figure 4.4 d). There was no statistical significance between lipid in and out as compared to either lipid in or lipid out. Close inspection of the concentration time distribution (figure 4.4 c) revealed a noisy distribution afforded by the SUV solution, where the initial rate appeared comparable to lipid in before then reaching equilibrium faster.

These results suggest that the presence of vesicles in the case of 'lipid in' can reduce the measured permeability across a DIB. The most likely explanation for these observations is the introduction of an additional permeant-vesicle interaction. A lipid in system introduces a secondary concentration gradient in dynamic equilibrium between the aqueous bulk and the vesicle interior, an additional kinetic step in equilibration of the DIB concentration gradient. The difference afforded by lipid in and lipid in and out, suggests packing differences between monolayers formed by either method. It is known that convection can affect the packing properties of lipid out monolayers and so due to droplet manipulation within the chip, additional consideration is required for comparison to static DSA measurements.²⁰⁹ In light of these results, lipid out was used throughout this work unless forming asymmetric membranes.

4.3.4. Forming bilayers of higher biomimicry and complexity with lipid in

Building biomimetic DIBs is key to achieving a physiologically relevant *in vitro* measure of membrane permeability. The PM exhibits leaflet asymmetry in lipid composition and phase, a significant challenge to reconstitute into DIBs.³ Lorent et al., uncovered the molar quantity of lipid species in each leaflet of the PM of human RBCs using mass spec (figure 1.1).¹ SM and PC are the major lipids in the outer leaflet and in the presence of cholesterol, the leaflet can phase separate.²¹⁰ To understand how lipid compositional complexity affected the measured permeability, DIBs were formed with increasing amounts of SM relative to DOPC (figure 4.5 a). To circumvent lipid specific monolayer formation rates in the case of lipid out,¹¹³ it was thought that pre-mixing the lipids with lipid in vesicles would ensure a representative monolayer lipid composition.

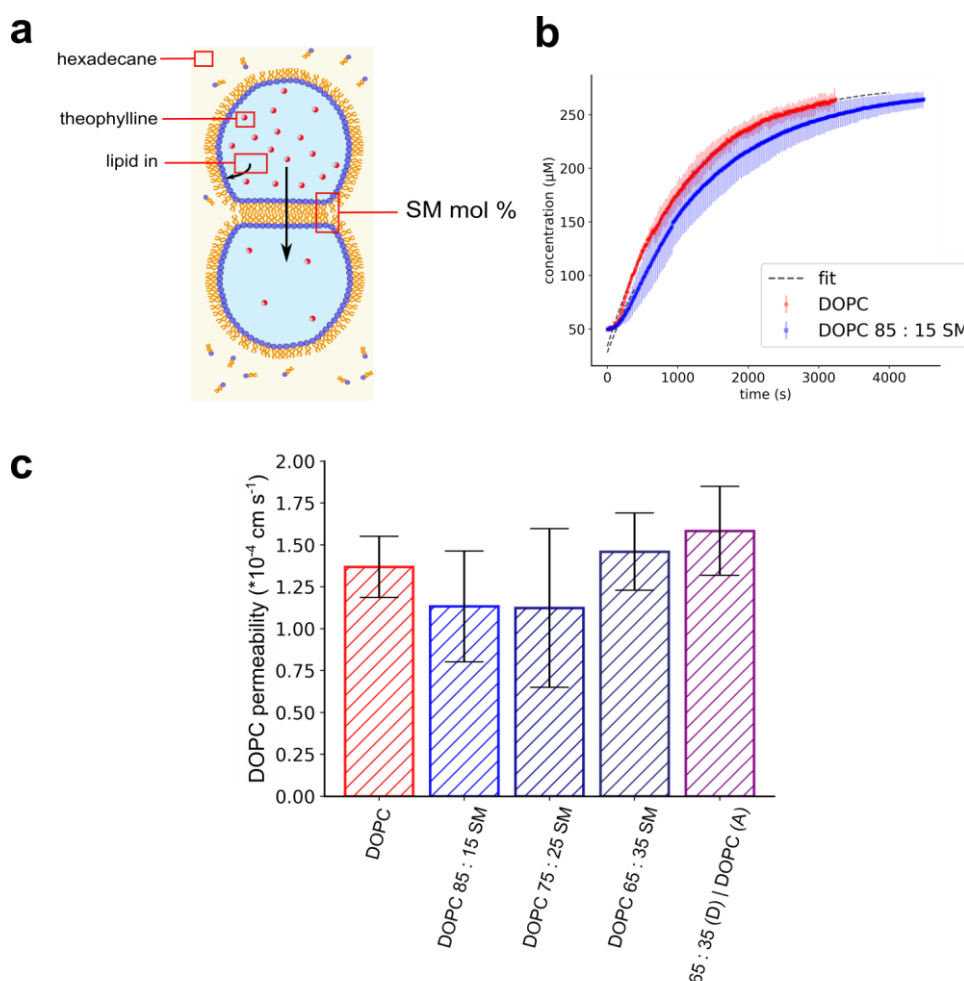


Figure 4.5: Adjusting SM content in DIBs using the lipid in approach **a** Cartoon indicating experimental parameters. Experiments were all performed with theophylline lipid in droplets in hexadecane with DOPC bilayers of varied mol % egg-SM. **b** Example concentration time distributions (n=3, error bars represent the standard deviation). Overlap was observed in the standard deviations. **c** Permeability measured as a function of system SM content (n=3, error bars represent the standard deviation, no significance observed between any pairing).

All samples were prepared with extrusion (100 nm), and at least 5 freeze thaw cycles. There was no statistical significance between the control (pure DOPC) and any of the SM containing conditions ($p > 0.05$). An apparent nonmonotonic relationship was observed with addition of SM content to the bilayer (figure 4.5 c). The permeability decreased from 0 to 25 % SM and then increased from 25 to 35 % SM. With increasing SM content, there was a marked reduction in DIB formation rate. Forming an asymmetric DIB (net total of 17.5% SM content) resulted in the fastest measured permeability.

A possible explanation for these results, is that SM failed to homogenously incorporate into fluid phase DOPC vesicles. Recent work has suggested that freeze thaw processes maintain the membrane composition of distinct vesicle populations, but can induce encapsulated

content exchange.¹⁵⁷ Repeating the above measurements with vesicles formed through electroformation may attenuate population inhomogeneity.²² Here, whilst some SM may have mixed with DOPC, as observed in the partial permeability reduction, it seems likely that instead two vesicle populations were present in the samples. Heat has been identified as a key experimental component for rendering the gel phase in DIBs.^{141,143} Non fluid vesicles typically require heating above the phase transition temperature to deposit at the w/o interface.²¹¹ Here, it seems likely that the DIB composition was formed from vesicles mostly comprised of DOPC whilst those with high SM content did not deposit at the interface. Biomimetic compositions are readdressed in chapter 8, with a novel system for heating DIBs above phase transition temperatures. As such, DOPC was primarily used throughout this work as ~50 % of the PM phospholipid content is PC based.

4.3.5. Zip up time – all conditions

The DIB zip up, is noted as a delay between droplet contact and bilayer formation. Zip up, therefore presents as an inflection point in the acceptor droplet concentration time distribution as the bilayer area grows, the flux increases until the equilibrium bilayer area is reached. The DIB zip up time was measured for each of the experiments in this section (figure 4.6 a) through assessment of each concentration time distribution, as in figure 4.6 b.

The DIB zip up time varied from ~65 to ~172 s, however there was considerable variance on most of the measurements (figure 4.6 c). A measurable negative correlation in zip up time was observed between 1 and 5% DMSO. Interestingly, there appeared to be minimal difference in the zip up time between hexadecane and squalene experiments despite the implication in literature that squalene retains more between leaflets.²⁰⁶ The trend in zip up time between lipid in, lipid in and out and lipid out follows a negative correlation with the zip up time further supporting the hypothesis of differences in monolayer packing. In this work, the variation in zip up time as a function of experimental factors, was significantly lower than the variation in zip up time as a function of permeant structure as measured by the Choi group (figure 1.9).⁶⁴

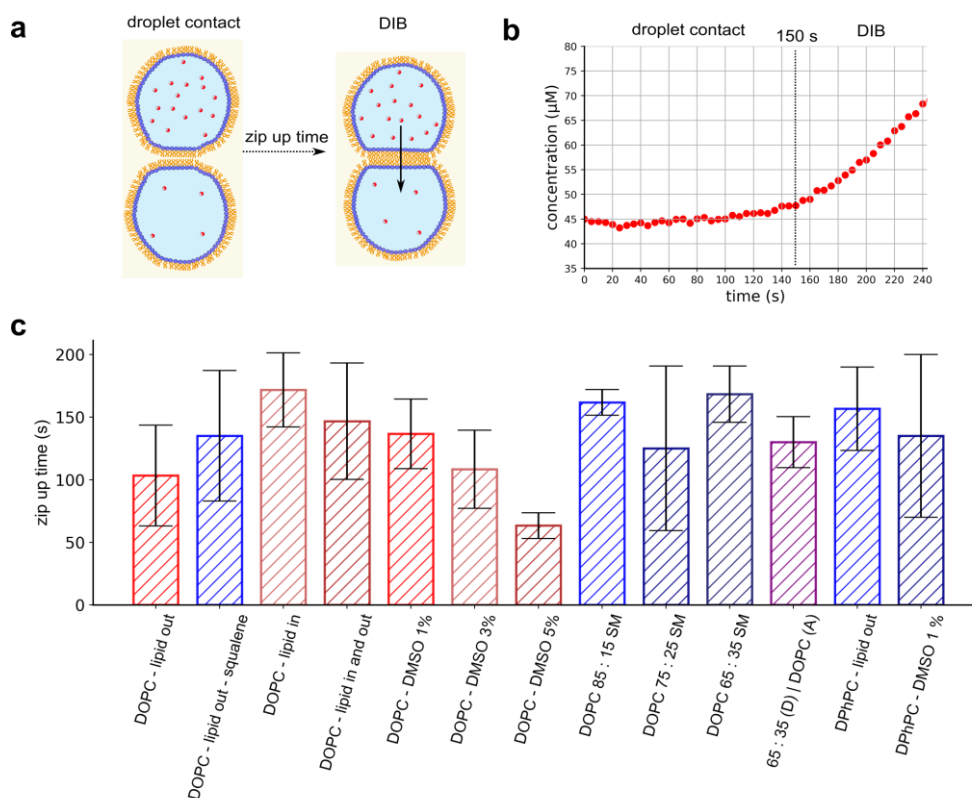


Figure 4.6: DIB zip up times **a** Cartoon showcasing mechanism of droplet zip up to form DIB **b** Example of zip up time determination. **c** Measured zip up time for each experiment in this section. Error bars represent the standard deviation.

4.3.6. Section conclusion

Of the measured trends within this section, statistically significant differences were only found between the permeation value for hexadecane vs squalene, for lipid out vs lipid in, and for DOPC vs DPhPC. The absolute % differences were 25, 35 and 57 % respectively. The first of these observations contributed to an ongoing discussion within the literature, the second was novel and the latter agreed strongly with literature findings in traditional fluorescent systems. Unsurprisingly the largest permeability difference was recorded between different lipids, however the difference between formation was surprisingly greater than the difference between oils. This rank ordering of biophysical factors is pertinent in light of the interpretation of the oil comparison, the lowest recorded significant difference.

The experiments within this section are non-exhaustive and there are considerable further experimental considerations to achieving higher biomimetic systems including buffer composition, pH, rheology and temperature. These aspects are discussed further in chapter 8. The experiments in this section targeted parameters which may affect drug molecules more immediately through compound partition to oil, interaction with vesicles in solution and

co-solvation. The impact of pH is highly permeant structure dependent and because of this, focus was placed on systems at a biological pH range (pH 7.4).

Nonetheless, a novel biophysical parameter space has been broached by this work. It is clear how pairing up some of these factors and studying the permeability of the resultant bilayers could further advance understanding of each factors mechanism in affecting *in vitro* bilayer permeability. For example, lipid formation mechanism combined with different oils; does changing lipid formation mechanism in a squalene-based system yield similar percentage differences between the comparative experiment in a hexadecane one (~35 %)? The conclusions yielded from the experiments in this section dictated the experimental conditions for investigating wide molecular space. Where possible, permeation measurements were conducted with lipid out DOPC hexadecane in PBS buffered droplets, pH 7.4 with 0 % DMSO. DMSO was not required to solubilise most compounds within this work and where required, its use has been highlighted.

4.4. Systematic permeant modifications – establishing permeability – structure relationships with commercial compounds

Considering the need to understand *in vivo* translocation, the growing cost of drug development and the vastness of chemical space, an improved understanding of how permeant structure affects bilayer transport is of utmost importance. Whilst it was evident that aspects of the bilayer and the DIB environ can affect the measured permeability – these aspects were expected to be minor when compared to the diversity of translocation behaviour presented in wide permeant chemical space.

4.4.1. The xanthine series

The xanthine series was introduced in chapter 3 through theophylline and caffeine. Both of these compounds are part of a larger, commercially available set (figure 4.7).

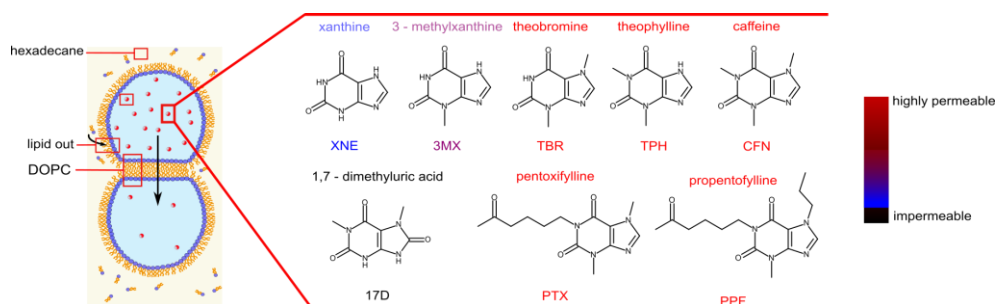


Figure 4.7: Summary of studied methyl xanthine derivative compounds including commercial and shorthand names used across section. Colours indicate relative compound permeability measured in this work.

The methylxanthines present a range of compounds with systematic functional group changes. Systematic methylations to the purine ring system exist between xanthine and caffeine. 1,7 dimethyluric acid is dimethylated derivative which contains an additional carbonyl group. Compound flexibility changes drastically from the trimethylated caffeine (0 RBN) to pentoxifylline (5 RBN), where a methyl group is replaced with a 2-hexone group. From pentoxifylline to propentofylline, an additional methyl is replaced with a propane group. Between xanthine and propentofylline, the MW and AlogP scale from 152 to 306 Da and - 0.71 to 1.65 respectively. As this series demonstrates a range of physicochemical properties within a narrow chemical structure range, the permeability of each compound was assessed in DIBs. The methylxanthine series thus acted as a model for a drug design pipeline, where, in a biological setting, small chemical structure changes can incur large changes in measurement.

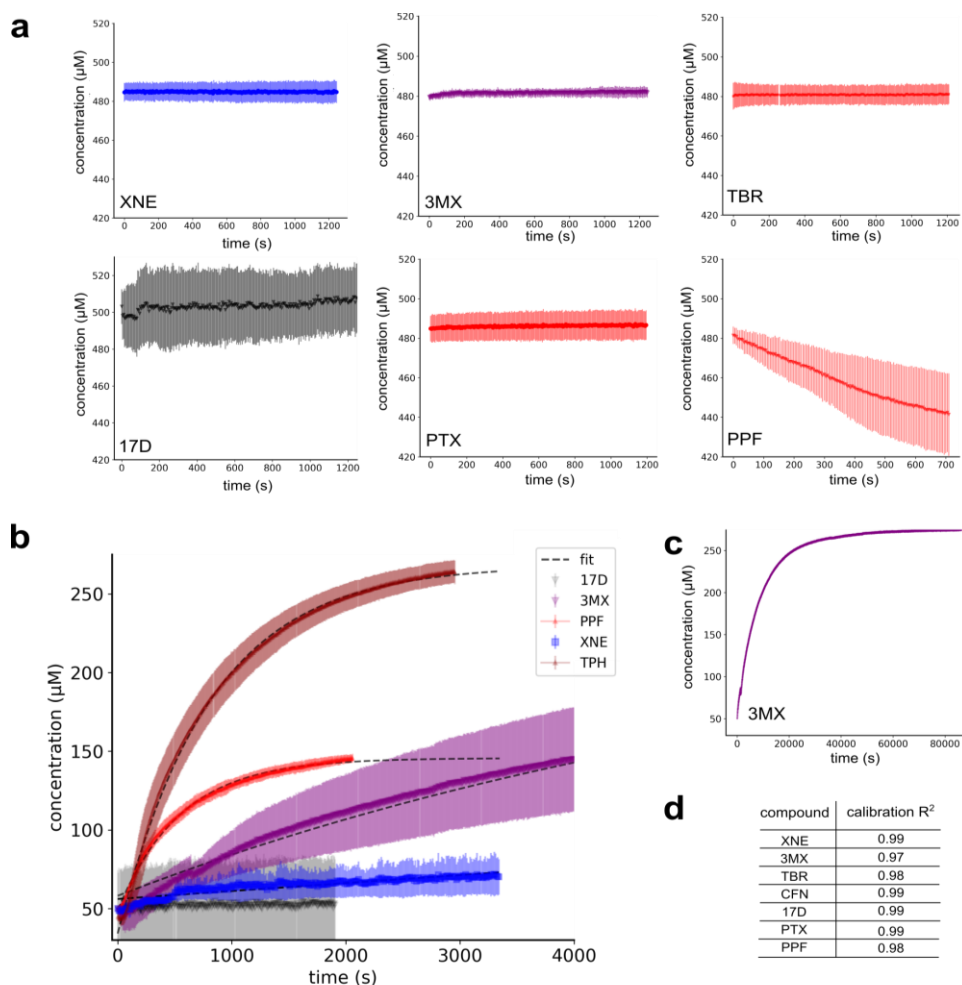


Figure 4.8: Measured distributions of xanthine series. **a** Oil partition plots for each xanthine compound. PPF showed significant partition. **b** Concentration time distributions for each permeant. 17D was deemed impermeable. PPF deviated from expected equilibrium as indicated by partition behaviour. TPH included for reference. PTX, TBR and CFN concentration time distributions are included in next section. (n =3, error bars represent the standard deviation). **c** Extended concentration time distribution to equilibrium for 3MX (n=1). **d** Calibration R^2 values for each xanthine compound.

As described in chapter 3, compound characterisation began with calibration of lipid out droplets. All DIBs were formed with an initial 50 μM and 500 μM permeant concentration gradient. A linear relationship was observed between these concentrations for each permeant (figure 4.8 d).

Apart from PPF, no oil partition was recorded for any of the xanthine series as indicated by flat concentration time distributions in figure 4.8 a.²¹² PPF demonstrated considerable oil partition, as indicated by a steady exponential concentration decay. This shape indicated concentration dependence on the rate of partition, indicating Fickian motion.

Apart from 17D, all methylxanthines translocated DOPC bilayers, as indicated by exponential rises in the concentration time distributions (figure 4.8 b). Error bars were generally larger for slower moving compounds (XNE and 3MX). The observation of minimal oil partition for all compounds apart from PPF was further ratified through the concentration time curves arriving at the expected equilibrium value (275 μM) (figure 4.8 c, d and figure 4.10 a). In analysing the shape of the PPF distribution, an apparent equilibrium at 147 μM was reached within 2100 s. This result inspired work in chapter 6 on simultaneous oil partition and bilayer permeability.

Due to the slow translocation of XNE, the equilibrium could not be observed. 3MX was the next slowest moving compound and an attempt was made to observe the 3MX equilibrium point. A DIB was recorded overnight, it was found that the system rose in the expected exponential fashion to equilibrium (280 μM) in 57000 s (figure 4.8 c). This experiment showcased how stable measurement, recording on the timescale of days, is possible with the method. Due to DIB stability and system throughput, measuring equilibrium for slower moving compounds was not always possible and a linear fitting approach was used to determine the permeability with the assumption of expected equilibrium as indicated by partition plots.

The relationship between structure and bilayer permeability was best showcased through assessment of measured permeability values. Figure 4.9 shows that from XNE to CFN, ring methylation greatly affected the measured DOPC permeability. Interestingly, it was noted that the biologically active compounds (TBR – PPF) were all highly bilayer permeable. Between each methylation (XNE – CFN), a significant difference was recorded in the permeability distribution. From CFN to PTX, the permeability was reduced, potentially as a function of higher MW. From PTX to PPF the permeability increased, however it is important to note that the PPF permeability value was overestimated with this method. As an assessment could not be made of the concentration in both compartments, accurate permeability values were thus limited to compounds which exhibited minimal oil partition. The PPF permeability value has been included here for completeness and was reassessed in chapter 6.

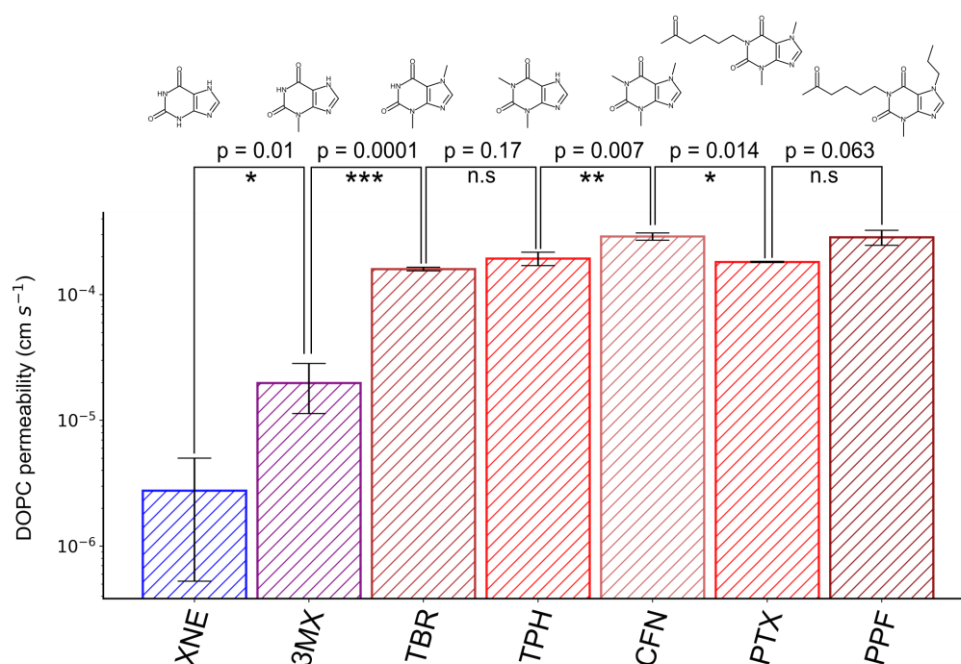


Figure 4.9: Measured permeability of xanthine series. The measured permeability is largely dependent upon permeant chemical structure, $n = 3 - 8$, error bars represent the standard deviation, statistical testing carried out with two tailed t test, *** = $p < 0.001$.

The measured CFN and TPH DOPC permeabilities showed strong agreement between a comparative supported bilayer system in Lee et al.⁶⁴ TPH and TBR are structural isomers of one another and showcased variation in their DOPC permeabilities, although this was not statistically significant. A clear focus exists within this set around the single to dimethylated compounds. Strikingly, between 3MX and TBR/TPH a single methylation afforded an order of magnitude change in the measured permeability however between TBR/TPH and 17D, a single carbonyl switched off the permeability entirely. All other dimethylated (and higher) compounds in the series were highly permeable. To the best of my knowledge, this set of results represents the first structure – permeability relationship uncovered with DIBs as compared to those traditionally derived with PAMPA.^{213,214}

The results within this section indicate a substantial advance toward DIBs for small molecule drug screening. Within a narrow chemical space, the measured DOPC permeability ranged across 3 orders of magnitude. An absolute percentage difference of 196 % was found between the fastest and slowest compound. These results indicated that, as compared to the findings in the previous section and within the experimental parameter space, permeant chemical structure had the largest effect on bilayer permeability.

4.4.2. DOPC vs DPhPC permeability reduction across the xanthine series

In section 4.3.1 it was found that the DPhPC permeability to theophylline was slower than DOPC. The role of permeant chemical structure on this variation was investigated through studying the highly permeable compounds from the methyl xanthine series. All experimental conditions were kept the same as in section 4.4.1 however DIBs were formed with DPhPC instead of DOPC.

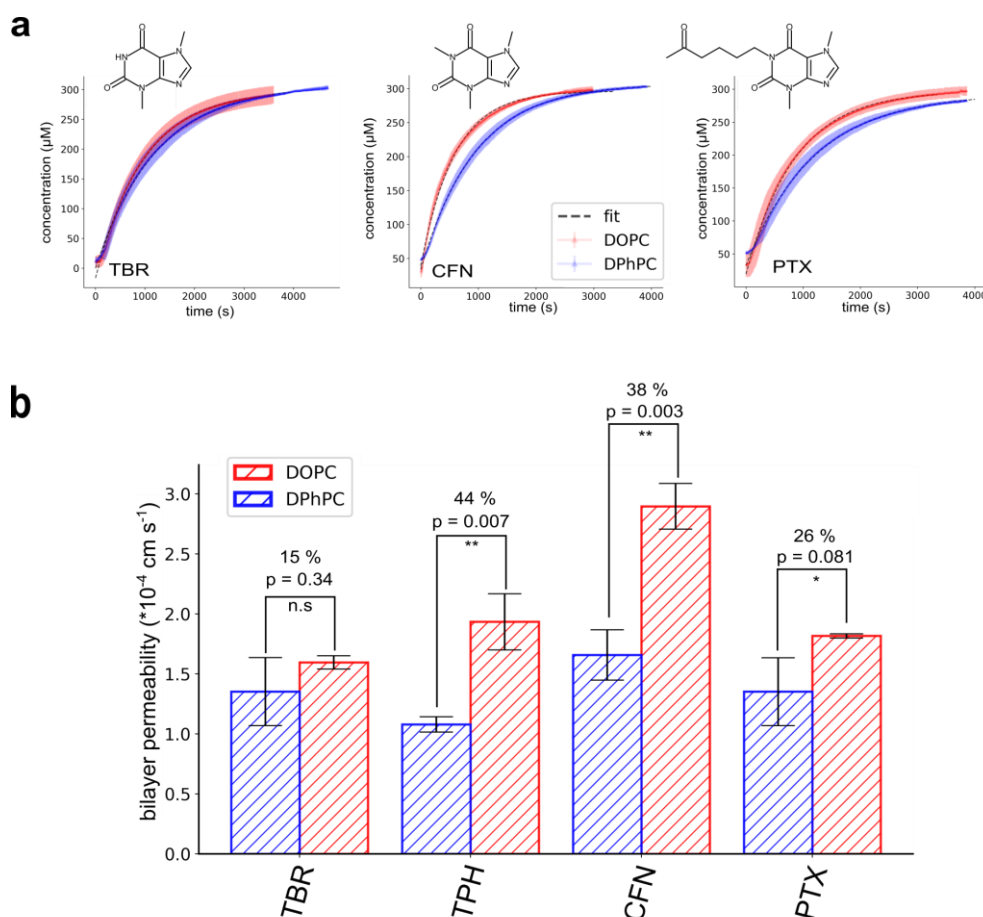


Figure 4.10: DOPC and DPhPC permeability compared across the xanthine series. **a** Concentration time distributions for each lipid and permeant. TPH included in figure 4.2 (n = 3, error bars represent the standard deviation). **b** Measured bilayer permeability values for permeable members of xanthine series, DPhPC bilayers were less permeable to all compounds (statistical testing carried out with two tailed t test, * = p < 0.05, n = 3, error bars represent the standard deviation, percentage values indicate % reduction from DOPC to DPhPC).

As was found for theophylline, DPhPC was less permeable than DOPC to theobromine, caffeine and pentoxifylline (figure 4.10) attributed to tail methylations in DPhPC. Within these compounds, the relative reduction in permeability changed (15 to 44 %) as a function of the chemical structure. Statistical significance was observed between lipids for caffeine and theophylline, which showed the greatest variations in permeability. Interestingly, the rank ordering of compounds changed between lipids too (table 4.1).

As most DIB platforms focus on a single fluorescent permeant, the role of lipids is often considered. It is well known that DPhPC bilayers are less permeable than DOPC ones, however the relative change between these can also be evidenced through comparing literature results. Table 4.1 showcases results from this work and a study within the literature, where DIBs have been used as the permeability model.

permeant	DOPC P (* 10 ⁻⁴ cm s ⁻¹)	DPhPC P (* 10 ⁻⁴ cm s ⁻¹)	% reduction	MW	reference
resorufin	1.98	0.81	59	213	¹⁴⁶
theobromine	1.60 (4)	1.36 (2)	15	180	this work
theophylline	1.94 (2)	1.08 (4)	44	180	this work
caffeine	2.90 (1)	1.66 (1)	38	194	this work
pentoxifylline	1.82 (3)	1.35 (3)	26	278	this work

Table 4.1 - DOPC and DPhPC permeability compared within DIBs. Comparison made between this work and literature finding. Bracketed numbers indicate rank ordering.

The reductions found in this work, were significantly lower as compared to the literature finding with resorufin. Whilst bilayer fluidity has a clear role to play in measured permeability,^{65,143} the role of electrostatic interactions at bilayers is often overlooked.²¹⁵ Specific lipid – permeant electrostatic interactions, may become pertinent in increasingly biomimetic bilayers of diverse lipid composition. Electrostatics may explain the results for TBR and TPH, which otherwise share identical physiochemical descriptors. These results showcased that structural isomerism could incur a change in the DOPC permeability, as well as the relative change in permeability between lipids (figure 4.1 b). The methylations are splayed in TBR as compared to TPH. This spreading out of hydrophobic groups may allow TBR to bypass the methylated tail core of DPhPC more readily. TBR may thus permeate DPhPC in a comparable mechanism to a bilayer like DOPC in which the area per lipid is lower and there is greater diffusional freedom within the tail region.

With respect to a multi parameter viewpoint, further comparisons can also be made within DIB permeability systems in which the oil varies. In Faugeras et al, lipid out DOPE and DPhPC DIBs formed in trioctanoate indicated a relative reduction of ~35% in the permeability of fluorescein.⁶⁵ In Faugeras et al, when the oil was switched to squalene the permeability lowered for both lipids with no statistical significance between them. This direction of change is different to the observations in section 4.3.2, where hexadecane DOPC DIBs were slower than squalene ones. In tandem, this suggests that oil can differentially affect the DIB permeability depending on lipid *and* permeant chemical structure.

Further exploration with lipid in vs lipid out may indicate the biophysical mechanism that leads to these observations. It is important to consider that oil experiments may also be affected by a measurement false positive as indicated in section 4.3.2.

4.4.3. Colchicine and chloramphenicol – oral drugs

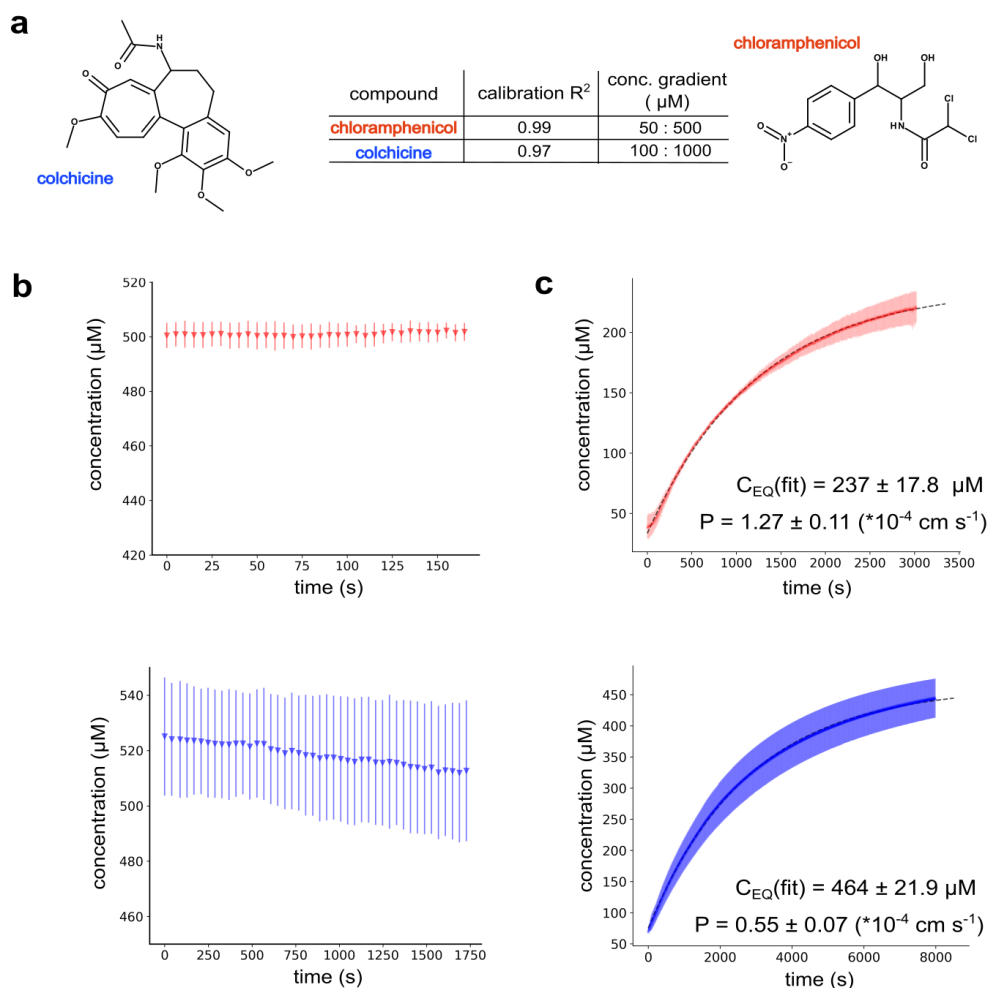


Figure 4.11: Measured distributions of commercial drugs. **a** Structure and calibration R² values for each drug compound. Note, that a 100 μM acceptor : 1000 μM donor initial concentration gradient (10*) was employed for colchicine due to 50 μM sitting outside of linear region. Colour coding independent of prior figures. **b** Oil partition plots for each drug compound. Colchicine partitioned slowly into oil. (n =3, error bars represent the standard deviation). **c** Concentration time distributions for each permeant. Both compounds deviated slightly from expected equilibrium as noted by fitted equilibrium values, permeability values included in inset. (n =3, error bars represent the standard deviation).

To further showcase the method, commercial orally available drugs were also tested.

Compounds were desired with divergent structural and physicochemical motifs as to explore permeability values in wider chemical space. Colchicine is a naturally occurring alkaloid anti-inflammatory, that impairs microtubule formation.²¹⁶ Colchicine was selected as its AlogP

value was similar to PPF (2.04 and 1.65 respectively). Chloramphenicol is a naturally occurring antibiotic that is bacteriostatic and bacteriocidal.²¹⁷ Chloramphenicol was selected as a model antibiotic and for its net neutral nitro group at pH 7.4.

Both colchicine and chloramphenicol were permeable within DOPC DIBs (figure 4.11). Colchicine was 57% less permeable than chloramphenicol. The translocation profiles for both compounds showcased deviation from the expected equilibrium value (yielded from the sum starting concentration). The relative reductions were 16 and 14 % respectively (figure 4.11 a and c). It is important to note that this C_{EQ} deviation was greater than the deviation from the measured and actual initial acceptor concentration.

A difference was observed in the oil partition behaviour of each compound (figure 4.11 a). Both PPF (figure 4.8) and colchicine partitioned into hexadecane at a measurable rate. Like with PPF, this observation accounts for the deviation in translocation equilibrium value for colchicine, where its partition rate was comparably slow to its permeation rate. For PPF, its partition dynamics were faster relative to its translocation dynamics, which thus yielded a greater deviation from the expected equilibrium (47 % reduction in C_{EQ} value). Despite the AlogP being similar, the difference in measured rates for these two compounds may be owed to their MW and TPSA, however measurable partition may be observed at an AlogP threshold >1.65.

Interestingly, chloramphenicol did not indicate any oil partition behaviour, and so the deviation in translocation C_{EQ} value was recognised as possible monolayer and bilayer retention of the compound. This is particularly pertinent considering the rapid translocation dynamics and the role of this phenomena for antibiotic compounds. The difference in retention behaviour between chloramphenicol and theophylline (figure 3.9) may be indicated by chloramphenicol's order of magnitude greater liposomal partition coefficient.⁸⁴ A comparative PAMPA (lipid mixture) and MDCK value for chloramphenicol is 0.088 and 0.031 ($\times 10^{-4}$ cm s⁻¹) respectively.^{214,195} Neither of these values otherwise reflect a 90% FA value for chloramphenicol.^{214,60}

4.4.4. Iodipamide and derivative – Radiolabelling molecules

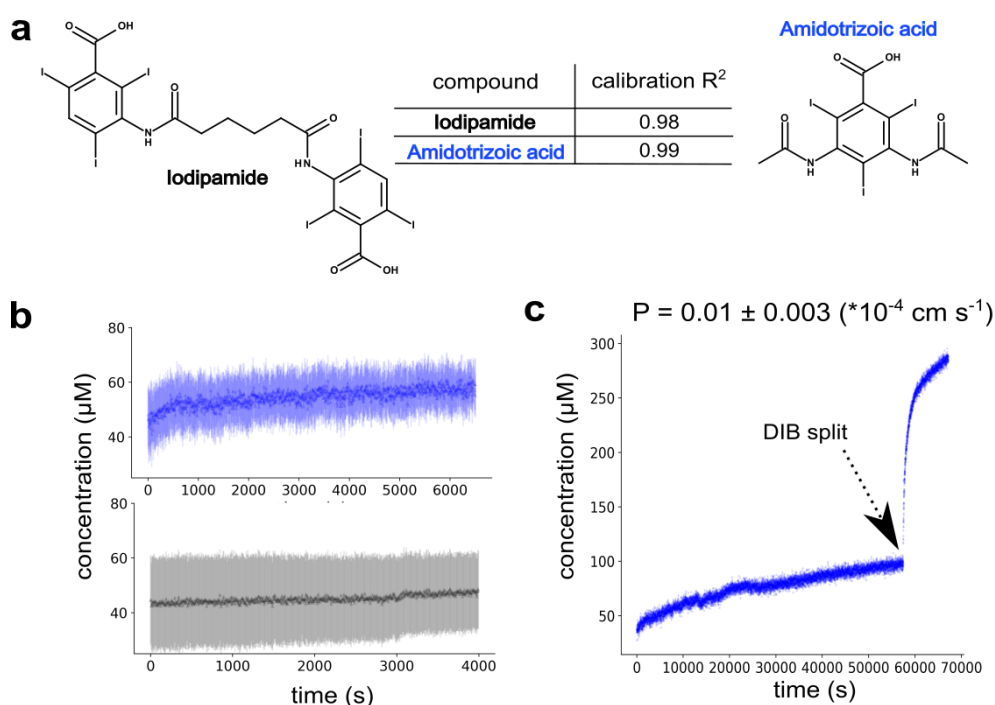


Figure 4.12: Measured distributions of radiolabelling compounds. **a** Structure and calibration R^2 values for each compound. **b** Concentration time distributions for each permeant. Iodipamide was deemed impermeable and amidotrizoic acid fractionally permeable ($n=3$, error bars represent the standard deviation). **c** Extended concentration time distribution for amidotrizoic acid ($n=1$). Permeability value derived from distributions in b.

Understanding the limits of bilayer permeability is highly important with respect to delineating *in vivo* translocation mechanisms. Two radiolabelling compounds were selected for assessment because of their unique structural features. Iodipamide and amidotrizoic acid are orally bioavailable contrast agents for MRI and XRay.²¹⁸ Both have high MWs (1139 and 613 Da) however iodine accounts for 64 and 62 % of their masses respectively. Despite this, both compounds are highly water soluble, which made them suitable candidates for probing the physicochemical limits of DOPC bilayer permeability.

Iodipamide was deemed impermeable as no statistical significance was found between gradients for calibration and translocation DIBs. Despite this, iodipamide has demonstrated appreciable cell permeability.²¹⁹ The AlogP of Iodipamide was calculated as 5.59. This high value indicates a compound that is highly hydrophobic and surprisingly iodipamide did not partition into the oil phase. Close inspection of amidotrizoic on the short and long timescale revealed a strikingly slow translocation profile (figure 4.12 b and c). All amidotrizoic acid permeability runs were classified as non-stationary via a Dickey - Fuller time series test.²²⁰ Slow diffusion post DIB rupture was observed for amidotrizoic acid owing to its high MW value (figure 4.12 c). These findings suggest that the plasma membrane transport of these compounds may thus be protein dominated.

4.5. GSK compounds – industrial compounds

4.5.1. Supplied compounds

The flexibility of the method was important with respect to industrial pharmaceutical, biotechnological or agrochemical applications. The screening range of PAMPA was translated into DIBs since both approaches rely on concentration measurement with UV spectroscopy.²⁰⁵ To further evidence the methods flexibility, a compound set was sent by GSK for assessment, summarised in figure 4.13.

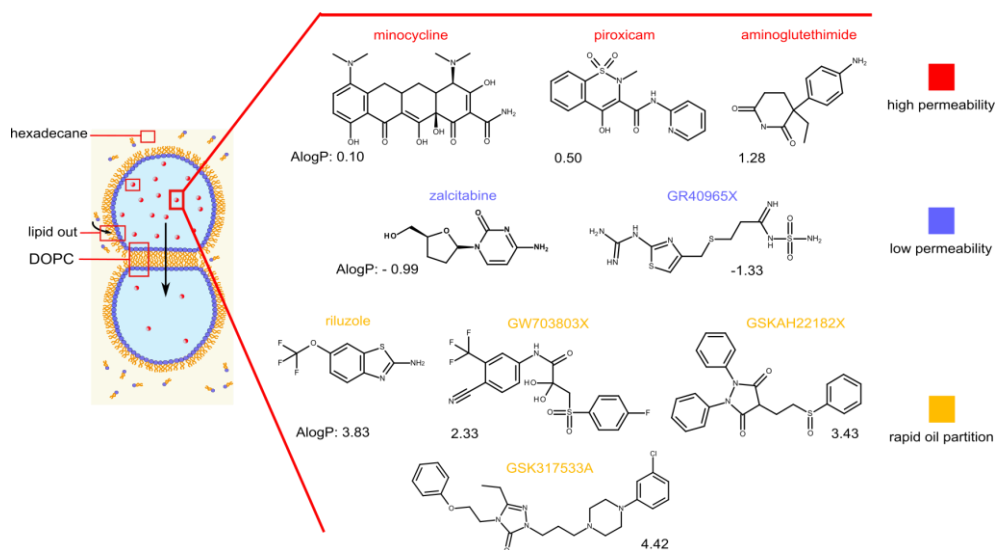


Figure 4.13: Summary of studied GSK compound set. Compounds organised by measured permeability and partition response. Values indicate compound lipophilicity, which broadly correlated with the measured response in this work.

4.5.2. Oil partition GSK compounds

Four of the nine supplied compounds appeared to partition rapidly into the surrounding hexadecane, which would have prevented an accurate measure of the DIB permeability (figure 4.14). These compounds each had AlogP values > 2.93, aminoglutethimide, which was deemed highly permeable, had the next highest AlogP (1.28). This suggests a permeant lipophilicity threshold between these two logP values at which, in this particular chip, permeability measurement may be prevented by oil partition. GSK703803X and GSK317533A did not appear to partition between phases, but instead solubilised into hexadecane within 1750 s. Riluzole demonstrated an apparent partition across the w/o interface, the decay slowed substantially at ~110 μM . This measurement was replicated in the absence of lipid, finding that the partition was unaffected by the presence of a monolayer at the w/o interface. Interestingly an initial assessment of the permeability of GSKAH22182X found identical rates of concentration decrease in the donor to that of the partition

(supplementary figure 2). This prevented confirmation of a bilayer mediated concentration increase in the acceptor droplet.

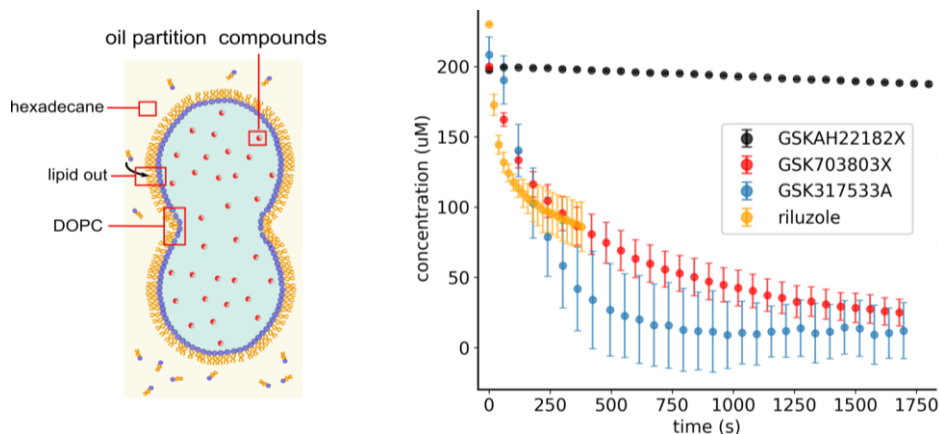


Figure 4.14: Compounds that demonstrated high partition. Concentration time distributions for each GSK compound, 1.8 μ L droplets in DOPC hexadecane oil. Due to rapid partition, distributions were normalised based on initial and end intensities. Riluzole demonstrated partition and a calibration was conducted with the initial intensity of a series of droplets ($R^2 = 0.99$). 1% DMSO was required to solvate GSKAH22182X, GSK703803X and GSK317533A.

4.5.3. Permeable GSK compounds

Piroxicam, aminoglutethimide and minocycline were deemed highly permeable across DOPC DIBs (figure 4.15 a and d). All compounds demonstrated a calibration R^2 value of 0.99 (figure 4.15 c). Significant leakage was measured for minocycline and piroxicam (inserts figure 4.15 a) however deviation from the expected C_{EQ} was only recorded for minocycline. This suggested that minocycline partitioned slightly from the aqueous phase, whilst piroxicam may have interacted strongly with the lipids. These three compounds were further studied in chapter 6. Zalcitabine and GSK4095X were minimally permeable across DOPC DIBs. Figure 4.15 b shows two approaches which confirmed the permeability of these compounds. As a measure of the equilibrium could not be established, the permeability was measured through linear fitting of the concentration time distribution. Neither zalcitabine or GSK40965X demonstrated measurable oil partition. These results are discussed further in the following section.

Single Droplet Label Free DIB Permeability Studies – DIB biophysical features, systematic chemical space and small molecule drugs

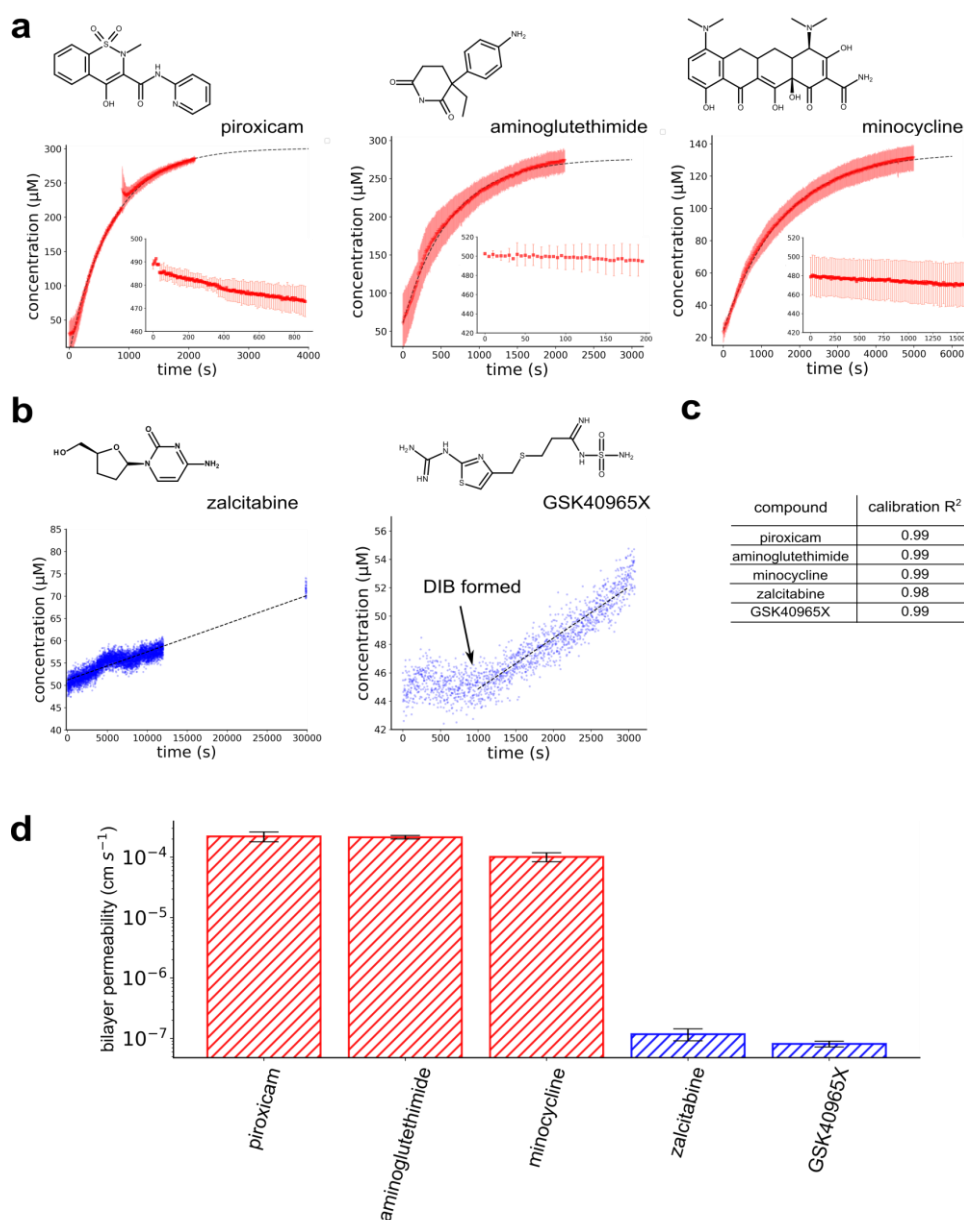


Figure 4.15: Permeable GSK compounds **a** Concentration time distributions for each of the compounds deemed highly permeable. Inserts show partition experiments with 1.8 μL droplets. Minocycline DIBs formed with a 10:1, 250:25 μM gradient. Partition measured for minocycline and piroxicam. Error bars represent standard deviation, $n \geq 3$. **b** Concentration time distributions for each of the compounds deemed slightly permeable. Two separate experiments were used to confirm bilayer translocation, on left a DIB was left overnight and recorded next morning, evidencing a clear linear change in the concentration. On the right, a droplet was monitored prior to forming a DIB. Upon DIB formation an inflection point in the concentration change was recorded. Each example $n=1$, permeability measured from 3 repeats each. **c** Calibration R^2 values for each compound between the acceptor and donor concentrations. **d** Measured permeability of each compound. A log scale has been applied to the y axis.

4.5.4. Section conclusion

Across this section, multiple commercially available small molecule drugs were studied in DOPC DIBs. To rationalise the impact of these results, comparison can be drawn with biological measures of compound availability and assay permeability.

permeant	oil partition	DOPC P (*10 ⁻⁴ cm s ⁻¹)	PAMPA P (*10 ⁻⁴ cm s ⁻¹)	t _{1/2} (hr)	<i>in vivo</i> mechanism	application
theophylline	no	1.94	0.002 ²²¹	8	passive ²²²	bronchodilator
caffeine	no	2.90	0.028 ²²³	5	passive ²²⁴	stimulant
chloramphenicol	no	1.27	0.039 ²²⁵	1.5	passive ²²⁶	antibiotic
colchicine	yes	0.55	0.008 ²²¹	27	n/a	antibiotic
piroxicam	yes	2.19	0.37 ²²⁵	30	OAT1 ²²⁷	anti-inflammatory
aminoglutethimide	no	2.13	0.182 ²²⁸	13	n/a	anticancer
minocycline	yes	1.01	0.600 ²²⁹	11	passive ²³⁰	antibiotic
zalcitabine	no	0.001	n/a	2	SLC ²³¹	antiretroviral

Table 4.2: DOPC permeability compared to biological findings. Rows coloured based on DOPC DIB permeability. PAMPA permeability recorded from measurements of hexadecane membranes, where possible. All measures of half-life from DrugBank. '*In vivo* mechanism' refers where evidence is available for passive transport processes, the statement of mechanism is non-exhaustive. Solute carrier proteins (SLC) – are a class of active transporters. n/a indicates lack of, or inconclusive evidence.

As compared to PAMPA, all DOPC DIB measures of the permeability were significantly faster. Up to a ~3 order of magnitude variance was recorded in this difference (theophylline and colchicine). It is worth considering the inhomogeneity of the referenced PAMPA membranes, in most instances the reported values are for hexadecane and decane oil slabs. Interestingly, for the compounds which demonstrated oil partition in the DIB system, this did not appear to translate into faster reported PAMPA values. The observation of oil partition was loosely correlated with increased biological half-life. These results highlight the intrinsic differences outlined in chapter 1 between the DIB and PAMPA interface. It is possible, that the simple, non-biomimetic nature of PAMPA, may have led to conflicting evidence as to the mechanistic role of passive, lipid diffusion at biological membranes.^{223,56,65} A significantly larger permeability dataset comparing between DIB and PAMPA could further uncover mechanistic considerations for the differences between these two methodologies.

The work in this section, which deployed the developments of chapter 3, showcased study in narrow (drug structural modifications) and wide (structurally diverse drugs) chemical space. This work broadly enables the outlook for a DIB based measurement which mimics the utility of PAMPA. Whether data determined from increasingly biomimetic membranes will improve

correlation with biological data remains unknown. The structure of small molecule drugs can be linked to membrane transporters, where protein specificity is coupled to molecular signatures.²³² Upon transporter gene knockout, there is often significant background permeability, for which a PAMPA based measurement is often used to investigate simple lipid diffusion. The advancements in this work suggest that a DIB based measurement could replace PAMPA and enable improved assessment with respect to decoupling drug permeation mechanisms *in vitro*.

4.6. Chapter conclusions

In this chapter, the developments of chapter 3 were employed to uncover subtle biophysical trends across bilayer composition, aqueous and oil phase composition as well as permeant chemical structure. The method was highly robust and successfully classified small molecule drug permeability and diffusive response in DIBs. The potential application of this method was demonstrated across three test datasets, the methylxanthine series, a range of commercial drug structures and a library of pharmaceutical compounds supplied by GSK. Compounds within these sets, were classified either impermeable or permeable, with the permeability measurement spanning three orders of magnitude. Bilayer permeability can be adjusted through many different factors, however quite clearly, permeant chemical structure affords a measurement range substantially larger than well understood biomimetic biophysical or compositional considerations.^{65,142} DPhPC bilayers were less permeable than DOPC ones, however the relative reduction was found to be permeant structure dependent. Owing to the massive diversity of biological lipids, it remains unclear to what end highly complex bilayer lipid compositions globally affect permeability across wide molecular space. Label free DIB methods have significant potential to unravel this important question through assessment of physiologically relevant compounds.

5 The Build and Design of a High Throughput Label Free Method – A custom spectroscopic platform

5.1. Chapter overview

Sections 5.4 and 5.5 of this chapter were conducted in collaboration with Dr. David Miller of Imperials Advanced Hackspace. Aspects of this chapter are in preparation for submission in early 2022.

5.2. Further development of label free concentration

assessment

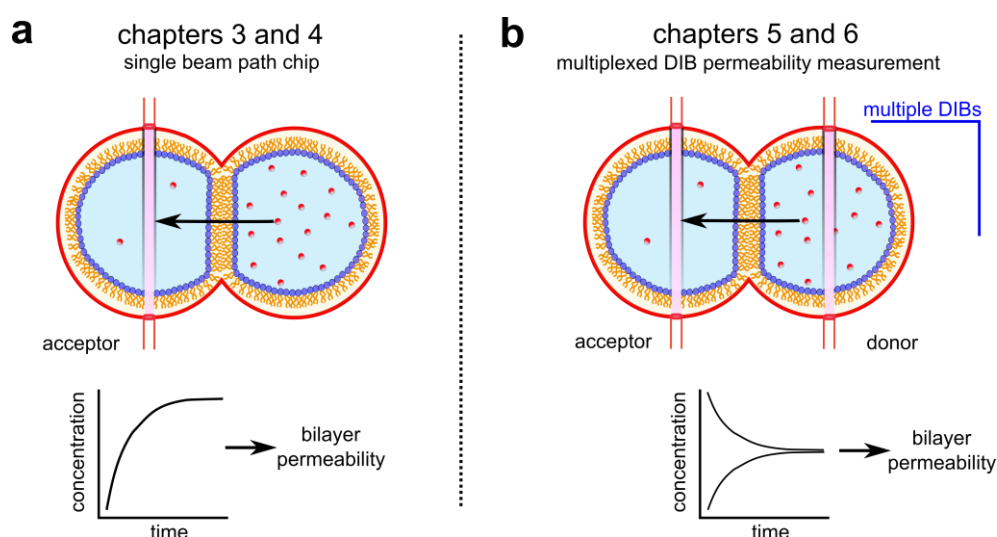


Figure 5.1: Overview of different measurement methods **a** A summary of the method introduced and utilised in chapters 3 and 4. **b** An overview of a multiplexed approach to label free concentration measurement in DIBs, developed in this chapter and utilised in chapter 6.

The single beam path chip, which was developed in chapter 3 and employed for a suite of experiments in chapter 4, had two key limitations during experimentation (figure 5.1 a). As a single droplet was recorded per experiment, the throughput of the measurement was inherently low and information, in the form of the reciprocal donor curve, was lost. In figure 5.1 b, a schematic is presented for how the measurement could be improved. An improvement was imagined such that a label free system would be outfitted with the same benefits as a traditional fluorescence microscopy DIB measurement.^{233,234,146} The ideal system would thus allow label free recording in both acceptor and donor droplets and would

be amenable to high throughput measurements. In most fluorescence microscopy platforms, these aspects are inherent to the experiment through; positionally resolved or broad droplet illumination and actuation of the microscope stage. Label free recording in both droplets has previously been performed with UV microspectroscopy in Nisisako et al..¹³⁷ This technique however is limited as it requires highly expensive and specialist equipment and can only record a single DIB.

Within this chapter approaches to engineer a high throughput label free system are described before reporting a bespoke spectroscopic platform which could rebuild the full DIB translocation time curve in a label free, high throughput manner.

5.3. Recording in both droplets - Dual beam path chip

This section details a system which recruited the same engineering principles as those that enabled the single beam path chip. This initial design presented a significantly low skill approach which utilised the same techniques as those in chapter 3. A chip was engineered with embedded immobilised fibers aligned across each DIB droplet. This design enabled droplet dependent recording. If multiple repeats of these chips were arranged in series, this would then achieve a high throughput system.

5.3.1. Commercial multiplexer

For this design, a commercial multiplexer (MPM – 2000) was purchased from OceanOptics (figure 5.2 a). A multiplexer controls the beam path from light source to spectrometer. The MPM-2000 has 2 inputs with 16 outputs split into 8 pairs. The multiplexer can control the beam path between each output. Fibers at one end can be attached at each multiplexer SMA port output with the other end arranged across a DIB droplet through embedded fibers (figure 5.2 b). The multiplexer's software allowed adjustment of output path and to set an output path loop.

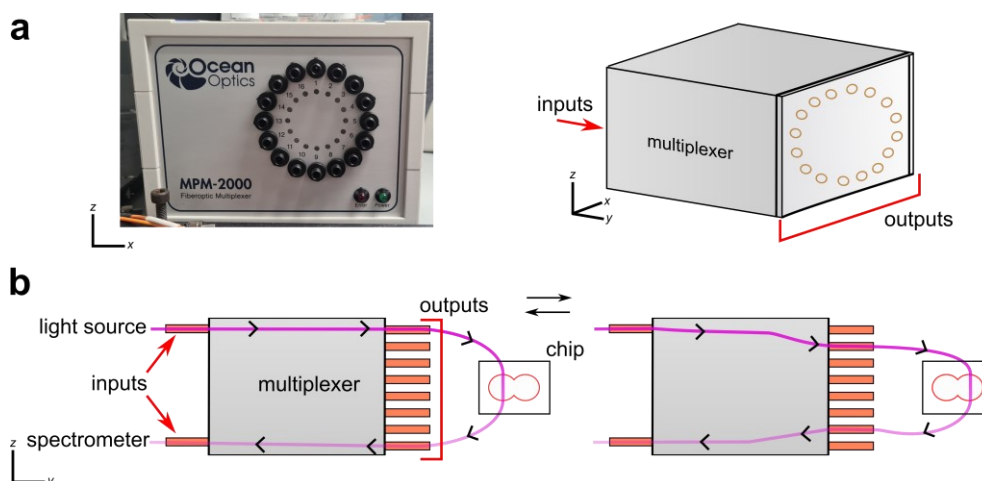


Figure 5.2: **a** Image and cartoon of purchased Ocean Optics UV fiber multiplexer MPM-2000. **b** Cartoon demonstrating control of beam path from light source to spectrometer. The device can shift back and forth between two output paths.

5.3.2. Dual beam path chip

An overview of a dual beam path chip system is presented in figure 5.3 a. Here, it can be seen how the multiplexer controlled the beam path which could pass across each droplet of the DIB.

A chip was engineered following the same laser cutting and heat-sealing procedure as in chapter 3. The single beam path chip utilised a cannula which attached to a fiber optic cable through a ceramic connection point. The attached cannula and fiber optic cable were too wide to arrange side by side whilst maintaining a straight embedded fiber. For the design outlined in the introduction of this section to be achieved, a solution was required where fibers could be arranged side by side without significant bending of the fiber. All other features (DIB well, droplet support area and alignment holes) apart from fiber implementation were kept the same (figure 5.3 c).

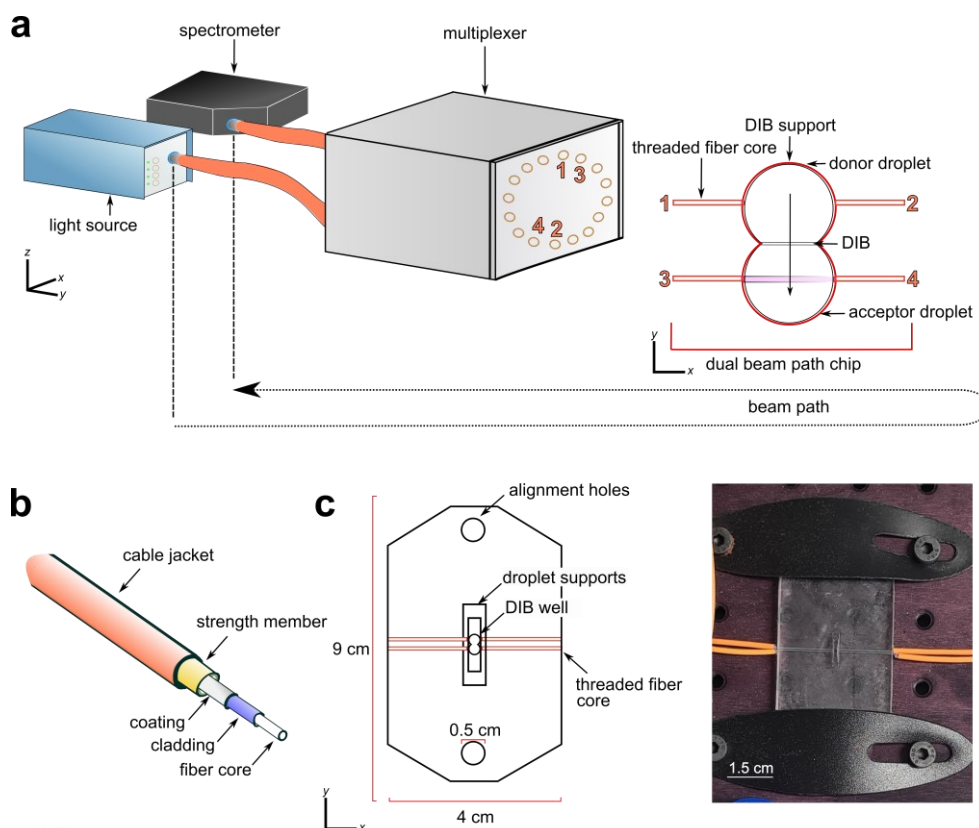


Figure 5.3: Dual beam path chip overview. **a** Cartoon of a dual beam path chip system. Orange numbers indicate where fiber optic cabling attached to multiplexer. **b** Overview of fiber optic cable anatomy. The fiber optic core had a diameter of 200 μm . **c** Cartoon schematic of dual beam path chip, features not drawn to scale. Photograph of actual fabricated dual beam path chip with threaded fiber optic cabling arranged side by side.

To achieve dual fiber arrangement, fiber optic cabling was stripped back to expose the fiber core (figure 5.3 b). Using a bespoke tool from ThorLabs (AFS900), fibers were stripped of their jacket, member, coating and cladding. The core was carefully cleaved using a diamond scribe (S90R) to present a clean cut, unfractured fiber end which was inspected with microscopy. Prior to heat pressing, a thin piece of bronze wire (280 μm Block, Germany) was arranged across etched 0.5 mm PMMA layers (this technique was also utilised in section 3.7). After heat pressing, this wire was withdrawn and a 280 μm channel was opened within the chip. Fiber cores were then easily threaded into this aperture and sealed at the chip interface with epoxy. This allowed fiber optic cables to align at the point at which fiber cores were threaded into the chip (figure 5.3 c) facilitating two aligned fibers arranged across each droplet support in the DIB well.

In testing the chip, the light transmittance was attenuated by 95 % (calculated from transmittance intensity at 280 nm) when passed from light source – multiplexer – chip path –

multiplexer – spectrometer (dual beam path chip) as compared to the same path passed from light source – chip path – spectrometer. To achieve a good signal to noise ratio in the measured spectra and resolve each wavelength, the integration time had to be increased from 40 ms (single beam path chip) to 1 s in the dual beam path chip. The multiplexer software did not sync with the recording from Oceanview. During data capture, two loops were running – the multiplexer cycling between outputs and spectra recording. To circumvent this, the mean spectra of 3 recordings were captured (3 s) and 4 sets (12 s) were captured at each path, with the spectra from the second of these sets recorded.

The system was tested with a caffeine translocation experiment (50:500 μ M acceptor: donor, respectively lipid in and out). The raw standardised intensity data (figure 5.4 a) showed that a complimentary exponential transmittance decay and increase were successfully captured with the system. Both curves reached an apparent equilibrium at 2500 s, however it can be seen how there was an absolute transmittance difference for each path at equilibrium (figure 5.4 a). In chapter 4, caffeine reached its expected concentration equilibrium and did not leak from the aqueous phase. The difference in equilibrium intensity was ascribed to an absolute transmittance difference between the two beam paths, possibly explained by threaded fiber alignment or imperfections in the fiber cleaving. In following path 1 and 2, two crossover regions can be seen between 400 - 900 s and 1600 – 2100 s. Here the returned spectra were rebuilt from the integration drifting across each beam path returning a spectral superposition between the two paths. Both regions crossed over at a comparative rate. This issue could therefore be attributed to, a communication fault between spectrometer and multiplexer or a potential mechanical fault in the temporal activation of the multiplexer. This problem was likely amplified by the required high integration time and lack of robust commercial software.

The distributions were processed to remove the crossover region and were min max normalised (figure 5.4 b). This processing followed the assumption of an equal concentration at equilibrium as was observed in the comparative system in the single beam path chip in chapter 4 section 4.4. It can be seen how, despite the issues presented by the multiplexer, an *in situ* dual droplet recording measurement was successfully demonstrated by the system.

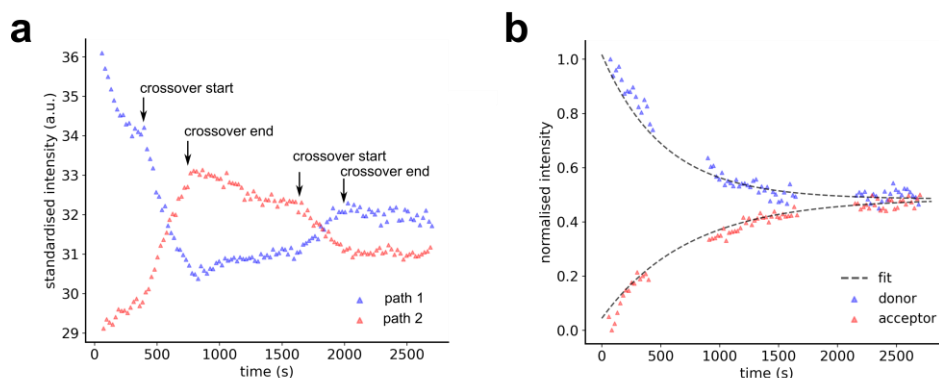


Figure 5.4: Caffeine translocation distributions collected with the dual beam path chip. **a** Raw standardised intensity time distribution recorded with dual beam path chip. Clear regions within the distribution were representative of spectral super positions. **b** A min max normalisation of each beam path with removed crossover regions. Each distribution was fitted to equation 19. This was repeated with two further experiments (total $n = 3$), each yielding a similar distribution with clear crossover regions. For clarity, a single repeat has been showcased, as the crossover region occurred at different times within the distribution, a mean intensity value could not be reported as a function of time.

Whilst this system was successful in part, significant processing was required for each experiment and the cross over region could only be ascertained as a function of the whole distribution. To achieve a suitable measurement, a large integration and capture time was required per droplet – leading to a low data recording interval (here - 12 s). This issue would scale with the number of multiplexer outputs in use, which would further limit experimental throughput and significantly complicate data processing. The commercial software did not successfully control the temporal activation of the multiplexer and spectrometer recording (figure 5.4 a). Due to these considerations, it was decided that a custom-made device and software would provide a bespoke solution to the central problem: a robust, label free, high throughput DIB permeability system.

5.4. Custom fabricated general fiber multiplexer

To improve upon the limitations of the multiplexer approach, an initial development focused on a custom designed multiplexer which would circumvent the signal attenuation of the commercial one (figure 5.5). Furthermore, by designing a bespoke fiber multiplexer, the mechanism of light path control would be better understood, and the platform could be made fully open source.

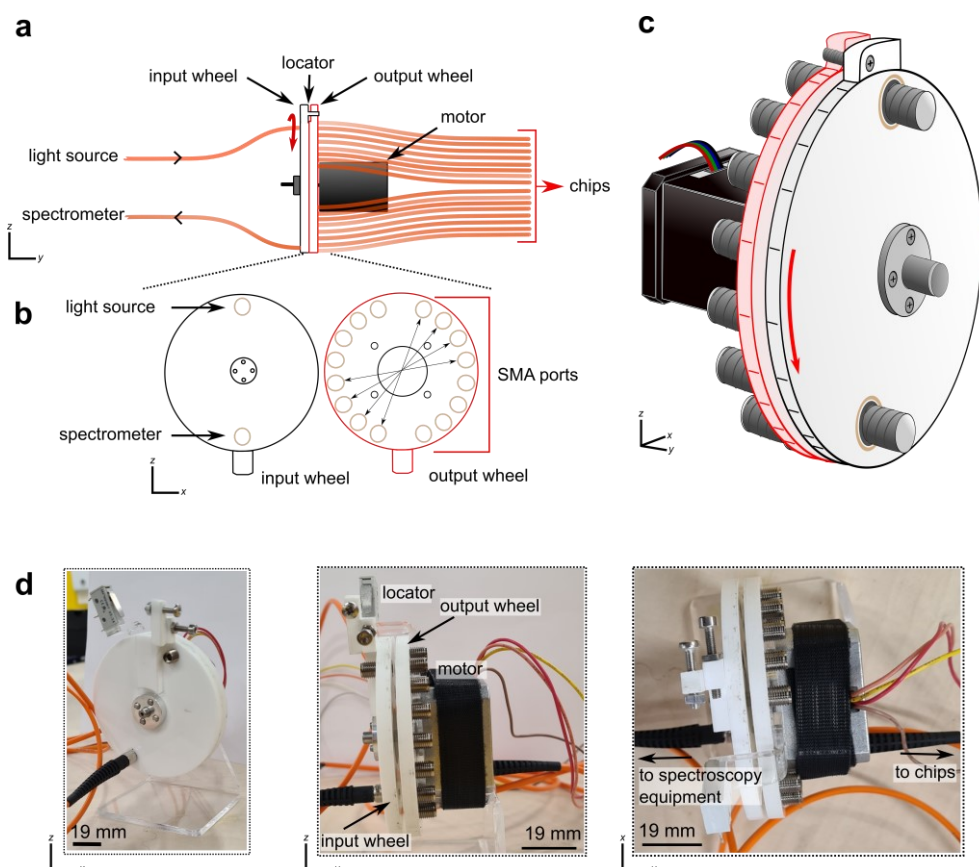


Figure 5.5: A bespoke design for a custom general fiber multiplexer **a** Cartoon showing fibers connected to multiplexer wheels with labelled components and parts. **b** Each wheel of the multiplexer, arrows indicate paired up paths, **c** A simplified view of the built device, rotation of the input wheel allows the passing of light to the output wheel. **d** Photos of engineered device.

The device was built around the concept of two axially aligned wheels cut with a CNC mill. One wheel contained the inputs for the light source and spectrometer and the other with outlets which could be plugged into any application (figure 5.5 a, c). The input wheel had two apertures (7.2 mm) separated by 180° where SMA ports (Farnell 905-120-5004) were set into each (figure 5.5 b). The output wheel was immobilised onto the back of a stepper motor and each output SMA port was separated by 20° , with pairs separated by 180° . Rotation of the input wheel would thread a light path into any of the 8 pairs of output SMA ports allowing beam path multiplexing.

Whilst this device successfully transmitted light to an output pair, the beam temporarily passed across a gap between the input and output wheel causing signal attenuation (figure 5.5 d). This constrained alignment of the beam path to the resolution of the stepping size and rotational axis of the motor (1.8° / step) and the width of beam ($200\text{ }\mu\text{m}$). A significant improvement of this prototype would be to fit a solenoid to the input wheel with translational movement in the shaft axis. The flat SMA apertures could be replaced with a conical entry which would allow end to end threading of the SMA fibers. This would ensure maximal light transmission through coupled fibers whilst overcoming the degree / step limitations of the design.

It was decided that whilst this prototype presented significant potential towards a generalised solution, a more immediate solution to the target problem could be achieved with a bespoke build.

5.5. Bespoke fabricated fiber multiplexer

5.5.1. System overview

The single beam path chip featured an embedded, immobilised beam path. A mobilised beam path was considered as a means to achieve the desired system elements outlined at the start of this chapter. Figure 5.6 shows a schematic of a bespoke approach to multi droplet measurement. A linear sequence of DIBs are arranged perpendicular to the beam path, similar to the individual geometry in the single beam path chip. In this system, after capturing the spectra at the first droplet (denoted t_0 in figure 5.6), the beam path is actuated across the DIB to the other droplet (t_1). The beam path can then be actuated to the next DIB droplet (t_2) before then across the DIB as in the prior step. As the measurement eventually returns back to the starting position, a trade-off between resolution and throughput is present - the number of linearly arranged DIBs dictates the throughput and the data recording interval.

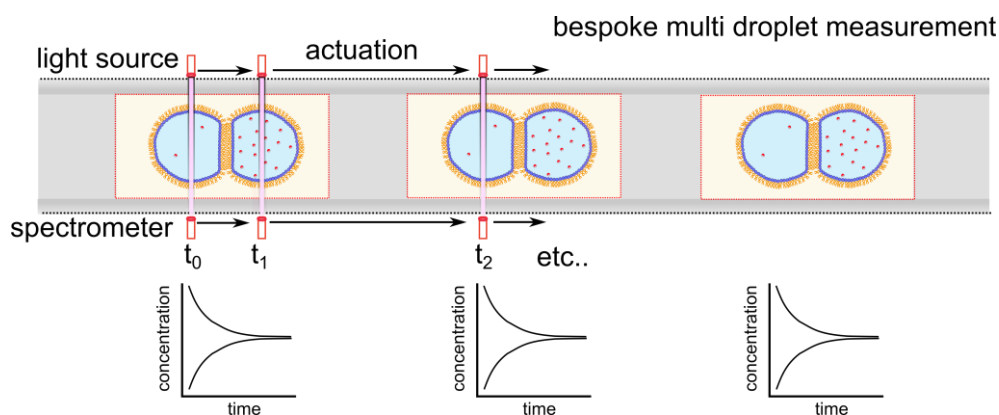


Figure 5.6: Overview of a bespoke method to achieve label free, high throughput, multi droplet DIB permeability assessment. Through actuation of a beam path, spectra can be collected at each droplet of a linearly arranged series of DIBs, determining the full translocation profile.

5.5.2. A bespoke, linearly actuated spectroscopic platform

To achieve this build, a platform for linear actuation was designed. It was imagined that fibers would sit atop a linearly actuated carriage and chips would be held perpendicular to the beam path by chip supports. Through stepping of the motor, linear actuation of the carriage was achieved. To yield each part in the designed system, a range of fabrication techniques were deployed, appropriate for the resolution and shape required per part. A short description of each part in figure 5.7 is as follows. A base plate was cut from a 5 mm PMMA sheet and 5.8 mm holes were tapped to an M6 thread with a cut tap and die. A laser cut rail support was glued to the base with epoxy and 2.8 mm holes were tapped to an M3 thread. A commercially available linear guide rail and carriage were attached to the rail support with M3 screws. A 3D printed motor support was glued to one end of the rail support and screwed onto the face of a stepper motor. A length of drive screw (M8 thread) was sawn and fitted to the stepper shaft with an adaptor. The adaptor was machined through drilling into one end to fit an M8 thread and both shaft and drive screw were secured with grub screws.

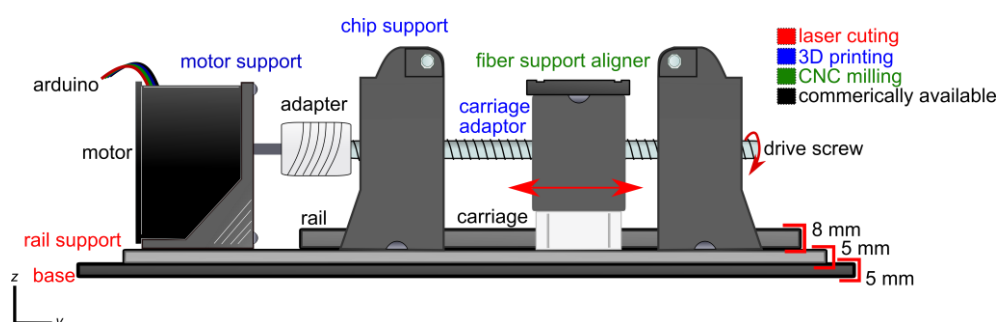


Figure 5.7: Cartoon side view of a linearly actuated platform. Colours indicate fabrication technique used for each part. Turning the drive screw resulted in the movement of the carriage.

To hold a chip in place perpendicular to the carriage, chip supports were 3D printed and screwed to the base plate with M6 screws. The chip supports were outfitted with a small locking mechanism used to secure a chip with an M3 nut and bolt and small holder piece. A carriage adaptor was designed which connected the rail carriage and drive screw. The carriage adaptor was 3D printed to secure two ends of a spring-loaded anti-backlash nut (Banggood – T8 anitbacklash nut) which fit to the drive screw. The nut was sealed into the carriage adaptor using a glue gun and the carriage adaptor screwed into the carriage. This assembly ensured a smooth actuation of the carriage upon rotation of the drive screw. A fiber support aligner was milled from a 5 mm PMMA sheet using a CNC. Grooves were milled into the top layer and holes were tapped for fitting fiber supports and for fitting to the carriage adaptor. In tandem, this build enabled a linearly actuated platform, upon which a fiber supporting assembly could be attached.

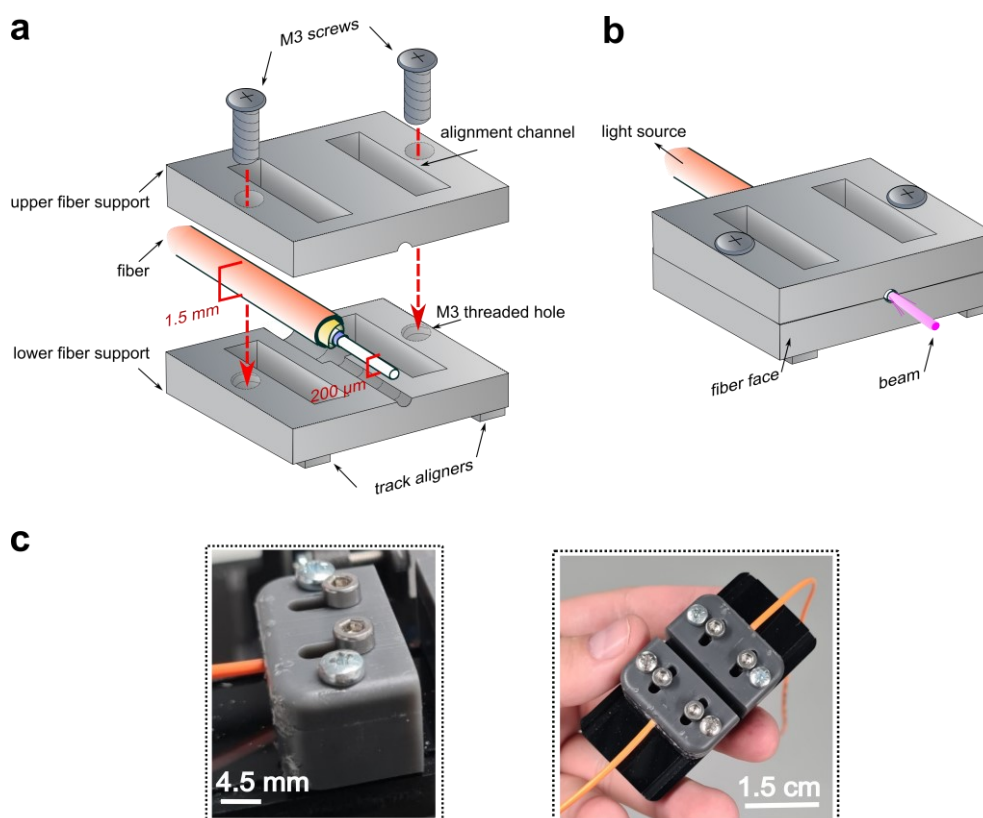


Figure 5.8: Custom fiber supports. **a** Exploded cartoon of fiber support assembly. **b** Cartoon of sealed fiber support, here plugged into a light source. **c** Photographs of actual assembly, left shows a single support, right shows two supports attached to fiber support aligner.

To attach fibers to the platform, a means to hold fiber ends in place was required (figure 5.8). A fiber support was 3D printed from two pieces. Both layers featured two semi spherical channels, one the diameter of the fiber cable jacket (1.5 mm) and the other, the exposed fiber core (200 μm) (figure 5.8 a). An alignment channel was included in both layers, which

would allow translational movement when fitted to the support aligner on the linear platform. Fibers were cut to the length of the fiber channel and prepped as in section 5.3.2 (see chapter 2). In the upper support layer, 3.2 mm clearance holes were set whilst in the lower fiber support, 2.8 mm holes were tapped to an M3 thread. The lower support layer was fit with aligners, which fit to the grooves in the fiber support aligner. The upper and lower layers were sealed with M3 screws running through both layers and a small bit of epoxy was applied on the inner surface (figure 5.8 b). To maximise light transmittance, the fiber end was polished such that the embedded fiber was flush up against the fiber face. The fiber face was made rectangular to allow ease of polishing with sacrificial material surrounding the embedded fiber. The fiber face was polished with increasing grade sandpaper (300 – 3000 grade) and then with polishing sheets (Thor Labs LF30D – LF6D – LF1D) and gently washed with DI water. The fiber was plugged into a halogen light source and the transmittance assessed with microscopy and where required; the fiber was re-polished.

Two fiber supports were attached to the fiber support aligner with M3 screws through the alignment channel, slotting the track aligners into the fiber support aligner (figure 5.8 c). This allowed for translational movement of the two fiber supports along the fiber support track. As such, the distance between fibers could be adjusted with the tightness of the alignment screw. Slight rotational movement was allowed, as the track aligners were made smaller than the track. The final spectroscopic platform is shown in figure 5.9. Individual CAD drawings for each engineered piece are included in the appendix. All CAD files are available via github link.

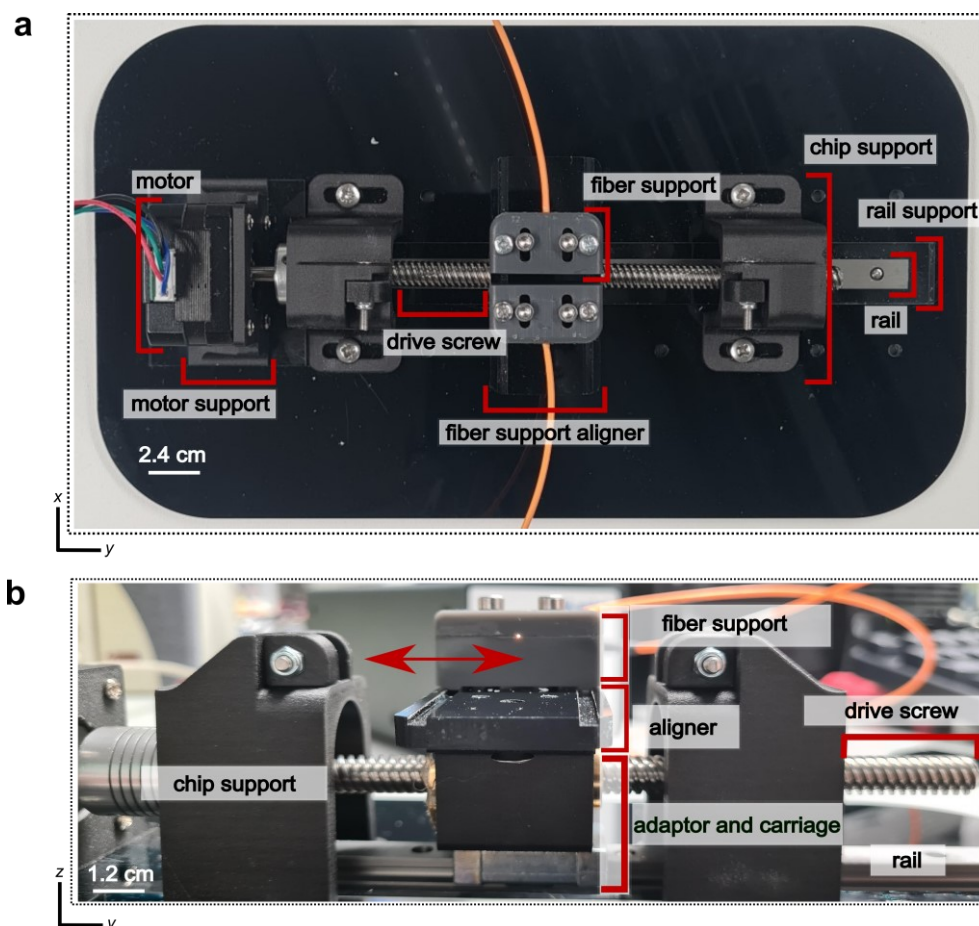


Figure 5.9: The assembled spectroscopic platform with fitted fiber optics. **a** Top-down photograph of complete spectroscopic platform. **b** Side view photograph of spectroscopic platform. One fiber support was removed and the other attached to an illuminated halogen light source as shown by small orange beam on the fiber support.

5.5.3. Fiber support alignment testing

The platform parts were designed with respect to modularity. In order to maximise light transmittance in the experimental system, the alignment of the optical path was optimised. A halogen beam was used to assess the system and spectra were collected using OceanView software. Fiber supports were set onto the spectroscopic platform and were tightened into arrangement.

The beam path was manually aligned between fiber supports. Figure 5.10 clearly shows that spectra were successfully captured with this bespoke build. The peak intensity was maximised through tightening of screws in the alignment channel, resulting in fine adjustments in the z axis. To investigate the optical path, fiber supports were separated, measured with a calliper and the spectra collected. The integration time was set to 7 ms for each separation. The distance between fiber supports, yielded a sigmoidal relationship with

peak intensity (figure 5.10 a). The signal intensity at 2 mm was roughly a third of the intensity at 0 mm – where fiber supports were in contact. For a 5 mm separation, a comparative spectrum to 0 mm was yielded with an 80 ms integration time, an 11* increase (blue line - figure 5.10 b). The width of a chip sat between the fiber supports was therefore at the cost of integration time and measurement resolution.

To understand the effect of a chips optical properties on the measurement, experiments were performed with various sheets of differing transparencies between the fiber supports. A non-transparent sheet attenuated the signal (black line – figure 5.10 b), however the signal intensity improved with a transparent sheet (red line – figure 5.10 b). This indicated slight beam collimation through a PMMA sheet.

Measurements were stable within the platform, as indicated by a flat intensity distribution when spectra were recorded as a function of time both with and without a PMMA sheet (figure 5.10 b). It was theorised that a method of continuous spectral collection during beam drive may have yielded significant measurement throughput. To test this, the fiber supports were actuated on the platform through rotation of the drive screw, with spectra continually collected. In comparing these two distributions, it can be seen that significant noise was introduced, as the beam moved along the linear axis. This noise was likely introduced from slight adjustments in fiber alignment during movement. All measurements with the platform were thus collected whilst the beam remained in a stationery position.

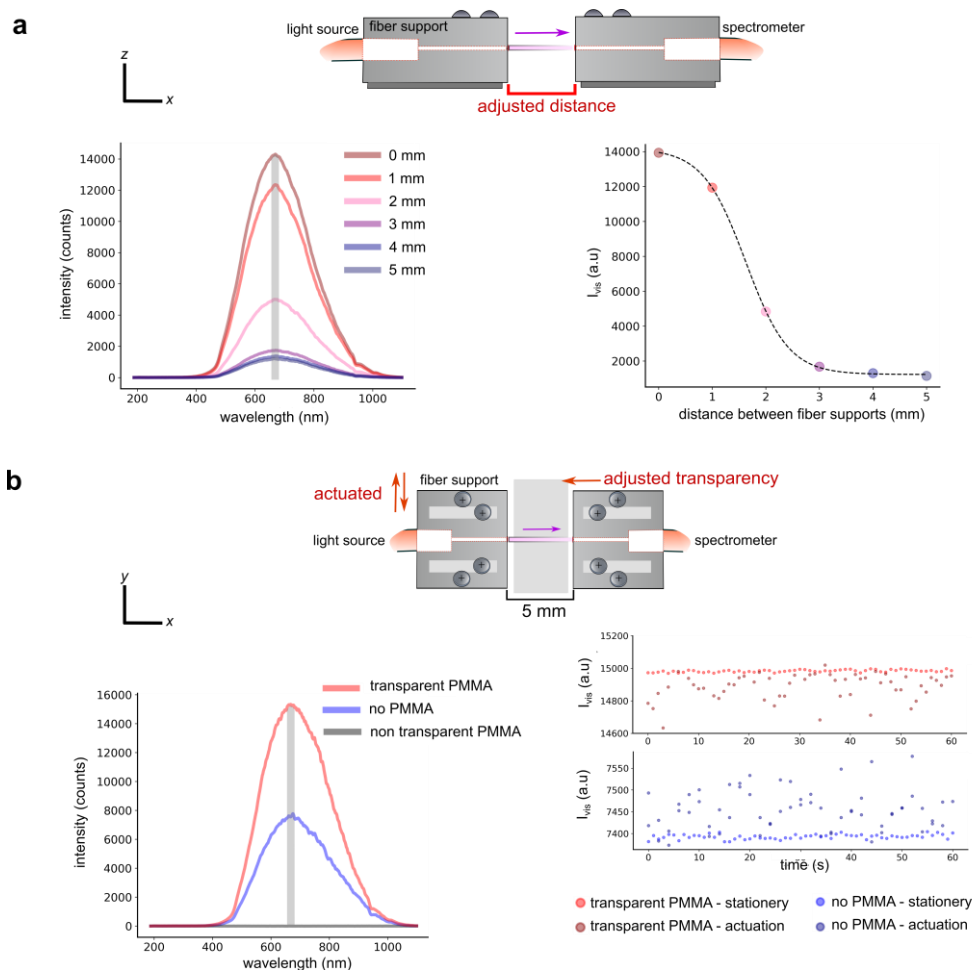


Figure 5.10: Fiber support alignment experiments **a** The distance between fiber supports was adjusted. Left shows collected spectra from a halogen beam, with grey area indicating processed peak region and right shows I_{abs} as a function of distance. Integration time = 7 ms. **b** The distance between fiber supports was set to 5 mm and sheets of different transparencies (all 5 mm) were slotted between the fiber supports. Left shows collected spectra for different sheets and right shows the effect of actuation. Integration time = 80 ms.

5.5.4. UV transparent removable chips

As the optics were aligned on the platform, a key advantage of the method was that the chip formation process was significantly de-skilled. This reduced the time of chip optimisation as compared to the same process for an embedded optics chip. It was also desired that chips could easily be removed from the platform and new chips inserted, either with different geometries or with further embedded analytical equipment. The chip support could be adjusted for an up to 5 mm chip thickness and each part of the platform could be customised if required.

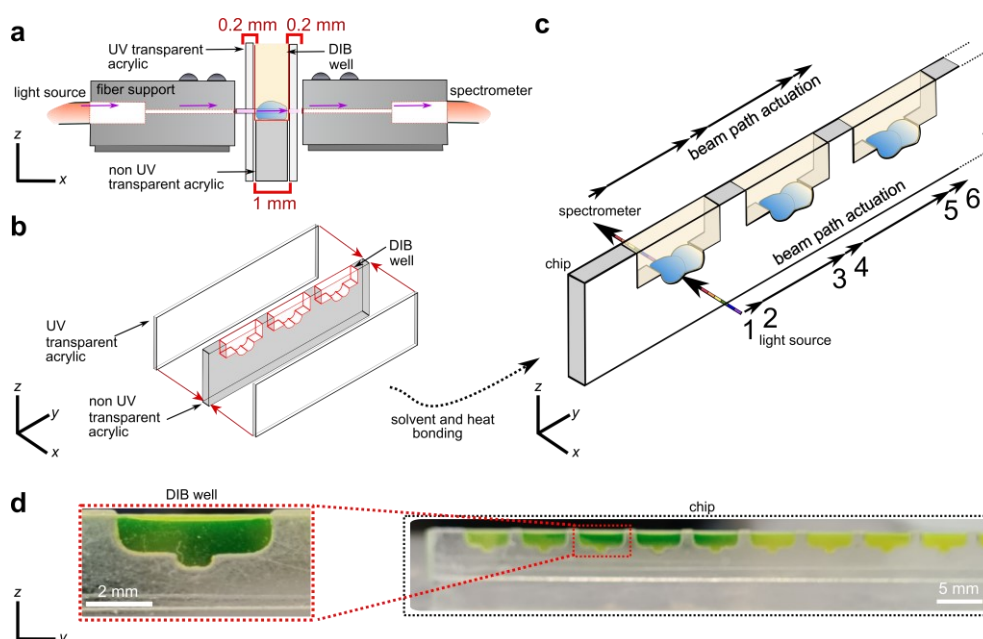


Figure 5.11: Chip formation for spectroscopic platform **a** Overview of light beam path from light source to spectrometer through a well containing a droplet. **b** Chip fabrication with layers of varied transparency arranged along x axis. **c** Cartoon schematic of beam path actuation to capture each DIB droplet in a linear series. **d** Photographs of chip with DIB well zoom in, note the small DIB arrangement feature in well centre. The ten wells were filled with calcein to highlight DIB well architecture.

The successful high throughput chip design is shown in figure 5.11. The beam path, as arranged across a single droplet is shown in figure 5.11 a. In the single beam path chip, layers were stacked in the z axis. In the previous section, it was shown that the measurement resolution (as dictated by the integration time) was optimised with as narrow a chip as possible (sigmoidal distribution – figure 5.11 b). To minimise the beam path length, chip layers were arranged in the same axis as the beam path (x axis in figure 5.11 b). In consideration, ten DIB wells were arranged with equal separation and cut into a 1 mm standard PMMA layer with transmittance $\sim 400 - 1200$ nm (figure 5.11 b). As compared to the single beam path chip, here a DIB support in each DIB well could only be introduced in line with the base of the bilayer (figure 5.11 b, c), reducing the overall control on DIB geometry. UV transparent PMMA, with transmittance from $\sim 250 - 1200$ nm was purchased from Weatherall (CLAREX Ultraviolet (UV) Transmission Filter). From commercial specification of the UV-transparent PMMA, the transmittance distribution per wavelength scaled from 250 – 300 nm where transmittance was then maximised from 300 – 1200 nm. A layer of UV transparent PMMA was arranged across the DIB well layer. This created a UV transparent ‘window’, along one side of the DIB wells. Chips were formed with either a 0.2 or 0.5 mm UV transparent PMMA layer. The final width of the chip was either 1.4 or 2 mm, well within the linear region of the sigmoidal thickness - intensity distribution (figure 5.10 a).

The layers were carefully held in place using a clamp and were sealed using heat and solvent bonding (figure 5.11 c). The final chip is shown in figure 5.11 d, where the linearly arranged DIB wells can be seen. One aspect of this fabrication that had to be controlled, was the clarity of the ‘window’, as shown in the zoom-in in figure 5.11 d. It can be seen that minor fractures and cloudiness were introduced to the window during the sealing process which was found to have a minor effect on the beam transmittance.

5.5.5. DIB formation in spectroscopic platform chips

DIBs were formed within the chip wells, where the ideal volume based on the geometry of the DIB well was 400 nL. Interestingly, due to the flow dynamics of the DIB well it was difficult to manipulate incubated droplets off the platform due to a backflow in the plane of the droplet. To avoid this, the monolayer evaporation technique introduced in Venkatesen et al. was used to aid monolayer stability and assembly time.¹⁵⁶ One droplet was pipetted into the DIB well and the other held at the air / oil interface for ~1 minute. At this point, the droplet often fell off the pipette tip due to reduction in IFT from monolayer assembly.^{209,168,164} The droplet was carefully dropped into the other side of the well where a DIB was formed (figure 5.12 a). The lipid out technique was employed for all DIBs within this system, allowing comparison to the single beam path chip.

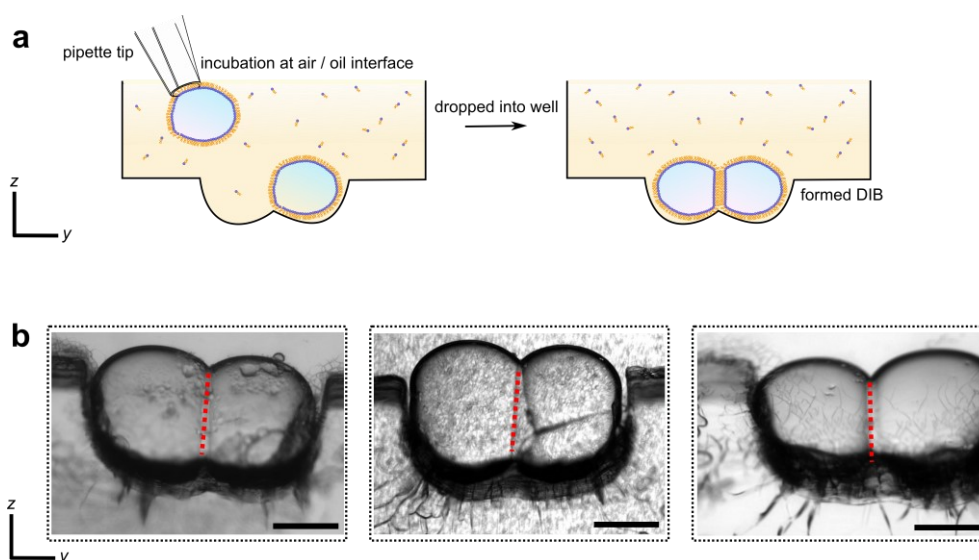


Figure 5.12: DIBs formed in spectroscopic platform chips. **a** Cartoon indicating monolayer compression method introduced in Venkatsen et al.¹⁵⁶ This method was used for all DIBs formed within this system. **b** Photographs of DIBs formed in chips. Images captured with Nikon Olympus microscope. In each case, black scale bar represents 0.5 mm and measured bilayer area is highlighted in red.

Multiple DIB diameters were measured with microscopy (figure 5.12 b), as in the prior method, a circular bilayer area was assumed. The mean bilayer area was found to be 0.00215 cm^2 with an uncertainty of 11.9% with 400 nL droplets. Due to the reduced constraint on DIB area, the uncertainty and the area: volume ratio is greater than in the comparative single beam path chip. Ideally each DIB would be measured individually with a camera. The implications of this assumption are further discussed and compared extensively in chapter 6.

5.5.6. Custom software for controlling the spectroscopic platform with example theophylline DIB permeability system

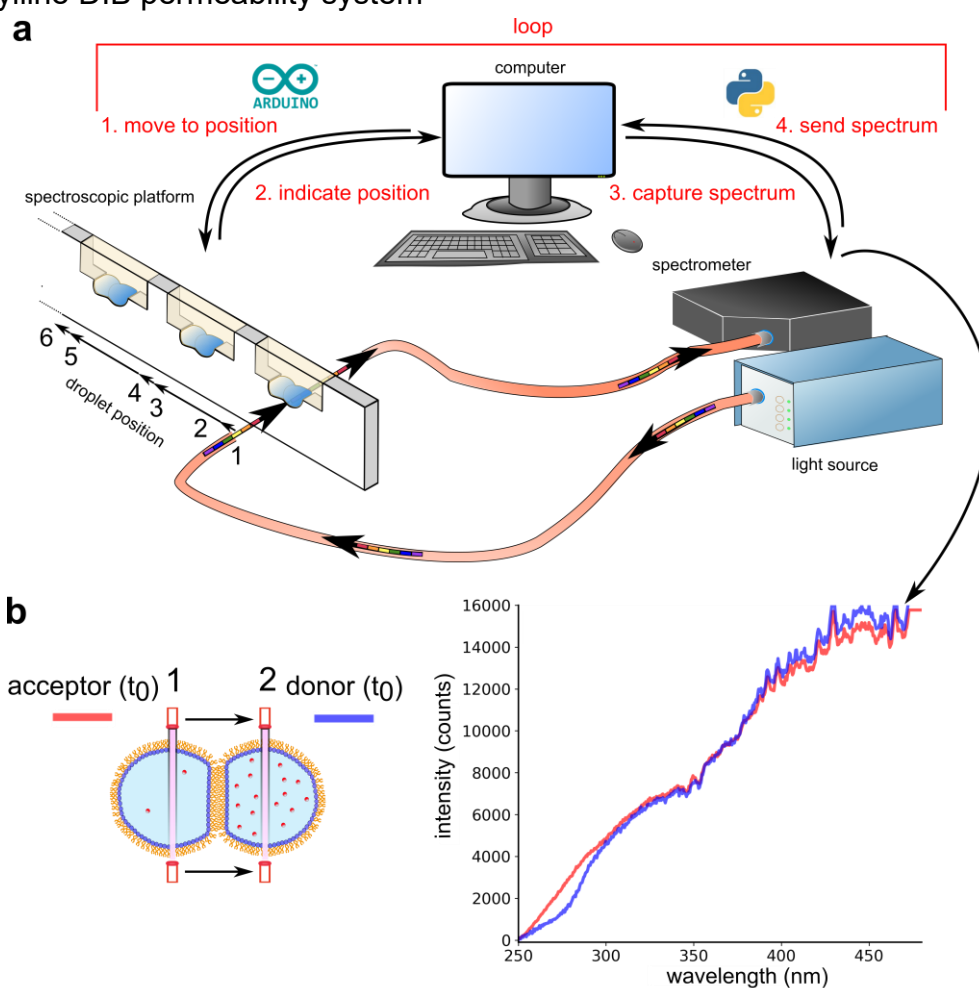


Figure 5.13: Operation of spectroscopic platform with custom software. **a** A cartoon schematic of the operation loop for the platform. The electrical circuit for control of the spectroscopic platform is included in supplementary figure 3. Operations 1 – 4 are executed per position with the sequence repeated position to position. Sequencing of each operation was achieved with a combination of Arduino scripting and Python programming. **b** Example initial spectra captured in each droplet either side of a DIB (here performed with a theophylline permeation system). The spectra have been truncated to show characteristic theophylline absorption region 250 – 302 nm.

A fully automated approach to measurement was desired such that the experimenter would be able to set a measurement loop, guiding the beam path between each DIB droplet (see

next section). This was incredibly important with respect to device throughput and ease of operation. For ease of use, the system had an *in-situ* readout of the translocation profile, allowing the experimenter to assess the outcome of the DIB experiment.

With respect to the above considerations, custom software was built utilising serial communication between Arduino and Python scripting. The importance of a stationery measurement was found through the optimisation experiments in section 5.5.3. As indicated with the commercial multiplexer in section 5.3.1, robust software was required to pull spectra at the correct time to prevent measurement drift. The relevant spectrum was one where integration began at the droplet after the platform had aligned the beam path across it. The spectrometer therefore had to be activated as defined by an automation loop (figure 5.13 a).

To achieve this, scripts were sent to an Arduino which set the fiber mount movement on a loop (for example 1 – 6 in figure 5.13 a). Data was sent to the computer via serial communication (9600 baud) depending on whether the beam was moving or had completed a move. Python software written using the standard libraries (os, time, numpy, scipy, matplotlib, regex, random, IPythondisplay) pulled this data from the serial (COM) channel (pyserial). The spectrometer was controlled through the open-source seabreeeze package,²³⁵ a python wrapped C++ OceanInsight library. Drivers for the spectrometer were installed and the spectrometer was set to 'trigger mode'. This mode set the spectrometer to receive a 'trigger' before integration, which differed from the standard 'free running' mode in which spectra were continuously pulled. The Python software orchestrated the activation of the spectrometer depending upon the data sent via serial communication. The software displayed the live *in-situ* spectra which were saved to folders sorted by each 'droplet position'. A separate script then allowed for the plotting of the standardised intensity time curve for each position during the experiment. In short, this software provided a robust and highly useful tool for the operation of the bespoke spectroscopic platform. The full code is provided in the appendix through a github repository.

Figure 5.13 b shows example broadband spectra collected using the spectroscopic platform with the custom software and with the same DIB experimental conditions as in figure 3.7. The fiber mounts were aligned by eye across each droplet and a spectrum was captured, showcasing the distinct absorption differences between each droplet. When directly comparing the spectra from each system, it was important to consider the beam path differences between the systems. Here, the beam path crossed a UV-transparent PMMA sheet either side of the droplet; spectra were thus affected by the wavelength dependent outer sheet transmittance. The transmittance of the sheets increased from 240 nm and

plateaued above 300 nm. To maximise signal intensity, the integration was increased to 400 ms, this maximised beam intensity between 250 – 450 nm however typically saturated 450 – 650 nm. Regardless, high quality spectra were clearly ascertained which were processed as described in chapter 3.

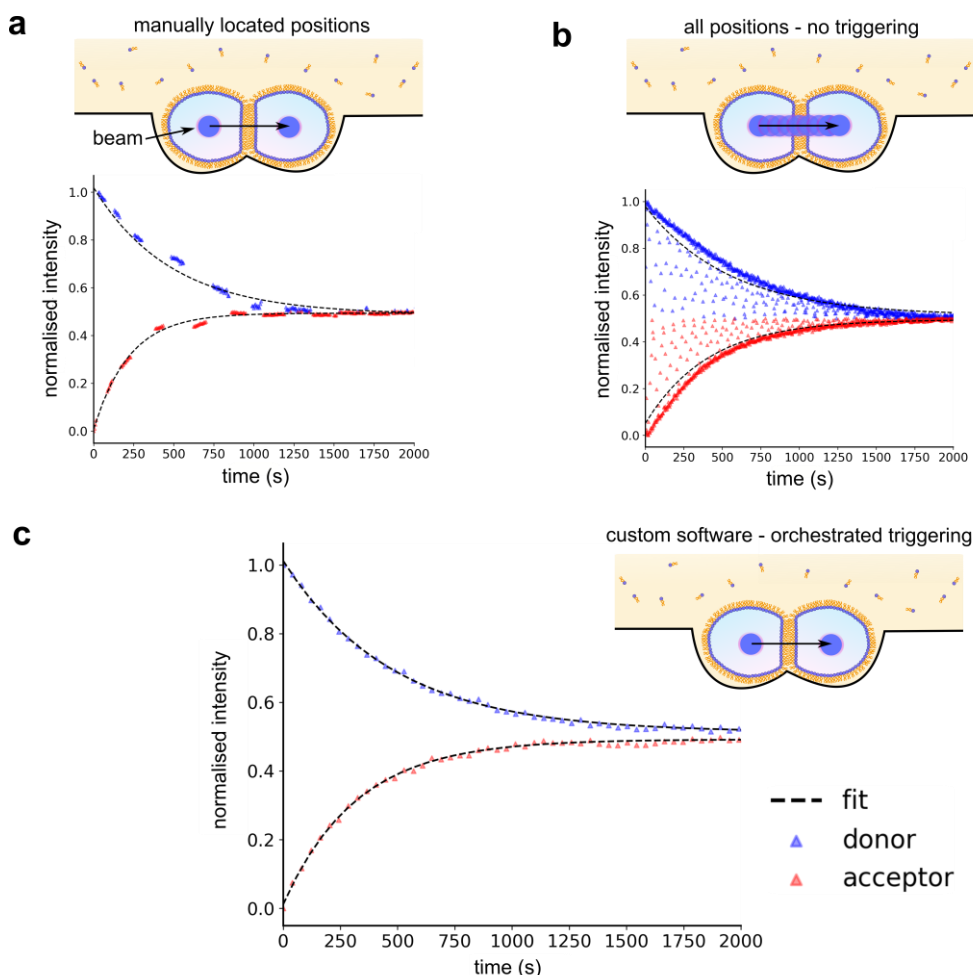


Figure 5.14: An illustration of the utility of custom software for permeation experiments. **a** The drive screw was manually adjusted to arrange the beam path at droplets, recording for ~60 s each time. **b** An automation loop was established setting a loop between two positions, however the spectrometer was left in 'free running' mode, which returned spectra with measurement drift as the beam passed across the DIB. **c** A translocation profile captured utilising the developed software with communication between spectroscopic platform and spectrometer. The beam path was successfully alternated between two positions and the correct spectra captured each time. This profile represents a significant advancement in the field.

Figure 5.14 shows a series of control experiments which provide an illustration of the measurement advantage conferred by the developed software. If the translocation profile was rebuilt from manual droplet location, this required significant physical involvement of the experimenter, limiting the systems application (figure 5.14 a). If the spectrometer and spectroscopic platform were not in sync, this returned spectral superpositions (figure 5.14 b). Unlike the results in section 5.3, these superpositions were built from the beam passing

across the DIB itself. Whilst this data could be filtered, this would incur significant data processing and this issue would multiply with DIB throughput as spectra were stored in a single folder. Finally, the translocation profile returned by the custom software clearly illustrated the potential of the method (figure 5.14 c). The software successfully triggered the spectrometer upon beam path alignment returning the correct spectra. The spectra obtained at each position were saved in separate directories, allowing for ease of *in-situ* and post experimental analysis. The translocation profile showed two complimentary exponential curves representative of growth and decay in droplet concentration across a DIB. To the best of my knowledge, this represents the first *in-situ*, low data interval, label free dual droplet measurement of the DIB translocation profile.

5.5.7. Droplet locating

Finally, an automation loop for collecting high throughput measurements was required. To achieve this, chips were scanned with the platform (figure 5.15 a). The stepper motor was driven with a 900 μ s delay between steps, and spectra were captured with custom software as described prior. The resultant plot revealed the transmittance distribution across the DIB wells (figure 5.15 b). A clear increase in the UV intensity allowed identification of where the beam passed across the well, as noted by a rise and fall in the mean UV intensity either side of the well.

Key features were present within the DIB well transmittance distribution. The bands in the zoom in of figure 5.15 b highlight these distinct regions. The blue bands show a region at which the width of the beam (200 μ m) clipped the edges of the well, which reduced the UV transmittance and absolute spectral intensity. As the beam began to pass across the first edge of the DIB well, a clear rise in the distribution was present, which at the peak indicated the beam had aligned across the bulk aqueous volume. From here, this region was denoted the 'measurement band', a ~350 μ m width within the droplet at which the signal was stabilised. Interestingly, even within this band, transmittance fluctuations were present owed to the chip fabrication (figure 5.15 b zoom in). Due to the width of the beam, a 200 μ m region existed either side of the DIB at which a reliable spectrum could not be collected due to the potential super position yielded from the beam clipping across both droplets (coloured red in figure 5.15 b).

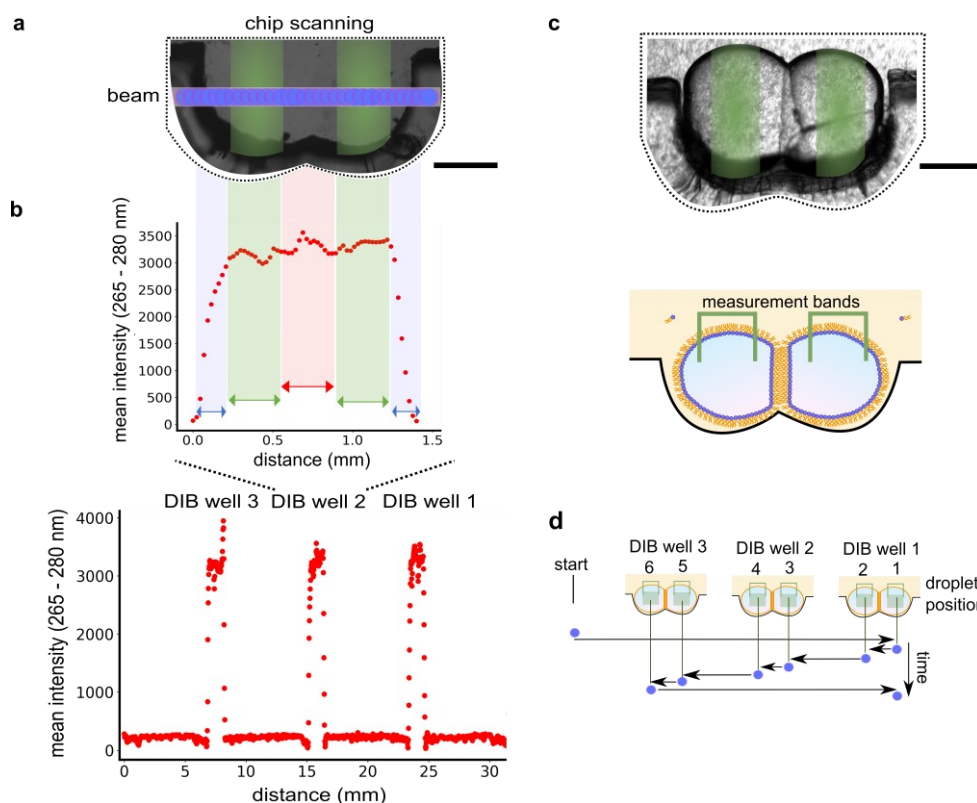


Figure 5.15: Droplet scanning revealed a suitable measurement band in DIBs. **a** Photograph of empty DIB well, cartoon indicates beam actuated across the well. **b** Scan data as a function of distance from starting point. Zoom in shows beam transmittance across a single DIB well (x axis corrected to well edges). Clear bands in the measurement were allocated. **c** Photograph and cartoon of DIB with highlighted measurement band region. Black scale bar = 0.5 mm. **d** Cartoon of automation loop for high throughput experiments, the measurement bands were located for each DIB well using the scan in b and stepped between sequentially.

The resolution of the spectroscopic platform ($\sim 4.5 \mu\text{m} / \text{step}$) was calculated from a measure of the DB well width and the step resolution of the plots in figure 5.15 b (5 steps / spectrum). Thus, with single step resolution there was approximately 280 assessable positions within a single DIB of which around 160 fell within the measurement bands (figure 5.15 c). To prevent clipping, a distance between the outer most edges of each band (195 steps) and between wells was calculated (1590 steps). This then defined an automation loop which was scripted in Arduino (figure 5.15 d). This loop located the measurement band of each droplet in a linearly arranged series of DIBs which allowed for full high throughput, reliable and label free measurement.

5.6. Chapter conclusions

The work within this chapter was built upon key developments made across chapters 3 and 4 toward a significantly advanced label free measurement of DIB permeability. Multiple approaches were tested toward this advance, each with significant potential. Through

deployment of extensive fabrication techniques including laser cutting, CNC milling, 3D printing and microcontrollers an instrument was engineered which could improve throughput and measurement integrity. This platform, which mobilised a beam path perpendicular to DIB droplets, allowed the collection of spectra in both droplets. This novel method was also significantly higher throughput, as it could measure multiple linearly arranged DIBs in a series of wells. The utility of this approach is significantly higher than the single beam path chip, despite a greater skill barrier for replication. The method was designed such that chip formation was de-skilled allowing custom chip architectures to be easily integrated into the device. Through the design of custom software, the entire method could be fully automated which allowed a full translocation profile to be determined, label free, both sides of a DIB. Chapters 3 and 4 are paired together and their contents are broadly system design, and experiments with the system respectively. This chapter and the next follow a similar pattern - in chapter 6, this newly developed approach was deployed for a novel range of DIB permeability studies.

6 Permeability Studies with a Multiplex Label Free Method – Complex drug oil partition, permeant chirality and DIB networks

6.1. Chapter overview

A central goal of this thesis was to engineer and utilise model artificial cell systems with significant potential for industrial translation. Understanding the diffusive behaviour of small molecule drugs would leverage DIBs as the platform technology for an industrially applied assay. Across this work, two novel methods were developed in chapters 3 and 5.

Understanding the differences between these systems, was pertinent to the reproducibility of DIB permeation systems as well as the role of DIB geometry in the measured permeability value. To this end, a range of permeants were assessed, and their diffusive behaviours in each method compared. The use of DIB networks uncovered how two droplet diffusive behaviour translated into a linearly arranged series of bilayers. The interaction of a permeant with a bilayer is affected by structural features of each. Literature findings from 2021,¹⁸² suggest that chirality can affect the rate of simple diffusion in DIB systems. In this work, enantioselective diffusion was studied across a series of systematically tagged amino acid like compounds. This chapter concludes with an assessment of the measured oil partition rate constants, finding no discernible relationship between the bilayer permeability and the measured oil partition. This finding has substantial impact in the field of high throughput drug design which can rely on crude correlations based on the Lipinski rule of five. Aspects of this chapter are in preparation for submission in early 2022.

6.2. Introduction

Understanding whether a particular structure can be screened in DIBs is limited by the structural range of previously screened permeants. Within the literature, this has been constrained to a limited range of well-studied fluorescent dyes. Of these dyes, permeable ones classically yield a mirrored exponential growth and decay in respective acceptor and donor DIB droplets. A flat distribution in both droplets classifies a dye as impermeable. In chapter 4 the diffusive behaviour across lipid mono and bilayers differed dependent on permeant and lipid chemical structure. A range of diffusive behaviours are expected across wide drug structure space and classifying permeability is thus amenable to a high throughput and label free measurement which can account for the dynamic behaviour at each presented

interface in DIB systems.⁶² In this chapter, afforded by exploration of wide chemical space, a third possible classification is introduced – that of simultaneous bilayer permeation and oil partition.

In chapter 3, a fully quantitative system was developed which supported robust characterisation of *in situ* small molecule drug concentration. Whilst this system enabled extensive label – free experimentation, one of the limitations was due to the immobilisation of the beam path which restricted assessment to a single DIB droplet. One theoretical approach to characterising DIB permeability is obtained as a three-compartment system comprising of the acceptor droplet, the donor droplet and the ‘surroundings’ – loosely made up of the bulk oil and the lipid mono and bilayers. In chapter 2, a system of ODEs was determined which model this multi-compartment approach for permeability assessment. For each permeant assessed with the single beam path chip, a secondary series of experiments characterising the ‘loss’ to surroundings had to be conducted, broadly referred to as the ‘oil partition’. In addition to recording droplets individually, this additional step reduced experimental throughput. To maintain breadth of experimentation, the permeability was determined from a single ‘compartment’ in the single beam path chip. The acceptor droplet was studied as the concentration initially increased - in the opposite direction to any loss mechanism.

An idealised system is therefore one in which a robust measurement of the concentration can be made either side of the bilayer - a central advantage of fluorescence microscopy. Replicating this advantage into a label free system has been a significant challenge within the literature and likely stymied significant maturation of DIB permeability screening platforms.^{123,137} The multi droplet recording method developed in chapter 5 afforded a system able to monitor a number of linearly arranged droplets. In the case of a two-droplet DIB system, this therefore meant a high throughput measure of all possible drug locations, where the ‘surroundings’ could be rebuilt from the sum intensities of the acceptor and donor droplets. Across the course of this chapter, attention was placed on readdressing oil partition within DIB systems and re-characterising compounds from chapter 4 which partitioned in the single beam path chip. Simultaneous measure of permeation and oil partition has not been readily addressed within DIB literature. Coupled to this, membrane retention is a highly attractive molecular design strategy, particularly for altering intrinsic membrane properties and its role in metabolism and clearance.^{42,153,236,237} The lack of significant study into oil partition and membrane retention in DIBs may be owed to the structure of commonly deployed fluorescent permeants such as calcein, fluorescein and rhodamine 6G.¹²³ The

work within this chapter, opens up the potential for these measurements to be made in DIBs with relevant biological and small molecule drug permeants.

6.3. Multi - droplet permeation assessment

6.3.1. Theophylline DIB permeability

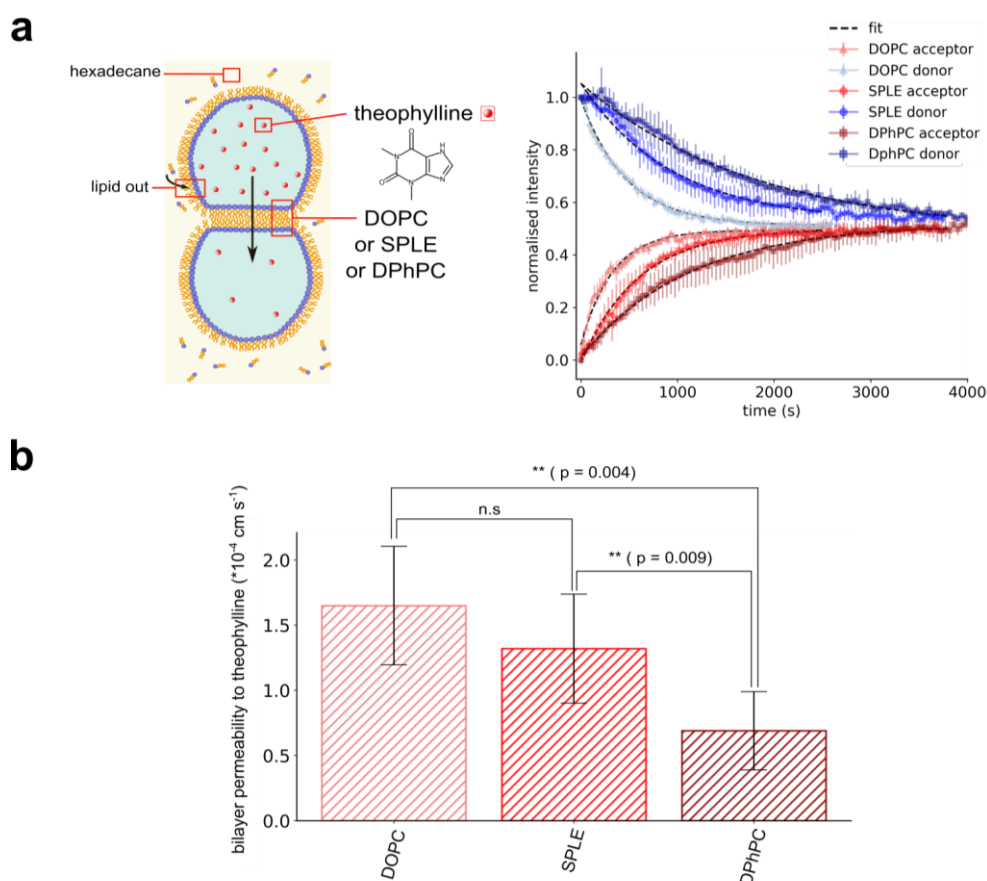


Figure 6.1: Dual droplet monitoring with different lipids. **a** Cartoon showing experimental conditions and translocation profiles for DIBs formed with various lipids. **b** The permeability calculated from each of the distributions in a. Error bars in all plots represent the standard deviation, $n > 3$.

Prior to the work in this chapter, theophylline had extensively been used as a model drug permeant, the data for which is presented in chapters 3 and 4. To confirm the capacity of the multi droplet method, experiments performed in the single beam path chip were replicated. Figure 6.1 shows the theophylline bilayer translocation and derived permeability values for DIBs comprising of DOPC, DPhPC and soy polar lipid extract (SPLE), a natural extract of the abundant lipids of plants.¹⁴² The translocation profile for each bilayer composition showcased a proportional exponential increase and decrease representative of the change in concentration either side of the bilayer (figure 6.1 a).

To maintain comparison across methods, each acceptor and donor distribution were independently fitted with the mean permeability presented in figure 6.1 b. Interestingly,

despite theophylline indicating negligible oil partition (section 3.5.4), a slight asymmetry in the rate constant for each acceptor and donor curve was observed, with the donor typically slower than the acceptor (figure 6.1 b). It can be imagined how, in a system with significant oil partition, the donor curve will demonstrate an apparent slower translocation as the distribution continues to decrease rather than plateauing. Equally, this particular asymmetry may be owed to slight non-linearities in the intensity read out, therefore making conclusions around small asymmetries difficult to quantify (such as with theophylline). This may explain why the reported DOPC permeability ($1.65 \times 10^{-4} \text{ cm s}^{-1}$) was slightly reduced as compared to comparative value in the single beam path chip ($1.94 \times 10^{-4} \text{ cm s}^{-1}$). Interestingly, for the dual droplet method, the mean acceptor permeability alone was $2.03 \times 10^{-4} \text{ cm s}^{-1}$, within a <5 % error between methods for the acceptor droplet despite a volume reduction from 900 to 400 nL. This also reflected the assumption that with this fitting approach, and for a compound with negligible oil partition, differences in bulk diffusion minimally affected the measurement. This is further ratified with previous findings in the literature which showcase comparative permeability values for resorufin across nL DIB droplet volumes (100 nL and 800 nL).^{142,146}

As compared to the single beam path chip and with a comparative fitting approach, a larger error in the permeability measurement was noted for the dual droplet method (figure 6.1 b and figure 4.9). The dual droplet method retained statistically significant differences between DOPC and DPhPC permeability values, with an apparent slight increase in the permeability reduction (45 % compared to 58 %). Despite this, no statistical significance was observed between the permeability datasets for both DOPC and DPhPC when comparing between each experimental method (figure 6.1 b and figure 4.10). It was concluded that the dual droplet system broadly replicated results obtained with the fully quantitative, single beam path chip method, ratifying the significantly advanced approach.

6.3.2. Theophylline DOPC networks

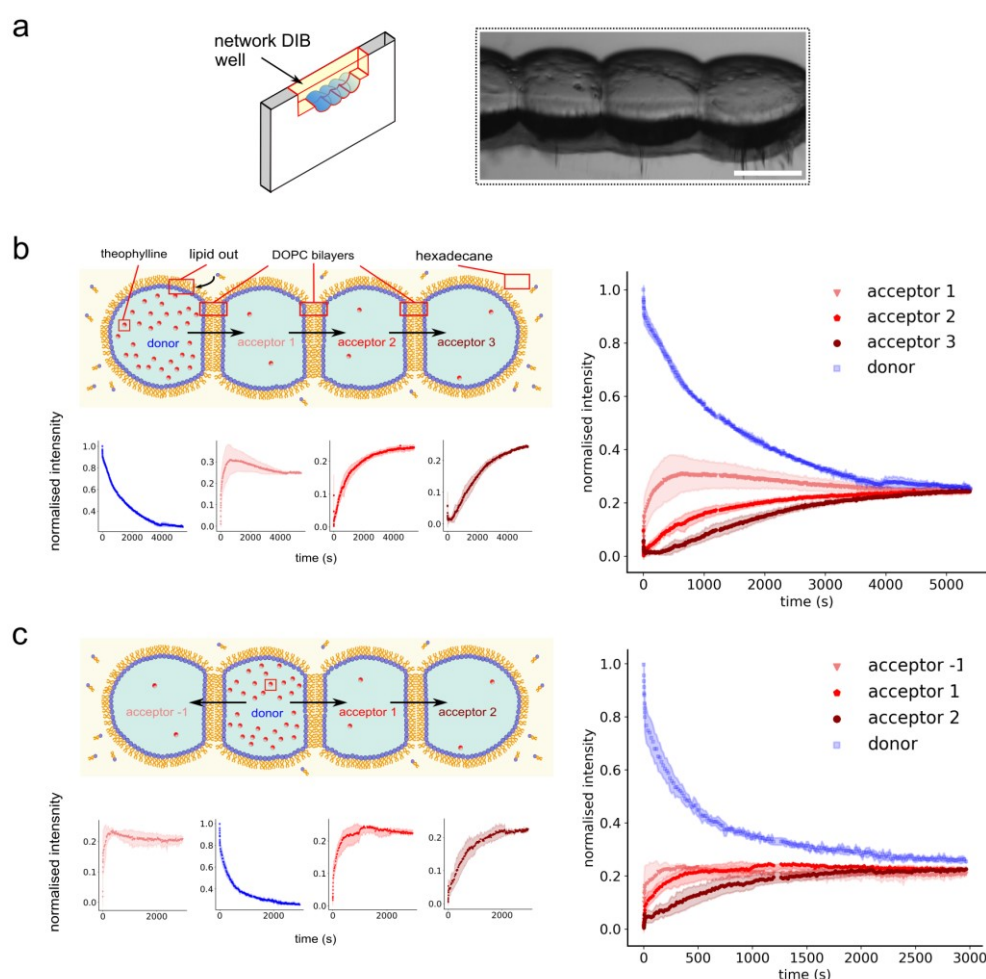


Figure 6.2: DIB networks with theophylline. **a** Cartoon of bespoke DIB network chip and photograph of actual DIB network formed in chip. Droplet volume = 300 nL, mean bilayer area = 0.0015 cm² with an uncertainty of 4.69 %. Scale bar in white = 0.5 mm. **b** Translocation profile for a four droplet DIB network with the donor droplet arranged at one end of the network. The translocation profile for each droplet is shown with the whole network on the right-hand side. **c** Translocation profile for a four droplet DIB network with the donor droplet arranged in the middle, presenting a diffusional junction. Cartoons represent experimental conditions. Error bars in all plots represent the standard deviation, $n = 3$.

Introduced in chapter 1, DIB networks are a highly attractive extension of DIB technology where the initial contents in each droplet can be leveraged for modelling biological, and enabling synthetic, compartmentalisation processes.^{115,125,238,239} The potential for DIB networks in a drug – discovery setting has been realised recently. By controlling initial pH and fluorescein concentration gradients as well as the lipid composition of each bilayer, Korner et al. mimicked the enterocyte mediated gut – blood diffusional pathway with a three droplet DIB network.²³⁸ Concentration assessment of non-fluorescent compounds within DIB networks has only been achieved with a tagging based strategy.¹⁸² Replicating such experiments with orally bioavailable permeants would substantially improve the utility of DIB

network permeability measurements. Modularity was engineered into the design of the multi droplet method - the droplet supporting chip could be tailored to the droplet arrangement and each droplet located by the scan approach demonstrated in section 5.5.7. In tandem, these considerations conferred significant novel functionality in the form of label free assessment in DIB networks. The formation of DIB networks in bespoke chips is illustrated in figure 6.2 a.

To investigate a label free DIB network measurement, the two-droplet theophylline system was extended into a four droplet DIB network comprising of a single donor droplet (1 mM) and three acceptor droplets (50 μ M). As the droplets were formed through pipetting, there was significant control over the location of the donor droplet within the network. Two network conformations were tested, one in which the donor sat at the end of the network (figure 6.2 b) and one in which the donor sat between two initial acceptor droplets (figure 6.2 c).

When the donor was arranged at one end of the network, theophylline diffused linearly into each acceptor droplet. The system equilibrium (~ 5300 s) was dictated by the diffusional pathway from the droplet to the furthest arranged acceptor droplet, denoted 'acceptor 3'. Due to the initial 20:1 concentration gradient, the exchange between donor and acceptor 1 demonstrated the fastest rate which overshot the bulk equilibrium value. As the rate of exchange increased between acceptor 1 and acceptor 2, the intensity of acceptor 1 tended downward to equilibration. At around 4000 s, after acceptor 1 and then acceptor 2 had reached equilibration, the concentration reached a steady state exchange between the donor and acceptor 3 which exhibited an initial lag (~ 460 s) before an increase in concentration. The graph in figure 6.2 b is strikingly similar to one yielded from a 5-droplet fluorescence-based measurement in Bachler et al. This similarity indicated how traditional approaches had successfully been replicated with a label free measurement.¹²³

If the donor was arranged between droplets of the network, this presented a diffusional junction where the concentration initially simultaneously diffused into the two neighbouring acceptor droplets (figure 6.2 c). Acceptor -1, which was only connected to the donor droplet, equilibrated slightly faster than acceptor 1 and demonstrated some back flux into the donor droplet. A steady state exchange was observed from ~ 1800 s, passing through acceptor 1 between the donor and acceptor 2. This droplet conformation reached equilibrium faster (~ 3000 s), than the linear droplet conformation (~ 5300 s). As expected, the qualities of the two-droplet system translated into the four droplet systems – theophylline did not partition out of the aqueous phase and translocated across each DOPC bilayer.

Compared to microfluidic approaches, this method of DIB network formation allows for reproducible and significant control over the initial contents and spatial arrangement of droplets.¹²⁴ In addition, the lipid compositional symmetry at each junction may be controlled through the lipid in approach. Due to the significant modularity of this method, a system could be designed with significant translational potential for testing small molecule drugs in biomimetic conditions within DIB networks. Owing to this ease of assembly, a DIB network could model diffusional processes such as organelle permeability, the permeation of biological molecules from particular organelles out of the cell and intestinal absorption as in Korner et al.²³⁸ From a biophysical perspective, patterning the diffusional pathway within three droplet DIB networks could be used to test the apparent increased permeability of asymmetric bilayers compared to symmetric or 'blended' compositions.^{117,123} A droplet diffusional junction separated by a symmetric and an asymmetric bilayer, would allow for a simultaneous assessment and comparison of the rate of crossing of each.

6.3.3. Bromocaffeine – assessed with all methods

Halogenation is known to increase the hydrophobicity of a molecule.²⁴⁰ A halogen group can interfere with the hydrogen bond forming network of the solute, a factor which can be exploited in drug design.²⁴¹ Understanding oil partition from a permeant structure perspective was a central drive of this work. 8-bromocaffeine is a bromine substituted caffeine that has been used as a radio sensitizing agent and as a precursor for further aryloxy – substituted caffeine molecules.^{242,243} It has been suggested that 8-bromocaffeine can pass the BBB.

The translocation behaviour of 8–bromocaffeine was assessed in DOPC DIBs (figure 6.3). The translocation profile of the two-droplet system indicated that 8-bromocaffeine crossed the bilayer but also rapidly solubilised into the surrounding oil phase (figure 6.3 a). The acceptor curve followed an initial exponential growth which failed to reach the expected equilibrium value before a delayed exponential decay. As the flux across the DIB slowed, the hexadecane flux became more apparent in the acceptor curve. The donor curve followed a classical exponential decay as the permeation and partition flux directions were the same. Both the donor and acceptor curve fit well to the ODE model outlined in chapter 2 and the quantified permeation and partition values are discussed in the next section. The oil partition curve was calculated from the acceptor and donor droplet distributions with equation 22. The oil partition showcased a clear exponential increase tending to full solubilisation of 8–bromocaffeine into hexadecane.

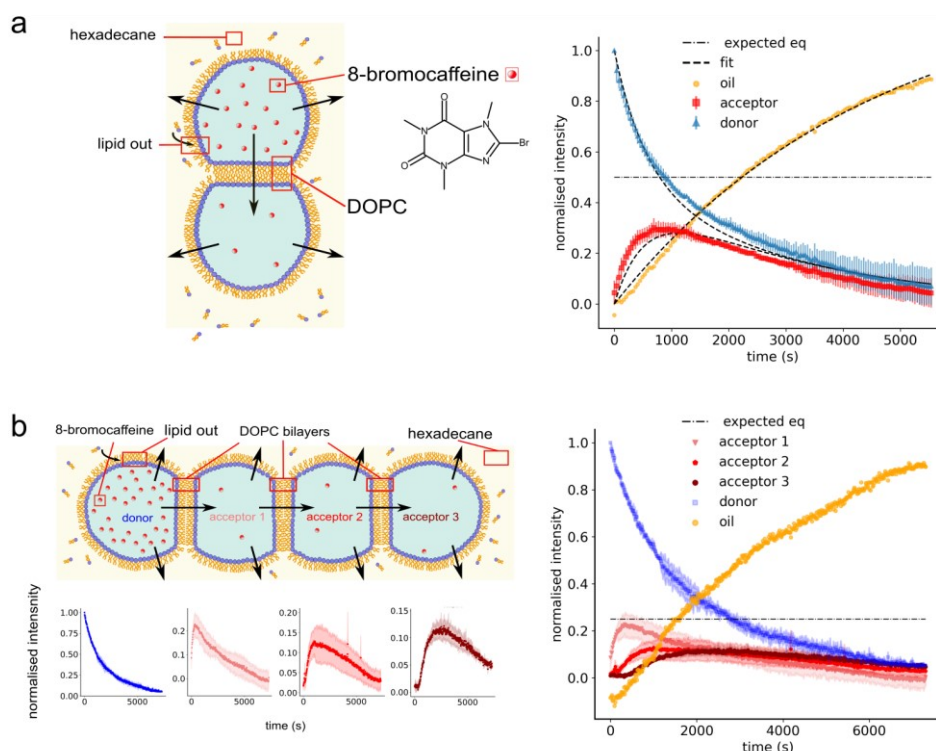


Figure 6.3: Experiments with 8-bromocaffeine – a compound with significant oil partition. **a** Translocation profile for a dual droplet system. **b** Translocation profile for a four droplet DIB network with 8-bromo caffeine. The translocation profile for each droplet is shown with the whole network on the right-hand side. Cartoons represent experimental conditions. Error bars in all plots represent the standard deviation, $n = 3$.

Comparing the two-droplet theophylline and 8-bromocaffeine profiles (figure 6.1 a and 6.3 a), highlights the drastic difference in permeant diffusional behaviour afforded by the addition of a methyl and a bromo group. This behavioural difference was further exemplified when comparing the diffusional dynamics in a four-droplet system (figure 6.2 b and figure 6.3 b). A linear DIB network diffusional pathway was established (figure 6.2 b). Just as in the two droplet 8-bromocaffeine system, diffusive behaviour was defined by whether the droplet was a donor or an acceptor. For each acceptor droplet, the initial rate of increase, the oil partition rate and the maximum intensity reduced in proportion to the distance from the donor droplet. As 8-bromocaffeine partitioned out of the aqueous system across the w/o interface, the concentration gradient across each successive bilayer became more gradual, reducing the net absolute amount of 8-bromocaffeine in each droplet.

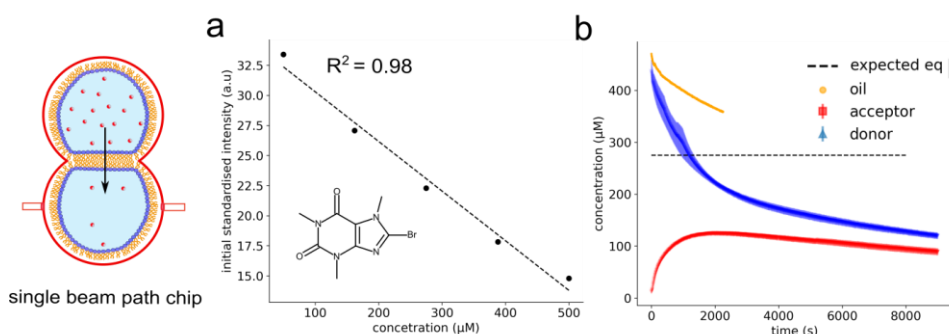


Figure 6.4: 8-bromocaffeine studied in the single beam path chip. **a** Calibration curve determined from the initial values of 1.8 μL droplets with varying concentration of 8-bromocaffeine. **b** The concentration time curves for a DIB translocation system rebuilt from individually measured droplets. Experiments were repeated where the acceptor and donor droplets ($n = 3$ each) were separately studied. Due to incubation of each droplet for 3 – 4 minutes prior, partition of 8-bromocaffeine caused a slight reduction in the measured concentration value. Error bars represent the standard deviation.

Having developed an understanding of the behaviour of 8-bromocaffeine in the multi droplet method, focus was next placed on comparing oil partition across different DIB morphologies in the single beam path and multi droplet chips. The diffusive behaviour of 8-bromocaffeine was favourable for this comparison as permeability and partition could simultaneously be characterised within a reasonable experimental timeframe. Figure 6.4 shows the rebuilt translocation distribution (donor and acceptor) for 8-bromocaffeine in the single beam path chip. The shape of the translocation profile is incredibly similar to the comparative experiment with the multi droplet method (figure 6.3 a). This similarity in result between a fully quantitative and a semi – quantitative method further ratified the approach to data processing and normalisation outlined in chapter 2. The initial droplet intensities were used to calibrate the system, due to immediate 8-bromocaffeine partition from aqueous droplets within the hexadecane reservoir. As with other permeants, there was a strong linearity in this measurement between 50 and 500 μM ($R^2 = 0.98$ – figure 6.4 a).

The key difference evidenced by these experiments, was a difference in the rate of oil partition between the methods. In the single beam path chip, at 8000 s the system was still far from partition equilibration, indicating that the rate of oil partition was reduced. In figure 6.4 b, the concentration values did not meet in the centre post permeation equilibration (>2500 s). It was difficult to determine if this droplet concentration difference was a result of the relationship between the bilayer and partition flux values or due to bilayer retention of the compound. Understanding and comparing between the two methods in conjunction with oil partition inspired the experiments in the following section.

6.4. Quantifying translocation behaviour – multi beam

experiments

6.4.1. Drug array

Work in section 6.3 highlighted that oil partition could lead to an asymmetry in the shapes of the acceptor and donor curves (figures 6.3 and 6.4). This asymmetry fit well to a system of ODEs which could quantify translocation and partition contributions (figure 6.3 b). In chapter 4, oil partition was evidenced by propentofylline, colchicine, minocycline and piroxicam with potential bilayer retention implicated for chloramphenicol (figures 4.8, 4.11 and 4.15). To further understand the apparent increased oil partition in the multi droplet chip, these compounds were reassessed with the method. The distributions are qualitatively discussed here, the measured permeability values discussed in 6.4.2 and the quantified oil partition discussed in section 6.6.

It is worth recalling the DIB well assemblies deployed for each method. In the single beam path chip, DIBs sat in an enclosed well. The droplet volume minimised the oil meniscus between droplet and PMMA. Only the top surface of the droplets had access to the bulk of the oil reservoir. The bilayer area to droplet volume ratio was 0.32. In the multi droplet method, only the base of the droplets were in direct contact with PMMA, providing a comparatively larger exposed w/o surface area. The bilayer area to droplet volume ratio was 0.53. It is these difference in DIB environment which were hypothesised to affect the rate of oil partition (as evidenced prior with 8-bromocaffeine).

The subtle relationship between a permeants chemical structure and diffusive behaviour in DIBs is shown in figure 6.5. Theophylline, aminogluthethimide and paracetamol previously exhibited negligible oil partition and this quality was retained in the multi droplet method (top row figure 6.5). The single beam path chip data for paracetamol was conducted by a colleague (Felix Sheffield) and included in a joint publication.²¹² The symmetry between the acceptor and donor curves indicated that the bilayer permeability was the only measurable property within the experimental window. Indeed, the multi compartment model yielded unreliable oil partition values which tended to 0 (k_2 – equations 34 and 35). Because of this, only the permeability values for these compounds were interpreted. For these compounds,

the equilibration time in the multi droplet method was ~40 % lower than in the single beam path chip, in agreement with the difference in bilayer area to droplet volume ratio.

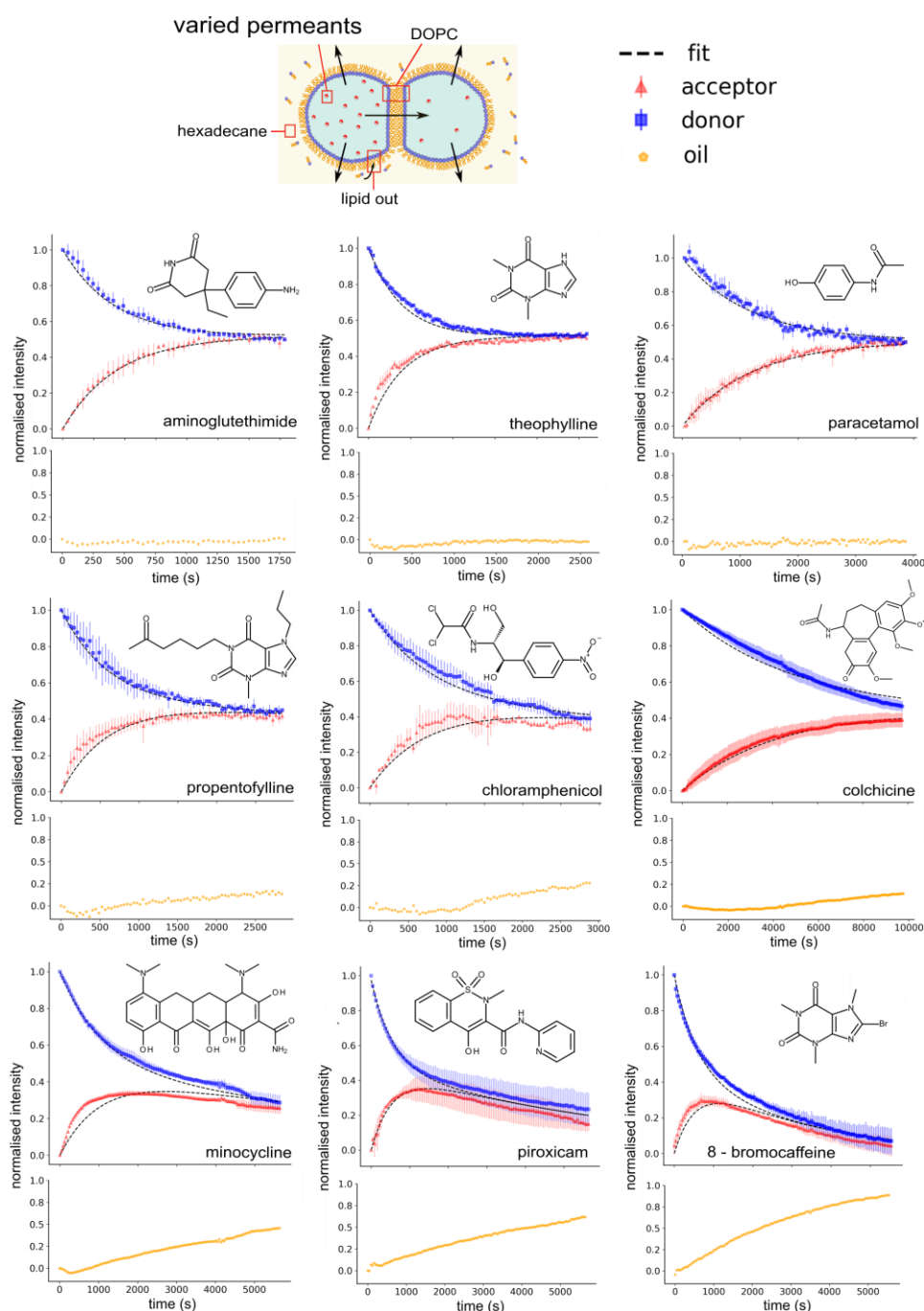


Figure 6.5: Partition behaviour across a range of drug structures. Compounds are arranged by their degree of partition behaviour. Compounds in the top row demonstrate negligible partition, in the middle row measurable partition and in the bottom row significant partition. Error bars in all plots represent the standard deviation. Orange plots indicate mass partition and were calculated with equation 22 from the acceptor and donor distributions of each compound.

The compounds in the middle and bottom rows of figure 6.5 showed measurable partition within the experimental window. In the middle row, propentofylline, chloramphenicol and

colchicine each showed a partition behaviour that was far slower than the measured permeability. The plateauing of the acceptor curve indicated bilayer flux equilibration which was followed by an exponential decay indicative of partition. For each compound, 10 - 15 % of the mass had partitioned from the aqueous phase at this point. In the single beam path chip, chloramphenicol did not indicate oil partition however its translocation distribution suggested the compound may retain within the bilayer. In the multi droplet method, chloramphenicol demonstrated a measurable partition behaviour. In tandem, the experiments with chloramphenicol suggest that the measured partition may be due to solubilisation into the bilayer. Colchicine was chosen in part due to its slow permeability ($5.5 \times 10^{-5} \text{ cm s}^{-1}$). With this method, partition could clearly be delineated across this longer time scale (10000 s), and colchicine had the smallest rate constant for oil partition.

In the bottom row of figure 6.5, minocycline, piroxicam and 8-bromocaffeine showed significant oil partition within the experimental window with around 20 – 30 % mass lost across the bilayer flux period. Due to the apparent difference in kinetics between the methods, it was difficult to decouple the exact mechanism of the partition behaviour. Minocycline showcased significant deviation from the fit in the acceptor distribution, potentially implicating significant lipid – permeant interactions. For piroxicam, a concentration inequality retained in the acceptor and donor droplets suggesting bilayer retention. This concurs with literature findings which measured a bilayer mass retention of ~23% for piroxicam in DOPC SLB bilayers.¹⁵³ The large difference in partition behaviour between theophylline, propentofylline and 8-bromocaffeine adds to the range of uncovered methylxanthine diffusive behaviours (section 4.4). 8-bromocaffeine has a lower lipophilicity than propentofylline, (AlogP: 0.88 and 1.38 respectively), however the rate constant for oil partition was around an order of magnitude greater (and 3.31 and $0.55 \times 10^{-5} \text{ s}^{-1}$ respectively).

6.4.2. Comparing permeability derived between methods

The determined permeability values for each of the small molecule drugs in figure 6.5 are presented in figure 6.6 a. All plots were fitted to the ODE model outlined in chapter 2, extracting the k_1 value (equations 34 and 35) with permeability values calculated with the DIB area and droplet volume (equation 11). This method yielded an inherently more rigorous permeability value, as the rate constants for oil partition (k_2) and bilayer flux (k_1) were decoupled.

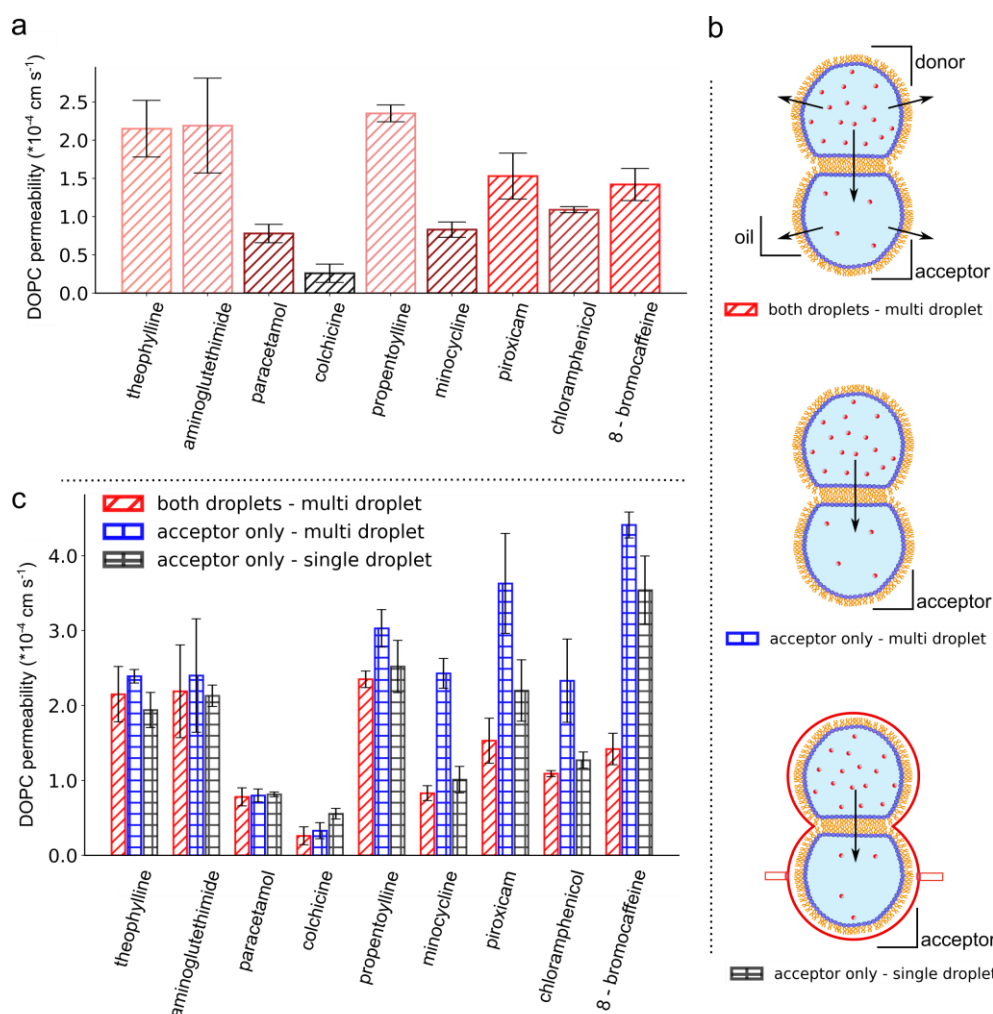


Figure 6.6: Permeability values compared across both multi droplet and single beam path methods. **a** Permeability values obtained from fitting equations 34 and 35 to each distribution in figure 6.5. Value calculated from a system of ODEs accounting for oil partition. **b** Cartoons of compared data collection and fitting methods, from top to bottom: fitting a system of ODEs (eqs 34 and 35) to acceptor and donor distributions obtained with multi droplet method, fitting an exponential profile to a normalised acceptor distribution (eq 19) obtained with multi droplet system and fitting an exponential profile to a quantitative measure of the concentration made with the single beam path chip (method outlined in chapters 3 and 4, normalised data included in supplementary figure 5). **c** The permeability value obtained for each compound, with each of the fitting and data collection methods outlined in b. Error bars represent the standard deviation ($n = \geq 3$ in all instances).

The permeability values in figure 6.6 are arranged from left to right by the magnitude of the k_2 partition value (theophylline, aminogluthethimide and paracetamol effectively 0). Here, propentofylline exhibited the fastest permeability ($2.35 \times 10^{-4} \text{ cm s}^{-1}$) which was in strong agreement with the value measured in the single beam path chip ($2.52 \times 10^{-4} \text{ cm s}^{-1}$). The slowest permeability was colchicine ($0.26 \times 10^{-4} \text{ cm s}^{-1}$) in reasonable agreement with the comparative single beam path chip value ($0.55 \times 10^{-4} \text{ cm s}^{-1}$). No statistical significance was observed between either of these sets. Care was taken when comparing permeability values between methods due to differences in fitting approach and DIB well structure. To better understand this comparison, each acceptor curve in figure 6.4 was normalised to the

apparent flux equilibration (for example 1100 s in 8-bromocaffeine). This distribution was then fitted to a typical exponential growth and the permeability extracted (all distributions showcased in the appendix). This simplified approach provided the optimal comparison between the single beam path and dual droplet methods. The difference in method and fitting approach are illustrated in figure 6.6 b with the full ODE model in red, the simplified approach in blue and the single beam path chip in black. The permeability values for each of these approaches are compared in figure 6.6 c.

For the compounds with negligible partition, the permeability values independent of measurement method (single beam path or multi droplet) and fitting approach (ODE model or exponential fit of acceptor) were highly similar. No statistical significance was observed between any pairing of permeability datasets for theophylline, aminoglutethimide and paracetamol.

The absolute and the percentage difference between the permeabilities extracted with the ODE model and the exponential fit of the acceptor (red vs blue figure 6.6) followed a negative correlation with the oil partition rate constant (figure 6.7 a). This relationship highlights a key oversight of DIB diffusional modelling which is not typically accounted for in the literature. If translocation curves are fit without quantifying permeant loss to the surroundings, this can lead to significant overestimation of the actual membrane permeability. This phenomenon was evidenced clearly for piroxicam and 8-bromocaffeine. In utilising DIBs as biophysical environments to assay fine permeant or membrane structural modifications, this oversight is particularly pertinent.^{202,117,182,65}

The difference in permeability value between the measurement methods appeared to positively correlate with the permeant MW (blue and black bars in figure 6.6 c, linear regression figure 6.7 b). This relationship should be considered in line with the partition bias in the multi droplet method. Colchicine, the slowest moving permeant, was identified as an outlier when comparing absolute difference, but not relative difference. Incidentally, the three non-partitioning permeants had the lowest MW and exhibited the least variation. The beam distance from the bilayer was 150 μm greater in the single beam path method. The accuracy of the permeability measurement was therefore bulk diffusion limited, where higher MW compounds introduced greater deviation. A further contributor to this difference may be due to the volume and bilayer area dependency on the size of the UWL.¹⁴⁶

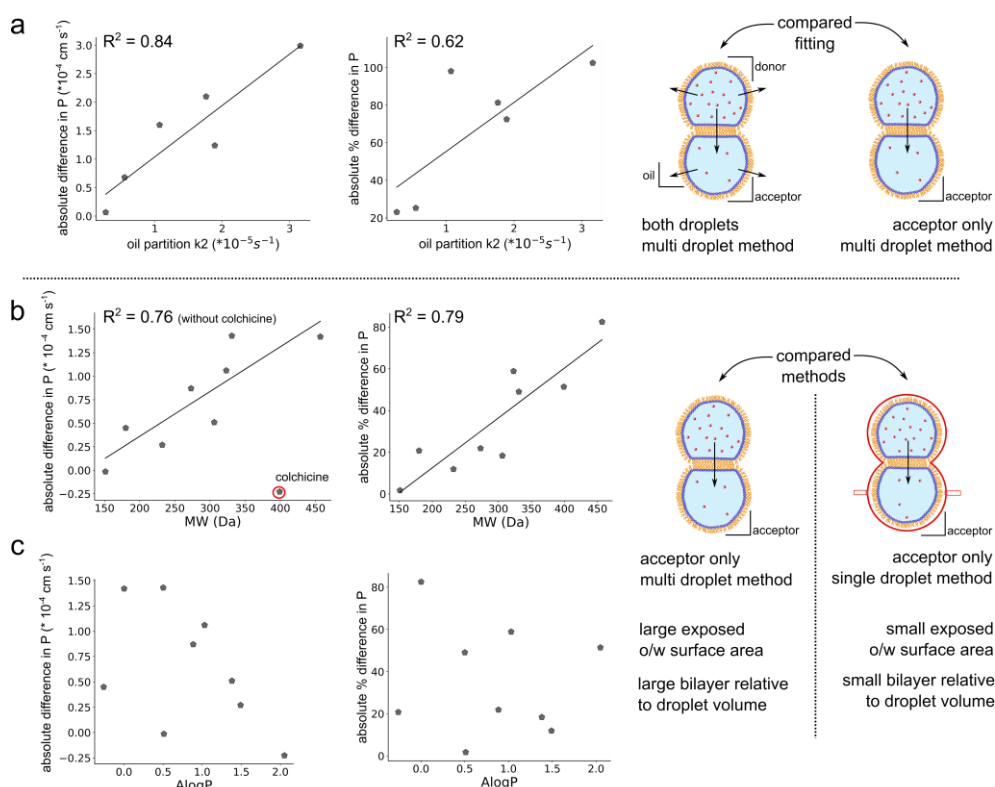


Figure 6.7: Uncovered trends across permeability systems and fitting approaches **a** Comparing the difference in permeability value between fitting approach with the measured oil partition for each of the six partitioning compounds. Permeability measured with multi droplet high throughput system. **b/c** Comparing the difference in permeability value between methods with the MW (**b**) and the AlogP (**c**). Concluded differences between systems indicated in cartoon inserts. Note that the linear regression for the ‘absolute difference’ plot in b was calculated without colchicine, an apparent outlier. All differences calculated with equations 34 / 35 and 19 for all compounds in figure 6.5. All linear least-squares regressions were performed with `scipy.stats.linregres` and R^2 values reported for each plot where appropriate.

For the partitioning compounds with faster permeability values (propentofylline, piroxicam and 8-bromocaffeine), there was a reasonable agreement between methods (blue and black). For the less permeable compounds (minocycline and chloramphenicol), there was an improved agreement between the ODE model and the single beam path chip (red and black). These correlations provide evidence toward the hypothesis of oil partition changing as a function of exposed w/o surface area. These trends also confirm that the distributions are attributable to partition and not photodegradation of the permeant. In the single beam path chip, the system was constantly irradiated whereas in the multi droplet method, each droplet was irradiated for 6 s per minute, yet partition was faster. Furthermore, the output power of the applied deuterium bulb was low (217 μW).

The accuracy of the measured permeability was therefore dependent on the absolute rate of permeability as well as the compounds MW, with more permeable compounds less sensitive to biased partition. Chloramphenicol and minocycline exhibited measurable partition in the

multi droplet method with minimal oil partition in the single beam path chip. For slower moving permeants, good agreement between the ODE model and the single beam path chip were obtained (colchicine, minocycline and chloramphenicol). This agreement was driven by two separate mechanisms – the permeability value obtained with the ODE model (red bar) was separated from the rate of partition, and in the single beam path chip (black bar) partition appeared to be attenuated by the DIB well geometry, both yielding similarly intrinsic measures of the bilayer permeability. The only outlier to this trend was 8-bromocaffeine, the most extreme example of partition in the drug set.

Across diverse small molecule drug structures, a range of diffusive properties persist. In comparing permeability values between systems, significant care must be taken with respect to the fitting approach and permeant oil partition. The utility of this model in determining simultaneous permeability and partition values, may enable enhanced modelling of drug retention, metabolism and crossing of the BBB.^{244,245}

6.5. The role of permeant chirality on translocation

Biological lipids are enantiomerically pure.¹⁵⁰ Avanti Polar Lipids supplied the DOPC used throughout this work. These stocks are synthesised from a biological, homochiral precursor, thus conferring enantiomeric purity of the biological (L / R) enantiomer. The next study carried out with this method was in part inspired by findings published in 2020 which suggest amino acid – like compounds demonstrate significant enantioselective permeability.¹⁸² In Hu et al., a DIB permeability measure was achieved through tagging permeants with an alkyne functional group. This tag enabled the formation of a fluorescent click chemistry product in the acceptor droplet. A difference in bilayer permeability between the biological (L) and abiological (D) enantiomer of up to 600 % was reported. Permeant dependent partition was not determined in Hu et al., and the permeability value was extracted through a typical exponential fit. Tagging is a significant concern with respect to experimental design across the biological sciences since the tag renders conclusions regarding the native structure unreliable.^{246,247} This viewpoint has been highlighted throughout this work, particularly with the diverse range of behaviours across subtle structural changes within the methylxanthine series. In light of the conclusions and limitations of Hu et al., chirality and its role in simple diffusion was investigated in both drug-like and biological molecules using the developed label free approach.

6.5.1. Amino acid permeability – systematic assessment of tagging and charge

Of the biological amino acids, only tryptophan and tyrosine could be screened with UV spectroscopy due to their aromatic functional groups. The translocation of native, untagged tryptophan and tyrosine were first assessed. All experiments were conducted at biological pH, capturing the zwitterionic nature of native amino acids. A flat intensity distribution was present in both acceptor and donor droplets of DIBs formed with a 10:1 concentration gradient. The L and D enantiomers of both tryptophan and tyrosine were bilayer impermeable (figure 6.8). The impermeability of amino acids across vesicle bilayers is well established, and these results agree strongly with such findings. ^{98,248}

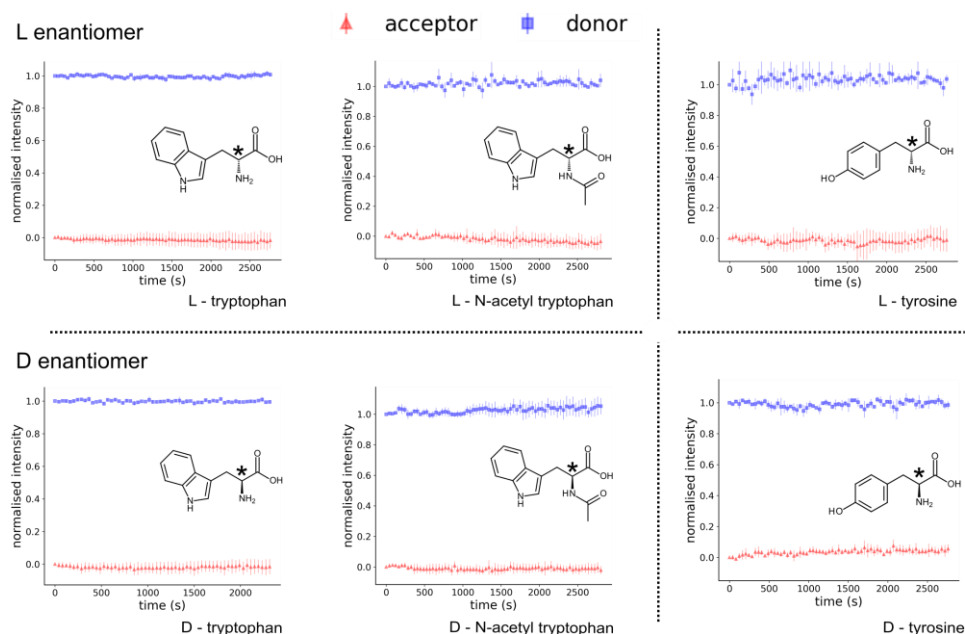


Figure 6.8: Tryptophan and tyrosine are DOPC bilayer impermeable. Impermeability was maintained upon acetylation of tryptophan, independent of enantiomer. Error bars in all plots represent the standard deviation, $n = 3$.

Next, the role of charge was investigated. The compound charge was modified by replacing either the carboxyl with a methyl ester (tryptophan methyl ester) or the amine with an acetyl group (N - acetyl tryptophan). If only the amine group was masked, the -ve compound remained impermeable, independent of enantiomer (figure 6.8). If only the carboxyl group was masked at the same functional group as in Hu Eet al., the +ve compound was highly permeable, shown in the intensity time graphs in figure 6.9 a. Depending on the molecular structure, markedly different translocation profiles were measured, evidencing how tagging strategies perturb the subtle relationship between permeant structure and bilayer permeability.

6.5.2. Chiral permeability – enantioselective diffusion of a tagged amino acid

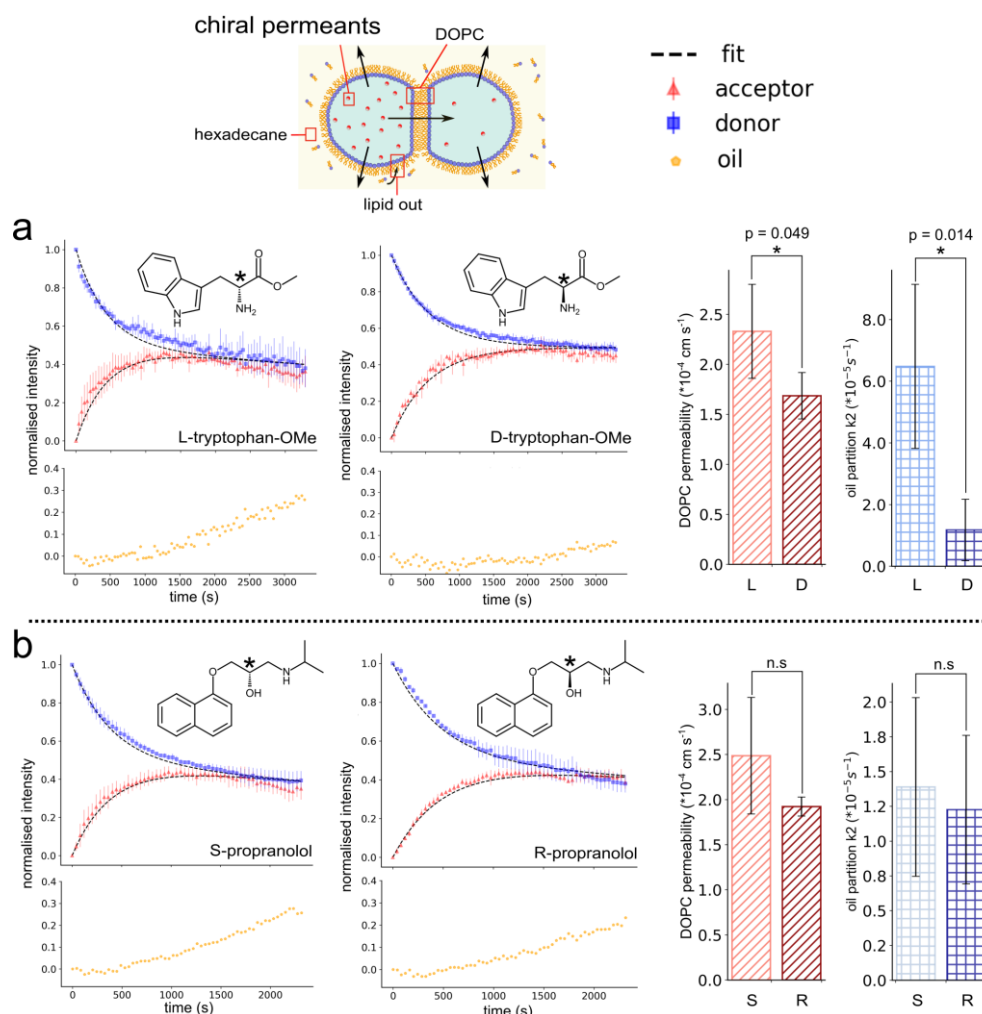


Figure 6.9: The permeability and oil partition for each enantiomer of two chiral permeants. **a** Translocation profiles for the L and D enantiomer of tryptophan methyl ester (n = 6 and 3 respectively). Profiles fit to multi compartment permeability model (equation 34 and 35). Bar charts show enantioselective diffusion for this permeant with statistically significant differences in the measured permeability and oil partition between each enantiomer. Cartoon at top shows experimental conditions. **b** Translocation profiles for the R and S enantiomer of propranolol (n = 3). Bar charts show no statistically significant difference in either measured property. Two tailed t test, n.s indicates p > 0.05. Error bars in all plots represent the standard deviation.

The translocation profiles for both enantiomers of tryptophan methyl ester indicated slight oil partition. There were statistically significant increases in the measured permeability and oil partition for the L as opposed to the D enantiomer of tryptophan methyl ester. The L tryptophan methyl ester DOPC permeability was 38 % faster and the rate of loss to surroundings 276 % faster than the D enantiomer (figure 6.9 a). As in the prior section, fitting the normalised acceptor curves to an exponential distribution, yielded a non-statistically significant increase in permeability of the L, as compared to the D enantiomer of 62 %. The L, biological enantiomer of a tagged amino acid enantioselectively diffused through DOPC bilayers of the R, biological enantiomer, which broadly replicated the findings of Hu et al. If

oil partition was not factored into the permeability measure, this led to a larger measure of the enantioselective diffusion suggesting that the measurements reported in Hu et al. may be significant overestimations. The impact of these findings is hard to quantify, particularly given the lack of such studies within the literature. Ishigami et al. suggest that L-amino acids can preferentially adsorb onto liposomal surfaces of the same chirality.²⁴⁹ Enantiomeric diffusion has been observed for some permeants across the PAMPA interface.²⁵⁰

It has been suggested that the size of functional groups at the chiral centre can affect the extent of enantiomeric lipid – permeant interactions.¹⁵⁰ Indeed, the magnitude of enantioselective diffusion positively correlated with larger R groups in Hu et al.¹⁸² Propranolol is an example of a well-studied commercial small molecule drug where the stereochemistry at the chiral centre is highly similar to tryptophan methyl ester. Passive diffusion is the dominant PM transport mechanism of propranolol.²⁵¹ Enantioselective diffusion of tagged amino acids has minimal direct physiological relevance however for propranolol this may have significant implications on metabolism, uptake and cell permeability. Reflecting biological studies, propranolol readily crossed DOPC bilayers (figure 6.9 b). The S enantiomer appeared to be slightly more permeable than the R enantiomer although this was not statistically significant. The rate of oil partition for both enantiomers was analogous. In tandem, this finding suggests that enantioselective diffusion should be quantified on a case-by-case basis and may not be a ubiquitous property of chiral permeants. Further understanding is required on how stereochemistry at the chiral centre affects this property.

A summary of tagging and enantioselective diffusion are provided in figure 6.10. The findings uncover the structural changes to a core amino acid which permit bilayer permeability. As highlighted across multiple sections of this thesis, there exists a subtle relationship between chemical structure and bilayer permeability which does not appear to correlate well with simple physicochemical descriptors. This particular study highlights this relationship in the context of tagging, where the physiological relevance of biological permeants can be lost if tagging is necessary to achieve an analytical readout. Native, zwitterionic amino acids were found to be bilayer impermeable, otherwise supported by similar literature experiments and the evolution of a human amino acid SLC transporter at the PM.²⁵² Here, a tag applied to the carboxyl group (as in Hu et al.) switched the permeability 'on'. These experiments highlight the oversight of conclusions on biological compounds as determined from tagged ones.

Lipid – permeant interactions appeared to be a significant driver of enantioselective diffusion. A differential response was also recorded in the oil partition across the w/o lipid monolayer interface, a significant novel result. Matching permeant and lipid chirality may yield

favourable interactions between chiral centres of the permeant and the lipid head group. In conclusion, such interactions in a DIB system, where the permeant is preferentially held close to lipid interfaces, may accelerate oil partition or bilayer crossing.²⁴⁹ Conclusions regarding the biological importance of enantioselective diffusion require significant scrutiny. To this end, label free methods which mitigate structure tagging should be deployed. Continued study of the subtleties between chemical structure and the permeability of model or real membranes could have largescale implications for biological experiments which are dependent upon interaction with lipid membranes.

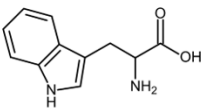
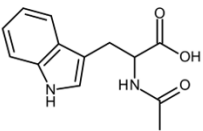
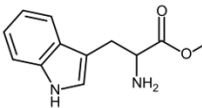
name	tryptophan	N-acetyl tryptophan	tryptophan methyl ester
charge (pH 7.4)	+/-	-	+
structure			
D enantiomer	impermeable	impermeable	$P = 1.69 \cdot 10^{-4} \text{ cm s}^{-1}$ oil partition $k_2 = 1.17 \cdot 10^{-5} \text{ s}^{-1}$
L enantiomer	impermeable	impermeable	$P = 2.33 \cdot 10^{-4} \text{ cm s}^{-1}$ oil partition $k_2 = 6.48 \cdot 10^{-5} \text{ s}^{-1}$

Figure 6.10: Summary of findings in this section, highlighting how subtle changes to the chemical structure can yield large differences in the measured permeability.

6.6. Oil partition – quantitative assessment

A range of oil partition values were measured across sections 6.4 and 6.5. For the ten compounds that demonstrated partition, the fitted k_2 values are presented in figure 6.11 a. Through fitting the ODE model, the partition and permeability could simultaneously be extracted from the translocation profile. This approach to modelling represents a considerable advance when compared to similar platforms which rebuild oil partition from separate experiments.^{76,153}

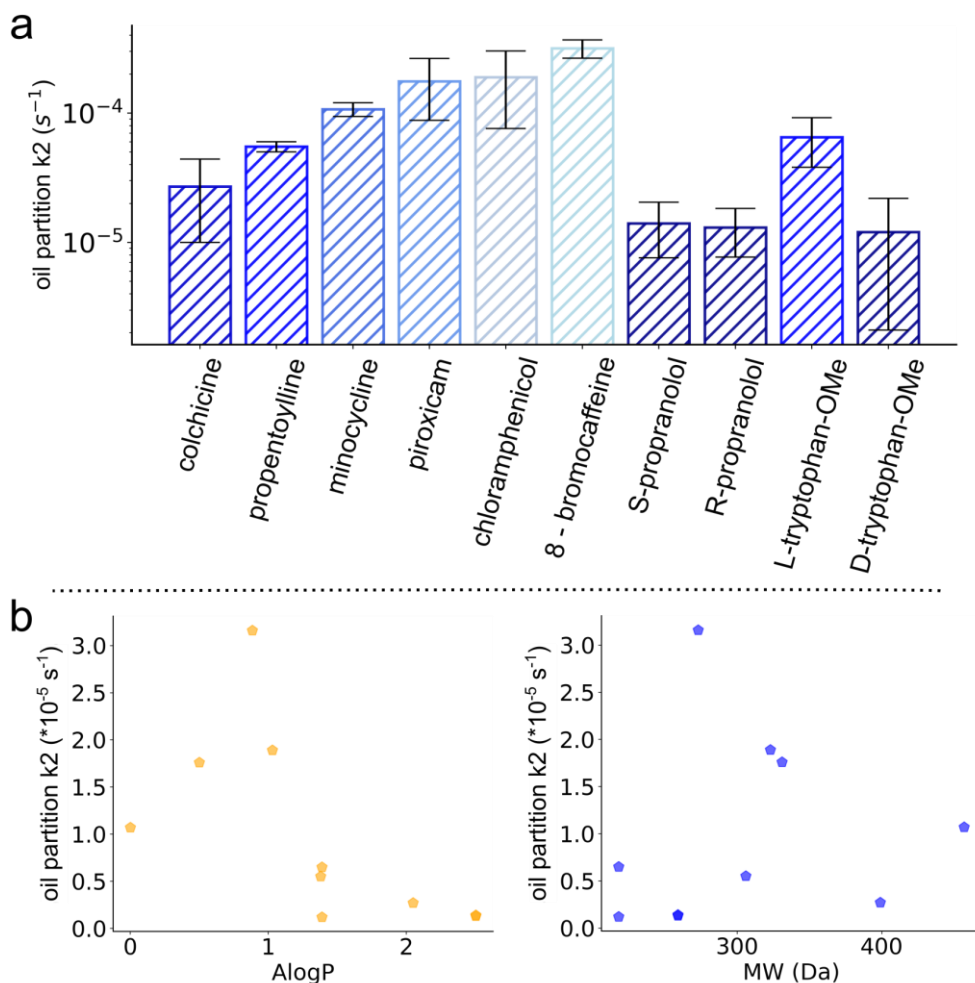


Figure 6.11: Oil partition across a wide physiochemical space. **a** Oil partition values obtained from fitting equation 34 and 35 to each distribution in figures 6.5 and 6.9. Value calculated from a system of ODEs. Error bars represent the standard deviation ($n \geq 3$ in all instances), log scale applied to y axis. **b** Oil partition plotted against AlogP and MW. Each plot demonstrates how oil partition did not appear to correlate across a wide physiochemical range.

A range of oil partition values were recorded, with the smallest and largest rate constants exhibited by the chiral permeants and 8-bromocaffeine respectively. This oil partition measure may act as a rough approximate of the PAMPA permeability allowing an *in-situ* comparison to the DIB permeability. As with similar PAMPA measures, the oil partition rate constant did not correlate with simple molecular descriptors (figure 6.11 b).^{205,253} No oil partition was measured for a compound with the hydrophilic classification of a negative partition coefficient. There also appeared to be no discernible relationship between the bilayer permeability and the rate of oil partition (k_1 / k_2) further implicating the poor biomimicry of the PAMPA interface.⁶⁵

As compared to the bilayer area, there was a larger w/o surface for permeant partition. Despite this, all k_1 values were at least double the respective measured k_2 values. In most instances this difference was at least an order of magnitude. Bilayer permeability was

therefore significantly faster than oil partition in every measured case. This observation contributes to suggestions that the PAMPA permeability is inherently slower than the DIB permeability.^{64,65,153} The hexadecane partition seen here, may be distinct from the comparative process in PAMPA. This may partly be explained by UWL effects and the lipid monolayer of emulsion droplets.^{148,153} As with permeability, understanding a relationship between oil partition and physiochemical metrics may support prediction of diffusive behaviour prior to assessment in DIBs.

6.7. Chapter conclusions

The utility of the multi droplet method is considerable, broadly capturing the benefits of fluorescence microscopy with a label free strategy. The findings in this chapter have relevance across a variety of fields and the translational potential of such an approach is significant. The full translocation profile for a range of small molecule drugs were assessed. Analysis yielded insight on the relationship between oil partition and translocation, finding negligible relation between these two properties. Partition was measured for 10 of the 19 screened permeants and 6 of the 19 were deemed impermeable (and did not partition). To better understand the limitations of the methods in chapters 3 and 5, comparison was drawn between the measured permeability values for 9 of the permeants. Oil partition can be affected by the surface area of the exposed w/o interface. The emergent view of this thesis is that the permeant chemical structure can drastically affect the translocation behaviour in DIBs. This viewpoint was highlighted in two studies. One, which uncovered the limitations of commonly deployed tagging strategies in the context of enantioselective diffusion and another, in which the diffusive behaviour of two structurally similar drug like molecules were characterised within DIB networks. The novelty and potential impact of these experiments highlight the deficiencies of PAMPA modelling which has failed to draw reliable conclusions regarding biological transport phenomena.^{48,254}

7 Mechanosensitive Channel Gating – A tool for controlling membrane permeability

7.1. Chapter overview

The work within this thesis has focused on passive transport through simple diffusion, with relatively minimal bilayers built from single or multiple fluid phase phospholipids. In this chapter, membranes of complex lipid and protein composition were developed. The membrane system leveraged asymmetric bilayer composition to control the net membrane permeability through the gating of a membrane protein. This unique mechanism was independent of the chemical concentration gradient across the membrane. To test this, the activity of the large mechanosensitive channel of conductance (MscL – G22C F93W) was studied in both DIB and vesicle experiments. In both cases, it was found that the gating of MscL, controlled membrane porosity and followed a concentration dependence with LysoPC asymmetrically distributed between membrane leaflets. Electrophysiological probing of the DIB environment revealed a tendency toward partial activation of the channel with full activation observed at high LysoPC concentration. A python analysis pipeline successfully uncovered a range of protein gating states which agreed strongly with literature findings. It was thought that LysoPC locally affected bilayer curvature, incurring a significant pressure differential across the membrane which met the channels activation energy. Results in this chapter were published in Chemical Science in 2021 and were performed in collaboration with Dr. James Hindley.

7.2. Mature synthetic bilayers with embedded flux control

7.2.1. The mechanosensitive channel of large conductance (MscL)

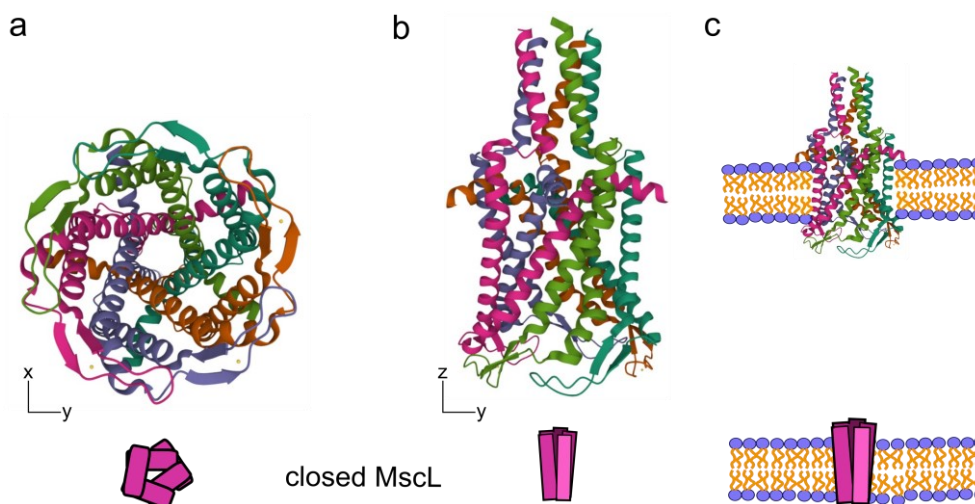


Figure 7.1: Structure of MscL. Periplasmic (a) and side on (b) view of MscL. c A view of membrane incorporation of MscL. In all sections, a structure of closed MscL from the protein data bank is shown with a cartoon alongside.

The mechanosensitive channel of large conductance (MscL) is a homopentameric membrane channel of bacterial origin (figure 7.1).²⁵⁵ Biologically, MscL acts as a release valve to prevent cell lysis under high osmotic pressures.²⁵⁶ Mechanistically, MscL responds to changes in the membrane tension. Depending on mutation and under various activation stimuli, MscL opens a membrane pore.²⁵⁷ This allows for the rapid expelling of cytoplasmic content, thus mitigating built up pressure. MscL can gate between its closed and fully open state, the non – selective 33 Å pore, through multiple conformational changes, sometimes referred to as ‘sub-conductance’ states.^{258,259} MscL has successfully been characterised in DIB and vesicle systems, where its activation has been well studied.^{122,260} Alpha haemolysin (αHL) pores have been widely reconstituted in DIBs to control membrane permeability.^{261,262,99,132} Unlike MscL, αHL pores are of a single conformer with a non-selective, 26 Å pore.¹³³

Much work has focused on loss / gain of function mutations of MscL to modulate its activity. The G22C and F93W loss of function mutations confer chemical activation and spectroscopic testing of MscL.²⁶³ One means of achieving MscL activation is through asymmetric lysophosphatidylcholines (LPC) incorporation. LPCs have significant positive curvature as they are derived from phosphatidylcholines with a singly cleaved fatty acid

group. Asymmetric membrane incorporation of these cone shaped, non-bilayer forming lipids thus gives rise to a significant curvature and membrane tension.^{264,265}

7.2.2. Overview – controlling membrane porosity with bilayer composition

Controlling and adjusting membrane permeability is a key element of *de novo* synthetic biology systems.^{266,267} Gaining significant control on the membranes permeability profile has potential in emergent synthetic membrane platforms such as droplet networks,²³⁹ designer tissues,²⁶⁸ and compartmentalised biological reactions.²⁶⁹ As shown in chapters 4 and 6, passive diffusion through lipid bilayers confers a limited permeant selectivity. A wide membrane permeability profile can be achieved with membrane proteins that lack solute specificity.^{123,132}

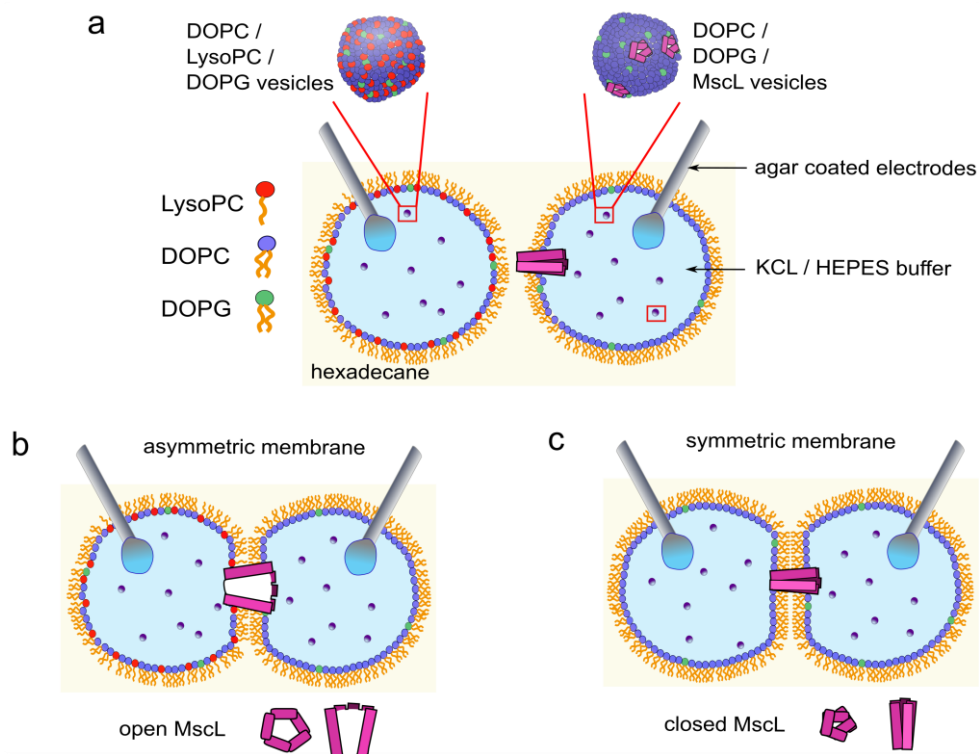


Figure 7.2: Activation of MscL with asymmetric DIBs – an electrophysiology approach. **a** DIBs were formed with two aqueous droplets suspended on agar coated electrodes. Each droplet was stabilised at the w/o interface with the lipid in technique, with one droplet containing reconstituted MscL protein. **b** Electrophysiology revealed that if bilayers were formed with an asymmetric concentration of 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (LysoPC - the specific LPC used in this work), MscL was activated. **c** If bilayers were formed with a symmetric distribution of lipids in the absence of LysoPC, MscL remained inactivated.

Asymmetric LPC mediated MscL activation has shown a concentration dependent activity in vesicles.²⁶⁰ Uniquely, this particular activation mechanism exploits the local MscL lipid environment and is therefore independent of the chemical concentration gradient across the

membrane. It has been shown in the prior four chapters how DIBs have significant potential as environments for membrane permeability assessment. The translation of this MscL activation mechanism to DIB, would allow for a membrane where the bulk permeability can be controlled. Coupled with this, an engineering consideration is how the intrinsic membrane environment of DIBs compared to vesicles, affects the bulk physical properties. Figure 7.2 summarises how a lipid in strategy successfully facilitated asymmetric LysoPC mediated MscL gating in DIBs.

7.2.3. Protein expression

MscL expression and characterisation were performed by Dr. James Hindley. In short, MscL (carrying G22C and F93W mutations) was expressed in *E. Coli* cells. MscL was isolated through cell lysis, centrifugation and re-solubilisation into DOPC: DOPG vesicles. All vesicle solutions were purified through size exclusion chromatography and stored on dry ice. To maintain protein activity, all experiments were performed within a day of sample prep. Further detail on this aspect of this chapter is included in the publication of this work.¹⁶³

7.3. Assessment of LysoPC mediated MscL activation - a vesicle assay

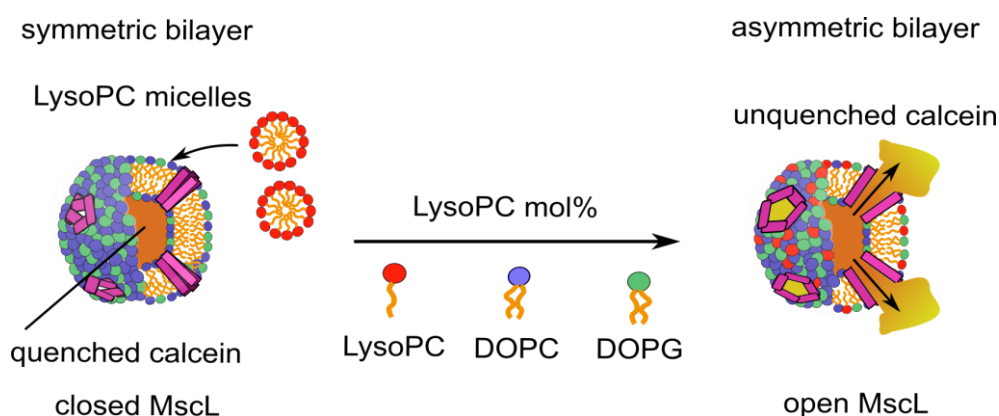


Figure 7.3: Overview of vesicular system for LysoPC mediated MscL activation.

To confirm MscL activity, a system first reported in the literature was replicated (described in figure 7.3).²⁶⁰ 1:1 DOPC: DOPG, 50:000:1 MscL: lipid molar ratio vesicles prepared with 50 mM of encapsulated, quenched calcein. Higher membrane charges have been implicated in MscL incorporation and activity.²⁷⁰ LysoPC micelles were added to the vesicle solution and fused with the outer membrane leaflet of the vesicles. A concentration gradient of LysoPC was applied and assessed as a percentage of the final lipid concentration. The MscL activity

was determined through the resultant calcein release. The bulk solution fluorescence was measured with fluorescence spectroscopy for 5 hours. All experiments were performed in triplicate.

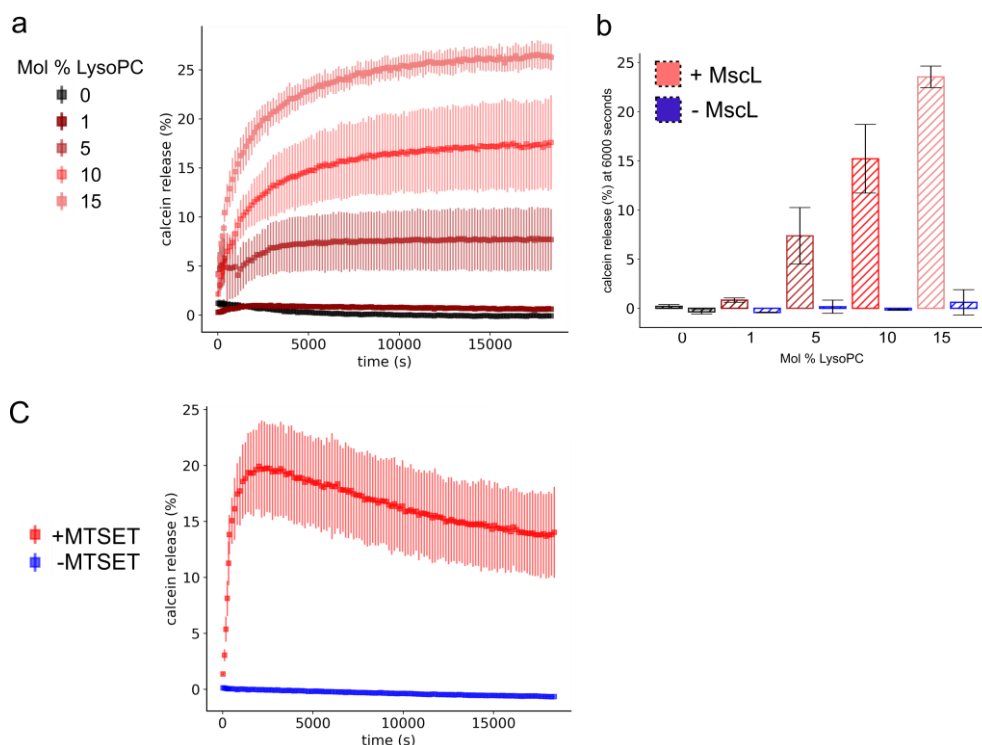


Figure 7.4: Results from vesicle assays of MscL. **a** Calcein flux from vesicles with varied applied molar % of LysoPC. **b** If the experiment in b was repeated in the absence of MscL, negligible calcein flux was recorded. This confirmed that calcein flux was due to LysoPC mediated activation of MscL. **c** Chemical activation of MscL with MTSET.

The activation of MscL, was confirmed through exponential rises in the bulk solution fluorescence (figure 7.4 a). As the quenched calcein diffused out of the MscL pore, it became unquenched. The equilibration time and the fluorescence peak intensity were dependent upon LysoPC concentration between 1 and 15 %. Peak intensity appeared to show a linear correlation. If the same concentration of LysoPC micelles were added to vesicles of the same lipid composition without MscL, negligible changes in fluorescence were recorded (figure 7.4 b). This was observed across the full LysoPC concentration range from 0 – 15 %. In tandem, these results indicated that a proportion of the vesicle population had been activated by the LysoPC and that the MscL activity threshold was between 1 – 5 % LysoPC. Previous work has estimated this threshold as less than 10 %.²⁶⁰ Finally, the baseline channel activity was confirmed with [2-(Trimethylammonium)ethyl]methanethiosulfate (MTSET – 1 mM). As with LysoPC, MscL was clearly activated by MTSET (figure 7.4 c).

7.4. Mechanosensitive channel sensing – a DIB assay

7.4.1. System controls

To translate LysoPC mediated MscL activity into DIBs, each droplet contained a separate vesicle composition which, through the lipid in approach allowed for the formation of symmetric and asymmetric bilayers. DOPG has been incorporated up to 25 mol % in DIBs however is known to invoke significant instability at high concentrations.¹¹⁰ As compared to the vesicle system, a reduced amount of DOPG was used - DOPC : DOPG 95: 5 molar ratio. It was anticipated that DIB stability and thus the Cl⁻ permeability may be affected by LysoPC and protein content. Figure 7.5 shows the Cl⁻ permeability of MscL-free LysoPC containing bilayers.

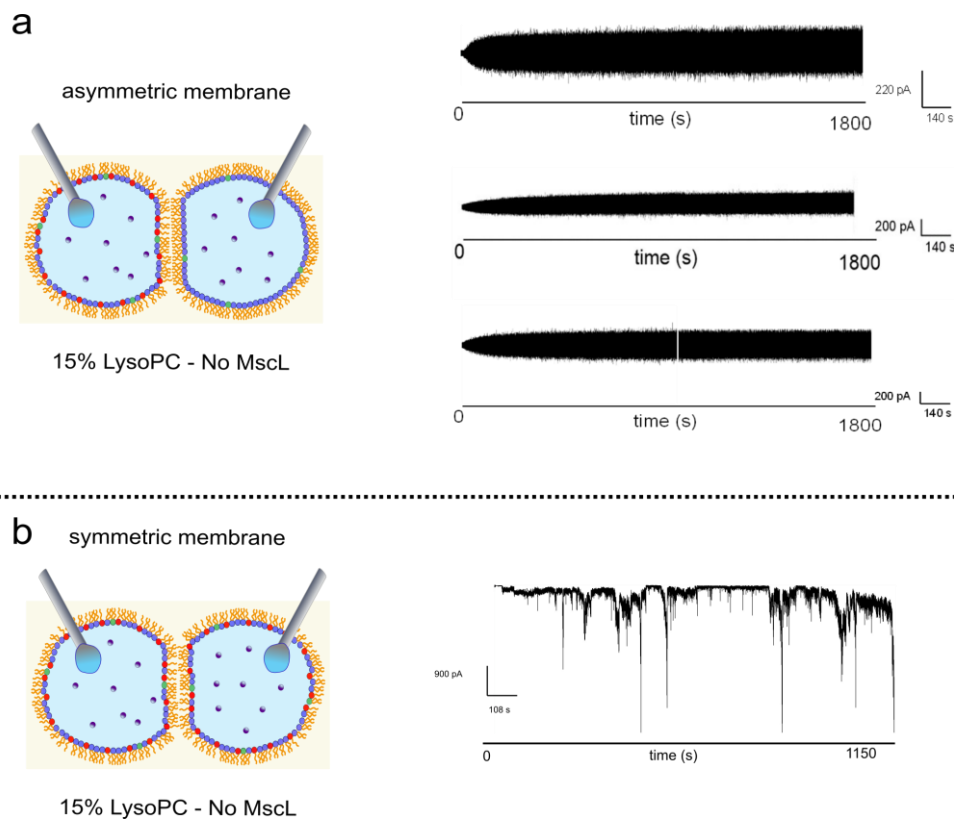


Figure 7.5: Control systems for the role of LysoPC in bilayer response. **a** Probing the role of asymmetry on bilayer porosity. Traces for bilayers formed with asymmetrically distributed LysoPC, without MscL. The traces indicated that these bilayers remained Cl⁻ impermeable. **b** Symmetrically distributed LysoPC resulted in a highly porous and unstable bilayer. Trace represents single stable DIB captured from at least 8 attempts. All DIBs coalesced.

As introduced in chapter 1, tension plays a central role in DIB formation. Bilayer tension is balanced by the monolayer surface tensions and droplet volume.¹¹³ This is typically measured from droplet contact angle with optical microscopy. A symmetric monolayer composition dictates a simple determination of the bilayer tension under the assumption of a

planar bilayer. However, at the global and local level, bilayer tension can be affected by asymmetric lipid composition.¹¹⁰ At high membrane concentrations, LPC's can act like a detergent and cause significant instability.²⁷¹ Significant positive curvature can result in membrane destabilisation, where pore formation can alleviate some of this stress, typically toward membrane failure.²⁷² In building a DIB based LysoPC activation system, a trade-off existed between DIB stability and capturing significant bilayer tension to maximise MscL activity. Tension is negligible in vesicle systems and so this issue was not present.

Asymmetric 15% LysoPC DIBs without MscL were stable. Upon droplet contact, all traces 'thickened' within 2 minutes, which was attributed to DIB zip up.¹²⁵ Interestingly, this was on a similar time scale to estimation of the DIB zip up made in chapter 3. All traces showed the typical electrophysiological response of a Cl^- impermeable lipid bilayer (figure 7.5) with no deviation from the baseline. These results mirrored those obtained in vesicle in the absence of MscL, at which 15% LysoPC without MscL resulted in no calcein flux (figure 7.4). The results of symmetrically distributed LysoPC in DIBs indicated significant bilayer instability. When attempting to form DIBs with 15 % LysoPC in either leaflet, 7 / 8 formation attempts fused immediately. The one DIB which formed, shown in figure 7.5 b, indicated a highly porous and unstable bilayer with massive noise and ion flux. Therefore, independent of the amount of absolute LysoPC percentage in the bilayer, it was concluded that symmetrically distributed LysoPC resulted in significant membrane stress and inherent ion permeability, thus unsuitable to assess MscL activity.

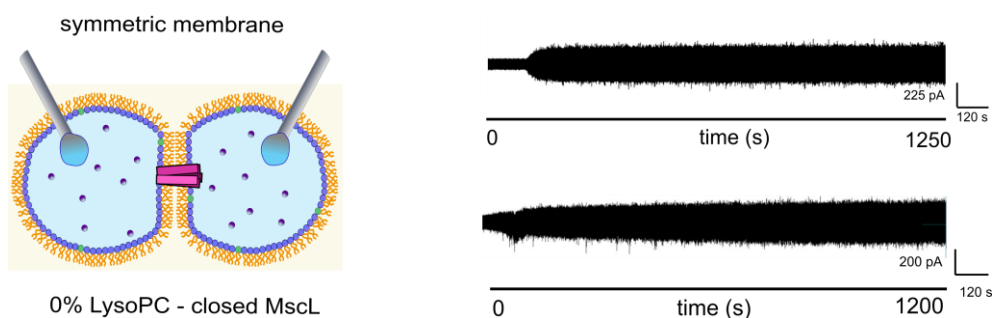


Figure 7.6: Control experiments for baseline MscL activation in symmetric membranes. MscL was inactive in a 0 % LysoPC membrane.

Finally, a protein reconstituted membrane system in which the local and global tension were controlled through monolayer symmetry was studied (figure 7.6). Here, MscL was incorporated into DIBs formed with symmetric DOPC: DOPG monolayers. The derived traces mirrored those from figure 7.5 a, which indicated that the membrane remained Cl^- ion impermeable. The surface tension in DIBs is on the order of $1 - 2 \text{ mN/m}$ ¹¹⁰ and a best estimate for the full gating MscL activation threshold is $(12 - 14 \text{ Mn /m})$.²⁷³ Therefore as

confirmed with this control, MscL remained inactive in a symmetric membrane and the baseline DIB tension was insufficient to gate the protein. These results are in strong agreement with those in Haylock et al., where MscL remained inactive in a symmetric membrane formed without a chemical activator (MTSET).¹²⁵

7.4.2. Concentration dependent LysoPC mediated MscL activation in DIBs

The activation of MscL was assessed in DIBs by forming asymmetric membranes with MscL and either 5, 10 or 15% LysoPC in one droplet monolayer (figure 7.7). A stark qualitative difference was found in the control and experimental traces (comparing figures 7.5 and 7.6 with figure 7.7). Large step changes in the current indicated significant membrane permeability of Cl⁻, yielded from conduction at the electrodes. At 15 % asymmetric LysoPC, significant MscL gating was clearly observed by the frequent step changes in the traces (figure 7.7 c). One gating event was found in the 5% LysoPC dataset and it was concluded that this condition failed to reliably gate MscL (figure 7.7 a). The unfiltered 10% LysoPC traces appeared to contain minimal gating events (figure 7.7 b). Upon filtering the traces, gating events were found within the dataset. The most apparent gating occurred in the 15% LysoPC dataset (figure 7.7 c), indicated by multiple conductance bands in each trace. Three biological replicates of this condition were performed, with gating observed in all experiments. These results showed that LysoPC mediated MscL activation was successfully performed in asymmetric DIBs, a significant result of this work. The concentration dependence of LysoPC on MscL activity also translated. The LysoPC MscL activation threshold changed from 1 – 5 to 5 – 10% from vesicle to DIB membranes respectively.

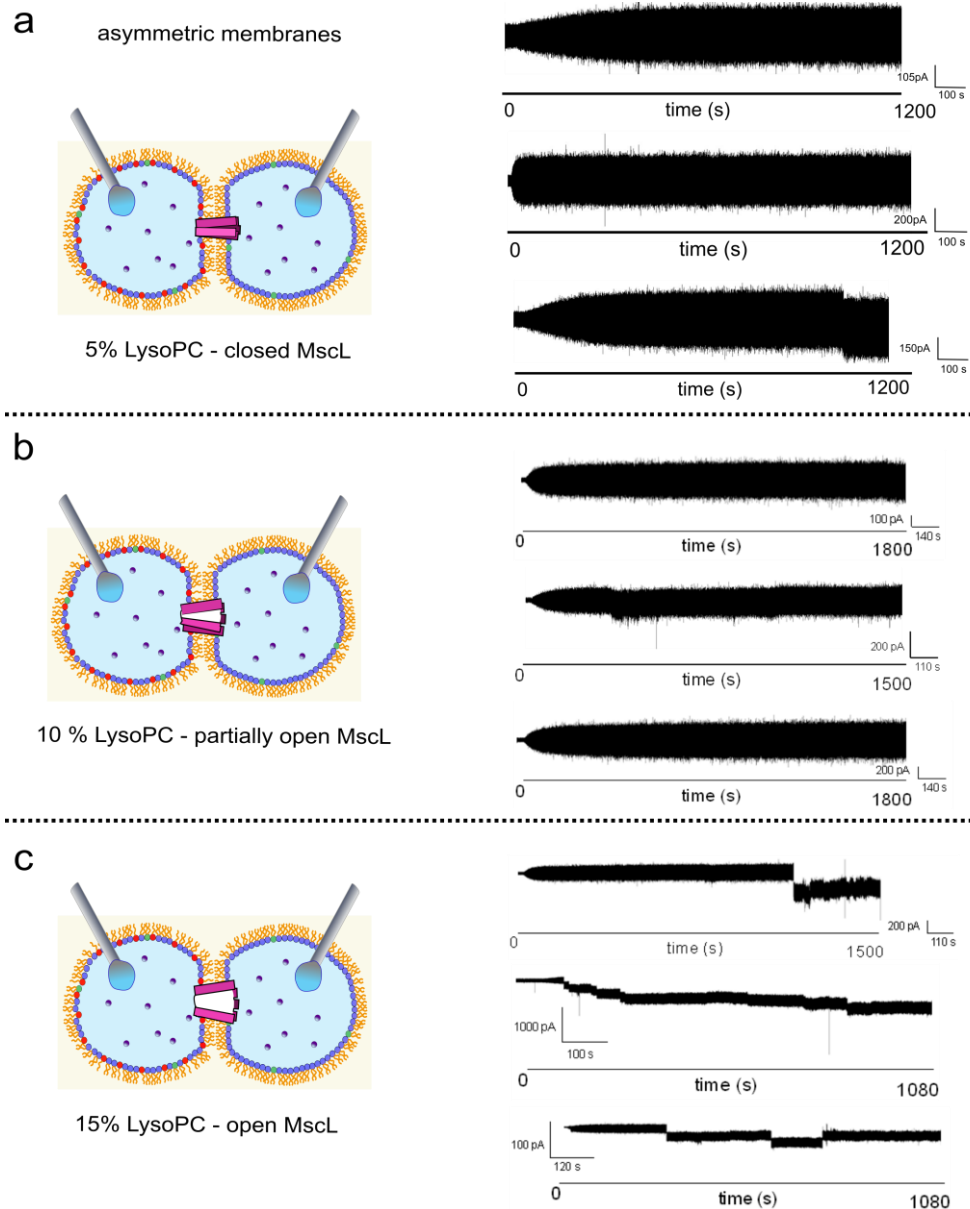


Figure 7.7: Increasing activation of MscL with increasing asymmetric LysoPC content. **a** Traces for asymmetric membranes formed with 5% LysoPC. A single gating event was recorded. **b** Traces for asymmetric membranes formed with 10% LysoPC. Multiple smaller gating events were recorded, discussed in next section. **c** Traces for asymmetric membranes formed with 15 % LysoPC. Multiple large gating events were readily observed in the unfiltered raw traces which highlighted clear MscL activation.

7.4.3. Coding pipeline to assess gating events – example of gating analysis

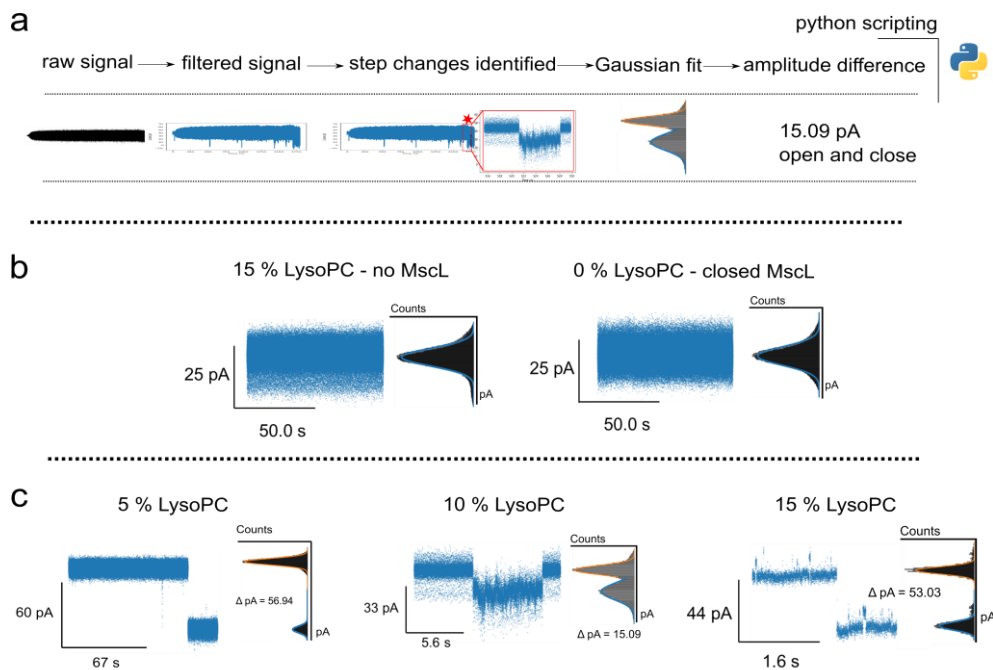


Figure 7.8: Analysing MscL gating activity. **a** A pipeline of the analytical procedure for each trace. A custom script was written to perform the analysis for each step change within the same analytical environment. **b** Example of trace analysis in control experiments, for each analysis, left shows analysed section of trace, right shows fitted Gaussian distribution. Across each control trace, no gating events were found. **c** Example MscL gatings found for each LysoPC condition, here showcasing clear step changes in the trace, with the difference in fitted amplitude shown for each. Note, that the single gating shown for 5% LysoPC was the only recorded gating at this condition.

It is thought that sub-conductance states can be stabilised by high potentials, where here a -100 mV potential was applied.²⁷⁴ Changes in conductance are proportional to the size of channel, where each step change within the recorded traces could be analysed to ascertain the MscL conformer associated with each gating activity. Multiple analytical software packages exist for trace analysis within the field, with Clampfit from Molecular Devices a well-regarded electrophysiology standard.^{275,276} Figure 7.8 a showcases a simple analytical pipeline for assessing the magnitude of a step change within a trace. This analytical pipeline is typical of the field. When traces were initially passed through Clampfit software, multiple gating events were found. It took around 10 minutes utilising Clampfit to analyse an isolated gating event in the trace and to export the distribution for advanced fitting in Origin.

Due to the volume of gating events and simplicity of the analysis, a python pipeline was designed which reduced analysis time to around 2 s per gating event once a step had been located within a trace. Data was loaded into python with the pyABF module.²⁷⁷ The traces were filtered with a low pass Chebyshev filter and reduced from 50 to 2.5 kHz. The trace

was split into sections and the difference between sections assessed. This allowed for an initial assessment of the indices of gating events before further manually scanning each trace. Upon identification of a gating event, a section either side was selected which provided sufficient datapoints for analysis (blue lines in figure 7.8 b and c). The datapoints within this section were converted into a histogram which was then fitted to Gaussian distributions, where each distribution accounted for the mean conductance before and after the MscL gating. Depending on the size of the analysed section, the histogram binning was adjusted to maximise the accuracy of the Gaussian fits. The difference in Gaussian amplitude (ΔpA) thus represented the difference in mean conductance, either side of the step change.

7.4.4. Partial activation

When analysed through the pipeline, all graphs and fitting results were returned immediately for each gating event. Across the experimental dataset, 47 gating events were analysed and classified by conductance. To aid discussion of the results within this chapter, the activity of MscL has been split into partial and full gating events.

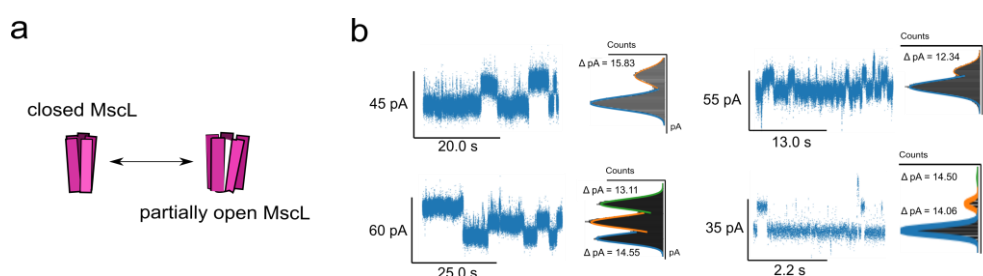


Figure 7.9: Flickering activity of MscL between the closed and smallest gating state at a consistent resolution. **a** Cartoon showing the rapid opening and closing of MscL. **b** Example traces found in the 10 and 15% LysoPC conditions of rapid MscL gating with the trace characteristically returning to the same 'level'. The top layer shows single gatings, as indicated by two Gaussians and the lower shows at least two gatings – as indicated by three Gaussians.

The smallest recorded gating was 14.6 ± 0.26 pA and was observed in both 10 and 15% LysoPC datasets (0.15 nS) (figure 7.8 c and figure 7.9 b). Some gating events at this level were classified as examples of 'flickering' activity where MscL appeared to rapidly open and close, as indicated by the trace returning to the same 'level', indicating a repetitious process (figure 7.9). All 'flickering' behaviour was within the resolution of this gating – which indicated that the activity was confined to a single conformer. Interestingly, this 'flickering' behaviour was also observed with three 'levels', which indicated co-operativity in MscL activity. Co-operativity was also evidenced by gating events which were amalgams of two levels. This is

best shown in figure 7.8 c – 15% LysoPC where ‘flickering’ behaviour either side of the major gating accompanied the ~40 pA gating, giving an apparent 53.33 pA transition.

The 0.15 nS gating level has not been observed in similar patch clamp LysoPC MscL (WT) electrophysiology systems.²⁷³ These results led to the notion that this gating may represent the resolution of minor conformational changes within the protein itself independent of actual conformer. The abundance of this level may suggest that individually reconstituted MscL proteins are only partially stimulated. Their activation may be in part due to rapid lateral diffusion of LysoPC within the incorporated leaflet where built up local curvature partially activated the protein. The identification of this sub conductance state may be at least partially explained by the high applied voltage (-100 mV), differences between MscL mutant and the inherent bilayer tension in DIBs compared to vesicles.

Δ pA (python pipeline)	Δ pA (Clampex software)
14.5 ($\pm$ 0.26)	14.9 ($\pm$ 0.28)
21.3 ($\pm$ 0.54)	21.9 ($\pm$ 0.65)

Table 7.1: Comparison of analytical process performed with commercial Clampex software. n = 8 and 4 for the ~14.9, 21.9 states respectively, dataset from samples within the 10 and 15 % LysoPC datasets. Error indicated by the SDOM.

The first two gating states (21.2 pA level introduced in next section) were used to compare the python pipeline to the same analysis performed with Clampex. For a range of gating events, the entire analytical procedure was repeated. Table 7.1 shows that minimal variation existed between the two approaches, with discrepancies attributed to slight variation in histogram binning. In tandem, this ratified the enhanced python based analytical procedure.

These first two gating events have previously been observed in DIBs with MTSET mediated MscL activation.¹²⁵ In another similar DIB system, calcein was seen to permeate the membrane through MscL.¹⁴⁹ In tandem with the results in this section, this indicates that the molecular weight cut off (MWCO) for these two opening states is likely at least <623 Da (the MW of calcein).

7.4.5. Full activation

Within the 15% LysoPC dataset, multiple large gating events were present (figure 7.7 c). A total of 7 gating states were derived based on statistical significance between Δ pA clustering's ($p < 0.001$). Following the 0.15 nS level, the next 6 states are summarised in figure 7.10 with an example of each gating included. Table 7.2 tabulates the conductivity of each determined gating state. Strong agreement was observed with similar literature assessments of the conductivity of each conformer.

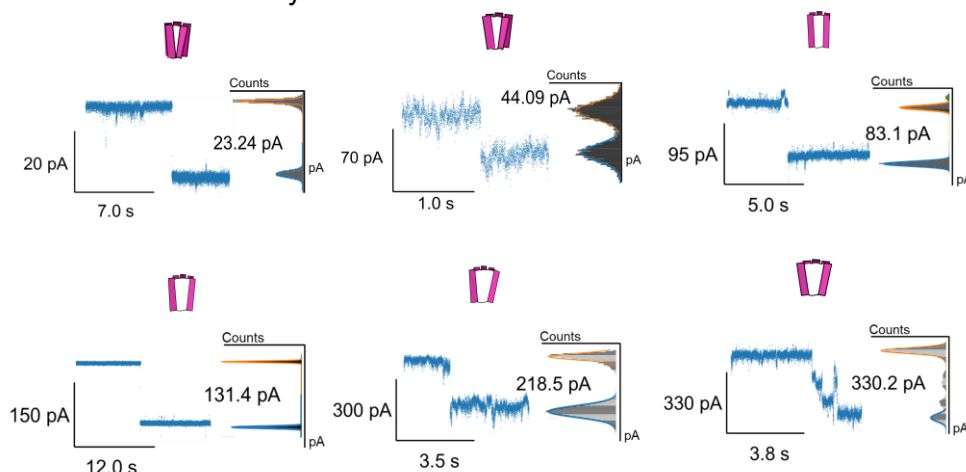


Figure 7.10: Gating of MscL from partial to fully open states. Example gating analyses for each opening state recorded in this work. The full opening event shows MscL passing through conformers. Note the 'flickering' behaviour present in the 83.1 pA example.

The conductance of the fully opened state of MscL has previously been reported as 3.13 nS,²⁵⁹ 3.5 – 3.8 nS and,¹²² ~2.8 nS.²⁷⁸ The largest gating event within the 15% LysoPC dataset was 3.23 nS, which due to the strong agreement with these three independent studies was deemed as the fully open state of MscL. Furthermore, it was observed that this gating often passed through sub conductance states toward full opening (figure 7.10). These sub states also agreed with the prior assessed levels, indicating distinct conformations between closed and open states. In prior DIB based MscL activation, full openings were rare.^{122,125} The potential of LysoPC mediated MscL activation to sustain sub-conductance states could prove useful for the study of these conformers and for dynamic control of the membrane permeability profile.

Δ pA	nS	n	SE (%)	% of open state	% of open state (^{259,274})
14.6	0.15	27	2.15	4.5	n/a
21.2	0.21	5	3.69	6.6	7
42.3	0.42	3	3.23	13	12
84.3	0.84	3	2.13	26	29
135	1.35	3	5.79	42	50
252	2.52	3	5.84	78	68 / 87
323 (open)	3.23	3	1.14	100	100

Table 7.2: All measured MscL gatings within this work compared to literature findings from a Makov analysis. Red indicates gatings in the 10 and 15% LysoPC datasets and blue indicates gatings in the 15% LysoPC dataset only.

Finally, the concentration dependence of the system was illustrated through summary of the found gating events in 10 and 15% LysoPC conditions in table 7.2. The smallest two gating events were found in the 10% LysoPC traces and examples – which indicated that the full opening threshold was not met. Each of the conformational states of MscL (between closed and opened states) were recorded within the 15% LysoPC traces. Due to the sustained activity of gating across all experimental timescales, the kinetics of LysoPC flip flop appeared negligible.

7.4.6. DSA findings

Having ascertained MscL activity, a brief investigation into the underpinning biophysics was performed. This was particularly pertinent to facilitate a narrative regarding translation of the activation mechanism from vesicle to DIB. The bilayer tension asymmetry was quantified with droplet shape analysis (DSA). Here, lipid monolayers were formed in a pendant drop tensiometer under similar conditions as in the electrophysiology platform. MscL free vesicles were used with lipid in. After 5 minutes of incubation, an image of the droplet was captured, and the IFT measured with OpenDrop software.

composition (DOPC:DOPG:LysoPC) (n)	IFT (mN/m)	SD (mN/m)	average volume (mm ³)	average Worthington (W ₀)
95:5:0 (6) – 0% LysoPC	1.83	0.23	0.37	0.28
90:5:5 (4) – 5% LysoPC	1.56	0.31	0.34	0.52
85:5:10 (6) – 10% LysoPC	1.20	0.51	0.57	0.43
80:5:1 (8) – 15% LysoPC	1.09	0.71	0.17	0.29

Table 7.3: DSA measured IFT values for LysoPC stabilised droplet monolayers.

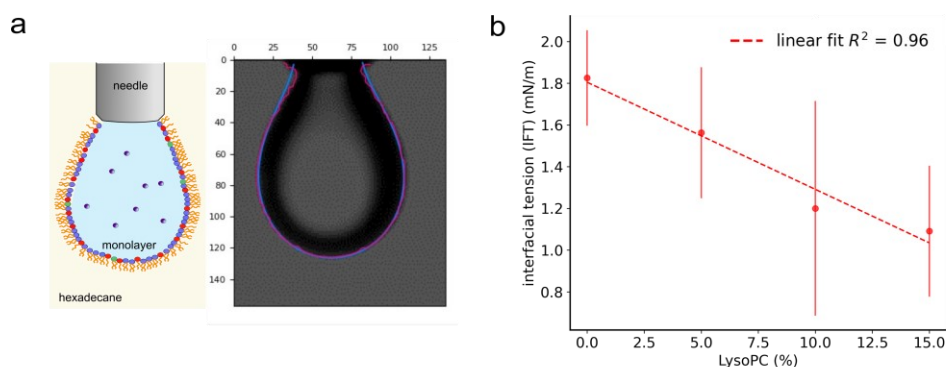


Figure 7.11: DSA results of LysoPC compositions. **a** Cartoon and photograph of a DSA analysis. A lipid monolayer is formed at the w/o interface, here performed with a lipid in approach. A fitting algorithm returns the droplet shape profile, from which the IFT can be calculated. **b** A plot showcasing the trend presented in table 7.2 – an apparent negative linear correlation in IFT as a function of increasing LysoPC in the monolayer.

Table 7.2 and figure 7.11 b show a negative, apparent linear correlation between increasing LysoPC content in the monolayer and the measured droplet IFT. This was expected as LysoPC acts like a detergent within the monolayer due to a positive curvature contribution. Similar trends have been observed with phosphatidic acid stabilised droplets.²⁷⁹ As the IFT difference between 15 and 0% LysoPC was on the order of 1 mN / m – it was concluded that local curvature must play a significant role in this activation mechanism. These results therefore indicate that local bilayer tension asymmetry is key to surpassing the MscL activation threshold in DIBs (12 – 14 mN / m).²⁷³

Indeed, this was further supported through the observation of concentration dependence across both DIB and vesicle systems. In DIBs, this concentration dependence may be due to lateral diffusion of the LysoPC concentration as the LysoPC was dispersed within the monolayer. Thus, for higher concentrations there was an increased likelihood of local curvature, affecting the lateral pressure profile and reaching the activation threshold.²⁶⁵ This would account for the response difference between 10% LysoPC and 15% LysoPC DIB systems. Alternatively, LysoPC may have partitioned from the monolayer into the hexadecane at a faster rate than the other lipids, leading to a lower monolayer concentration than expected and thus the apparent reduced activity for 5 and 10% LysoPC in DIBs.

It is difficult to compare the MscL gating activity between vesicle and DIB systems, as analysed by these methods. It has been inferred that MscL is fully open between 20 – 30 LPC% in vesicles.²⁶⁰ A sweep of experiments testing the release of larger solutes (>kDa) would allow for a rough assessment of the MWCO of the vesicle membrane and thus the stabilisation of MscL sub-conducting states. Direct comparison is further complicated due to

the electrostatics present in the electrophysiology DIB experiments. Electrostatics can promote the potentiality of sub conductance states.²⁷⁰ In the vesicle system, the concentration dependence was owed to a finite amount of fusogenic LysoPC micelles, thus activating a proportion of the vesicle population. As the LysoPC micelles fused into a DOPC: DOPG vesicle membrane, this may have led to a region of immediately high local curvature. This would have activated MscL rapidly - which was indicated by the high rate of initial fluorescence increase; however, activity would reduce as a function of LysoPC lateral diffusion – which may describe the concentration dependence in equilibration point.

7.4.7. The potential utility of LysoPC mediated MscL activation in DIBs

The results within this chapter have successfully shown that protein activation studies derived from vesicles can be translated into a DIB system. A conclusion of the work is showcased in figure 7.12.

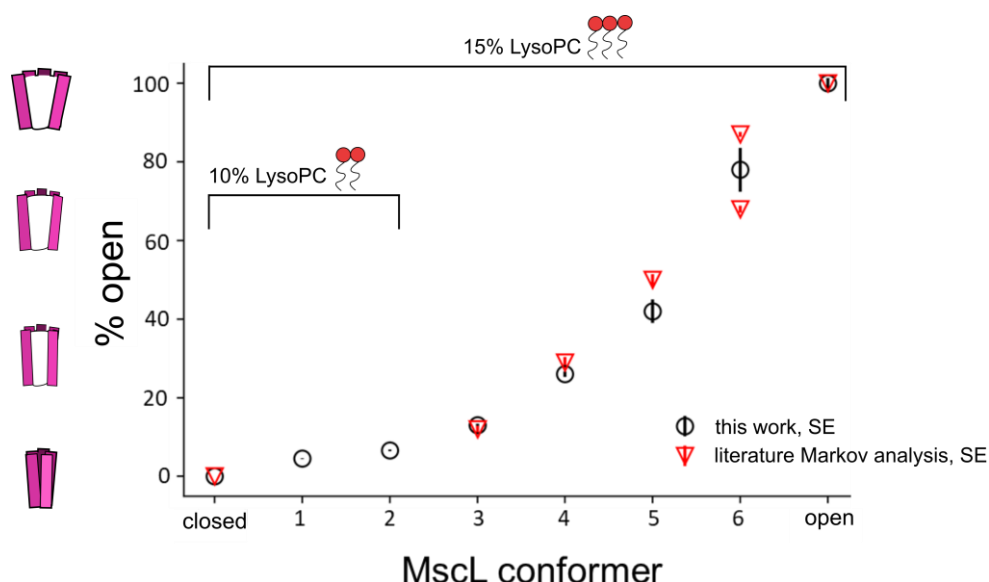


Figure 7.12: All recorded MscL gatings for 10 and 15 % LysoPC asymmetric activation in DIBs. Data from table 7.2 plotted against comparative Markov analysis dataset in Almanjahie et al.²⁵⁹ A strong agreement between the datasets was observed.

Whilst evidence within literature exists for the first two MscL conformers (denoted 1 and 2 in figure 7.12), strong agreement was observed in the % open value (calculated as a percentage of the nS of the fully open state) for the larger gatings. Interestingly, the gating with the largest error (conformer 6) appeared to indicate a blend of two identified conformers which may resolve with further gating data.

This particular system enables control of membrane permeability profile dependent upon asymmetric LysoPC content. The concentration of LysoPC in the membrane can be increased by secretory phospholipase A2 (sPLA₂) - which cleaves a single fatty acid tail from a phospholipid,¹⁰⁰ and decreased by bovine serum albumin (BSA) - which chelates LPCs.²⁶⁰ The incorporation of these additional components would present a dynamic system with tuneable control over synthetic membrane permeability, leveraging the biological function of MscL to *de novo* applications. DIB network communication pathways are traditionally controlled through α HL inserted into particular membranes, thus constructing a transport pathway through the droplets.^{129,280,281} Instead, this activation mechanism would allow transport pathways through the network which can be patterned with the monolayer composition with proteins residing in all membranes. If a system is imagined in which LysoPC is contained in certain droplets, then MscL activation may also act as a measure of LysoPC diffusive time. As LysoPC flip flops and laterally diffuses around the network, droplet communication pathways are opened. Understanding the timescale of interleaflet lipid diffusion is a challenging measurement to make in a label free fashion.

7.5. Chapter conclusions

In this chapter, a unique membrane protein gating mechanism was successfully studied in both DIB and vesicle systems. The mechanism utilised MscL mechanosensitivity to membrane tension, to control the net membrane permeability through the membrane composition. This functionality exhibited significant concentration dependence when studied in both DIB and vesicles with different mechanisms postulated for the dependence in each. Most pertinently, in DIBs it was found that the degree of channel opening was controlled through LysoPC concentration, thus allowing control over the channel pore size and therefore the MWCO of the membrane permeability. Understanding how these aspects interlink with modifications to bilayer permeability as studied in chapters 3 – 6 may allow for significantly advanced inter droplet communication systems to be established. Digital MscL activation may be yielded by further gain of function mutations, where activity can easily be assessed with droplet electrophysiology. When deployed in DIBs, this system presents a parameter space with significant potential for controlled permeation of >1KDa solutes, such as biological macromolecules and DNA. Whilst the permeation enabled through MscL is an example of further passive transport, replication of such studies with human transporters should allow for increasingly biomimetic reconstitution of active transport processes. These results further exemplify the role DIBs may play in the future of *in vitro* modelling biological processes and indeed engineering *de novo* synthetic biology technologies.

8 Conclusions and outlook – Toward improving biomimicry in DIB systems

8.1. Conclusion - Major findings of this research

This thesis aimed to contribute toward the growing field of DIB based permeability measurements. A core aim was to develop a method which enabled study of physiologically relevant compounds within nL volume droplet DIBs. To achieve this, a range of analytical and fabrication techniques were utilised including UV spectroscopy, electrophysiology, laser cutting, 3D printing and droplet microfluidics. In this chapter, conclusions are drawn with respect to the intentions of the work and where future progressions could be made.

8.1.1. Two novel methods for label free DIB permeability measurement

To date, DIBs have mostly relied on fluorescence microscopy-based measurements. This work engineered fiber-based, label free approaches with an *in situ* read out of small molecule drug concentration. One method performed fully quantitative measurements and another, high throughput experiments in linear droplet arrangements. These approaches unlock novel experimentation in diverse permeant space facilitating the translational potential of DIB technology.

8.1.2. Structure – permeability relationships derived from DIB interfaces

The methods delineated structure – permeability relationships within narrow physicochemical spaces and the assessed range summed across wide chemical space. The results herein interrogate the relationship between bilayer permeability and permeant chemical structure, broadly capturing the experimental utility of PAMPA with DIBs. As outlined above, it is anticipated that the developed methods may contribute toward understanding of *in vivo* cellular translocation, a major bottleneck of biomedical and drug discovery research.

8.1.3. Controlling permeability with membrane composition

The lipid composition of a membrane is coupled to the permeability; however, membrane proteins are integral players in the permeant selectivity of a membrane. In this work, MscL, a non-selective membrane pore, was gated in a concentration dependent manner based on the lipid composition of a membrane. This system was engineered as a novel part of the

synthetic biologist's toolkit to enable control of chemical communication pathways within *de novo* membrane systems. Such feats of protein reconstitution, highlight the significant potential for DIBs as the chassis for *in vitro* study of human membrane transporters, major permeability regulators of biological membranes.

8.2. Conclusion - Correlating DIB permeability to structural signatures

8.2.1. Summary of measured permeability response

Across the course of this work, the permeability of a wide range of small molecule drugs and endogenous compounds were assessed. In building toward a QSAR model of bilayer permeability derived from a DIB interface – the following figures interrogate the dataset produced within this work.

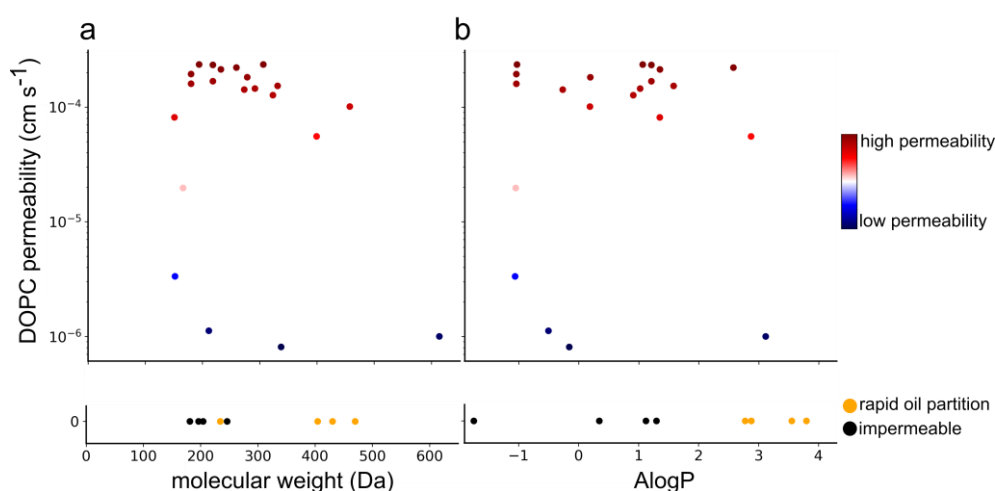


Figure 8.1: Summary of permeability values measured within this thesis. Permeability as a function of molecular weight **(a)** and AlogP **(b)**. The top graph (log scale) shows the measured DIB DOPC permeability. The bottom graph shows the physicochemical range of compounds which were either impermeable or partitioned rapidly into hexadecane.

In chapters 4 and 6, a total of 30 physiologically relevant compounds were assessed in DOPC DIBs. Figure 8.1 showcases the range of measured behaviour across wide physiochemical space. The permeability spanned 3 orders of magnitude. For MW, the compounds with high permeability occupied a similar range as those with low permeability. The rate of translocation was therefore uncorrelated with MW in a range of 151 – 614 Da. For the AlogP range of -1.06 and 3.11, lower permeability occupied below AlogP 0 and above AlogP 3. In understanding the difference between permeable and impermeable, the impermeable compounds were clustered within a MW and AlogP range which also included

compounds which exhibited high and low permeability. This suggested that the permeability of a compound within these physiochemical ranges could not be anticipated by a simple assessment of MW or AlogP. The oil partition compounds clustered toward the highly lipophilic values, indicating that this property may be anticipated at an AlogP threshold >3.55. However, no partition was recorded for amidotrizoic acid, a poorly permeable compound with an AlogP of 3.11. Indeed, the certainty of such assessments could be further supported by the measurement of compounds at the extremes of the AlogP distribution, for which plenty of small molecule drugs reside. Within contained and wide structural space, lipophilicity metrics have shown strong correlations with PAMPA permeability.^{282,36} These results generally indicate that bilayer permeability is low for more polar compounds and that physiochemical metrics can only loosely indicate whether a compound has access to bilayer permeability.

8.2.2. PCA

As introduced in chapter 1, the Lipinski guidelines attempt to correlate a compounds metabolism with a series of physiochemical descriptors. In light of this, a principal component analysis (PCA)²⁸³ was performed to assess any potential correlation between physiochemical properties and permeability response. PCA is a dimensionality reduction technique which is an example of unsupervised learning; the intrinsic features of a dataset may support classification within data clusters in two-dimensional vector space. To perform the PCA (scikit-learn), physicochemical metrics were calculated (RDkit) and min-max normalised.

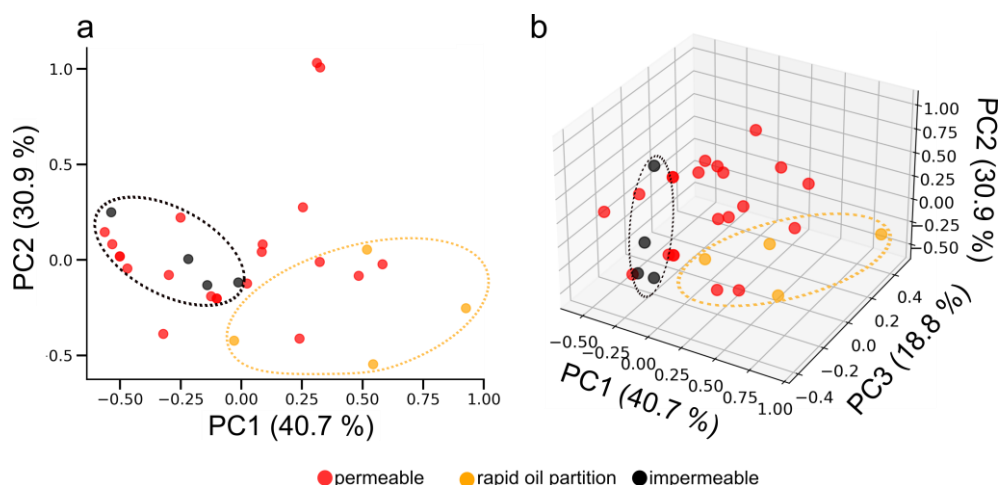


Figure 8.2: PCA analysis of Lipinski descriptors (HBA, HBD, MW, AlogP, TPSA and RBN) of all measured compounds in 2 (a) and 3 (b) dimensions mapped to the DOPC DIB classification. An apparent separation was observed for impermeable and oil partition compounds. The percentage variance explained by each component is given in the bracket of the axis label. Note, iodipamide, which was deemed impermeable in chapter 4 was not included in the analysis in this section (MW 1139 Da, AlogP 6.84).

It was thought that if the Lipinski properties of the permeants were accounted for, this may yield clustering that would map to the diffusive classification (permeable, impermeable or oil partition). Figure 8.2 shows minimal clustering in 2 and 3 dimensions, with a wide data spread for the compounds deemed permeable. An apparent separation was noted between the oil partition and impermeable classification, which was maintained in 3 dimensions. It is important to consider the sparsity of data, and the lack of apparent clustering. This further supports the conclusion that the DIB DOPC translocation rate could not be predicted from the Lipinski range of physicochemical descriptors. This lack of correlation was further observed with a multi-dimensional linear regression of the same metrics with the permeability value (R^2 of 0.42). Further exploration with uniform manifold approximation and projection (UMAP),²⁸⁴ and t-distributed stochastic neighbour embedding (t-SNE)²⁸⁵ algorithms produced similar results.

The analyses in this section highlight a central outlook of this work which evolved across chapters 3 to 6; that the permeant behaviour at an oil interface is substantially different from that at a bilayer. The simplicity of the Lipinski descriptors fails to accurately capture the relevant structural features which dictate the DIB DOPC permeability response despite finding mixed agreement within PAMPA systems.²⁰⁵ Correlation may be improved by a consideration of the compounds charge state and pKa with a more robust metric such as logD (pH 7.4).²⁸⁶ These observations provide the groundwork for building more robust, models from large datasets yielded by high throughput DIB methodologies and to further interrogate the role of simple diffusion at biological membranes.

8.3. Outlook - The use of DIBs as an alternative to PAMPA

Continuing from the outlook established in the prior section, a further viewpoint is that DIB systems may advance toward wholesale replacement of PAMPA measurements. To illustrate this potential, the case is made for application of this technology in a divergent chemical space.

8.3.1. Application in novel small molecule drug development space – the case for PROTAC permeability

The physicochemical space of small molecule drugs has historically been constrained to within the Lipinski guidelines (Ro5). As drug discovery has expanded, novel physicochemical spaces outside of Ro5 have been broached, denoted beyond rule of 5 (bRo5).⁹³ Compounds

in this area are thus fertile ground for understanding the biophysical constraints of biological permeability.

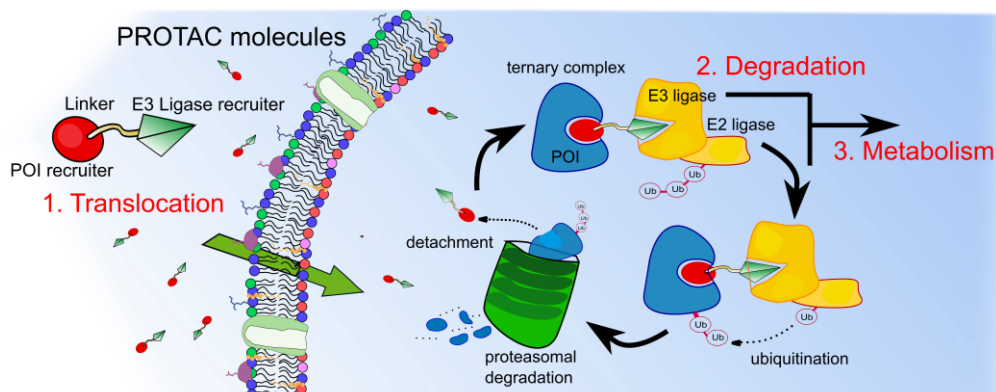


Figure 8.3: A cartoon overview of the novel therapeutic mechanism of PROTAC compounds. From administration to clearance, PROTACs interact with the PM of the target cell. To understand compound efficacy, it is important to decouple the kinetics of translocation from those of the protein degradation mechanism.

Proteolysis targeting chimeras (PROTACs) are a class of bRo5 compounds with huge potential in the clinic.^{287,288} The functionality of PROTACs is coupled with their structural signature; two inhibitor molecules joined by a flexible linker. *In vivo*, PROTACs recruit a target protein in close proximity to an E3 ligase protein. The ternary complex between proteins and PROTAC facilitates ubiquitination of the target protein for recognition and destruction by the proteasome. As opposed to a classical inhibitory modality, the PROTAC efficacy follows a catalytic cycle which effectively downregulates the target gene at the post-translational level (figure 8.3).²⁸⁹

Across multiple cell lines and across the large chemical space afforded in development, PROTAC compounds are readily permeable across biological membranes.^{290,291} The PAMPA permeability of PROTACs is often close to the limit of detection with successful measurements typically on the order of $10^{-8} \text{ cm s}^{-1}$.^{291,292} *In vivo*, the maximal degradation is normally reached on the order of hours, conflicting with PAMPA measurements.²⁹³ Due to the poor understanding of membrane permeability in bRo5 space, the debate over the biological mechanism of PROTAC permeability has significant focus. It has been suggested that drugs 'hitchhike' into cells via membrane transporter recognition of shared structural motifs between synthetic and endogenous compounds.⁵⁶ There exists no strong link for membrane transporters in influx, which may be explained by the lack of endogenous compounds with a similar molecular shape to PROTACs. Due to the non-specificity of p-GP, evidence is growing for p-GP mediated efflux of PROTACs.²⁹⁴ Interestingly, PROTACs have been designed for the degradation of SLC transporters.²⁹⁵ PROTACs are highly large,

flexible structures and 2-D molecular descriptors such as those in Ro5 space fail to capture their dynamic conformational properties.²⁴⁶ Intramolecular hydrogen bonds (IMHBs) have provided a design handle for improving biological permeability in bRo5 space. IMHBs can enable so called chameleonic properties, where a compounds size and polarity can be altered by its conformation.^{95,296,291} This facilitates passive lipid diffusion at the PM by dynamic conformational changes famously showcased by cyclosporins.²⁹⁷ Thus, a pervasive view in the literature is that PROTACs undergo a pseudo mechanistic, passive diffusion interaction with the lipids of biological membranes.

Biological and *in vitro* studies point toward a contribution of passive processes in the permeability of PROTACs at biological membranes. An emergent opinion is that PAMPA is an inappropriate assay for *in vitro* assessment of PROTAC permeability.²⁹⁸ The systems built herein may present a solution. Preliminary work has evidenced measurable DOPC DIB PROTAC permeability on the order of $10^{-5} \text{ cm s}^{-1}$, a considerably high value, potentially closer to biological observations. Through assessment of the functional group modifications which evolve during the design of a new PROTAC candidate, a structure permeability relationship could be uncovered. Such an experimental scope could provide an advanced analysis on the role of passive diffusion in novel, extended drug space and provide a useful assay for classifying the mechanism of biological permeability.

8.4. Outlook - The relationship between biomimetic bilayer composition and permeability response

8.4.1. A method for heating droplets

As discussed in section 4.3.4 – it is well known that heat is integral to reconstituting highly biomimetic lipid bilayer compositions. Lipids in the aqueous phase heated above their transition temperature (gel to fluid phase transition) enable monolayer assembly. In order to render such a system, a first pass custom heating block was engineered (figure 8.4). The block was designed around the modularity of the spectroscopic platform, fitting an individual, temperature controlled well.

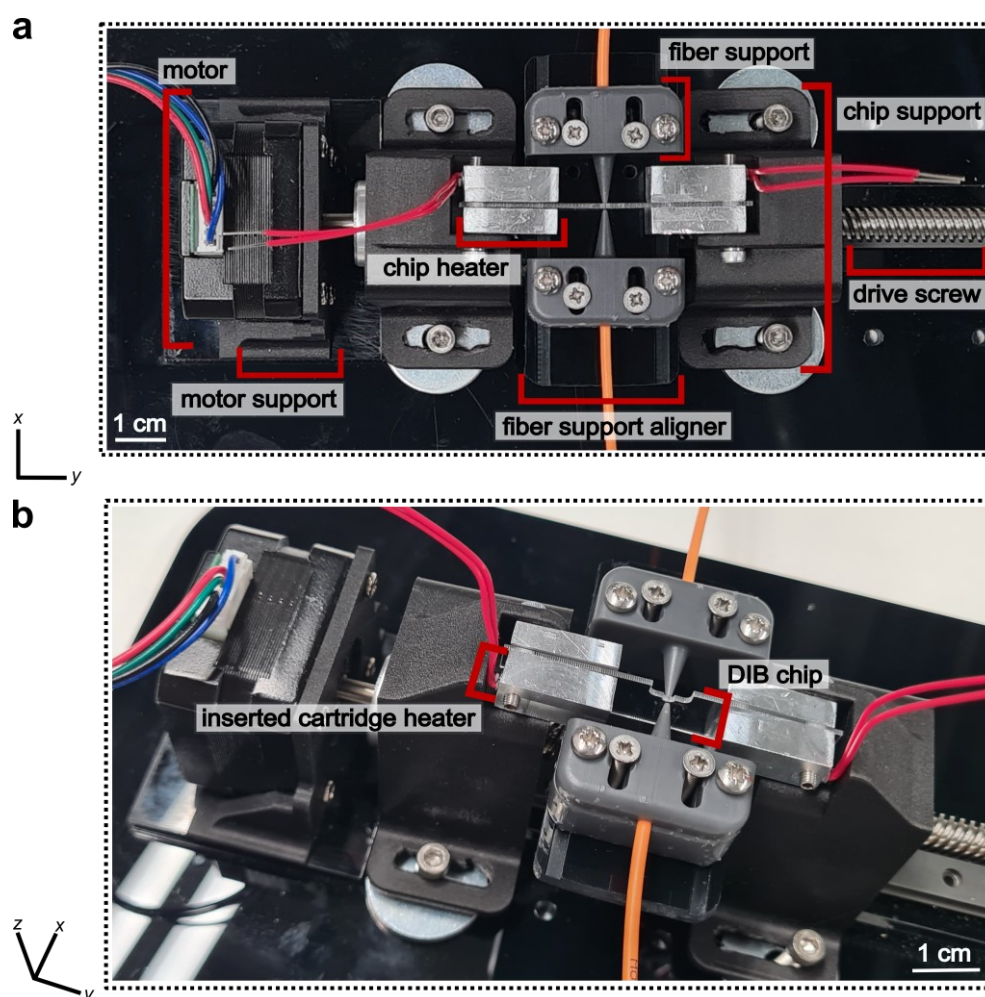


Figure 8.4: Custom heating block for temperature-control in label free DIB permeability measurements. **a** Top-down view of device. Aluminium chip heaters, made with metal working, align and support a DIB chip, a cartridge heater (Thorlabs HT15W – 15 W miniature cartridge heating element) was embedded in each. The fiber supports were designed to prevent clashing of the beam with chip heaters during actuation. **b** Side on view of device highlighting fabrication details.

Future work was required to enable a fully functioning heating stage. For example, by further outfitting the rig with a proportional integral derivative (PID) temperature controllers in the oil and at each chip heater. Through linking each PID to the same microcontroller, a feedback loop controlling the oil temperature could be established. This would enable a reliable measure of the temperature of the oil, which is anticipated to be highly sensitive to local laboratory conditions.

8.4.2. Permeability response of a range of small molecule drugs to membranes of high compositional complexity and biomimicry – an outlook for the future of translational DIB permeability assays

The approach outlined in this section, pairs a label free measurement with control of the system temperature. From an experimental perspective, this would couple wide drug chemical space with complex lipid composition. Such an experimental scope may facilitate major utility as a translational technology and serves as an appropriate framework to conclude this thesis.

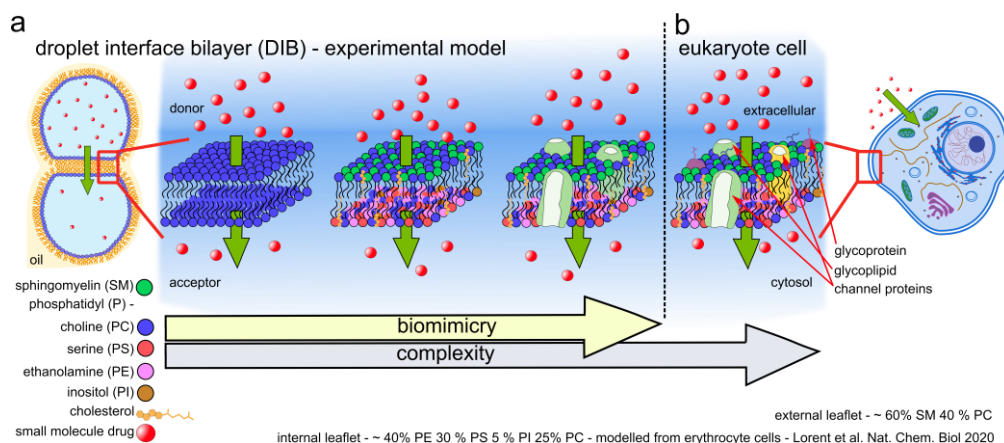


Figure 8.5: An outlook for building biological complexity in DIBs – toward an improved understanding of membrane permeability **a** Cartoon overview of experimentally reconstituting highly biomimetic conditions into DIBs, the membrane biomimicry increases in line with complexity. From left to right; a single lipid composition (most of this work), an asymmetric biomimetic lipid composition and a membrane consisting of a biomimetic lipid composition with embedded proteins (of relevance to transport). **b** Cartoon of eukaryote cell membrane composition, based on RBC membrane composition in Lorent et al. (figure 1.1).

Perhaps one of the most important questions with respect to cellular permeability is; **what is the physiological relevance of passive diffusion through the lipid matrix of biological membranes?** The work in this thesis was borne out of a desire to build a new approach for an alternative *in vitro* assay for membrane permeability. This thesis contributes to a field which has highlighted DIBs as an experimental chassis for the study of key features of the plasma membrane. DIBs are primed to readdress this question, since much research is based around overly simplistic, non-biomimetic assays. Figure 8.5 highlights a future direction for such systems. A question that emerged from the results of experiments in chapter 4, was **how do aspects of DIB compositional complexity act in tandem and in isolation?** Since the experimental conditions in DIBs can be controlled, such a case-by-case approach to experimentation may contribute toward improved understanding of the governing forces at biological membranes. With respect to biomimicry, an important question is **which features of *in vitro* membranes best capture the biophysical properties of biological ones, and what is the simplest lipid composition which can achieve this?** In chapters 4, 6 and 7 DIBs were formed with variable lipid compositions as well as the membrane protein MscL. Figure 8.5 highlights a potential roadmap for achieving an

asymmetric DIB (ideally modelled off a biological membrane) that may act as a highly relevant synthetic membrane for examining passive lipid diffusion. Indeed, from the perspective of drug design, all of this must also be understood in conjunction with the permeant chemical structure and so; **how does the permeability of a range of small molecule drugs respond to increasingly biomimetic membrane composition?** In chapters 3 and 5, novel methods were presented for the study of relevant drug chemical structures in DIBs. By measuring the permeability of a range of drugs (for example the compounds studied in chapter 6), as a function of membrane biomimicry, the relative permeability of biological membranes to wide chemical space may be better understood. A key question that may be answered by a sweep of membrane compositions with small molecule drug compounds is **does the behaviour of subsets of chemical space diverge?**

Finally, future challenges are anticipated in answering such questions. These include, although are not limited to; ensuring that the lipid concentration external from the monolayer is representative of the monolayer itself, understanding changes to the dependence of lipid structure on oil retention and ensuring that complex bilayers are non-porous and do not have defects (such as at phase boundaries). Highly lipophilic compounds will inevitably exhibit oil partition. Polymerising the oil phase may slow or possibly abrogate oil partition entirely, simplifying the transport kinetics.²⁶¹ Finally, higher throughput could be achieved by engineering in an additional linear rail for beam actuation across 2 axes and for integration of automated droplet pipetting strategies.

8.5. Final remarks

Scientific evidence is continuously reassessed as methods mature. This work has explored the delicate relationship between scope of measurement and the capacity for yielding novel findings. Through operating in a multidisciplinary manner, delicately treading the line between engineer and scientist, new experimental spaces were uncovered by carefully engineered methodologies. Hopefully, the tools presented herein, can be employed toward the long-term goal of supplementing drug discovery and unravelling the astonishing complexity of biological membranes.

9 References

1. Lorent, J. H. *et al.* Plasma membranes are asymmetric in lipid unsaturation, packing and protein shape. *Nat. Chem. Biol.* **16**, 644–652 (2020).
2. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nat. Rev. Mol. Cell Biol.* **9**, 112–124 (2008).
3. Doktorova, M., Symons, J. L. & Levental, I. Structural and functional consequences of reversible lipid asymmetry in living membranes. *Nat. Chem. Biol.* **16**, (2020).
4. Tanford, C. & Tanford, C. The Hydrophobic Effect and the Organization of Living Matter. *Science (80-.)*. **200**, 1012–1018 (1978).
5. Mills, T. T. *et al.* Order parameters and areas in fluid-phase oriented lipid membranes using wide angle x-ray scattering. *Biophys. J.* **95**, 669–681 (2008).
6. Pomorski, T. G. & Menon, A. K. Lipid somersaults: Uncovering the mechanisms of protein-mediated lipid flipping. *Progress in Lipid Research* **64**, 69–84 (2016).
7. Symons, J. L. *et al.* Lipidomic atlas of mammalian cell membranes reveals hierarchical variation induced by culture conditions, subcellular membranes, and cell lineages. *Soft Matter* **17**, 288–297 (2021).
8. Kamp, F., Zakim, D., Zhang, F., Noy, N. & Hamilton, J. A. Fatty Acid Flip-Flop in Phospholipid Bilayers Is Extremely Fast. *Biochemistry* **34**, 11928–11937 (1995).
9. Ma, Y., Poole, K., Goyette, J. & Gaus, K. Introducing membrane charge and membrane potential to T cell signaling. *Front. Immunol.* **8**, 1–11 (2017).
10. Tarling, E. J., Vallim, T. Q. d. A. & Edwards, P. A. Role of ABC transporters in lipid transport and human disease. *Trends in Endocrinology and Metabolism* **24**, 342–350 (2013).
11. Bagatolli, L. A. & Mouritsen, O. G. Is the fluid mosaic (and the accompanying raft hypothesis) a suitable model to describe fundamental features of biological membranes? What may be missing? *Frontiers in Plant Science* **4**, (2013).
12. McMahon, H. T. & Boucrot, E. Membrane curvature at a glance. *J. Cell Sci.* **128**, 1065–1070 (2015).
13. Shearman, G. C., Ces, O., Templer, R. H. & Seddon, J. M. Inverse lyotropic phases of lipids and membrane curvature. *J. Phys. Condens. Matter* **18**, S1105–S1124 (2006).
14. Shearman, G. C. *et al.* A 3-D hexagonal inverse micellar lyotropic phase. *J. Am. Chem. Soc.* **131**, 1678–1679 (2009).
15. Quinn, P. J. & Wolf, C. The liquid-ordered phase in membranes. *Biochim. Biophys. Acta - Biomembr.* **1788**, 33–46 (2009).
16. M'Baye, G., Mély, Y., Duportail, G. & Klymchenko, A. S. Liquid ordered and gel phases of lipid bilayers: Fluorescent probes reveal close fluidity but different

- hydration. *Biophys. J.* **95**, 1217–1225 (2008).
17. Mills, T. T. *et al.* Liquid-liquid domains in bilayers detected by wide angle x-ray scattering. *Biophys. J.* **95**, 682–690 (2008).
18. Nicolson, G. Update of the 1972 Singer-Nicolson Fluid-Mosaic Model of Membrane Structure. *Discoveries* **1**, 3 (2013).
19. Singer, S. J. & Nicolson, G. L. The fluid mosaic model of the structure of cell membranes. *Science (80-.)*. **175**, 720–731 (1972).
20. Gupta, A., Korte, T., Herrmann, A. & Wohland, T. Plasma membrane asymmetry of lipid organization: Fluorescence lifetime microscopy and correlation spectroscopy analysis. *J. Lipid Res.* **61**, 252–266 (2020).
21. Heberle, F. A. & Feigenson, G. W. Phase separation in lipid membranes. *Cold Spring Harb. Perspect. Biol.* **3**, 1–13 (2011).
22. Bezlyepkina, N., Gracià, R. S., Shchelokovskyy, P., Lipowsky, R. & Dimova, R. Phase diagram and tie-line determination for the ternary mixture DOPC/eSM/Cholesterol. *Biophys. J.* **104**, 1456–1464 (2013).
23. Aghaaminiha, M., Farnoud, A. M. & Sharma, S. Quantitative relationship between cholesterol distribution and ordering of lipids in asymmetric lipid bilayers. *Soft Matter* **17**, 2742–2752 (2021).
24. Steck, T. L. & Lange, Y. How slow is the transbilayer diffusion (Flip-Flop) of cholesterol? *Biophysical Journal* **102**, 945–946 (2012).
25. Podolsky, K. A. & Devaraj, N. K. Synthesis of lipid membranes for artificial cells. *Nature Reviews Chemistry* **5**, 676–694 (2021).
26. Allender, D. W., Giang, H. & Schick, M. Model Plasma Membrane Exhibits a Microemulsion in Both Leaves Providing a Foundation for “Rafts”. *Biophys. J.* **118**, 1019–1031 (2020).
27. Frewein, M., Kollmitzer, B., Heftberger, P. & Pabst, G. Lateral pressure-mediated protein partitioning into liquid-ordered/liquid-disordered domains. *Soft Matter* **12**, 3189–3195 (2016).
28. Hanafusa, K., Hotta, T. & Iwabuchi, K. Glycolipids: Linchpins in the Organization and Function of Membrane Microdomains. *Front. Cell Dev. Biol.* **8**, 1–9 (2020).
29. Ollila, O. H. S. & Vattulainen, I. Chapter 2. Lateral pressure profiles in lipid membranes: dependence on molecular composition. in *Molecular Simulations and Biomembranes: From Biophysics to Function* 26–55 (2010).
30. Fick, A. V. On liquid diffusion. *London, Edinburgh, Dublin Philos. Mag. J. Sci.* **10**, 30–39 (1855).
31. Johansson, I., Karlsson, M., Johanson, U., Larsson, C. & Kjellbom, P. The role of aquaporins in cellular and whole plant water balance. *Biochim. Biophys. Acta* -

- Biomembr.* **1465**, 324–342 (2000).
32. Takata, K., Matsuzaki, T. & Tajika, Y. Aquaporins: Water channel proteins of the cell membrane. *Prog. Histochem. Cytochem.* **39**, 1–83 (2004).
 33. Seddon, A. M. *et al.* Drug interactions with lipid membranes. *Chem. Soc. Rev.* **38**, 2509–2519 (2009).
 34. Waghray, D. & Zhang, Q. Inhibit or Evade Multidrug Resistance P-Glycoprotein in Cancer Treatment. *Journal of Medicinal Chemistry* **61**, 5108–5121 (2018).
 35. Overton, C. E. Studies of Narcosis. *Vierteljahrsschr Naturforsch Ges Zurich* **41**, (1896).
 36. Chi, C. T., Lee, M. H., Weng, C. F. & Leong, M. K. In silico prediction of PAMPA effective permeability using a two-QSAR approach. *Int. J. Mol. Sci.* **20**, (2019).
 37. Orsi, M. & Essex, J. W. Chapter 4. Passive Permeation Across Lipid Bilayers: a Literature Review. in *Molecular Simulations and Biomembranes* 76–90 (Royal Society of Chemistry, 2010). doi:10.1039/9781849732154-00076
 38. Leo, A., Hansch, C. & Elkins, D. Partition coefficients and their uses. *Chemical Reviews* **71**, 525–616 (1971).
 39. Parisio, G., Stocchero, M. & Ferrarini, A. Passive membrane permeability: Beyond the standard solubility-diffusion model. *J. Chem. Theory Comput.* **9**, 5236–5246 (2013).
 40. Marrink, S. J., de Vries, A. H. & Tieleman, D. P. Lipids on the move: Simulations of membrane pores, domains, stalks and curves. *Biochim. Biophys. Acta - Biomembr.* **1788**, 149–168 (2009).
 41. Khan, D. R., Rezler, E. M., Lauer-Fields, J. & Fields, G. B. Effects of drug hydrophobicity on liposomal stability. *Chem. Biol. Drug Des.* **71**, 3–7 (2008).
 42. Sekiya, Y. *et al.* Electrophysiological Analysis of Membrane Disruption by Bombinin and Its Isomer Using the Lipid Bilayer System. *ACS Appl. Bio Mater.* **2**, 1542–1548 (2019).
 43. Stewart, M. P., Langer, R. & Jensen, K. F. Intracellular delivery by membrane disruption: Mechanisms, strategies, and concepts. *Chemical Reviews* **118**, 7409–7531 (2018).
 44. Shinoda, W. Permeability across lipid membranes. *Biochim. Biophys. Acta - Biomembr.* **1858**, 2254–2265 (2016).
 45. Mills, J. K. & Needham, D. Lysolipid incorporation in dipalmitoylphosphatidylcholine bilayer membranes enhances the ion permeability and drug release rates at the membrane phase transition. *Biochim. Biophys. Acta - Biomembr.* **1716**, 77–96 (2005).
 46. Krämer, S. D. *et al.* When barriers ignore the “rule-of-five”. *Advanced Drug Delivery Reviews* **101**, 62–74 (2016).
 47. Tse, C. H., Comer, J., Wang, Y. & Chipot, C. Link between Membrane Composition

- and Permeability to Drugs. *J. Chem. Theory Comput.* **14**, 2895–2909 (2018).
48. Berben, P. *et al.* Drug permeability profiling using cell-free permeation tools: Overview and applications. *Eur. J. Pharm. Sci.* **119**, 219–233 (2018).
49. Pathak, K. & Raghuvanshi, S. Oral Bioavailability: Issues and Solutions via Nanoformulations. *Clin. Pharmacokinet.* **54**, 325–357 (2015).
50. Veber, D. F. *et al.* Molecular properties that influence the oral bioavailability of drug candidates. *J. Med. Chem.* **45**, 2615–23 (2002).
51. Kim, M. T., Sedykh, A., Chakravarti, S. K., Saiakhov, R. D. & Zhu, H. Critical evaluation of human oral bioavailability for pharmaceutical drugs by using various cheminformatics approaches. *Pharm. Res.* **31**, 1002–1014 (2014).
52. Newby, D., Freitas, A. A. & Ghafourian, T. Decision trees to characterise the roles of permeability and solubility on the prediction of oral absorption. *Eur. J. Med. Chem.* **90**, 751–765 (2015).
53. Kadry, H., Noorani, B. & Cucullo, L. A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity. *Fluids Barriers CNS* **17**, 1–24 (2020).
54. Swift, R. V & Amaro, R. E. Back to the Future: Can Physical Models of Passive Membrane Permeability Help Reduce Drug Candidate Attrition and Move Us Beyond QSPR? *Chem. Biol. Drug Des.* **81**, 61–71 (2013).
55. Al-Husseini, J. K., Stanton, N. J., Selassie, C. R. D. & Johal, M. S. The Binding of Drug Molecules to Serum Albumin: The Effect of Drug Hydrophobicity on Binding Strength and Protein Desolvation. *Langmuir* **35**, 17054–17060 (2019).
56. Kell, D. B. Hitchhiking into the cell. *Nat. Chem. Biol.* 1–2 (2020).
57. Girardi, E. *et al.* A widespread role for SLC transmembrane transporters in resistance to cytotoxic drugs. *Nat. Chem. Biol.* **16**, 469–478 (2020).
58. Lee, B. L. & Kuczera, K. Simulating the free energy of passive membrane permeation for small molecules. *Mol. Simul.* **44**, 1147–1157 (2018).
59. Refsgaard, H. H. F. *et al.* In silico prediction of membrane permeability from calculated molecular parameters. *J. Med. Chem.* **48**, 805–811 (2005).
60. Kansy, M., Senner, F. & Gubernator, K. Physicochemical high throughput screening: Parallel artificial membrane permeation assay in the description of passive absorption processes. *Journal of Medicinal Chemistry* **41**, 1007–1010 (1998).
61. Wohnsland, F. & Faller, B. High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. *J. Med. Chem.* **44**, 923–930 (2001).
62. Sun, H. *et al.* Highly predictive and interpretable models for PAMPA permeability. *Bioorganic Med. Chem.* **25**, 1266–1276 (2017).

-
63. Velický, M., Bradley, D. F., Tam, K. Y. & Dryfe, R. A. W. In situ artificial membrane permeation assay under hydrodynamic control: Permeability-ph profiles of warfarin and verapamil. *Pharm. Res.* **27**, 1644–1658 (2010).
64. Lee, Y., Lee, H.-R., Kim, K. & Choi, S. Q. Static and Dynamic Permeability Assay for Hydrophilic Small Molecules Using a Planar Droplet Interface Bilayer. *Anal. Chem.* **90**, 1660–1667 (2018).
65. Faugeras, V., Duclos, O., Bazile, D. & Thiam, A. R. Membrane determinants for the passive translocation of analytes through droplet interface bilayers. *Soft Matter* **16**, 5970–5980 (2020).
66. Van Breemen, R. B. & Li, Y. Caco-2 cell permeability assays to measure drug absorption. *Expert Opinion on Drug Metabolism and Toxicology* **1**, 175–185 (2005).
67. Smith, D. *et al.* Passive lipoidal diffusion and carrier-mediated cell uptake are both important mechanisms of membrane permeation in drug disposition. *Molecular Pharmaceutics* **11**, 1727–1738 (2014).
68. Takács-Novák, K. & Avdeef, A. Interlaboratory study of log P determination by shake-flask and potentiometric methods. *J. Pharm. Biomed. Anal.* **14**, 1405–1413 (1996).
69. Isac-García, J., Dobado, J. A., Calvo-Flores, F. G. & Martínez-García, H. Determining Physical and Spectroscopic Properties. in *Experimental organic chemistry* 145–175 (Elsevier, 2016).
70. Ghose, A. K., Viswanadhan, V. N. & Wendoloski, J. J. Prediction of hydrophobic (lipophilic) properties of small organic molecules using fragmental methods: An analysis of ALOGP and CLOGP methods. *J. Phys. Chem. A* **102**, 3762–3772 (1998).
71. Alvarez Núñez, F. A. & Yalkowsky, S. H. Correlation between log P and ClogP for Some Steroids. *J. Pharm. Sci.* **86**, 1187–1189 (1997).
72. Kristl, A., Pečar, S. & Kmetec, V. Are calculated log P values for some guanine derivatives by different computer programs reliable? *Int. J. Pharm.* **181**, 219–226 (1999).
73. Mannhold, R., Poda, G. I., Ostermann, C. & Tetko, I. V. Calculation of Molecular Lipophilicity: State-of-the-Art and Comparison of LogP Methods on more than 96,000 Compounds. *J. Pharm. Sci.* **98**, 861–893 (2009).
74. Johnstone, C. Medicinal chemistry matters - A call for discipline in our discipline. *Drug Discov. Today* **17**, 538–543 (2012).
75. Moriguchi, Ikuo, Hirano Shuichi, Liu, Qian, Nakagome, I. Simple Method of Calculating Octanol/Water Partition Coefficient. *Chem. Pharm. Bull.* **40**, 127–130 (1992).
76. Toulmin, A., Wood, J. M. & Kenny, P. W. Toward prediction of alkane/water partition coefficients. *J. Med. Chem.* **51**, 3720–3730 (2008).

-
77. Caron, G. & Ermondi, G. Calculating virtual log P in the alkane/water system (log P Nalk) and its derived parameters Δ log P Noct-alk and log DpHalk. *J. Med. Chem.* **48**, 3269–3279 (2005).
78. Abraham, M. H. & Mitchell, C. An Analysis of Water-Octanol and Water-Alkane Partitioning and the Alog P Parameter of Seiler. *J. Pharm. Sci.* **83**, (1994).
79. Kenny, P. W., Montanari, C. A. & Prokopczyk, I. M. ClogPalk: A method for predicting alkane/water partition coefficient. *J. Comput. Aided. Mol. Des.* **27**, 389–402 (2013).
80. Tsai, R. S. *et al.* Solute-Water Interactions in the Organic Phase of a Biphasic System. 1. Structural Influence of Organic Solutes on the “Water-Dragging” Effect. *J. Am. Chem. Soc.* **115**, 9632–9639 (1993).
81. Dainty, J. The polar permeability of plant cell membranes to water. *Protoplasma* **57**, 220–228 (1963).
82. Cass, A. & Finkelstein, A. Water Permeability of Thin Lipid Membranes. *J. Gen. Physiol.* **50**, 1765–1784 (1967).
83. Braziel, S., Sullivan, K. & Lee, S. Quantitative Raman microspectroscopy for water permeability parameters at a droplet interface bilayer. *Analyst* **143**, 747–755 (2018).
84. Esteves, F., Moutinho, C. & Matos, C. Correlation between octanol/water and liposome/water distribution coefficients and drug absorption of a set of pharmacologically active compounds. *Journal of Liposome Research* **23**, 83–93 (2013).
85. Dickson, C. J., Hornak, V., Bednarczyk, D. & Duca, J. S. Using Membrane Partitioning Simulations To Predict Permeability of Forty-Nine Drug-Like Molecules. *J. Chem. Inf. Model.* **59**, 236–244 (2019).
86. Levin Victor A, Doglinow Doug, Landahl Herebert, Yorke Craig, C. J. Relationship of Octanol/Water Partition Coefficient and Molecular Weight to Cellular Permeability and Partitioning in S4 9 Lymphoma Cells. in *Pharmaceutical Research* 259–266 (1984).
87. Stewart, A. M. & Grass, M. E. Practical Approach to Modeling the Impact of Amorphous Drug Nanoparticles on the Oral Absorption of Poorly Soluble Drugs. *Mol. Pharm.* **17**, 180–189 (2020).
88. Lakshminarayana, S. B. *et al.* Comprehensive physicochemical, pharmacokinetic and activity profiling of anti-TB agents. *J. Antimicrob. Chemother.* **70**, 857–867 (2015).
89. Bockus, A. T. *et al.* Probing the Physicochemical Boundaries of Cell Permeability and Oral Bioavailability in Lipophilic Macrocycles Inspired by Natural Products. *J. Med. Chem.* **58**, 4581–4589 (2015).
90. Lipinski, C. A., Lombardo, F., Dominy, B. W. & Feeney, P. J. Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv. Drug Deliv. Rev.* **23**, 3–25 (1997).

91. Young, R. J. & Leeson, P. D. Mapping the Efficiency and Physicochemical Trajectories of Successful Optimizations. *Journal of Medicinal Chemistry* **61**, 6421–6467 (2018).
92. Rossi Sebastiano, M. *et al.* Impact of Dynamically Exposed Polarity on Permeability and Solubility of Chameleonic Drugs beyond the Rule of 5. *J. Med. Chem.* **61**, 4189–4202 (2018).
93. Matsson, P. R., Doak, B. C., Over, B. & Kihlberg, J. Cell permeability beyond the rule of 5. *Adv. Drug Deliv. Rev.* **101**, 42–61 (2016).
94. Doak, B. C., Over, B., Giordanetto, F. & Kihlberg, J. Oral druggable space beyond the rule of 5: Insights from drugs and clinical candidates. *Chemistry and Biology* **21**, 1115–1142 (2014).
95. Whitty, A. *et al.* Quantifying the chameleonic properties of macrocycles and other high-molecular-weight drugs. *Drug Discovery Today* **21**, 712–717 (2016).
96. Trantidou, T. *et al.* Engineering Compartmentalized Biomimetic Micro- and Nanocontainers. *ACS Nano* **11**, 6549–6565 (2017).
97. Vutukuri, H. R. *et al.* Active particles induce large shape deformations in giant lipid vesicles. *Nature* **586**, 52–56 (2020).
98. Biedermann, F., Ghale, G., Hennig, A. & Nau, W. M. Fluorescent artificial receptor-based membrane assay (FARMA) for spatiotemporally resolved monitoring of biomembrane permeability. *Commun. Biol.* **3**, 383–393 (2020).
99. Booth, M. J., Cazimoglu, I. & Bayley, H. Controlled deprotection and release of a small molecule from a compartmented synthetic tissue module. *Commun. Chem.* **2**, 142–150 (2019).
100. Hindley, J. W. *et al.* Building a synthetic mechanosensitive signaling pathway in compartmentalized artificial cells. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 16711–16716 (2019).
101. Zhang, S. *et al.* Engineering motile aqueous phase-separated droplets via liposome stabilisation. *Nat. Commun.* **12**, 1673–1684 (2021).
102. Funakoshi, K., Suzuki, H. & Takeuchi, S. Lipid bilayer formation by contacting monolayers in a microfluidic device for membrane protein analysis. *Anal. Chem.* **78**, 8169–8174 (2006).
103. Sarles, S. A. & Leo, D. J. Physical encapsulation of droplet interface bilayers for durable, portable biomolecular networks. *Lab Chip* **10**, 710 (2010).
104. Holden, M. A., Needham, D. & Bayley, H. Functional bionetworks from nanoliter water droplets. *J. Am. Chem. Soc.* **129**, 8650–8655 (2007).
105. Bayley, H. *et al.* Droplet interface bilayers. *Mol. Biosyst.* **4**, 1191–1208 (2008).
106. Dixit, S. S., Pincus, A., Guo, B. & Faris, G. W. Droplet shape analysis and

- permeability studies in droplet lipid bilayers. *Langmuir* **28**, 7442–7451 (2012).
107. Taylor, G. J., Venkatesan, G. A., Collier, C. P. & Sarles, S. A. Direct in situ measurement of specific capacitance, monolayer tension, and bilayer tension in a droplet interface bilayer. *Soft Matter* **11**, 7592–7605 (2015).
108. Marquardt, D. *et al.* Lipid bilayer thickness determines cholesterol location in model membranes. *Soft Matter* **12**, 9417–9428 (2016).
109. Gross, L. C. M., Heron, A. J., Baca, S. C. & Wallace, M. I. Determining membrane capacitance by dynamic control of droplet interface bilayer area. *Langmuir* **27**, 14335–14342 (2011).
110. Barlow, N. E. *et al.* Measuring bilayer surface energy and curvature in asymmetric droplet interface bilayers. *J. R. Soc. Interface* **15**, 20180610 (2018).
111. Yanagisawa, M., Yoshida, T. A., Furuta, M., Nakata, S. & Tokita, M. Adhesive force between paired microdroplets coated with lipid monolayers. *Soft Matter* **9**, 5891–5897 (2013).
112. Carreras, P. *et al.* A microfluidic platform for size-dependent generation of droplet interface bilayer networks on rails. *Biomicrofluidics* **9**, 064121 (2015).
113. Guiselin, B., Law, J. O., Chakrabarti, B. & Kusumaatmaja, H. Dynamic Morphologies and Stability of Droplet Interface Bilayers. *Phys. Rev. Lett.* **120**, 238001 (2018).
114. Barlow, N. E. *et al.* Multiplexed droplet Interface bilayer formation. *Lab Chip* **16**, 4653–4657 (2016).
115. Villar, G., Graham, A. D. & Bayley, H. A Tissue-Like Printed Material. *Science* (80-.). **340**, 48–52 (2013).
116. Stanley, C. E. *et al.* A microfluidic approach for high-throughput droplet interface bilayer (DIB) formation. *Chem. Commun.* **46**, 1620 (2010).
117. Stephenson, E. B. & Elvira, K. S. Biomimetic artificial cells to model the effect of membrane asymmetry on chemoresistance. *Chem. Commun.* **57**, 6534–6537 (2021).
118. Gupta, R. & Rai, B. Electroporation of Skin Stratum Corneum Lipid Bilayer and Molecular Mechanism of Drug Transport: A Molecular Dynamics Study. *Langmuir* **34**, 5860–5870 (2018).
119. Allen, M. E., Albon, J. & Elani, Y. Layer-by-layer assembly of multi-layered droplet interface bilayers (multi-DIBs). *Chem. Commun.* **58**, 60–63 (2022).
120. Makhoul-Mansour, M. M. & Freeman, E. C. Droplet-Based Membranous Soft Materials. *Langmuir* **37**, 3231–3247 (2021).
121. Nguyen, M. A., Srijanto, B., Collier, C. P., Retterer, S. T. & Sarles, S. A. Hydrodynamic trapping for rapid assembly and: In situ electrical characterization of droplet interface bilayer arrays. *Lab Chip* **16**, 3576–3588 (2016).
122. Najem, J. S. *et al.* Activation of bacterial channel MscL in mechanically stimulated

- droplet interface bilayers. *Sci. Rep.* **5**, 13726 (2015).
123. Bachler, S., Ort, M., Krämer, S. D. & Dittrich, P. S. Permeation Studies across Symmetric and Asymmetric Membranes in Microdroplet Arrays. *Anal. Chem.* **93**, 5137–5144 (2021).
124. Elani, Y., Demello, A. J., Niu, X. & Ces, O. Novel technologies for the formation of 2-D and 3-D droplet interface bilayer networks. *Lab Chip* **12**, 3514–3520 (2012).
125. Haylock, S. *et al.* Membrane protein mediated bilayer communication in networks of droplet interface bilayers. *Commun. Chem. (In Rev.)* (2020).
126. Bayley, H., Cazimoglu, I. & Hoskin, C. E. G. Synthetic tissues. *Emerging Topics in Life Sciences* **3**, 615–622 (2019).
127. Downs, F. G. *et al.* Multi-responsive hydrogel structures from patterned droplet networks. *Nat. Chem.* (2020).
128. Villar, G., Heron, A. J. & Bayley, H. Formation of droplet networks that function in aqueous environments. *Nat. Nanotechnol.* **6**, 803–808 (2011).
129. Alcinesio, A., Krishna Kumar, R. & Bayley, H. Functional Multivesicular Structures with Controlled Architecture from 3D-Printed Droplet Networks. *ChemSystemsChem* **4**, (2022).
130. Baxani, D. K. *et al.* Bilayer Networks within a Hydrogel Shell: A Robust Chassis for Artificial Cells and a Platform for Membrane Studies. *Angew. Chemie - Int. Ed.* **55**, 14240–14245 (2016).
131. Friddin, M. S. *et al.* Single-channel electrophysiology of cell-free expressed ion channels by direct incorporation in lipid bilayers. *Analyst* **138**, 7294–7298 (2013).
132. Allen-Benton, M., Findlay, H. E. & Booth, P. J. Probing membrane protein properties using droplet interface bilayers. *Experimental Biology and Medicine* **244**, 709–720 (2019).
133. Song, L. *et al.* Structure of staphylococcal α -hemolysin, a heptameric transmembrane pore. *Science (80-.)*. **274**, 1859–1866 (1996).
134. Thomas, J. M., Friddin, M. S., Ces, O. & Elani, Y. Programming membrane permeability using integrated membrane pores and blockers as molecular regulators. *Chem. Commun.* **53**, 12282–12285 (2017).
135. Findlay, H. E., Harris, N. J. & Booth, P. J. In vitro synthesis of a Major Facilitator Transporter for specific active transport across Droplet Interface Bilayers. *Sci. Rep.* **6**, 39349 (2016).
136. Czekalska, M. A. *et al.* A droplet microfluidic system for sequential generation of lipid bilayers and transmembrane electrical recordings. *Lab Chip* **15**, 541–548 (2015).
137. Nisisako, T., Portonovo, S. A. & Schmidt, J. J. Microfluidic passive permeability assay using nanoliter droplet interface lipid bilayers. *Analyst* **138**, 6793–6800 (2013).

-
138. Foley, S., Miller, E., Braziel, S. & Lee, S. Molecular organization in mixed SOPC and SDPC model membranes: Water permeability studies of polyunsaturated lipid bilayers. *Biochim. Biophys. Acta - Biomembr.* **1862**, 183365 (2020).
139. Milianta, P. J., Muzzio, M., Denver, J., Cawley, G. & Lee, S. Water permeability across symmetric and asymmetric droplet interface bilayers: Interaction of cholesterol sulfate with DPhPC. *Langmuir* **31**, 12187–12196 (2015).
140. Hwang, W. L., Chen, M., Cronin, B., Holden, M. A. & Bayley, H. Asymmetric droplet interface bilayers. *J. Am. Chem. Soc.* **130**, 5878–5879 (2008).
141. Taylor, G. J. & Sarles, S. A. Heating-enabled formation of droplet interface bilayers using escherichia coli total lipid extract. *Langmuir* **31**, 325–337 (2015).
142. Barlow, N. E. *et al.* Engineering plant membranes using droplet interface bilayers. *Biomicrofluidics* **11**, 24107 (2017).
143. Korner, J. L. & Elvira, K. S. The role of temperature in the formation of human–mimetic artificial cell membranes using droplet interface bilayers (DIBs). *Soft Matter* **17**, 8891–8901 (2021).
144. Makhoul-Mansour, M. M., El-Beyrouthy, J. B., Mumme, H. L. & Freeman, E. C. Photopolymerized microdomains in both lipid leaflets establish diffusive transport pathways across biomimetic membranes. *Soft Matter* **15**, 8718–8727 (2019).
145. Ben M'barek, K. *et al.* ER Membrane Phospholipids and Surface Tension Control Cellular Lipid Droplet Formation Article ER Membrane Phospholipids and Surface Tension Control Cellular Lipid Droplet Formation. *Dev. Cell* **41**, 591–604 (2017).
146. Barlow, N. E. *et al.* Rheological Droplet Interface Bilayers (rheo-DIBs): Probing the Unstirred Water Layer Effect on Membrane Permeability via Spinning Disk Induced Shear Stress. *Sci. Rep.* **7**, 17551 (2017).
147. Chiou, W. L. Effect of 'unstirred' water layer in the intestine on the rate and extent of absorption after oral administration. *Biopharm. Drug Dispos.* **15**, 709–717 (1994).
148. Hidalgo, I. J., Hillgren, K. M., Grass, G. M. & Borchardt, R. T. Characterization of the unstirred water layer in caco-2 cell monolayers using a novel diffusion apparatus. *Pharm. Res. An Off. J. Am. Assoc. Pharm. Sci.* **8**, 222–227 (1991).
149. Barriga, H. M. G. *et al.* Droplet interface bilayer reconstitution and activity measurement of the mechanosensitive channel of large conductance from Escherichia coli. *J. R. Soc. Interface* **11**, 20140404 (2014).
150. Martin, H. S., Podolsky, K. A. & Devaraj, N. K. Probing the Role of Chirality in Phospholipid Membranes. *ChemBioChem* **22**, 3148–3157 (2021).
151. Neklesa, T. K. *et al.* Small-molecule hydrophobic tagging–induced degradation of HaloTag fusion proteins. *Nat. Chem. Biol.* **7**, 538–543 (2011).
152. Brudno, Y. *et al.* In Vivo Targeting through Click Chemistry. *ChemMedChem* **10**, 617–

- 620 (2015).
153. Lee, Y. & Choi, S. Q. Quantitative analysis for lipophilic drug transport through a model lipid membrane with membrane retention. *Eur. J. Pharm. Sci.* **134**, 176–184 (2019).
154. de Bruin, A. *et al.* A transparent 3D printed device for assembling droplet hydrogel bilayers (DHBs). *RSC Adv.* **7**, 47796–47800 (2017).
155. Gadallah, M. H. & Abdu, H. M. Modeling and optimization of laser cutting operations. *Manuf. Rev.* **2**, (2015).
156. Venkatesan, G. A. *et al.* Evaporation-induced monolayer compression improves droplet interface bilayer formation using unsaturated lipids. *Biomicrofluidics* **12**, 024101 (2018).
157. Litschel, T. *et al.* Freeze-thaw cycles induce content exchange between cell-sized lipid vesicles. *New J. Phys.* **20**, 055008 (2018).
158. Banwell, C. N. & McCash, Elaine M. *Fundamentals of Molecular Spectroscopy*. (McGraw-Hill Publishing Company, 1994).
159. Manafirad, A. Single Ion-Channel Analysis in Droplet Interface Bilayer. in *Nanopore Technology* 187–195 (2021).
160. Kono, N. & Arakawa, K. Nanopore sequencing: Review of potential applications in functional genomics. *Development Growth and Differentiation* **61**, 316–326 (2019).
161. Capel, A. J., Rimington, R. P., Lewis, M. P. & Christie, S. D. R. 3D printing for chemical, pharmaceutical and biological applications. *Nature Reviews Chemistry* **2**, 422–436 (2018).
162. Morgan, A. J. L. *et al.* Simple and versatile 3D printed microfluidics using fused filament fabrication. *PLoS One* **11**, 1–17 (2016).
163. Strutt, R. *et al.* Activating mechanosensitive channels embedded in droplet interface bilayers using membrane asymmetry. *Chem. Sci.* **12**, 2138–2145 (2021).
164. Berry, J. D., Neeson, M. J., Dagastine, R. R., Chan, D. Y. C. & Tabor, R. F. Measurement of surface and interfacial tension using pendant drop tensiometry. *Journal of Colloid and Interface Science* **454**, 226–237 (2015).
165. Kamaruddin, H. D. & Koros, W. J. Some observations about the application of Fick's first law for membrane separation of multicomponent mixtures. *J. Memb. Sci.* **135**, 147–159 (1997).
166. Schaich, M. *et al.* An Integrated Microfluidic Platform for Quantifying Drug Permeation across Biomimetic Vesicle Membranes. *Mol. Pharm.* **16**, 2494–2501 (2019).
167. Friberg, S. E. & Langlois, B. Evaporation From Emulsions. *J. Dispers. Sci. Technol.* **13**, 223–243 (1992).
168. Demond Avery, L. A. S. Estimation of interfacial tension between organic liquids and

- water. *Environ. Sci. Technol.* **43**, 7754–7761 (1992).
169. Goebel, A. & Lunkenheimer, K. Interfacial tension of the water/n-alkane interface. *Langmuir* **13**, 369–372 (1997).
170. Duffy, D. C., McDonald, J. C., Schueller, O. J. A. & Whitesides, G. M. Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). *Anal. Chem.* **70**, 4974–4984 (1998).
171. Dr Ziyi Yu. PDMS Microfluidic Device Fabrication. (2016).
172. Trantidou, T. *et al.* Mask-Free Laser Lithography for Rapid and Low-Cost Microfluidic Device Fabrication. *Anal. Chem.* **90**, 13915–13921 (2018).
173. Raj M, K. & Chakraborty, S. PDMS microfluidics: A mini review. *J. Appl. Polym. Sci.* **137**, (2020).
174. Mohammadi, M., Fazli, H., Karevan, M. & Davoodi, J. The glass transition temperature of PMMA: A molecular dynamics study and comparison of various determination methods. *Eur. Polym. J.* **91**, 121–133 (2017).
175. Soldera, A., Metatla, N., Beaudoin, A., Said, S. & Grohens, Y. Heat capacities of both PMMA stereomers: Comparison between atomistic simulation and experimental data. *Polymer (Guildf)*. **51**, 2106–2111 (2010).
176. Toepke, M. W. & Beebe, D. J. PDMS absorption of small molecules and consequences in microfluidic applications. *Lab on a Chip* **6**, 1484–1486 (2006).
177. Mukhopadhyay, R. When PDMS isn't the best. *Anal. Chem.* **79**, 3248–3253 (2007).
178. Campbell, S. B. *et al.* Beyond Polydimethylsiloxane: Alternative Materials for Fabrication of Organ-on-a-Chip Devices and Microphysiological Systems. *ACS Biomaterials Science and Engineering* **7**, 2880–2899 (2021).
179. Liga, A., Morton, J. A. S. & Kersaudy-Kerhoas, M. Safe and cost-effective rapid-prototyping of multilayer PMMA microfluidic devices. *Microfluid. Nanofluidics* **20**, 164 (2016).
180. Schafer, D. N. *et al.* Microfluidic cell counter with embedded optical fibers fabricated by femtosecond laser ablation and anodic bonding. *Opt. Express* **17**, 6068 (2009).
181. Urakubo, K., Iwamoto, M. & Oiki, S. Drop-in-well chamber for droplet interface bilayer with built-in electrodes. in *Methods in Enzymology* **621**, 347–363 (Elsevier Inc., 2019).
182. Hu, J., Cochrane, W. G., Jones, A. X., Blackmond, D. G. & Paegel, B. M. Chiral lipid bilayers are enantioselectively permeable. *Nat. Chem.* **13**, 786–791 (2021).
183. Singh, N., Shreshtha, A. K., Thakur, M. S. & Patra, S. Xanthine scaffold: scope and potential in drug development. *Heliyon* **4**, e00829 (2018).
184. Drugs.com. Theophylline. *Drugs.com* 1–36 (2021).
185. Ito, K. *et al.* A molecular mechanism of action of theophylline: Induction of histone deacetylase activity to decrease inflammatory gene expression. *Proc. Natl. Acad. Sci.*

- U. S. A. **99**, 8921–8926 (2002).
186. Fredholm, B. B. On the mechanism of action of theophylline and caffeine. *Acta Med. Scand.* **217**, 149–153 (1985).
187. Drugs.com. Caffeine. *Drugs.com* (2021).
188. Lingala, S. M. & Ghany, M. G. M. Mhs. The biophysics and cell biology of lipid droplets. **25**, 289–313 (2016).
189. Debon, A. P., Wootton, R. C. R. & Elvira, K. S. Droplet confinement and leakage: Causes, underlying effects, and amelioration strategies. *Biomicrofluidics* **9**, (2015).
190. Moore, J. S. & Jensen, K. F. Automated multitrajectory method for reaction optimization in a microfluidic system using online IR analysis. *Org. Process Res. Dev.* **16**, 1409–1415 (2012).
191. Jankowski, P., Kutaszewicz, R., Ogończyk, D. & Garstecki, P. A microfluidic platform for screening and optimization of organic reactions in droplets. *J. Flow Chem.* **10**, 397–408 (2020).
192. King, R. & Miskelly, G. The inhibition by amines and amino acids of bleach-induced luminol chemiluminescence during forensic screening for blood. *Talanta* **67**, 345–353 (2005).
193. Karabchevsky, A., Mosayyebi, A. & Kavokin, A. V. Tuning the chemiluminescence of a luminol flow using plasmonic nanoparticles. *Light Sci. Appl.* **5**, 1–7 (2016).
194. Bennion, B. J. *et al.* Predicting a Drug's Membrane Permeability: A Computational Model Validated with in Vitro Permeability Assay Data. *J. Phys. Chem. B* **121**, 5228–5237 (2017).
195. Zhu, C., Jiang, L., Chen, T. M. & Hwang, K. K. A comparative study of artificial membrane permeability assay for high throughput profiling of drug absorption potential. *Eur. J. Med. Chem.* **37**, 399–407 (2002).
196. Gironi, B. *et al.* Effect of DMSO on the Mechanical and Structural Properties of Model and Biological Membranes. *Biophys. J.* **119**, 274–286 (2020).
197. Yu, Z. W. & Quinn, P. J. The modulation of membrane structure and stability by dimethyl sulphoxide. *Molecular Membrane Biology* **15**, 59–68 (1998).
198. Notman, R., Den Otter, W. K., Noro, M. G., Briels, W. J. & Anwar, J. The permeability enhancing mechanism of DMSO in ceramide bilayers simulated by molecular dynamics. *Biophys. J.* **93**, 2056–2068 (2007).
199. Venkatraman, R. K. & Baiz, C. R. Ultrafast Dynamics at the Lipid-Water Interface: DMSO Modulates H-Bond Lifetimes. *Langmuir* **36**, 6502–6511 (2020).
200. Kundu, S. *et al.* A Comparative Study on DMSO-Induced Modulation of the Structural and Dynamical Properties of Model Bilayer Membranes. *Langmuir* **37**, 2065–2078 (2021).

201. Paternò, G. M. *et al.* The Effect of an Intramembrane Light-Actuator on the Dynamics of Phospholipids in Model Membranes and Intact Cells. *Langmuir* **36**, 11517–11527 (2020).
202. Bachler, S., Haidas, D., Ort, M., Duncombe, T. A. & Dittrich, P. S. Microfluidic platform enables tailored translocation and reaction cascades in nanoliter droplet networks. *Commun. Biol.* **3**, 769 (2020).
203. Tristram-Nagle, S. *et al.* Structure and water permeability of fully hydrated diphytanoylPC. *Chem. Phys. Lipids* **163**, 630–637 (2010).
204. Loftsson, T. & Brewster, M. E. Physicochemical properties of water and its effect on drug delivery. A commentary. *Int. J. Pharm.* **354**, 248–254 (2008).
205. Avdeef, A., Nielsen, P. E. & Tsinman, O. PAMPA - A drug absorption in vitro model: 11. Matching the in vivo unstirred water layer thickness by individual-well stirring in microtitre plates. *Eur. J. Pharm. Sci.* **22**, 365–374 (2004).
206. Richens, J. L., Lane, J. S., Mather, M. L. & O'Shea, P. The interactions of squalene, alkanes and other mineral oils with model membranes; effects on membrane heterogeneity and function. *J. Colloid Interface Sci.* **457**, 225–231 (2015).
207. Tarun, O. B., Eremchev, M. Y. & Roke, S. Interaction of Oil and Lipids in Freestanding Lipid Bilayer Membranes Studied with Label-Free High-Throughput Wide-Field Second-Harmonic Microscopy. *Langmuir* **34**, 11305–11310 (2018).
208. Ekanayake Mudiyanse, S., Hamburger, M., Elsner, P. & Thiele, J. J. Ultraviolet A induces generation of squalene monohydroperoxide isomers in human sebum and skin surface lipids In Vitro and In Vivo. *J. Invest. Dermatol.* **120**, 915–922 (2003).
209. Venkatesan, G. A. *et al.* Adsorption Kinetics Dictate Monolayer Self-Assembly for Both Lipid-In and Lipid-Out Approaches to Droplet Interface Bilayer Formation. *Langmuir* **31**, 12883–12893 (2015).
210. Sych, T., Gurdap, C. O., Wedemann, L. & Sezgin, E. How Does Liquid-Liquid Phase Separation in Model Membranes Reflect Cell Membrane Heterogeneity? *Membranes (Basel)*. **11**, 323 (2021).
211. Lee, S., Kim, D. H. & Needham, D. Equilibrium and dynamic interfacial tension measurements at microscopic interfaces using a micropipet technique - 2. Dynamics of phospholipid monolayer formation and equilibrium tensions at the water-air interface. *Langmuir* **17**, 5544–5550 (2001).
212. Strutt, R. *et al.* UV-DIB: label-free permeability determination using droplet interface bilayers. *Lab Chip* **22**, 972–985 (2022).
213. Larregieu, C. A. & Benet, L. Z. Distinguishing between the permeability relationships with absorption and metabolism to improve BCS and BDDCS predictions in early drug discovery. *Mol. Pharm.* **11**, 1335–1344 (2014).

-
214. Yu, H. *et al.* A New PAMPA Model Proposed on the Basis of a Synthetic Phospholipid Membrane. *PLoS One* **10**, (2015).
215. Lukacova, V. *et al.* Drug-membrane interactions studied in phospholipid monolayers adsorbed on nonporous alkylated microspheres. *J. Biomol. Screen.* **12**, 186–202 (2007).
216. Robinson, K. P. & Chan, J. J. Colchicine in dermatology: A review. *Australas. J. Dermatol.* **59**, 278–285 (2018).
217. Eliakim-Raz, N. *et al.* Efficacy and safety of chloramphenicol: Joining the revival of old antibiotics? Systematic review and meta-analysis of randomized controlled trials. *J. Antimicrob. Chemother.* **70**, 979–996 (2014).
218. Hastings-Janies, R., and J Glazebrook, M. A. & Boniface, S. Cholografin. *London Med. Assoc. J.* **72**, (1955).
219. Joppen, C., Petzinger, E. & Frimmer, M. Properties of iodipamide uptake by isolated rat hepatocytes. *Naunyn. Schmiedeberg's Arch. Pharmacol.* **331**, 393–397 (1985).
220. Cheung, Y. W. & La, K. S. Lag order and critical values of the augmented dickey-fuller test. *J. Bus. Econ. Stat.* **13**, 277–280 (1995).
221. Shin, S. *et al.* Development of an aryloxazole derivative as a brain-permeable anti-glioblastoma agent. *Pharmaceutics* **11**, (2019).
222. Belleman, P. & Scholz, H. Relationship between theophylline uptake and inotropic effect in the guinea-pig heart. *Br. J. Pharmacol.* **52**, 265–274 (1974).
223. Galinis-Luciani, D., Nguyen, L. & Yazdanian, M. Is PAMPA a useful tool for discovery? *J. Pharm. Sci.* **96**, 2886–2892 (2007).
224. Mukhtar, I. *et al.* The Competitive Absorption by the Gut Microbiome Suggests the First-Order Absorption Kinetics of Caffeine. *Dose-Response* **19**, 1–8 (2021).
225. Fortuna, A., Alves, G., Soares-Da-Silva, P. & Falcão, A. Optimization of a Parallel Artificial Membrane Permeability Assay for the Fast and Simultaneous Prediction of Human Intestinal Absorption and Plasma Protein Binding of Drug Candidates: Application to Dibenz[b,f]azepine-5-Carboxamide Derivatives. *J. Pharm. Sci.* **101**, 530–540 (2012).
226. Nurbaeti, S. N. *et al.* Active Mediated Transport of Chloramphenicol and Thiamphenicol in a Calu-3 Lung Epithelial Cell Model. *J. Pharm. Sci.* **107**, 1178–1184 (2018).
227. Jung, K. Y. *et al.* Characterization of ochratoxin A transport by human organic anion transporters. *Life Sci.* **69**, 2123–2135 (2001).
228. Lomize, A. L. & Pogozeva, I. D. Physics-Based Method for Modeling Passive Membrane Permeability and Translocation Pathways of Bioactive Molecules. *J. Chem. Inf. Model.* **59**, 3198–3213 (2019).

-
229. Yamauchi, S., Inoue, D. & Sugano, K. Permeation characteristics of tetracyclines in parallel artificial membrane permeation assay II: Effect of divalent metal ions and mucin. *ADMET DMPK* **8**, 129–138 (2020).
230. Chopra, I. & Hacker, K. Uptake of minocycline by *Escherichia coli*. *J. Antimicrob. Chemother.* **29**, 19–25 (1992).
231. Giacomini, K. M. *et al.* Membrane transporters in drug development. *Nature Reviews Drug Discovery* **9**, 215–236 (2010).
232. Kermani, A. A. *et al.* The structural basis of promiscuity in small multidrug resistance transporters. *Nat. Commun.* **11**, 6064–6073 (2020).
233. Leptihn, S. *et al.* Constructing droplet interface bilayers from the contact of aqueous droplets in oil. *Nat. Protoc.* **8**, 1048–1057 (2013).
234. Baxani, D. K., Jamieson, W. D., Barrow, D. A. & Castell, O. K. An Encapsulated Droplet Interface Bilayer Array for the High-throughput Optical Measurement of Lipid Membranes with Single Bilayer Resolution. *Biophys. J.* **114**, 686a (2018).
235. Poehlmann, A. python-seabreeze. (2012).
236. Rothe, H. *et al.* Comparison of protocols measuring diffusion and partition coefficients in the stratum corneum. *J. Appl. Toxicol.* **37**, 806–816 (2017).
237. Figueira, T. N. *et al.* Quantitative analysis of molecular partition towards lipid membranes using surface plasmon resonance. *Sci. Rep.* **7**, 45647 (2017).
238. Korner, J. L., Stephenson, E. B. & Elvira, K. S. A bespoke microfluidic pharmacokinetic compartment model for drug absorption using artificial cell membranes. *Lab Chip* **20**, 1898–1906 (2020).
239. Friddin, M. S. *et al.* Optically assembled droplet interface bilayer (OptiDIB) networks from cell-sized microdroplets. *Soft Matter* **12**, 7731–7734 (2016).
240. Priimagi, A., Cavallo, G., Metrangolo, P. & Resnati, G. The Halogen Bond in the Design of Functional Supramolecular Materials: Recent Advances. *Acc. Chem. Res.* **46**, 2686–2695 (2013).
241. Lu, Y., Wang, Y. & Zhu, W. Nonbonding interactions of organic halogens in biological systems: Implications for drug discovery and biomolecular design. *Phys. Chem. Chem. Phys.* **12**, 4543–4551 (2010).
242. Sauer, R. Therapy of Malignant Brain Tumors. in *Radiation Therapy of Brain Tumours* 195–276 (Springer, 1987).
243. Kadi, A. A., El-Tahir, K. E. H., Jahng, Y. & Rahman, A. F. M. M. Synthesis, biological evaluation and Structure Activity Relationships (SARs) study of 8-(substituted)aryloxycaffeine. *Arab. J. Chem.* **12**, 2356–2364 (2015).
244. Pajouhesh, H. & Lenz, G. R. Medicinal chemical properties of successful central nervous system drugs. *NeuroRx* **2**, 541–553 (2005).

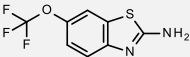
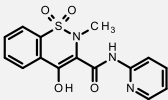
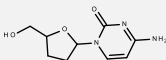
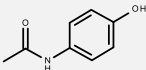
-
245. Chen, M. L. & Yu, L. The use of drug metabolism for prediction of intestinal permeability. *Mol. Pharm.* **6**, 74–81 (2009).
246. Ermondi, G., Garcia Jimenez, D., Rossi Sebastiano, M. & Caron, G. Rational Control of Molecular Properties Is Mandatory to Exploit the Potential of PROTACs as Oral Drugs. *ACS Med. Chem. Lett.* **12**, 1056–1060 (2021).
247. Grimm, J. B. & Lavis, L. D. Caveat fluorophore: an insiders' guide to small-molecule fluorescent labels. *Nat. Methods* **19**, 149–158 (2022).
248. Chakrabarti, A. C. Permeability of membranes to amino acids and modified amino acids: Mechanisms involved in translocation. *Amino Acids* **6**, 213–229 (1994).
249. Ishigami, T., Suga, K. & Umakoshi, H. Chiral Recognition of L -Amino Acids on Liposomes Prepared with L -Phospholipid. *ACS Appl. Mater. Interfaces* **7**, 21065–21072 (2015).
250. Arranz-Gibert, P. *et al.* Lipid Bilayer Crossing-The Gate of Symmetry. Water-Soluble Phenylproline-Based Blood-Brain Barrier Shuttles. *J. Am. Chem. Soc.* **137**, 7357–7364 (2015).
251. Zheng, Y., Benet, L. Z., Okochi, H. & Chen, X. Ph dependent but not p-gp dependent bidirectional transport study of s-propranolol: The importance of passive diffusion. *Pharm. Res.* **32**, 2516–2526 (2015).
252. Scalise, M., Pochini, L., Console, L., Losso, M. A. & Indiveri, C. The Human SLC1A5 (ASCT2) amino acid transporter: From function to structure and role in cell biology. *Front. Cell Dev. Biol.* **6**, 1–17 (2018).
253. Miyachi, H. *et al.* Structure, solubility, and permeability relationships in a diverse middle molecule library. *Bioorganic Med. Chem. Lett.* **37**, 127847 (2021).
254. Barlow, N., Chalmers, D. K., Williams-Noonan, B. J., Thompson, P. E. & Norton, R. S. Improving Membrane Permeation in the Beyond Rule-of-Five Space by Using Prodrugs to Mask Hydrogen Bond Donors. *ACS Chem. Biol.* **15**, 2070–2078 (2020).
255. Sukharev, S. I., Blount, P., Martinac, B. & Kung, and C. Mechanosensitive channels of Escherichia Coli: The MscL gene, protein, and activities. *Annu. Rev. Physiol.* **59**, 633–657 (1997).
256. Booth, I. R. & Blount, P. The MscS and MscL Families of Mechanosensitive Channels Act as Microbial Emergency Release Valves. *J. Bacteriol.* **194**, 4802–4809 (2012).
257. Cruickshank, C. C., Minchin, R. F., Le Dain, A. C. & Martinac, B. Estimation of the pore size of the large-conductance mechanosensitive ion channel of Escherichia coli. *Biophys. J.* **73**, 1925–1931 (1997).
258. Khan, R. N. *et al.* Hidden Markov analysis of mechanosensitive ion channel gating. *Math. Biosci.* **193**, 139–158 (2005).
259. Almanjahie, I. M., Khan, R. N., Milne, R. K., Nomura, T. & Martinac, B. Hidden Markov

- p analysis of improved bandwidth mechanosensitive ion channel data.
- Eur. Biophys. J.*
- 44**
- , 545–556 (2015).
260. Dimitrova, A. *et al.* In situ, reversible gating of a mechanosensitive ion channel through protein-lipid interactions. *Front. Physiol.* **7**, 1–9 (2016).
 261. Challita, E. J., Najem, J. S., Monroe, R., Leo, D. J. & Freeman, E. C. Encapsulating networks of droplet interface bilayers in a thermoreversible organogel. *Sci. Rep.* **8**, 6494 (2018).
 262. Czekalska, M. A., Kaminski, T. S., Makuch, K. & Garstecki, P. Passive and parallel microfluidic formation of droplet interface bilayers (DIBs) for measurement of leakage of small molecules through artificial phospholipid membranes. *Sensors Actuators, B Chem.* **286**, 258–265 (2019).
 263. Yoshimura, K., Batiza, A. & Kung, C. Chemically charging the pore constriction opens the mechanosensitive channel MscL. *Biophys. J.* **80**, 2198–2206 (2001).
 264. Nomura, T. *et al.* Differential effects of lipids and lyso-lipids on the mechanosensitivity of the mechanosensitive channels MscL and MscS. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 8770–8775 (2012).
 265. Perozo, E., Kloda, A., Cortes, D. M. & Martinac, B. Physical principles underlying the transduction of bilayer deformation forces during mechanosensitive channel gating. *Nat. Struct. Biol.* **9**, 696–703 (2002).
 266. Szostak, J. W., Bartel, D. P. & Luisi, P. L. Synthesizing life. *Nature* **409**, 387–390 (2001).
 267. Schwille, P. Jump-starting life? Fundamental aspects of synthetic biology. *J. Cell Biol.* **210**, 687–690 (2015).
 268. Cazimoglu, I., Booth, M. J. & Bayley, H. A lipid-based parallel processor for chemical signals. *bioRxiv* (2021).
 269. Shetty, S. C. *et al.* Directed Signaling Cascades in Monodisperse Artificial Eukaryotic Cells. *ACS Nano* **15**, 15656–15666 (2021).
 270. Zhong, D. & Blount, P. Electrostatics at the membrane define MscL channel mechanosensitivity and kinetics. *FASEB J.* **28**, 5234–5241 (2014).
 271. Henriksen, J. R., Andresen, T. L., Feldborg, L. N., Duelund, L. & Ipsen, J. H. Understanding detergent effects on lipid membranes: A model study of lysolipids. *Biophys. J.* **98**, 2199–2205 (2010).
 272. Fuller, N. & Rand, R. P. The Influence of Lysolipids on the Spontaneous Curvature and Bending Elasticity of Phospholipid Membranes. *Biophys. J.* **81**, 243–254 (2001).
 273. Mukherjee, N. *et al.* The activation mode of the mechanosensitive ion channel, MscL, by lysophosphatidylcholine differs from tension-induced gating. *FASEB J.* **28**, 4292–4302 (2014).

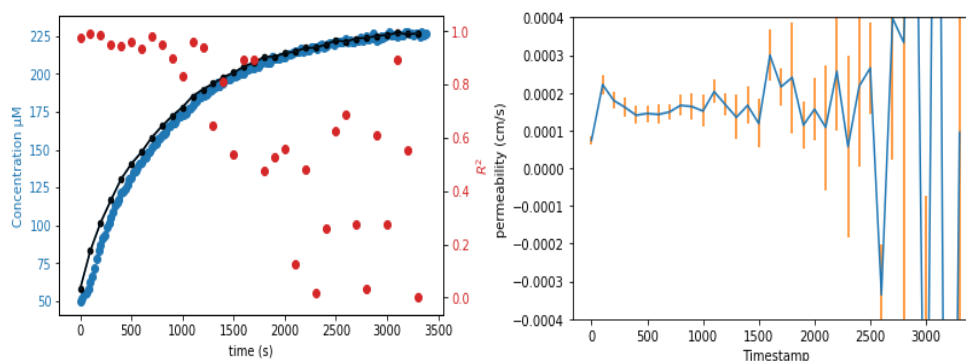
-
274. Chiang, C. S., Anishkin, A. & Sukharev, S. Gating of the Large Mechanosensitive Channel in Situ: Estimation of the Spatial Scale of the Transition from Channel Population Responses. *Biophys. J.* **86**, 2846–2861 (2004).
275. García-López, V. *et al.* Molecular machines open cell membranes. *Nat. Publ. Gr.* **548**, 567–572 (2017).
276. Bartsch, A. *et al.* High-resolution experimental and computational electrophysiology reveals weak β -lactam binding events in the porin PorB. *Sci. Rep.* **9**, 1–11 (2019).
277. Harden, S. pyABF. (2020).
278. Freeman, E. C., Najem, J. S., Sukharev, S., Philen, M. K. & Leo, D. J. The mechanoelectrical response of droplet interface bilayer membranes. *Soft Matter* **12**, 3021–3031 (2016).
279. Ben M'barek, K. *et al.* ER Membrane Phospholipids and Surface Tension Control Cellular Lipid Droplet Formation. *Dev. Cell* **41**, 591-604.e7 (2017).
280. Hwang, W. L., Holden, M. A., White, S. & Bayley, H. Electrical behavior of droplet interface bilayer networks: Experimental analysis and modeling. *J. Am. Chem. Soc.* **129**, 11854–11864 (2007).
281. Schild, V. R. *et al.* Light-Patterned Current Generation in a Droplet Bilayer Array. *Sci. Rep.* **7**, (2017).
282. Naylor, M. R. *et al.* Lipophilic Permeability Efficiency Reconciles the Opposing Roles of Lipophilicity in Membrane Permeability and Aqueous Solubility. *J. Med. Chem.* **61**, 11169–11182 (2018).
283. David, C. C. & Jacobs, D. J. Principal component analysis: A method for determining the essential dynamics of proteins. *Methods Mol. Biol.* **1084**, 193–226 (2014).
284. McInnes, L., Healy, J. & Melville, J. UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. *arXiv* (2018).
285. Van Der Maaten, L. & Hinton, G. Visualizing data using t-SNE. *J. Mach. Learn. Res.* **9**, 2579–2625 (2008).
286. Treyer, A., Walday, S., Boriss, H., Matsson, P. & Artursson, P. A Cell-Free Approach Based on Phospholipid Characterization for Determination of the Cell Specific Unbound Drug Fraction (fu,cell). *Pharm. Res.* **36**, (2019).
287. Sakamoto, K. M. *et al.* Protacs: Chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8554–8559 (2001).
288. Arvinas. A phase 1 clinical trial of ARV-110 in patients with metastatic castration-resistant prostate cancer. *Clinical trial* (2019).
289. Bondeson, D. P. *et al.* Catalytic in vivo protein knockdown by small-molecule PROTACs. *Nat Chem Biol* **617**, 611–617 (2015).

-
290. Wang, L. *et al.* A New Class of Selective Estrogen Receptor Degraders (SERDs): Expanding the Toolbox of PROTAC Degrons. *ACS Med. Chem. Lett.* **9**, 803–808 (2018).
291. Klein, V. G. *et al.* Understanding and Improving the Membrane Permeability of VH032-Based PROTACs. *ACS Med. Chem. Lett.* **11**, 1732–1738 (2020).
292. Scott, D. E. *et al.* Systematic Investigation of the Permeability of Androgen Receptor PROTACs. *ACS Med. Chem. Lett.* **11**, 1539–1547 (2020).
293. DeMars, K. M., Yang, C., Castro-Rivera, C. I. & Candelario-Jalil, E. Selective degradation of BET proteins with dBET1, a proteolysis-targeting chimera, potently reduces pro-inflammatory responses in lipopolysaccharide-activated microglia. *Biochemical and Biophysical Research Communications* **497**, 410–415 (2018).
294. Cecchini, C., Pannilunghi, S., Tardy, S. & Scapozza, L. From Conception to Development: Investigating PROTACs Features for Improved Cell Permeability and Successful Protein Degradation. *Frontiers in Chemistry* **9**, 672267 (2021).
295. Bensimon, A. *et al.* Targeted Degradation of SLC Transporters Reveals Amenability of Multi-Pass Transmembrane Proteins to Ligand-Induced Proteolysis. *Cell Chem. Biol.* **27**, 728–739 (2020).
296. Atilaw, Y. *et al.* Solution Conformations Shed Light on PROTAC Cell Permeability. *ACS Med. Chem. Lett.* **12**, 107–114 (2021).
297. Wang, C. K., Swedberg, J. E., Harvey, P. J., Kaas, Q. & Craik, D. J. Conformational Flexibility Is a Determinant of Permeability for Cyclosporin. *J. Phys. Chem. B* **122**, 2261–2276 (2018).
298. Cantrill, C. *et al.* Fundamental aspects of DMPK optimization of targeted protein degraders. *Drug Discov. Today* **25**, 969–982 (2020).
299. Wishart, D. S. *et al.* DrugBank 5.0: a major update to the DrugBank database for 2018. *Nucleic Acids Res.* **46**, 1074–1082 (2018).
300. Mendez, D. *et al.* ChEMBL: towards direct deposition of bioassay data. *Nucleic Acids Res.* **47**, 930–940 (2019).

10 Appendix

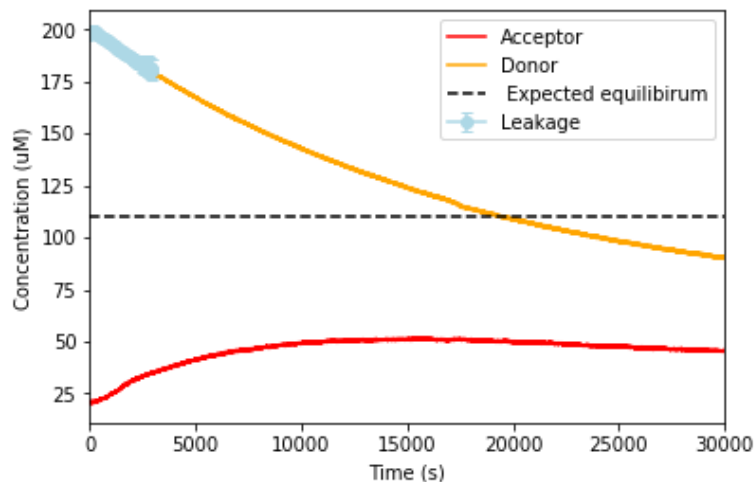
Compound	Structure	BIOVIA (AlogP)	ChemAxon (Marvin - clogP)	ChemBL (AlogP)	DrugBank (logP)
Caffeine		-0.091	-0.55	-1.0	-0.07
Riluzole		3.8	3.4	2.78	2.3
Piroxicam		0.50	0.60	1.58	3.0
Zalcitabine		-0.99	-1.2	-0.5	-1.3
Paracetamol		0.71	0.91	1.4	0.91

Supplementary table 1: A sample of compounds studied within this thesis illustrating the discrepancy in octanol – water partition coefficient depending on experimental determination ($\log P$) against calculated ($\log P$), with various algorithms. Values taken from DrugBank²⁹⁹ and ChemBL³⁰⁰ databases with BIOVA values determined

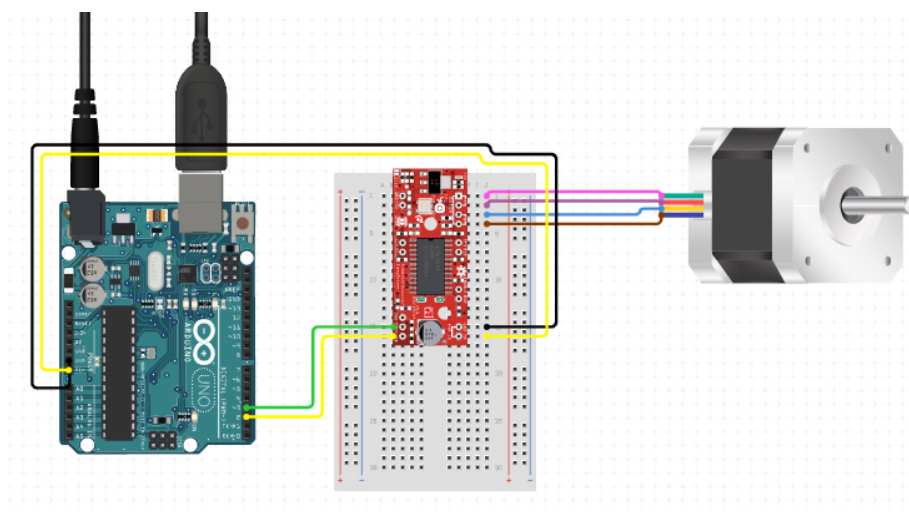


Supplementary figure 1: Assumption of linearity breaks down, as shown by a high throughput result from a permeability pipeline. An example permeation profile of caffeine using a donor concentration of 500 μM and an acceptor concentration of 50 μM . Black lines indicate linear fits performed within a time interval of 100 s, as the concentration time curve moves toward equilibrium, the R^2 value decreases. This relationship is reflected in the permeability determined for each linear regression (averaged for each concentration point within the regression), a stable region in P is observed from

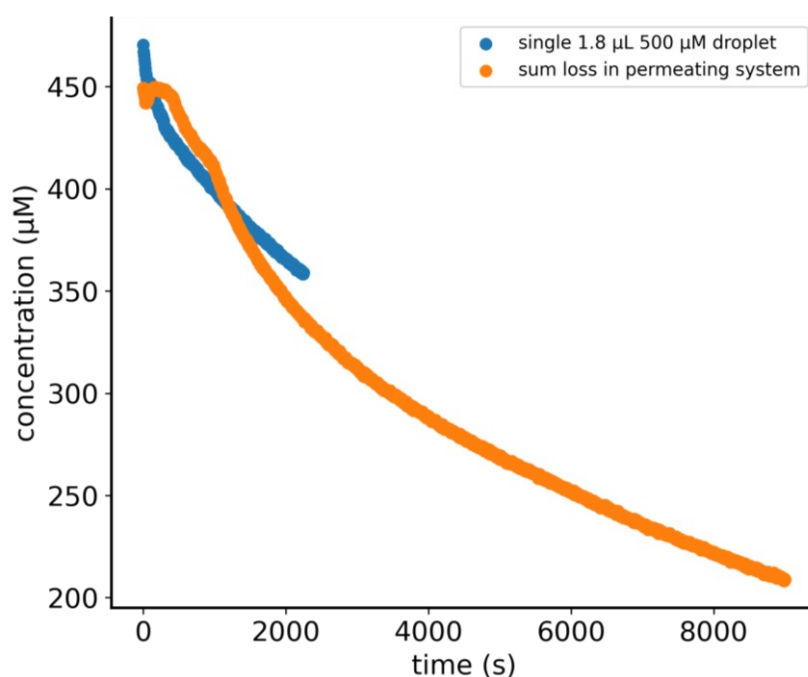
around 200 – 800 s (after DIB zip up) whilst R^2 remains high, significant noise in P is observed as a function of a low R^2 . Error bars represent propagated error.



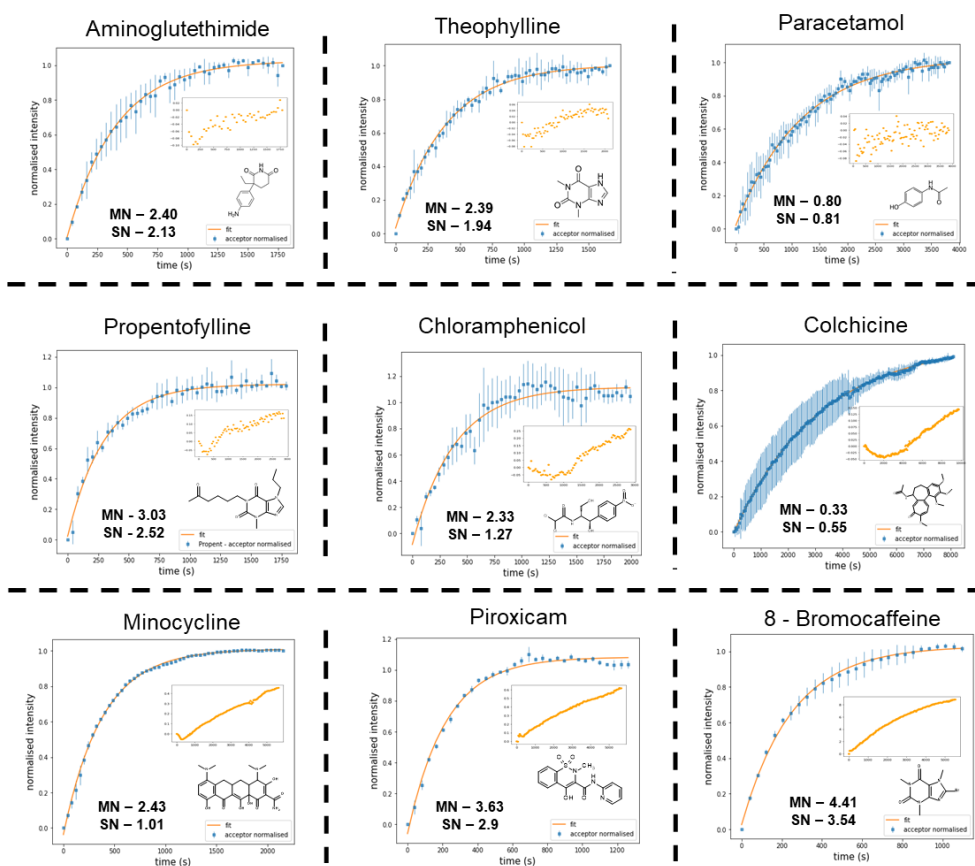
Supplementary figure 2: DIB permeability assessment of AH22182X – the compound demonstrated slow permeability, however the rate of donor decrease appeared to be identical to that of a partition experiment. Due to this, the characteristics of the increase of concentration in the acceptor curve could not be confirmed.



Supplementary figure 3: Circuit for controlling a stepper motor with an EasyDriver stepper motor driver. The system was powered by an external 12 V DC 2A power source. Circuit diagram built with circuito.



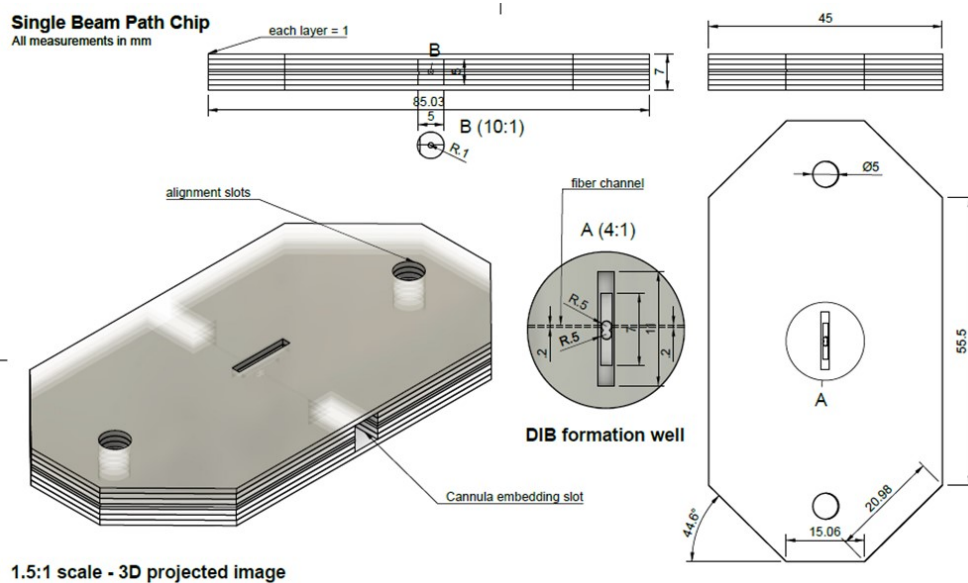
Supplementary figure 4: Comparing the partition rates derived for 8 – bromocaffeine in the single beam path chip. This figure shows the measured partition from single large droplets as compared to the measured loss from a permeating DIB system. If photodegradation was significant for this compound, the blue line would be expected to show a faster rate than the orange, as roughly double the amount of compound is present. (blue = 500 μM , orange = 275 μM). Instead, the orange curve is slightly faster, likely owed to the presence of the lipid bilayer in the system acting as an additional compound sink. Further consideration should be taken comparing the orange curve here, to that in figure 6.3, where the above orange curve is significantly slower despite the results above conducted under continuous illumination. These observations reflect the notion that the exposed interfacial w/o surface area is critical in affecting comparisons between the single beam path and multi droplet systems and that photodegradation within the system is minimal.



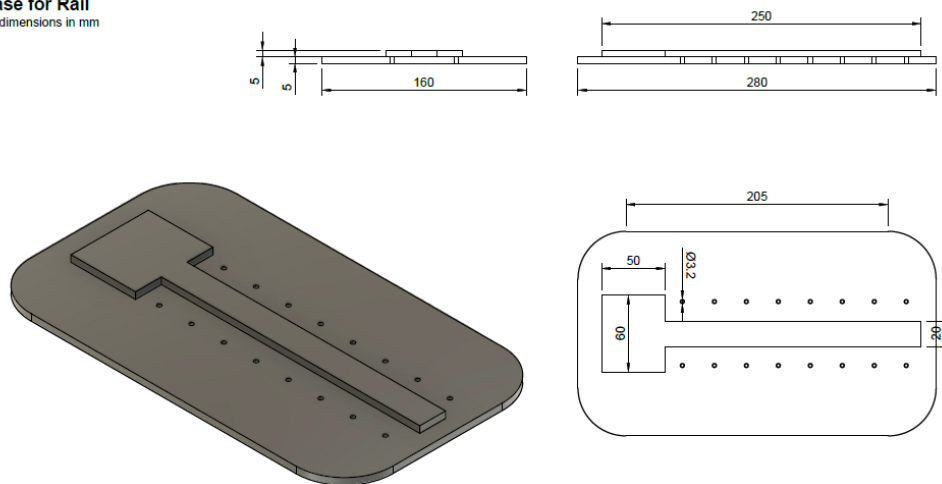
Supplementary figure 5: Normalised acceptor curves from figure 6.5 normalised based on apparent permeation equilibrium and fit to classical exponential model.

11 Appendix 2 - CAD drawings

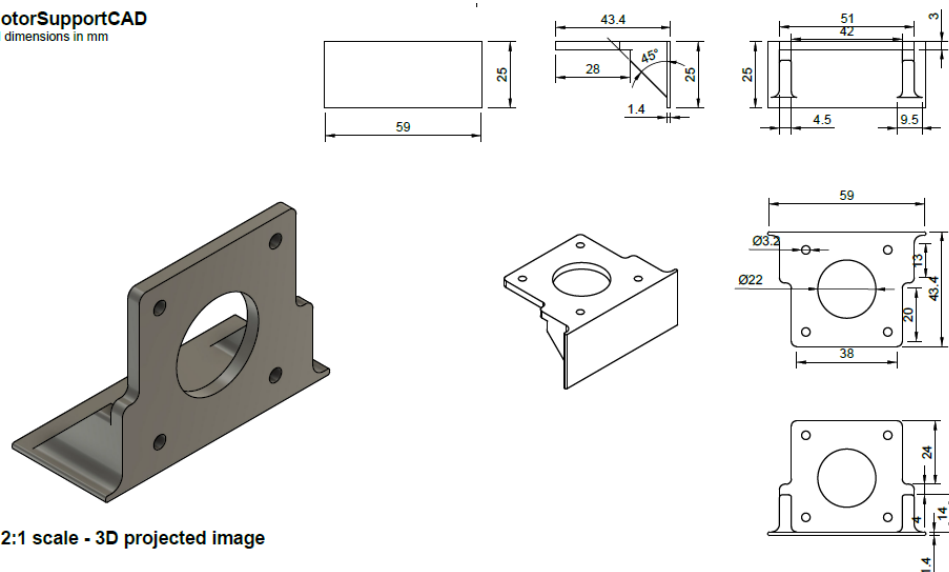
Single Beam Path Chip
All measurements in mm



Base for Rail
All dimensions in mm



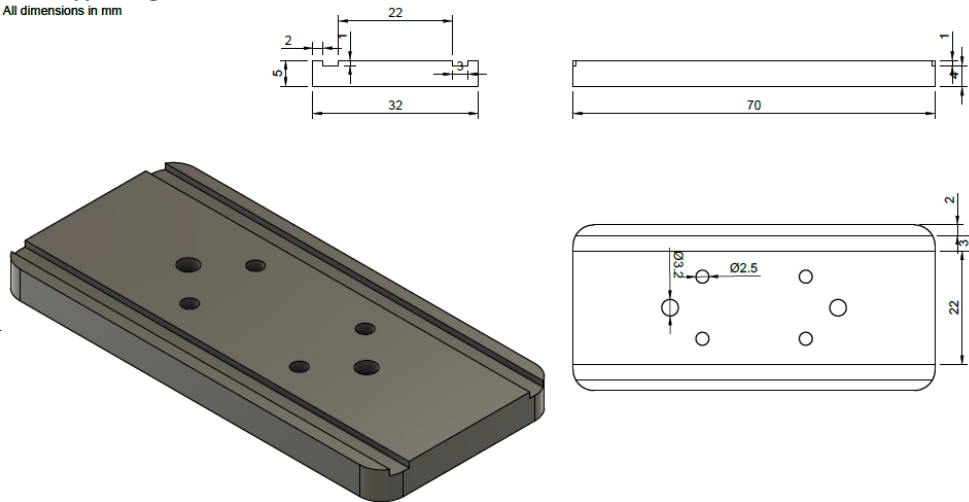
MotorSupportCAD
All dimensions in mm



Appendix 2 - CAD drawings

Fiber Support Aligner

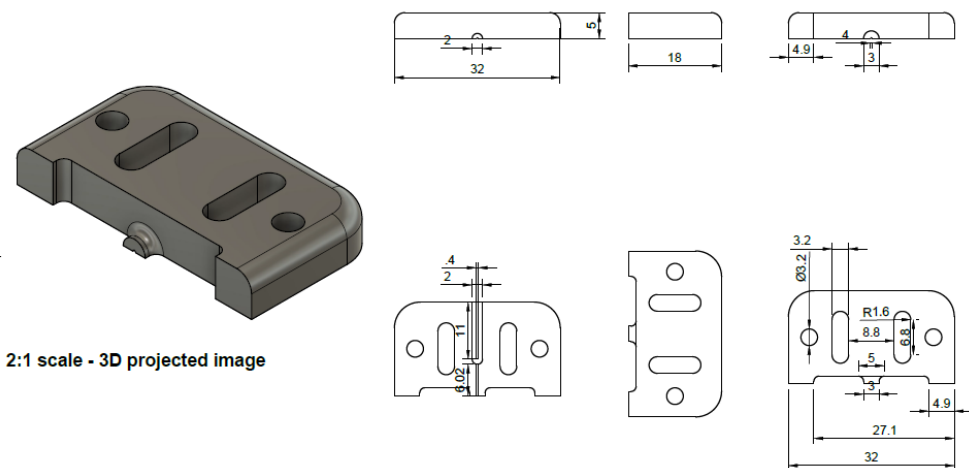
All dimensions in mm



2:1 scale - 3d projected image

Fiber Support Upper Piece

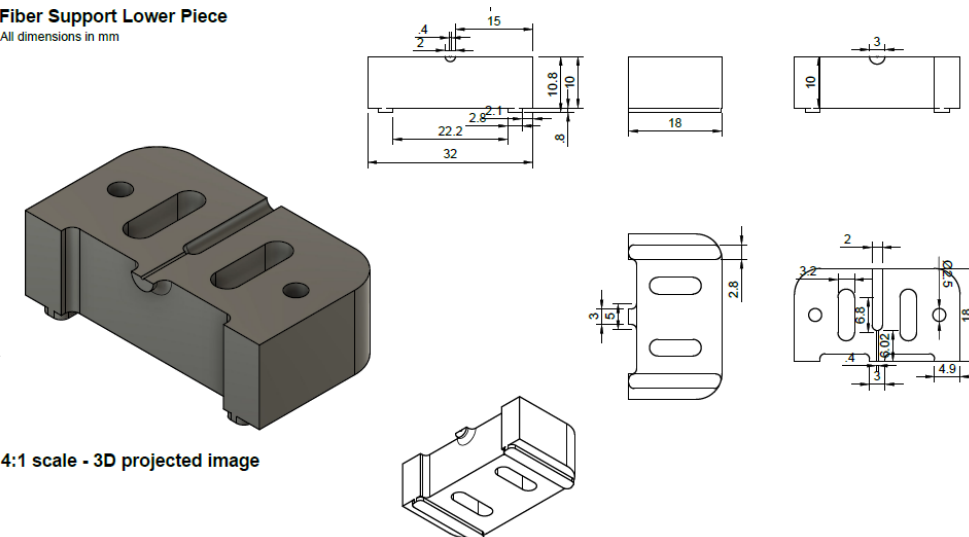
All dimensions in mm



2:1 scale - 3D projected image

Fiber Support Lower Piece

All dimensions in mm

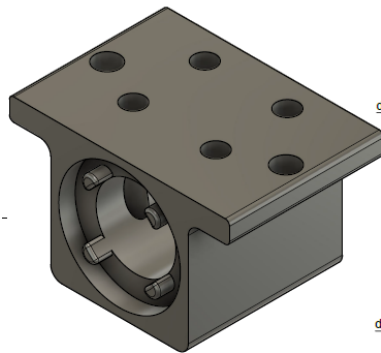


4:1 scale - 3D projected image

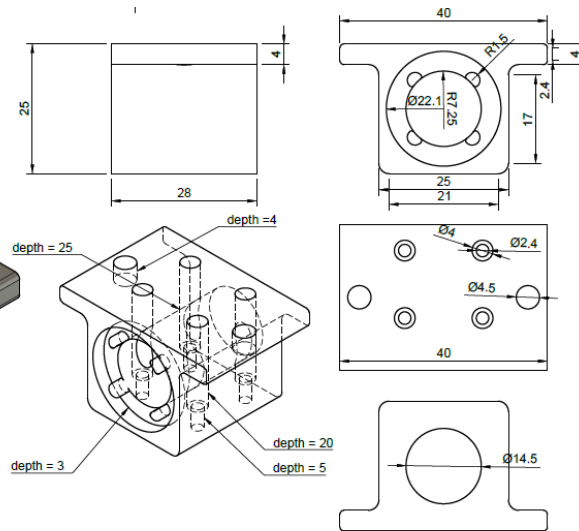
Appendix 2 - CAD drawings

Carriage Adaptor

All dimensions in mm

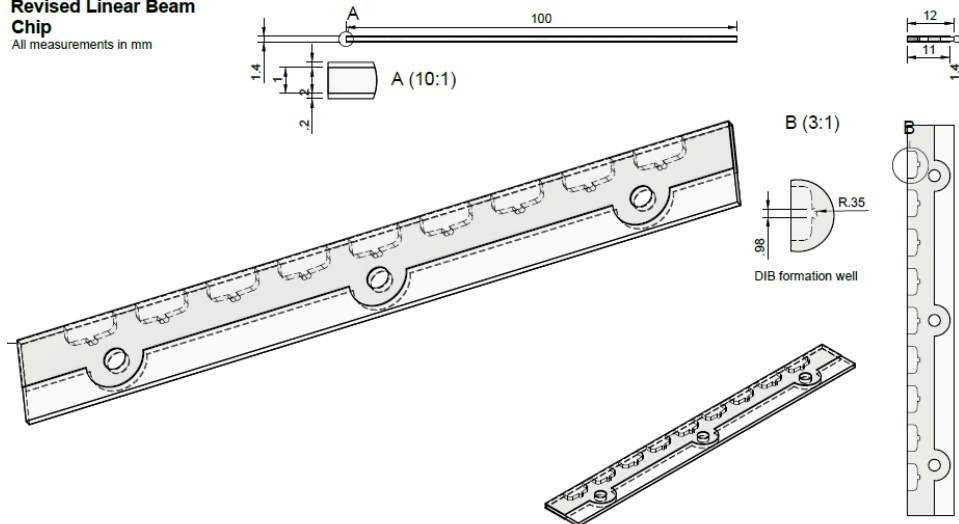


1.5:1 scale - 3D projected image



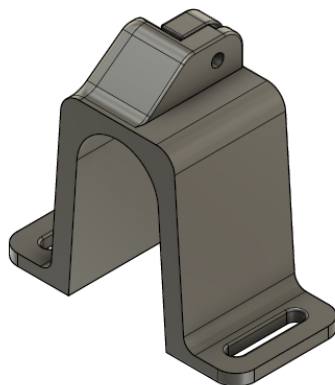
Revised Linear Beam Chip

All measurements in mm



Chip Mount with Support Piece

All dimensions in mm



2:1 scale - 3D projected image

