

**Using Trophoblast Cells to Develop Biotechnological Approaches that
Advance Prenatal and Women's Health**

By

Christina Mary Bailey-Hytholt

B.S., Worcester Polytechnic Institute, 2015

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Engineering at Brown University

PROVIDENCE, RHODE ISLAND

February 2020

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Dedicated to my husband, Erik Hytholt,
and my parents Ronald and Theresa Bailey

This dissertation by Christina M. Bailey-Hytholt is accepted in its present form by the Biomedical Engineering program, a joint program in the Division of Biology and Medicine and the School of Engineering as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date

Anita Shukla, Ph.D., Advisor

Date

Anubhav Tripathi, Ph.D., Advisor

Recommended to the Graduate Council

Date

Eric Darling, Ph.D., Reader

Date

Gary Wessel, Ph.D., Reader

Date

Morey Kraus, Ph.D., Reader

Approved by the Graduate Council

Date

Andrew Campbell, Ph.D., Dean of the Graduate School

ABSTRACT

The placenta plays a crucial role during pregnancy, yet it remains one of the least understood human organs. Trophoblast cells comprise the placenta with functions including nutrient and waste transport, invading the endometrium to anchor the placenta, and remodeling vasculature for adequate blood flow. These placental trophoblast cells have great potential for use in developing non-invasive prenatal testing and *in vitro* models of the maternal-fetal interface. In this thesis we utilized biomaterial and diagnostic techniques to (1) develop a rapid and inexpensive method for enriching trophoblast cells from clinical cervical samples for non-invasive prenatal testing, (2) develop lipid models representative of trophoblast cells at various gestational time points, and (3) consider therapeutic design of pharmaceuticals for improving drug efficacy with the potential to mitigate placental interaction. Our enrichment method led to an ~700% enrichment of trophoblast cells from cervical samples with the ability to then isolate a single trophoblast cell using automated cell picking. Our *in vitro* trophoblast lipid models mimic 1st, 3rd, and full-term trophoblasts and were used to study antifungal pharmaceutical and environmental toxicant interactions. Additionally, we contributed to the fundamental lipid bilayer field through studies focusing on the role of flow on rupturing vesicles into supported lipid bilayers and investigating phthalate interactions with lipid structures. Finally, we developed antifungal nanoparticles by encapsulating the antifungal therapeutic, anidulafungin, into liposomes, also for future use by pregnant women that are prone to fungal infections. These nanoparticles demonstrated increased efficacy against planktonic and biofilm *Candida albicans* while also improving survival of *Candida* infected *Galleria mellonella* compared to free drug. Ultimately, this thesis provides new methods to understand and use trophoblast cells for

the improvement of maternal and fetal health. The biotechnological approaches we have developed have the ability to minimize risk to a developing fetus, enable better informed treatment plans, and allow for screening of new therapies.

CURRICULUM VITAE

Christina M. Bailey-Hytholt

EDUCATION

Ph.D. in Biomedical Engineering Sept. 2015 – Dec. 2019
National Science Foundation Graduate Research Fellow
Brown University, Providence, RI
Using Trophoblast Cells to Develop Biotechnological Approaches that Advance Prenatal and Women's Health
Thesis Co-advisors: Anubhav Tripathi, Ph.D. and Anita Shukla, Ph.D.

B.S. in Chemical Engineering 2011 - 2015
Worcester Polytechnic Institute, Worcester, MA
Graduated with High Distinction

HONORS

- Women in Chemical Engineering (WIC) Travel Award, AIChE Annual Meeting, Orlando, FL 2019
1 of 15 awarded conference attendees
- Biomedical Engineering Society Career Development Award 2019
1 of 20 awarded conference attendees
- National Science Foundation Leadership Workshop, Worcester Polytechnic Institute 2018
Competitive selection process, 1 of 26 awarded participants
- Biomedical Engineering Society Travel Award, BMES Annual Meeting, Phoenix, Arizona 2017
1 of 20 awarded conference attendees
- Poster Competition 1st Place Organic Biochemistry division, Central MA ACS Meeting, Worcester, MA 2015
- National Science Foundation Graduate Research Fellowship 2015
- Outstanding Greek Student Award 2015
Awarded to one senior student in the Greek life community for the highest academic achievement
- Poster Competition 1st Place Environmental I division, AIChE Annual Student Conference, Atlanta, GA 2014
- Poster Competition 1st Place, AIChE Northeast Student Conference, University of Connecticut, Storrs, CT 2014
- Paper Competition 3rd Place, AIChE Northeast Student Conference, University of Connecticut, Storrs, CT 2014

- Bonnie-Blanche Schoonover Outstanding Women Student Award, 2014
Worcester Polytechnic Institute
Awarded to one junior woman student annually
- Omicron Delta Kappa National Leadership Honor Society, 2014
Worcester Polytechnic Institute
- Tau Beta Pi Engineering Honor Society, Worcester Polytechnic Institute 2013
- Gertrude R. Rugg Outstanding Student Award, Worcester 2012
Polytechnic Institute
Awarded to one freshman woman student annually
- Charles O. Thompson Scholar, Worcester Polytechnic Institute 2012
Recognition of first year academic excellence
- Daniel M. Clemson Scholarship for Young Engineers, 2011-2015
Stratus Technologies, Inc., Maynard, MA
Awarded to one student annually
- Dean's List, Worcester Polytechnic Institute 2011-2015

PUBLICATIONS

JOURNAL PUBLICATIONS

6. **Bailey-Hytholt, C.M.**, Puranik, T., Tripathi, A., Shukla, A., *Investigating the Impact of Phthalate Environmental Toxicants on Lipid Structures*, in review.
5. Vera-Gonzalez, N,* **Bailey-Hytholt, C.M.**,* Langlois, L., de Camargo Ribeiro, F., Luzia de Souza Santos, E., Campos Junqueira, J., Shukla, A., *Anidulafungin nanoparticles exhibit antifungal activity against planktonic and biofilm Candida albicans*, in review. *Co-first authors
4. **Bailey-Hytholt, C.M.**, Sayeed, S., Kraus, M., Joseph, R., Shukla, A., Tripathi, A., *A Rapid Method for Label-Free Enrichment of Rare Trophoblast Cells from Cervical Samples*, **Scientific Reports**, 9, 12115, 2019.
Featured in Brown News: <https://www.brown.edu/news/2019-08-26/trophoblasts>
3. **Bailey, C.M.**, Tripathi, A., Shukla, A., *Effects of Flow and Bulk Vesicle Concentration on Supported Lipid Bilayer Formation*, **Langmuir**, 33(43), 11986-11997, 2017.
2. **Bailey, C.M.**, Kamaloo, E., Waterman, K. L., Wang, K. F., Nagarajan, R., Camesano, T. A. *Size dependence of gold nanoparticle interactions with a supported lipid bilayer: A QCM-D study*. **Biophysical chemistry**, 203, 51-61, 2015.
1. **Bailey, C.M.**, Waterman, K., Macedonio, J., and Camesano, T.A. *Characterizing Gold Nanoparticle Interactions with Model Cell Membranes*, **Northeast Bioengineering**

Conference (NEBEC), 40th Annual, 2014, pp. 1-2 (10.1109/NEBEC.2014.6972717), 2014.

BOOK CHAPTERS

2. **Bailey, C.M.**, Nagarajan, R., Camesano, T. A., *Designing polymer micelles of controlled size, stability and functionality for siRNA delivery*, **Control of Amphiphile Self-Assembling at the Molecular Level: Supra-Molecular Assemblies with Tuned Physicochemical Properties for Delivery Applications**, pp. 35-70, American Chemical Society, 2017. *Featured figure on book cover.*

1. Kamaloo, E., **Bailey, C.M.**, Camesano, T. A. *Effect of Concentration on the Interactions of Gold Nanoparticles with Model Cell Membranes: A QCM-D Study*. **Nanotechnology to Aid Chemical and Biological Defense** (pp. 67-76). Springer, Dordrecht, 2015.

TO BE SUBMITTED

2. **Bailey-Hytholt, C.M.**,* Sayeed, S.,* Shukla, A., Tripathi, A., *Enrichment of JEG-3 trophoblast cells from cervical samples using inclined plane flow*. *Co-first authors

1. **Bailey-Hytholt, C.M.**, Shen, T.L., Tripathi, A., Shukla, A., *Development and application of a placental trophoblast lipid bilayer for cell-free molecular interaction studies*.

RESEARCH EXPERIENCE

NSF Graduate Research Fellow

Sept. 2015 - Present

Center for Biomedical Engineering, School of Engineering
Brown University, Providence, RI

Co-advisors: Professor Anubhav Tripathi, Professor Anita Shukla

Using Trophoblast Cells to Develop Biotechnological Approaches that Advance Prenatal and Women's Health

Aim 1: Advance methods for trophoblast cell enrichment and isolation from clinical cervical samples for non-invasive prenatal testing
Collaborated with mentors from PerkinElmer, Inc.: Morey Kraus, Chief Scientific Officer and Richard Joseph, Senior Assay Development Scientist, PerkinElmer, Waltham, MA

- Developed methods for fetal trophoblast cell enrichment and isolation from clinical cervical samples
- Developed techniques to enrich rare cells from a heterogeneous population using RareCyte CyteFinder imaging and cell picking platform

Aim 2: Develop a placental lipid bilayer for molecular interaction studies to inform risk to a developing fetus

- Investigated influence of flow and bulk vesicle concentration on supported lipid bilayer formation
- Investigated the impact of environmental toxicants on lipid structures
- Developed a placental mimetic lipid bilayer for cell-free molecular interaction studies

Aim 3: Develop drug delivery vehicles to improve therapeutic efficacy with potential to mitigate interactions during pregnancy

Collaborated with Noel Vera-Gonzalez, Ph.D. Candidate in the Shukla lab, Brown University

- Developed antifungal, anidulafungin, loaded liposomes for improved efficacy and solubility compared to free drug
- Investigated liposomes with planktonic and biofilm *Candida albicans* as well as *Candida* infected *Galleria mellonella* model

Collaboration projects:

- Isolated single MCF-7 breast cancer cells using a centrifugal force device
Collaboration with Adam Snider, Ph.D. candidate in the Tripathi lab and Ileana Pirozzi, undergraduate researcher in the Tripathi lab, Brown University
- Extracted metabolites from trophoblast cells for data storage
Collaboration with Eamonn Kennedy, Post-doc in the Rosenstein lab, Brown University

Skills: Lipid vesicle and liposome formation, extrusion, quartz crystal microbalance with dissipation (QCM-D), dynamic light scattering (DLS), zeta potential, microscopy techniques including fluorescence and confocal, spectroscopy, mammalian cell culture (BeWo, JEG-3, TCL-1, HTR-8, MCF-7, HUVEC cell lines), clinical cervical and blood sample handling, microfluidic chip fabrication, mammalian cell enrichment, automated cell picking, quantitative polymerase chain reaction (qPCR), immunomagnetic cell isolation

Undergraduate Researcher

June 2012 – Aug. 2015

Department of Chemical Engineering

Worcester Polytechnic Institute, Worcester, MA

Advisors: Professor Terri A. Camesano and Dr. Ramanathan Nagarajan (Senior Research Scientist, Soldier Nanomaterials, Natick Soldier Research, Development and Engineering Center)

- Investigated gold nanoparticle interactions with supported lipid bilayers using quartz crystal microbalance with dissipation
- Developed environmental models using humic acid and polymer substances
- Determined the effect of nanoparticle size and functionalization on their interaction with supported lipid bilayers

Worcester Polytechnic Institute Major Qualifying Project

Sept. 2014 – May 2015

Department of Chemical Engineering

Worcester Polytechnic Institute, Worcester, MA

Advisors: Professor Terri A. Camesano, Dr. Ramanathan Nagarajan (Senior Research Scientist, Soldier Nanomaterials, Natick Soldier Research, Development and Engineering Center)

Improving the Delivery of Therapeutic Agents: Creating Enhanced siRNA Carriers using Block Copolymers

- Developed block copolymer micelle complexation with siRNA
- Used fluorescence resonance energy transfer (FRET) and dynamic light scattering techniques to monitor micelle complexation

Worcester Polytechnic Institute Interactive Qualifying Project Oct. 2013 – Dec. 2013

United States Coast Guard, Anacostia, Washington, D.C.

Advisors: Lt. Joshua Buck and Anthony Morris, Assistant Branch Chief, Mission Management System

Team: **Bailey C.M.**, Caisse M.W., Kirichenko E., Smith N.R.

Risk-Based Marine Inspection Performance Measures

WPI Electronic Projects Collection, E-Project-121913-180809, December 2013.

- Interacted with U.S. Coast Guard personnel and WPI teammates of various majors to develop a new method to determine risk analysis during inspections of marine vessels
- Developed a computer program to help assign risk values to categories of the inspection process

TEACHING EXPERIENCE

Undergraduate Research Advisor

2015 - Present

Center for Biomedical Engineering, School of Engineering, Brown University

- Advised six Brown University undergraduates on prenatal and biomaterial research

Teaching Assistant

Center for Biomedical Engineering, School of Engineering, Brown University

- TA for undergraduate core course, *Biomedical Engineering Design and Innovation 2 (ENGN 1930/0930)* (Enrollment of 20 students) Spring 2019
- TA for undergraduate core course, *Biomedical Engineering Design and Innovation (ENGN 1930/0930)* (Enrollment of 40 students) Fall 2018
- TA for undergraduate core course, *Transport and Biotransport Processes (ENGN 1110)* (Enrollment of 45 students) Spring 2017
- TA for undergraduate core course, *Transport and Biotransport Processes (ENGN 1110)* (Enrollment of 40 students) Spring 2016

Frontiers Biomedical Engineering Exploration

2014, 2015

Summer Program Lecturer

Worcester Polytechnic Institute, Worcester, MA

- Lead lecturer for two week long biomedical engineering summer program for 30 high school students
- Organized lesson plans and lead lecturers and activities on biomedical engineering topics including fluid flow, stress-strain, immunocytochemistry, rapid prototyping, and translational product development

Innovations Math and Science Summer Program Mentor

2013

Worcester Polytechnic Institute, Worcester, MA

- Mentored 15 middle school girls on research projects and presentation skills

Math and Science Tutor

2012

The Knowledge Quest, Worcester, MA

- Tutored approx. 10 elementary and high school students in math and science topics and SAT preparation

INTELLECTUAL PROPERTY

3. Shukla, A., Cowles, S., Vera-Gonzalez, N., **Bailey-Hytholt, C.**, Silvert, E., Brown University, Antifungal Nanoparticles for Targeted Treatment of Fungal Infections, U.S. patent application number: 62/852,095, filed: May 23, 2019.

2. **Bailey-Hytholt, C.**, Tripathi, A., Sayeed, S., Shukla, A., Inclined Plane Enrichment Device, U.S. patent application number 62/850,881, filed: May 21, 2019.

1. Shukla, A., **Bailey-Hytholt, C.**, Tripathi, A., Brown University, Placental lipid bilayer for cell-free molecular interaction studies, U.S. patent application number: 62/850,188, filed: May 20, 2019.

CONFERENCE PRESENTATIONS

ORAL PRESENTATIONS

14. **Bailey-Hytholt, C.M.**, Puranik, T., Tripathi, A., Shukla, A., Effect of Di(2-ethylhexyl) Phthalate and Mono(2-ethylhexyl) Phthalate Interactions with a Lipid Membrane, *2019 American Institute of Chemical Engineers Annual Meeting*, Orlando, FL, November, 2019.

13. **Bailey-Hytholt C.M.**, Sayeed, S., Kraus, M., Joseph, R., Shukla, A., Tripathi, A., A Rapid Method for Label-Free Enrichment of Rare Trophoblast Cells from Cervical Samples, *2019 American Institute of Chemical Engineers Annual Meeting*, Orlando, FL, November, 2019.

12. **Bailey-Hytholt C.M.**, Shen, T.L., Tripathi, A., Shukla, A., Award Submission: Development of a Model Placental Lipid Bilayer, *2019 American Institute of Chemical Engineers Annual Meeting*, Orlando, FL, November, 2019.
11. **Bailey-Hytholt C.M.**, Tripathi, A., Shukla, A., Investigating Molecular Interactions of the Placenta for Prenatal Treatment and Testing, *2019 American Institute of Chemical Engineers Annual Meeting*, Orlando, FL, November, 2019.
10. **Bailey-Hytholt, C.M.**, Shen, T.L., Tripathi, A., Shukla, A., Development of a Model Placental Lipid Bilayer, *2019 American Institute of Chemical Engineers Annual Meeting*, Orlando, FL, November, 2019.
9. **Bailey-Hytholt, C.M.**, Puranik, T., Tripathi, A., Shukla, A., Impact of Phthalate Environmental Toxicants with Lipid Structures, *Biomedical Engineering Society Annual Meeting*, Philadelphia, PA, October, 2019.
8. **Bailey-Hytholt C.M.**, Shen, T.L., Tripathi, A., Shukla, A., Development of a Model Placental Lipid Bilayer, *Biomedical Engineering Society Annual Meeting*, Philadelphia, PA, October, 2019.
7. Sayeed, S., **Bailey-Hytholt, C.M.**, Shukla, A., Tripathi, A. Trophoblast Enrichment Using Surface Adhesion Properties, *45th Annual Northeast Bioengineering Conference*, Rutgers, New Brunswick, NJ, March 2019.
6. **Bailey-Hytholt, C.M.**, Kamaloo, E., Waterman, K., Swana, K., Camesano, T.A., Nagarajan, R., Nanotoxicology: Exploring Nanoparticle-Model Membrane Interactions, *257th ACS National Meeting & Exposition*, March, 2019. *underline notes presenter
5. **Bailey, C.M.**, Tripathi, A., Shukla, A., Flow Induced Liposome Rupture into a Lipid Bilayer on Solid Surfaces using QCM-D, *Biomedical Engineering Society 2017 Annual Meeting*, Phoenix, Arizona, October, 2017.
4. Puranik, T., **Bailey, C.M.**, Shukla, A., Investigating Cholera Toxin Infection During Pregnancy Using an In Vitro Placental Model, *Biomedical Engineering Society 2017 Annual Meeting*, Phoenix, Arizona, October 2017.
3. Cowles, S.C., **Bailey, C.M.**, Vera-Gonzalez, N.A., Shukla, A., A Novel Liposomal Formulation Targeting *Candida albicans*, *Biomedical Engineering Society 2016 Annual Meeting*, Minneapolis, MN, October 2016.

2. Cowles, S.C., **Bailey, C.M.**, Vera-Gonzalez, N.A., Shukla, A., Functionalized Liposome Delivery Targeting Opportunistic Fungi, *American Institute of Chemical Engineering Annual Student Meeting*, San Francisco, CA, November, 2016.

1. **Bailey, C.M.**, Kamaloo, E., Waterman, K., Macedonio, J., Wang, K., and Camesano T.A., Gold Nanoparticle Interactions with Model Biological Membranes. *American Institute of Chemical Engineers Northeast Student Regional Conference*, Storrs, CT, April, 2014. **Received 3rd Place Paper Competition**

POSTER PRESENTATIONS

24. **Bailey-Hytholt, C.M.**, Advancing Technologies for Prenatal and Women's Health, 2019 *American Institute of Chemical Engineers Annual Meeting*, Orlando, FL, November, 2019.

23. **Bailey-Hytholt C.M.**, Sayeed, S., Kraus, M., Joseph, R., Shukla, A., Tripathi, A., A Rapid Method for Label-Free Enrichment of Rare Trophoblast Cells from Cervical Samples, *Biomedical Engineering Society Annual Meeting*, October, 2019.

22. Nie, B., **Bailey-Hytholt, C.M.**, Tripathi, A., Shukla, A., Investigating Placental Lipid Bilayer Interactions using QCM-D, *Undergraduate Research and Teaching Symposium*, Brown University, August, 2019.

21. Langlois, L. M., **Bailey-Hytholt, C.M.**, Vera-Gonzalez, N. A., Junqueira, J., Shukla, A., Targeted Antifungal Liposomes for the Treatment of Systemic Fungal Infections, *Undergraduate Research and Teaching Symposium*, Brown University, August, 2019.

20. **Bailey-Hytholt, C.M.**, Sayeed, S., Kraus, M. Joseph, R., Shukla, A., Tripathi, A., Rare Trophoblast Cell Enrichment from Cervical Samples by Differential Cell Settling, *2019 Scientista Symposium*, Boston, MA, March, 2019.

19. **Bailey-Hytholt, C.M.**, Puranik, T., Sayeed, S., Tripathi, A., Shukla, A., Investigating Molecular Interactions of the Placenta for Prenatal Treatment and Testing, *Young Scholar's Conference*, Brown University, Providence, RI, October, 2018.

18. Sayeed, S., **Bailey-Hytholt, C.M.**, Shukla, A., Tripathi, A., JEG-3 Cell Enrichment Using Surface Adhesion Properties, *Sigma Xi Annual Meeting & Student Research Conference*, October, 2018.

17. Sayeed, S., **Bailey-Hytholt, C.M.**, Shukla, A., Tripathi, A., Enrichment of JEG-3 Cells by Intrinsic Surface Adhesion Properties, *Biomedical Engineering Society 2018 Annual Meeting*, Atlanta, GA, October, 2018.
16. Sayeed, S., **Bailey-Hytholt, C.M.**, Shukla, A., Tripathi, A., JEG-3 Adhesion Using Intrinsic Surface Properties, *Undergraduate Research and Teaching Symposium*, Brown University, August, 2018.
15. Puranik, T., **Bailey, C.M.**, Shukla, A., Investigating Cholera Toxin Infection During Pregnancy using an in vitro Placental Model, *Undergraduate Research and Teaching Symposium*, Brown University, August, 2017.
14. Cowles, S., Vera-Gonzalez, N., **Bailey, C.M.**, Shukla, A., A Novel Liposomal Formulation Targeting Opportunistic Fungi, *Undergraduate Research and Teaching Symposium*, Brown University, August, 2016.
13. **Bailey, C.M.**, Tripathi, A., Shukla, A., The role of convective properties on model cell membrane assembly mechanisms using QCM-D, *Indian Institute of Technology 2016 Leadership Conference*, August, 2016.
12. **Bailey, C.M.**, Nagarajan, R., and Camesano, T.A., Kinetic release of micellized PEG-PLL block copolymer complexed with siRNA using FRET assay, *American Chemical Society 250th National Meeting*, Boston, MA, August, 2015.
11. **Bailey, C.M.**, Nagarajan, R., and Camesano, T.A., Improving the delivery of therapeutic agents: creating siRNA carriers using PEG-PLL block copolymers, *Central Massachusetts American Chemical Society Meeting*, April 2015. ***Received 1st place in organic biochemistry division.***
10. **Bailey, C.M.**, Kamaloo, E., Waterman, K., Wang, K., Nagarajan, R., Camesano, T.A., Size Dependence of Gold Nanoparticle Interactions with Supported Lipid Bilayer: A QCM-D Study, *249th ACS National Meeting*, March, 2015.
9. **Bailey, C.M.**, Kamaloo, E., Waterman, K., Wang, K., and Camesano, T.A. Gold Nanoparticle Interactions with Model Biological Membranes. *American Institute for Chemical Engineers Annual Meeting*, Atlanta, GA, November, 2014.
8. **Bailey, C.M.**, Kamaloo, E., Waterman, K., Wang, K., and Camesano, T.A. Gold Nanoparticle Interactions with Model Cell Membranes. *American Institute for Chemical*

Engineers Annual Student Conference, Atlanta, GA, November 2014. **Received 1st Place Environmental I Division Poster**

7. **Bailey, C.M.**, Kamaloo, E., and Camesano, T.A., Interactions of Functionalized Gold Nanoparticles with Humic Acid. *Third Sustainable Nanotechnology Organization Conference*, Boston, MA, November, 2014.

6. **Bailey, C.M.**, Waterman, K., Kamaloo, E., Logan, T, Ly, M., Wang, K., and Camesano, T.A., Characterizing Gold Nanoparticle Interactions with a Supported Lipid Bilayer in the Presence of Humic Acid. *Biomedical Engineering Society 2014 Annual Meeting*, San Antonio, Texas, October, 2014.

5. **Bailey, C.M.**, Waterman, K., Kamaloo, E., Logan, T, Ly, M., Wang, K., and Camesano, T.A., Interactions of Surface Charged Gold Nanoparticles with a Supported Lipid Bilayer: The Role of Humic Acids. *NATO Advanced Research Workshop in Nanotechnology to Aid Chemical and Biological Defense*, Antalya, Turkey, September, 2014.

4. **Bailey, C.M.**, Kamaloo, E., Waterman, K., Wang, K., and Camesano, T.A., Characterizing Gold Nanoparticle Interactions with Biological Membranes. *International Translational Nanomedicine Conference*, Boston, MA, July, 2014.

3. **Bailey, C.M.**, Kamaloo, E., Waterman, K., Macedonio, J., and Camesano, T.A., Characterizing Gold Nanoparticle Interactions with Model Cell Membranes. *40th Annual Northeast Bioengineering Conference*, Northeastern University, Boston MA, April, 2014.

2. **Bailey, C.M.**, Kamaloo, E., Waterman, K., Macedonio, J., Wang, K., and Camesano T.A., Gold Nanoparticle Interactions with Model Biological Membranes. *American Institute of Chemical Engineers Northeast Student Regional Conference*, Storrs, CT, April, 2014. **Received 1st Place Poster**

1. **Bailey, C.M.**, Waterman, K., Macedonio, J, and Camesano, T.A., Gold Nanoparticle Interactions with Model Cell Membranes. *Biomedical Engineering Society 2013 Annual Meeting*, Seattle, WA, September, 2013.

PROFESSIONAL ACTIVITIES AND SERVICE

Brown University

- Panel member, Undergraduate Professional Workshop for Women & Minorities, Women in Chemical Engineering, AIChE Annual Meeting, Orlando, FL, 2019
- Engineering Research Fair, Brown University, Providence RI, Nov. 2016, 2017, 2018

- Judge, Rhode Island Science and Engineering Fair, Community College of Rhode Island, Warwick, RI, 2016-2018
- Quartz Training for Engineering Administrative Assistants, May 2018
- Lecture/demonstrations, Vartan Gregorian Elementary School Science Conference, Providence, RI, 2016, 2019

Worcester Polytechnic Institute

- Corresponding Secretary, Tau Beta Pi Engineering Honor Society, 2012-2013
- American Institute of Chemical Engineers major mentor, WPI, 2014-2015
- Alpha Gamma Delta Philanthropy efforts supporting Diabetes Research, WPI, 2011-2015
- Introduce a girl to engineering day, WPI, 2014, 2015
- Middle school chemical reaction program, Framingham, MA, 2015
- Boy Scout troop science day camp, Troop 151, West Boylston, MA, 2014
- Emergency room assistant, University of Massachusetts Memorial Hospital, 2012-2013

PROFESSIONAL DEVELOPMENT

- Future Faculty Workshop, Princeton University, August 2019
- Young Scholar's Conference: Communicating Your Science, Brown University, October 2018
- National Science Foundation Leadership Workshop, Worcester Polytechnic Institute, June 2018
- Biomedical Engineering Seminar, Brown University, 2015-Present

STUDENTS MENTORED

- Bonnee Nie, Biochemistry, Class of 2020 2019 – Present
 - Senior Honors Thesis, 2020
- Luc Langlois, Chemistry, Class of 2020 2019 – Present
 - Undergraduate Teaching and Research Award recipient, 2019
 - Accepted BMES Annual Meeting poster presentation, 2019
 - Accepted Sigma Xi Annual Meeting poster presentation (1 of 4 selected Brown students), 2019
 - 1st place in engineering Sigma Xi poster competition, 2019
- Sumaiya Sayeed, Biomedical Engineering, Class of 2020 2017 – Present
 - Accepted podium talk at NEBEC, 2019
 - Undergraduate Teaching and Research Award recipient, 2018
 - Supplemental Funding for UTRA project awarded, 2018

- Accepted BMES Annual Meeting poster presentation, 2018
- Accepted Sigma Xi Annual Meeting poster presentation (1 of 4 selected Brown students), 2018
- Senior Honors Thesis, 2019
- Eli Silvert, Materials Science and Engineering, Class of 2020 2017 – 2019
- Tanaya Puranik, Biomedical Engineering, Class of 2019 2016 – 2019
- Current Sc.M. Student at Brown University*
 - Undergraduate Teaching and Research Award recipient, 2017
 - Accepted BMES undergraduate podium talk, 2017
 - Recipient of BMES travel award, 2017
- Sarah Cowles, Chemical Engineering, Class of 2017 2016 – 2017
- Current Ph.D. Student and NSF Graduate Research Fellow at Massachusetts Institute of Technology*
 - Undergraduate Teaching and Research Award recipient, 2016
 - Senior Honors Thesis, 2016
 - Accepted AIChE Annual Meeting podium talk, 2016
 - Accepted BMES Annual Meeting podium talk, 2016
 - NSF Graduate Research Fellowship recipient, 2017

AFFILIATIONS

- Biomedical Engineering Society 2013 - Present
- American Institute of Chemical Engineers 2011 - Present
- American Chemical Society 2015 - Present
- Tau Beta Pi Engineering Honor Society 2014 - Present
- Corresponding Secretary (2014-2015)*
- Alpha Gamma Delta – Zeta Zeta, active sister 2011 - 2015
- Publications Coord. (2012 – 2013)*
- Alpha Gamma Delta – Zeta Zeta, alumni sister 2015 - Present
- Omicron Delta Kappa National Leadership Honor Society 2014-2015
- Sustainable Nanotechnology Organization 2014-2015

ACKNOWLEDGEMENTS

I would like to start by thanking my PI's Dr. Anita Shukla and Dr. Anubhav Tripathi. Thank you for always giving me freedom in starting new and exciting projects in the lab. Dr. Tripathi, thank you for inspiring me and giving me confidence to always be innovating. Dr. Shukla, thank you for teaching me so much and for your tremendous amount of support and guidance. You have been an excellent role model to me. A large part of my success in graduate school is due to having both of your mentorship and encouragement.

In addition to my advisors, I am grateful to have a committee of talented researchers. I would like to thank my committee members Dr. Eric Darling, Dr. Gary Wessel, and Dr. Morey Kraus for your instrumental support and suggestions throughout this thesis.

I have fully enjoyed my time at Brown and a big reason for this is my labmates. To every member of the Shukla and Tripathi labs I would like to extend my thanks for your help in lab work and for your friendship. To the past and present postdoctoral researchers (Akram, Alessia, Chao, Mary, and Roya) and graduate students (Adam, Alexis, Bethany, Carly, Cassi, Celeste, Dahlia, Francis, Kiara, Lindsay, Lei, Luke, Nisha, Noel, Quentin, Rachel, Samantha, Shashank, Veronica, Yingying) in the Shukla and Tripathi labs, thank you for making time in the lab fun with daily lunches together, lazy book club, and many other adventures (holiday parties, conference travels, pasta making, and eating more cake than I ever have in my life). Thank you for making this experience special.

Dr. Shukla and Dr. Tripathi always encouraged me to be a mentor to undergraduate students in the lab, which is something I am very passionate about. It has been a pleasure

to mentor students (Bonnee, Eli, Luc, Sarah, Sumaiya, Tanaya) and I am so proud to have seen your growth into independent researchers.

I have also had a chance to collaborate with many people during my projects. I would like to thank the mass spectrometry facility at Brown (Dr. Tun-Li Shen), the Kilguss Research Institute at Women & Infants Hospital (Dr. Surendra Sharma), the R.I. Consortium for Nanoscience and Nanotechnology (Dr. Irene Andreu), the Univ. Estadual Paulista, Unesp (Dr. Juliana Campos Junqueira, Felipe de Camargo Ribeiro, and Evelyn Luzia de Souza Santos), and PerkinElmer, Inc. (Dr. Morey Kraus and Richard Joseph).

There are also many people from my undergraduate institution, Worcester Polytechnic Institute, that I would like to thank. Dr. Terri Camesano, Dr. Ramanathan Nagarajan and the members of the Camesano lab (in particular Kellie, Lindsay, and Todd), you were each instrumental towards finding my passion for research and continuing to graduate school.

I would like to thank my entire family and friends for countless gatherings and memories that added balance to my Ph.D. work. To my Hytholt family (Valerie, Dale, and Tom), I would like to extend my thanks for welcoming me into your family and for your support and encouragement. I would also like to thank my grandmothers, Cecile ‘Sis’ D’Elia and Bertha Bailey. Grammie Sis, you have been my biggest supporter and I am so thankful for your faith, wisdom, and ability to brag to everyone about how proud you are of me. I also know that my grandfathers (Alfred D’Elia and Ronald Bailey, Sr.) are watching over and I know that Grampie Al is beaming with pride. Grampie Al, your kindness and character has been a large influence on me and has inspired me throughout my life.

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Chapter 1

Background

1.1 Motivation

Currently, less than 10% of U.S. Food and Drug Administration (FDA) approved pharmaceuticals have enough information to determine risks towards a developing fetus (Figure 1.1a).¹ However, 9 out of 10 women in the U.S. take at least one medication during the course of their pregnancy (Figure 1.1b).¹ The use of the medications results in 5.4 million pregnancies exposed to medications each year.¹ The Centers for Disease Control and Prevention (CDC) currently has a Treating for Two program¹ that recognizes the importance of this issue and aims to improve the health of women and babies by identifying safe treatment options.

Unknown risks (both those associated with medication use and others) can lead to a variety of health concerns throughout pregnancy (Figure 1.1c).¹ Currently, methods to diagnose these potential abnormalities are often invasive procedures that carry risk of miscarriage and infection.^{2,3} New non-invasive prenatal testing methods are needed to provide safer options to expecting mothers.

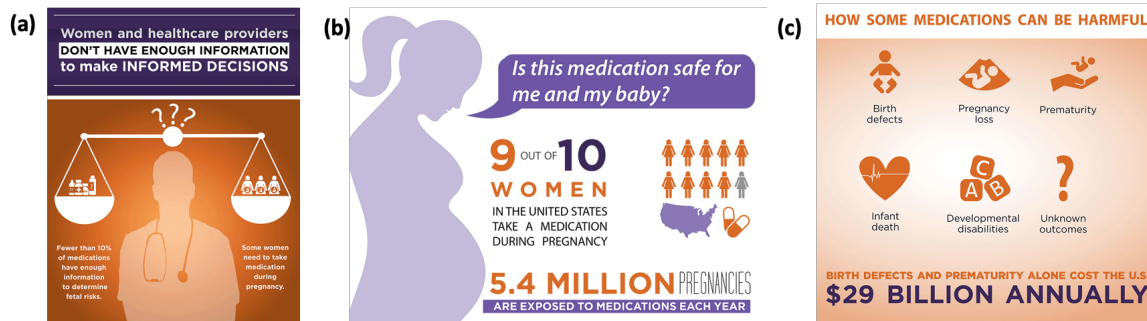


Figure 1.1: Infographics from the CDC Treating for Two program depicting the need for more information and diagnostic techniques to improve safety during pregnancy.¹

These facts motivated this thesis into exploring the development of techniques to improve non-invasive prenatal testing options, developing models to investigate small molecule interactions during pregnancy, and considering new delivery vehicles to improve both safety and efficacy of a pharmaceutical.

1.2 The Placenta

The placenta is the primary site of nutrient and waste transport and acts as a barrier to protect a developing fetus from foreign molecules.⁴ Despite this crucial role, the placenta is one of the least understood human organs.⁵ The placenta is the first organ to develop after the implantation of the blastocyst.⁶ The outer layer of the blastocyst is the trophoctoderm, which differentiates into placental cells, known as trophoblasts.^{4,6} These trophoblasts then proceed to invade the endometrium.

Trophoblast cells proliferate and differentiate into either villous trophoblasts or extravillous trophoblasts (EVTs) (Figure 1.2).^{4,6,7} The villous trophoblast cells can fuse to form multinucleated cells known as syncytiotrophoblasts.^{4,8} These syncytiotrophoblasts form the outer most layer of the developing placenta and form the barrier between maternal

and fetal blood.^{4,9} They cover the entire chorionic villi, which are elongated branches allowing maximum surface area for contact with maternal blood.^{4,10} The syncytiotrophoblast is responsible for placental hormone production including human chorionic gonadotropin (hCG), estrogen, and progesterone.^{4,6} As EVT's undergo epithelial-mesenchymal transitions, they either become migratory endovascular trophoblasts or invasive interstitial trophoblasts.⁴ They penetrate into the maternal decidua and myometrium, which is the smooth muscle tissue of the uterus.¹¹ This invasion is necessary to dilate maternal arteries to allow adequate blood flow. This transformation creates low-resistance channels, which is important to increase placental perfusion leading to greater nutrient transport.^{4,12}

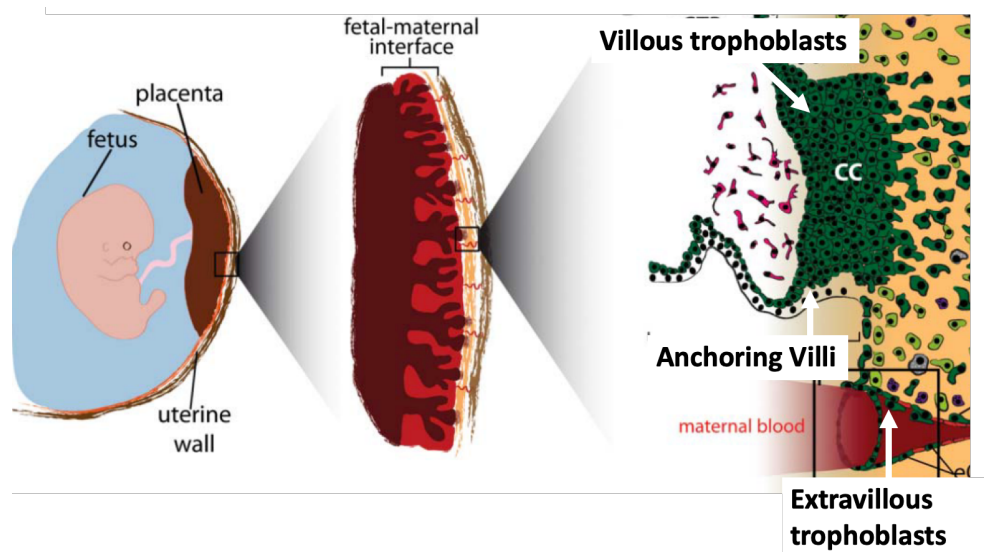


Figure 1.2: Structure of the placenta adapted from Velicky *et al.*, Cell Adhesion and Migration, 2016⁶ with permission under creative commons (CC BY).

1.3 Pregnancy-related complications

Over the course of pregnancy, complications can arise requiring medical intervention. Over 130 million babies are born each year, and 20% of pregnancies result in complications.¹³ Some common pregnancy-associated conditions include preeclampsia, gestational diabetes, intrauterine growth restriction, and preterm birth (Table 1.1). These complications further motivate the need for early prenatal testing methods and the need for improved understanding and development of pharmaceuticals during pregnancy.

Preeclampsia occurs in 4-8% of pregnancies.^{14,15} Complications of preeclampsia result in a death every 6 minutes around the world.¹⁶ While it is still not fully understood, preeclampsia is believed to be a complication with deep implantation of trophoblasts into the inner third of the myometrium.^{14,15,17} During a normal pregnancy, the spiral arteries are invaded by trophoblast cells and dilated, which is necessary for adequate blood supply to the fetus.^{14,15} However, during preeclampsia, there is limited or no invasion of trophoblasts into the spiral arteries and limited dilation of the arteries.^{14,15} Preeclampsia is often diagnosed in the third trimester due to clinical symptoms of high blood pressure. While medications may help to lower blood pressure, the most effective treatment is delivery.^{14,15} However, this can be a challenge in some settings where there is less advanced equipment to keep a premature baby alive.

Gestational diabetes mellitus (GDM) occurs in ~4% of all pregnancies and is a result of glucose intolerance with first onset being during pregnancy.¹⁸ ~15-45% of babies born to a mother with GDM results in macrosomia, which is increased birth weight above the 90th percentile.¹⁸ This can result in short and long term complications to both mother

(e.g. cesarean delivery and postpartum hemorrhage) and baby (e.g. increased risk of obesity and development of type II diabetes).¹⁸ GDM is often diagnosed at 20-24 weeks.¹⁸ The mechanisms for how GDM develops is still not fully understood, but rises in hormone levels (e.g. estrogen, progesterone, cortisol, placental lactogen) resulting in increased insulin resistance is thought to play a large role.¹⁸ Most often after diagnosis, diet and exercise changes can manage GDM.¹⁹ If diet and exercise alone does not help, insulin treatments, including glyburide and metformin, are prescribed for GDM management.¹⁹ GDM is typically resolved for the mother after delivery.

In the U.S., intrauterine growth restriction (IUGR) is defined as birth weight less than the 10th percentile for gestational age.²⁰ In order to diagnose IUGR, ultrasound is the most common technique used by obstetricians. IUGR can be caused by a number of complications from either the fetus (e.g. chromosomal abnormalities, infection, congenital malformations), placenta (e.g. small size), or mother (e.g. malnutrition and vascular disease).²⁰ An IUGR fetus is at increased risk of mortality²⁰, and the prevalence is greatly dependent on geographical location.²¹ Management of IUGR is dependent on the gestational age at diagnosis.²⁰ At earlier time points, some treatments have been tried including nutritional supplementation, low-dose aspirin, and maternal oxygen therapy; however, evidence for the efficacy of these treatments is limited.²⁰ At later gestational time points, the obstetrician may decide for delivery.

Preterm birth often occurs due to preeclampsia or intrauterine growth restriction.²² Other causes of preterm birth can include inflammation, infection, or vascular disease, which are often referred to as spontaneous preterm birth.²² Preterm birth is defined as delivery prior to 37 weeks of gestation²² and occurs in ~10% of births in the U.S. Preterm

birth results in the largest contribution to infant death.²³ Further, life-long complications can result, including cardiovascular disease, neurological disorders, asthma, and infections.^{24,25} Upon symptoms of preterm labor, expecting mothers may receive tocolytics or progesterone treatment to slow or stop contractions, often delaying labor a few days.²⁶ Antenatal corticosteroids may be then provided for the baby's lung development or antibiotics if preterm labor is onset by infection.²⁶ Recent investigations are looking towards novel approaches to mitigate preterm labor, by increasing the therapeutic options with delivery vehicles. For example, an investigation published in 2019 developed mucoinert progesterone nanosuspensions to increase effectiveness for preterm birth prevention.²⁷

Table 1.1: Common pregnancy-related complications.

Pregnancy-related complication	Defining characteristics	Prevalence	Common treatment
Preeclampsia	Inadequate dilation of spiral arteries leading to clinical symptoms of high blood pressure typically in the 3 rd trimester ^{14,15,17}	4-8% ^{14,15}	Medication to lower blood pressure, delivery ^{14,15}
Gestational diabetes	Glucose intolerance first onset during pregnancy at 20-24 weeks gestation ¹⁸	~4% ¹⁸	Diet and exercise modifications, insulin ¹⁸
Intrauterine growth restriction	Birth weight less than 10 th percentile as a result of complications from either the fetus, placenta, or mother leading to increased risk of fetal mortality ²⁰	Varies widely among countries ²¹	Numerous approaches with little evidence ²⁰

Preterm birth	Delivery prior to 37 weeks of gestation ²²	~10% ²³	Limited therapeutics include tocolytics, progesterone, and antenatal corticosteroids. Bed rest can also be recommended. ²⁶
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1.4 Prenatal screening and diagnostics

3-5% of pregnancies are complicated by birth defects due to genetic disorders with 1 in 150 live births presenting chromosomal abnormalities.^{28,29} Chromosomal abnormalities, including aneuploidy, translocations, duplications, and deletions, are some of the most common reasons for prenatal testing currently.²⁹ Recently, in 2007, the American College of Obstetricians and Gynecologists (ACOG) released the “ACOG Practice Bulletin No. 77” which recommended making aneuploidy screening available for all women.³⁰ This has led to prenatal screening being performed in ~67-72% of pregnancies.³¹

There are a number of screening and diagnostic techniques currently available. Screening techniques are typically performed to determine risk prior to a diagnostic technique. Current screening methods include the first trimester screen, quadruple marker screen, and integrated, stepwise sequential, and contingent screen. The screen positive rate for these methods is ~ 5%.²⁹ All of these methods involve a serum sample and combine various biomarkers and risk factors. More details are listed in Table 1.2.

Cell-free fetal DNA screening became commercially available in 2011 and is gaining popularity with offering screening for trisomy 21, trisomy 18, and trisomy 13 with improved detection rates compared with the previously discussed screening methods. For example the trisomy 21 detection rate using cell-free fetal DNA is >99% with a false-positive rate of <1%.² However, cell free fetal DNA is mixed with the cell free maternal

DNA, making additional genetic testing challenging.³² Furthermore, this is not a diagnostic technique and follow up procedures are still recommended pending a positive result.

Clinically, prenatal diagnostic techniques are limited to two invasive procedures: amniocentesis and chorionic villus sampling (CVS). Amniocentesis is performed in the second trimester and involves removing amniotic fluid from the uterus, which contains fetal cells and proteins.³³ CVS is performed earlier than amniocentesis, during the first trimester.³⁴ CVS involves removing a chorionic villi sample either transcervical or transabdominal.³⁴ These techniques are often considered if a prenatal screening test comes back with a positive result. For example, there are ~200,000 amniocentesis procedures performed in the U.S. per year.^{35,36} The major complications associated with these procedures are risks for miscarriage and infection.³⁷ The miscarriage rate for amniocentesis is 1/900, while the miscarriage rate for CVS is 1/455.²

Table 1.2: Clinically available prenatal testing options.

Prenatal test	Invasive vs. Non-invasive	Diagnostic vs. Screening	Method	Detection rates	Gestation age	Potential for pregnancy loss
First trimester screen	Non-invasive	Screening	Serum screening for human chorionic gonadotropin (hCG) and pregnancy-associated plasma protein A and ultrasound for nuchal translucency ²⁹	Trisomy 21: 82-87% ²⁹	10-13 weeks ²⁹	No
Quadruple Marker Screen	Non-invasive	Screening	Serum screening for hCG, α -fetoprotein, inhibin A, and unconjugated	Trisomy 21: 81% ²⁹	15-22 weeks ²⁹	No

			estriol with assessment of patients' age, race, weight, number of fetuses, diabetes status, and gestational age. This method can screen for open neural tube defects ²⁹			
Integrated, Stepwise Sequential, and Contingent Screening	Non-invasive	Screening	Combines first trimester screen followed up by quad screening ²⁹	Trisomy 21 Integrated : 96% Stepwise: 80% Contingent: 94% ²⁹	10-22 weeks ²⁹	No
Cell-Free DNA	Non-invasive	Screening	Can be used for screening trisomy 21, trisomy 18, and trisomy 13 ²⁹	Trisomy 21: 99.2% with false positive rate (FPR) 0.09% Trisomy 18: 96.3% with FPR 0.13% Trisomy 13: 91.0% with FPR 0.13% ^{38,39}	< 10 weeks ²⁹	No
Chorionic Villus Sampling	Invasive	Diagnostic	Sample of chorionic villi ³⁴	All aneuploidies >99% detection rate; >95% detection rate for other genetic conditions ²	10-14 weeks ³⁴	1 in 455 ²

Amniocentesis	Invasive	Diagnostic	Sample of amniotic fluid ³³	All aneuploidies >99% detection rate; >95% detection rate for other genetic conditions ²	> 15 weeks ³³	1 in 900 ²
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1.4.1 Trophoblast retrieval from maternal samples

Given the risks associated with diagnostic procedures, there is a need to develop new non-invasive approaches. The ability to capture entire fetal cells non-invasively compared to only fetal DNA has emerged to enhance the genetic testing and diagnostic capabilities.^{40–42} Fetal cells can be found either in maternal blood or in cervical samples.^{43,44} These non-invasive samplings have the potential for diagnostic use in chromosomal abnormalities and pregnancy complications as early as the first trimester. Trophoblast capture from cervical samples also would allow for earlier detection compared with current invasive procedures (Figure 1.3).⁴⁵ However, the quantity of these cells is low (~6-20 cells/mL of maternal blood⁴⁶ and ~1 in 2,000 cervical cells⁴⁷) and separation for both cervical and blood samples remains a large hurdle to become a technique used in the clinic.

One method to separate whole trophoblast cells from a heterogeneous population is through cell sorting. Various methods of cell sorting exist based on intrinsic properties of morphology, size, density, and dielectric or magnetic susceptibility.^{48–51} However, trophoblasts are difficult to sort by intrinsic size properties due to their affinity to clump together non-uniformly. Other techniques involving labeling with biological markers including fluorescence-activated cell sorting and magnetic-activated cell sorting.⁵²

However, these labeling techniques can come with unwanted potential changes to the cell genetic information as well as high cost and low-throughput.⁵³

New studies are also investigating the use of prenatal testing to detect pregnancy complications, such as those detailed in section 1.3 *Pregnancy related complications*. For example, preeclampsia clinical symptoms arise during the 3rd trimester. However, shallow trophoblast invasion causing preeclampsia takes place during the 1st trimester. Current studies have shown that trophoblast yield from cervical samples was decreased with preeclampsia or intrauterine growth restriction compared to healthy term pregnancies.⁵⁴

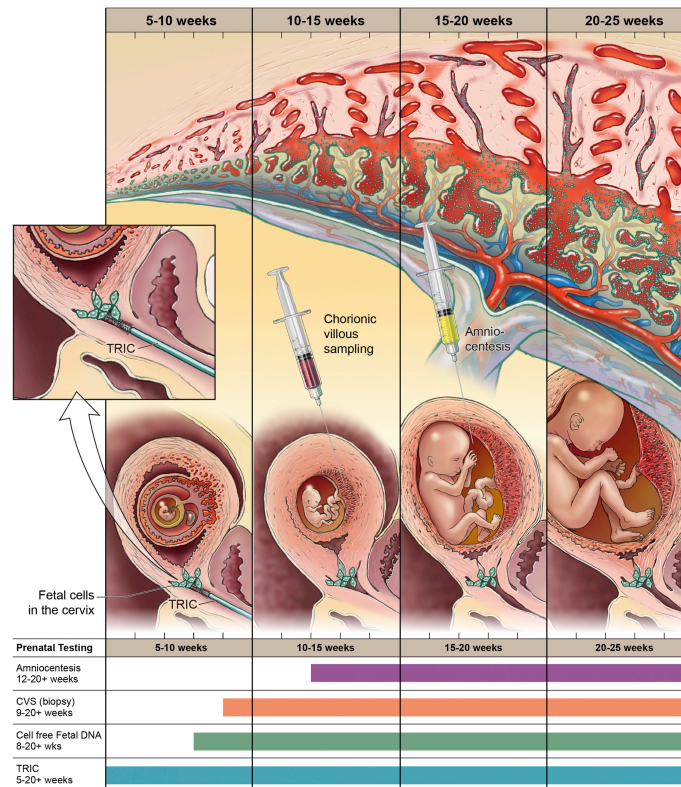


Figure 1.3: Comparison of gestational age required for performing prenatal tests. Reprinted from Drewlo *et al.*, Placenta, 2017⁴⁵ with permission under creative common attribution-noncommercial-no derivatives license (CC BY NC ND).

1.5 Molecular interactions during pregnancy

Pharmaceuticals, environmental toxicants, and nanomaterials are all common exposures during pregnancy with limited information on their effects. More specifics are detailed below and further motivate the need for new *in vitro* tools to assess their safety during pregnancy.

1.5.1 Pharmaceuticals

As previously motivated, only approximately 10% of medications approved by the U.S. Food and Drug Administration (FDA) from 2000 to 2010 have sufficient data on the risk when used during pregnancy.¹ However, many women (~90%) take at least one medication during pregnancy.¹ Reasons for this include medications that women may not be able to stop during pregnancy (e.g. epilepsy, narcolepsy, and depression medications) and conditions that arise during pregnancy (e.g. infection, gestational diabetes, and preterm birth). Many medications are small molecules, which can cross the placenta via passive diffusion.⁵⁵ Medications crossing the placenta can result in fetotoxicity including hemorrhage, teratogenicity, and adenocarcinoma.⁵⁵

As of 2015, the FDA updated requirements to prescription medication information from its previous letter grades to a more detailed requirement called pregnancy and lactation labelling rule (PLLR).⁵⁶ This rule now necessitates manufacturers to provide written description of risks during the entire pregnancy including labor, delivery, and lactation.⁵⁷ This will now require data-based evidence and literature surrounding the risks, which is currently limited.⁵⁷ This further motivates the swift need for tools to investigate pharmaceuticals.

1.5.2 Environmental toxicants

Phthalates are plasticizers used to make plastic material flexible. They are used in numerous consumer products including adhesives, detergents, personal care products (e.g. soaps, shampoos, hair sprays), plastic packaging film, children's toys, blood-storage containers, and medical tubing. The U.S. Environmental Protection Agency's (EPA's) current management plan includes these eight phthalates: dibutyl phthalate (DBP), diisobutyl phthalate (DIBP), butyl benzyl phthalate (BBP), di-*n*-pentyl phthalate (DnPP), di(2-ethylhexyl) phthalate (DEHP), di-*n*-octyl phthalate (DnOP), diisononyl phthalate (DINP), and diisodecyl phthalate (DIDP).⁵⁸ There is a need to further investigate how these phthalates influence biological properties.

One recent study investigated the impact of a common phthalate, DEHP, on its influence during pregnancy. This study found that miscarriage risk was highly associated with acquired concentration of DEHP.⁵⁹ Phthalates have also been associated with additional reproductive health concerns including decreasing oocyte yield^{60,61} and increasing risk for GDM.⁶²

1.5.3 Nanomaterials

Nanoparticle exposure during pregnancy can result in disruption of fetal development.⁶³ These nanomaterials can lead to pulmonary inflammation, placental inflammation, neurotransmitter interactions, and oxidative stress.⁶³ Exposure to nanomaterials can be achieved either passively through environmental exposure or by nanoparticle pharmaceutical delivery. Although they can have negative effects, nanomaterials are also

being investigated for their use to mitigate interactions of pharmaceuticals during pregnancy or targeted treatment to a developing fetus. Nanoparticle pharmaceutical delivery is further discussed in *1.8 Drug delivery for use during pregnancy*.

1.6 Current methods for studying the placenta

Current methods for studying interactions with trophoblast cells and the placenta include animal models, perfused placenta models, trophoblast cell lines, and new emerging biotechnologies. These are all further detailed below.

1.6.1 Animal models

The placenta is the most species specific organ.^{64,65} This complicates *in vivo* options for studying this dynamic organ and interactions with it. The mouse is currently the most often used *in vivo* model for placental studies (Figure 1.4). Similarities between mouse and human placenta exist. Trophoblast giant cells in the mouse are invasive and result in implantation similar to the extravillous trophoblasts in human placenta.⁶⁶ The labyrinth of the mouse placenta performs the same function as the chorionic villi of the human placenta.⁶⁶ However, differences are also observed. In the mouse, the maternal blood comes into direct contact with the trophoblast surface due to the removal of the endometrium during implantation.⁶⁶ Additionally, the mouse model has three trophoblast cell layers while the human placenta has one trophoblast cell layer.⁶⁶

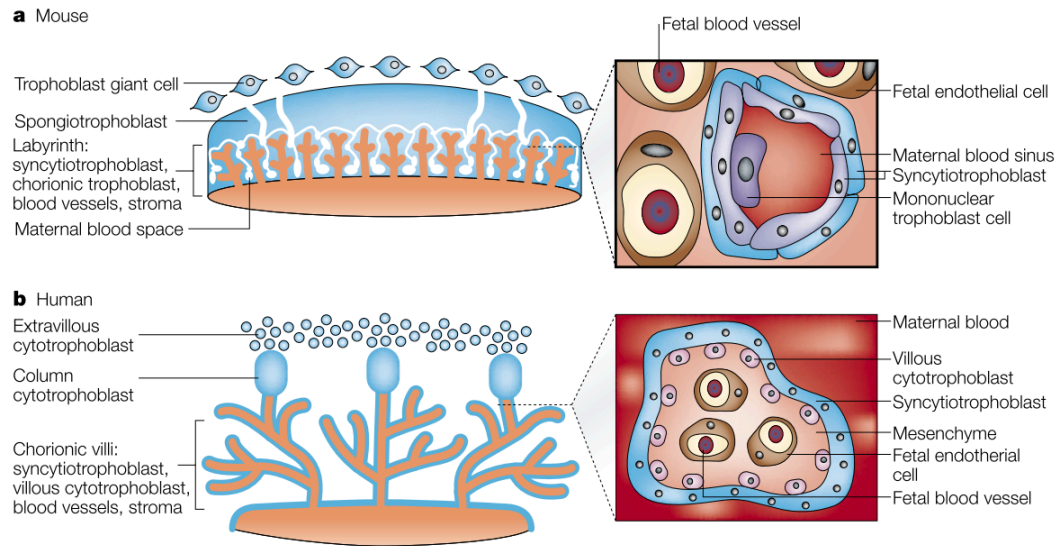


Figure 1.4: Structural differences between (a) mouse and (b) human placenta. Figure reprinted from Rossant *et al.*, Nature Reviews Genetics, 2001⁶⁶ with permission.

1.6.2 Perfused placenta model

Human placental perfusion model is another technique to investigate transport across the placenta. This method entails dual perfusion of a placental lobule and has been shown to correlate with *in vivo* results.⁶⁷ This model also includes key elements such as passive and active transport as well as placental metabolism which can affect substance transport.⁶⁷ Thus, this makes this model the most closely resembling the human placenta architecture. However, experiment systems can vary lab to lab with variations including cannulation method, isolation procedure, perfusion fluid and flow, and oxygenation.⁶⁷ Additionally, studies can only be performed on term placenta. This model does not allow for transport investigations at different gestational ages. Finally, experiments with perfused placenta typically need to be performed directly after delivery.⁶⁸

1.6.3 Placental cell lines

Trophoblast cell lines are often used to investigate trophoblast cell biology, immunology, endocrine function, and placental development.⁶⁹ Commonly used trophoblast cell lines include the TCL-1, HTR-8, BeWo, JEG-3, and JAR cell lines, but many more exist.⁶⁹ TCL-1 and HTR-8 were both derived from human trophoblast cells that have been immortalized, where HTR-8 were obtained from 1st trimester villous explants and TCL-1 were obtained from 3rd trimester choriondecidua.^{69,70} BeWo, JEG-3, and JAR were derived from malignant choriocarcinoma cell lines and have been shown to express trophoblast markers.⁶⁹ Trophoblast markers often used to investigate trophoblast nature include hCG, human placental lactogen, cytokeratins, vimentin, and human leukocyte antigens.⁶⁹

1.6.4 Emerging technologies

A recent study developed a microfluidic “placenta-on-a-chip” device using a co-culture of JEG-3 and human umbilical vein endothelial cells (HUVECs) (Figure 1.5a).⁷¹ Since the development of placenta-on-a-chip published in 2016, studies have investigated using this device for nanoparticle interactions⁷² and caffeine transport⁷³, which were recently published in 2019. The nanoparticle interaction study was able to analyze factors including reactive oxygen species production, cell apoptosis, and permeability and concluded that exposure to titanium dioxide nanoparticles led to cell damage.⁷²

Placental microtissues have also been developed for nanotoxicity studies using placental fibroblasts and trophoblast cells (Figure 1.5b).⁷⁴ These microtissues are advantageous for monitoring changes in hormone production, such as hCG.⁷⁴ An increased

level of hCG production was observed in 3D cultured microtissues compared to 2D cell cultures or monolayers, indicating enhanced trophoblast differentiation.⁷⁴

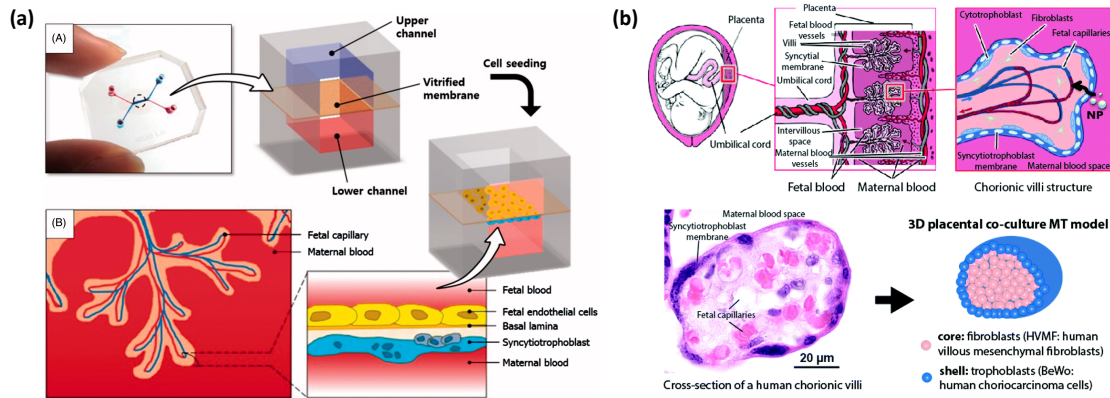


Figure 1.5: Examples of emerging technologies for placental models. (a) Placenta-on-a-chip microdevice adapted from Lee *et al.*, Journal of Maternal-Fetal and Neonatal Medicine, 2016⁷¹ with permission. (b) Human placenta microtissue model adapted from Muoth *et al.*, Nanoscale, 2016⁷⁴ with permission.

1.7 Model lipid bilayers

Model lipid bilayers include liposomes, supported lipid bilayers (SLBs), and suspended lipid structures (Figure 1.6). SLBs are a model of the phospholipid cell membrane commonly used in biotechnology applications where lipid vesicles are ruptured on a supported substrate material.^{75–77} SLBs mimicking aspects of cell membrane functions are used to study molecular interactions of lipid bilayers with various macromolecules and small molecules.^{78–81} Previous work has investigated SLB formation with varying lipid compositions,⁸² incorporation of lipid proteins,^{83–85} and utilizing polymer cushions,⁸⁶ yielding SLBs capable of mimicking aspects of cell membrane function. The great potential of lipid bilayers for biomedical applications has been demonstrated with investigations spanning molecular and nanoparticle transport,^{78,79} protein assembly and

function,^{83–85} peptide arrangement and insertion,^{80,81} drug screening,⁸⁷ and microfluidic platforms.⁸⁸ One common technique used to monitor this formation is quartz crystal microbalance with dissipation (QCM-D) which is able to monitor the adsorption of vesicles in comparison to the bulk liquid properties.^{79,81,89–95}

In order to investigate the permeability of nanomaterials, pharmaceuticals, or toxins across a lipid bilayer model, the parallel artificial membrane permeability assay (PAMPA) has been used where a lipid bilayer is suspended across a porous hydrophobic insert.^{96–99} By combining both the SLB and PAMPA techniques, a model can be developed that allows for the ability to investigate adsorption and permeability of compounds with lipid components of a desired tissue or cell type. Given the need for more models to study the placenta and placental complications, lipid bilayer techniques can be a suitable option for developing high-throughput interaction and permeability studies.

Previous investigations have modeled the liver, red blood cell, myelin, mitochondrion, endoplasmic reticulum, and *E. Coli* bacterium cell membranes all by alterations in phospholipids, cholesterol, and glycolipids.¹⁰⁰ PAMPA lipid models have also been developed for different biological interfaces including the blood-brain, buccal, intestinal, and transdermal interfaces.¹⁰¹ Lipid compositions have also been shown to vary in disease states, for example, liver disease has demonstrated different composition of lipids compared to normal livers.¹⁰²

The largest limitation in developing lipid bilayers is that they do not include transporter proteins and thus represent passive transport. More fundamental lipid bilayer studies are currently being conducted to understand how to include transmembrane proteins into these synthetic structures with correct protein confirmation.^{83,103}

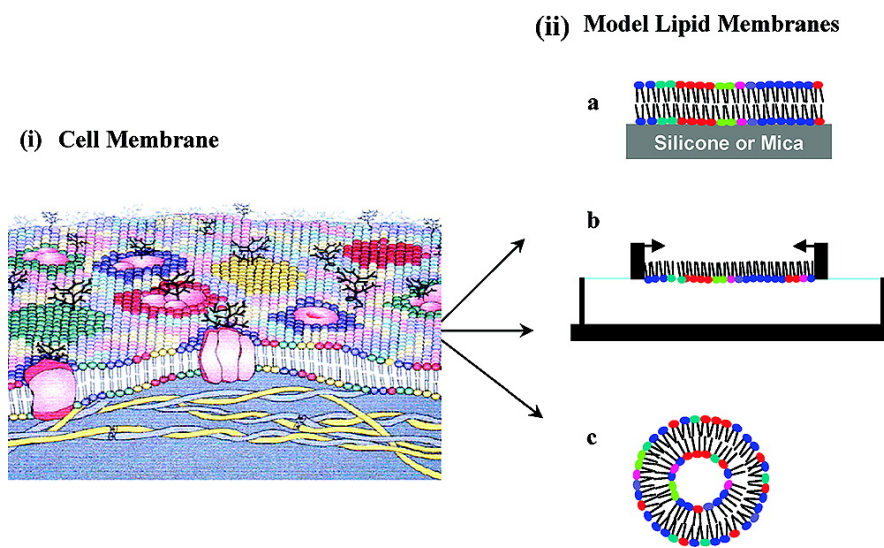


Figure 1.6: Model lipid membranes showing supported bilayers, suspended lipid layers, and vesicles that represent the cell membrane. Reproduced with permission from Peetla *et al.*, Molecular Pharmaceutics, 2009.¹⁰⁴

1.8 Drug delivery for use during pregnancy

Currently there is limited information on nanoparticle transport across the placenta. Drug delivery can be useful for therapeutic design during pregnancy.⁵⁷ Delivery carriers can be used to mitigate interactions with the placenta for treating maternal conditions and alleviate potential fetal toxicity. For example, liposomal nanomedicine has potential to be used to treat maternal cancer with limited fetal exposure.⁵⁷

Alternatively, delivery carriers can also be designed to target the placenta to treat fetal conditions, such as congenital heart disease, *in utero*.^{57,105} For example, positively charged particles have been shown to bind to trophoblast cells.⁵⁷ Additionally, surface proteins expressed on the placenta can be used for targeted delivery.^{55,57} Placental chondroitin sulfate A is expressed only on the placenta and a binding peptide has been investigated for active targeting.⁵⁵ A number of other nanoparticles with ligand and peptide decorations have been investigated, with a majority being liposomes.⁵⁵ The application of these

targeted liposomes have been to help pregnancy complications including preterm birth and fetal growth restriction.⁵⁵ However, despite the significance treatment for pregnancy complications would have, few therapeutics are available and more research is needed in this area.

In this thesis we became particularly interested in considering therapeutic design as it relates to the treatment of fungal infections. Vulvo-vaginal candidiasis occurs in up to 20% of pregnant women and systemic fungal infections are more severe during pregnancy.¹⁰⁶ Further, antifungal prescriptions are challenging in pregnant women due to potential fetal toxicity.¹⁰⁶ Many antifungal therapeutics are toxic to mammalian cells, which is in part due to their hydrophobicity and similarities with mammalian cells as fungi are eukaryotes. Incorporating an antifungal into a liposome may improve not only its antifungal efficacy but have potential to mitigate fetal toxicity during pregnancy.

1.9 Summary and research overview

This thesis focuses on new methods to improve non-invasive prenatal testing, developing models for investigating placental interactions, and therapeutic design to improve both drug efficacy and potentially mitigate placental interactions.

Chapter 2 describes a new method to rapidly enrich trophoblast cells from cervical samples. Our method resulted in a ~700% enrichment of trophoblast cells from the heterogeneous sample in less than 5 minutes through differences in cell settling properties. This enrichment was great enough to then enable single cell picking to obtain a trophoblast cell free of maternal genetic material for downstream prenatal diagnostics, which we were

able to successfully perform following our enrichment technique. Currently, there is no clinical non-invasive prenatal diagnostic technique.

Chapter 3 discusses another method to enrich trophoblast cells from cervical samples using inclined plane flow on a slide surface. In this study, we were able to obtain enrichments of ~300-400%. This study focused on investigating parameters such as angle of incline, surface material, incubation time prior to surface incline, and volume of fluid. We found that an incubation time of 5 min prior to incline at 50° on polystyrene slides were important parameters.

Chapter 4 begins our work towards developing a placental lipid bilayer. First, we performed a fundamental study to further the understanding of supported lipid bilayers as a tool. We conducted an investigation looking at the impact of flow and vesicle concentration on rupture of uni-lipid phosphatidylcholine lipid vesicles into supported lipid bilayers. Previous studies have indicated the importance of critical vesicle coverage to lead to vesicle rupture. In this study we note a paradigm shift, as we observed that critical vesicle coverage could be achieved under a no flow condition, but result in vesicles remaining adsorbed to the surface and un-ruptured until the re-introduction of flow.

Chapter 5 discusses our work on investigating phthalate interactions with lipid structures. In particular, we investigated a commonly used phthalate, DEHP and its metabolite mono(2-ethylhexyl)phthalate (MEHP). To the best of our knowledge, this was the first direct study to investigate how phthalates interact with lipid structures. Further, we identified that the interactions of DEHP and MEHP with the lipid structures resulted in changes to common small molecules interaction and permeation of the lipid bilayers. We

also determined that incubating the phthalate with chitosan particles could mitigate the vesicle size changes observed with phthalates alone.

Chapter 6 examines the development of placental lipid bilayer models. Here we isolated and quantified the lipid composition from trophoblast cells representing 1st, 3rd, and term placenta. With this quantified composition, we developed synthetic lipid vesicles, SLBs, and suspended lipid bilayers. We then performed studies investigating how DEHP, amphotericin B, a common antifungal, and AmBisome, the liposomal formulation of amphotericin B, interact with the trophoblast bilayers. Interestingly, we observed that AmBisome interacts significantly less with the trophoblast bilayers compared to amphotericin B. This result indicated the importance of drug delivery vehicle on potential interactions with the placenta.

Chapter 7 discusses the development of a new antifungal liposome, encapsulating anidulafungin. We characterized the liposomes and demonstrate stability of the liposomes for up to 140 days. The liposomal formulation of anidulafungin demonstrated effectiveness against both planktonic and biofilm *Candida albicans* as well as improving survival of *Candida* infected *Galleria mellonella* compared to free drug.

Chapter 8 concludes this thesis and provides future perspectives on each of the project areas investigated in the work. Final remarks are discussed about the field of biomedical engineering in women's health and how this work has contributed to the field.

Chapter 2

A Rapid Method for Label-Free Enrichment of Rare Trophoblast Cells from Cervical Samples

Chapter 2 has been published as an original research article.

Bailey-Hytholt, C.M., Sayeed, S., Kraus, M., Joseph, R., Shukla, A., Tripathi, A., *A Rapid Method for Label-Free Enrichment of Rare Trophoblast Cells from Cervical Samples*, **Scientific Reports**, 9, 12115, 2019.

2.1 Abstract

Extravillous trophoblasts (EVTs) have the potential to provide the entire fetal genome for prenatal testing. Previous studies have demonstrated the presence of EVT in the cervical canal and the ability to retrieve a small quantity of these cells by cervical sampling. However, these small quantities of trophoblasts are far outnumbered by the population of cervical cells in the sample, making isolation of the trophoblasts challenging. We have developed a method to enrich trophoblast cells from a cervical sample using differential settling of the cells in polystyrene wells. We tested the addition of small quantities of JEG-3 trophoblast cell line cells into clinical samples from standard Pap tests taken at 5 to 20 weeks of gestation to determine the optimal work flow. We observed that a 4 min incubation in the capture wells led to a maximum in JEG-3 cell settling on the surface ($71 \pm 10\%$ of the initial amount added) with the removal of $91 \pm 3\%$ of the cervical cell population, leading to a 700% enrichment in JEG-3 cells. We hypothesized that settling of mucus in the cervical sample affects the separation. Finally, we performed a proof-of-concept study using our work flow and CyteFinder cell picking to verify enrichment and pick individual JEG-3 and trophoblast cells free of cervical cells. Ultimately, this work provides a rapid, facile, and cost-effective method for enriching native trophoblasts from cervical samples for use in subsequent non-invasive prenatal testing using methods including single cell picking.

2.2 Introduction

Extravillous trophoblasts (EVTs) are cells that originate from the placenta and invade the endometrium. These rare cells have the potential to enhance non-invasive prenatal testing (NIPT).^{107,108} NIPT is an important method for detecting fetal complications as it has lower cost and risks compared to invasive measures.¹⁰⁹ Biomarker techniques and cell free fetal DNA from patient blood samples have shown promise in some prenatal testing, particularly for aneuploidy screening.¹¹⁰ However, these techniques are limited by the genetic information available in the samples. The capture of the entire fetal genome contained in intact fetal cells would be a significant improvement over current testing capabilities.

During trophoblast invasion EVTs enter the endocervical canal,⁴⁷ which can be sampled by a cervical swab. Previous studies have demonstrated the ability for trophoblast retrieval and isolation from the cervix (TRIC).^{47,54,111–114} With the frequency of one EVT per 2,000 cervical cells,⁴⁷ novel isolation methods are needed for downstream testing to provide a quality sample that is not overwhelmed by maternal cells. Common cell separation techniques are based on cell density, size, shape, piezoelectric effects, electric capacitance, magnetic susceptibility, hydrodynamic properties, and affinity to antibodies.^{52,115} However, many of these techniques are not suitable for capturing EVTs with minimal cell loss or equipped to handle the cervical matrix.¹¹⁶ In our investigation, we have utilized the intrinsic properties of these rare cells and their matrix in order to yield an EVT enriched sample based on differential settling of the cells in polystyrene wells.

In order to isolate putative fetal cells, studies to date have commonly used antibodies for human leukocyte antigen G (HLA-G), cytokeratin, beta human chorionic gonadotrophin (β -hCG), and X and Y chromosome probing using fluorescence in situ

hybridization (FISH) and polymerase chain reaction (PCR).^{54,117,118} Isolation methods in the literature have used HLA-G coupled to magnetic beads to elute trophoblast cells from the maternal cell population.^{113,114} However, any amount of maternal cells or DNA present in a sample can pose further challenge during analysis of the genome. Single cell picking, in which a single fetal cell is identified and selected from a mixed population of both maternal and fetal cells, is one advantageous strategy to eliminate the presence of maternal cells and isolate pure trophoblasts.^{119,120} This is a similar approach to previous investigations aiming to isolate rare tumor cells.^{121,122} However, a major issue of picking a single trophoblast cell from a cervical sample with no clean-up is the overwhelming density of cervical cells, which makes picking challenging and near impossible. Our strategy allows enrichment to a degree that improves the ability to pick and isolate a single trophoblast cell while effectively removing maternal contamination.

The goal of this work was to enrich a cervical sample to increase the trophoblast frequency for optimal single cell picking. In this study we provide a facile workflow that eliminates at least 90% of squamous cervical cells and captures at least 70% of fetal cells (Figure 2.1). We used cervical cells from clinical Papanicolaou (Pap) tests stored in ThinPrep® PreservCyt® and supplemented with a known number of JEG-3 cells (a common trophoblast cell line) for parameter optimization. To achieve enrichment, we allowed the JEG-3 and cervical cells to settle in a polystyrene well for a variable amount of time. After the settling time, we removed the supernatant, which contained a large majority of cervical cells. Remaining in the capture well was the enriched population of trophoblast cells. We also performed a proof-of-concept on an imaging and picking platform to show the ability to pick single trophoblast cells for whole genome amplification. This is the first study to

use cell settling for enriching trophoblast cells from a heterogeneous cervical cell population. Ultimately, we provide a technique that is quick, inexpensive, minimizes cell loss, and results in retrieval of individual trophoblast cells.

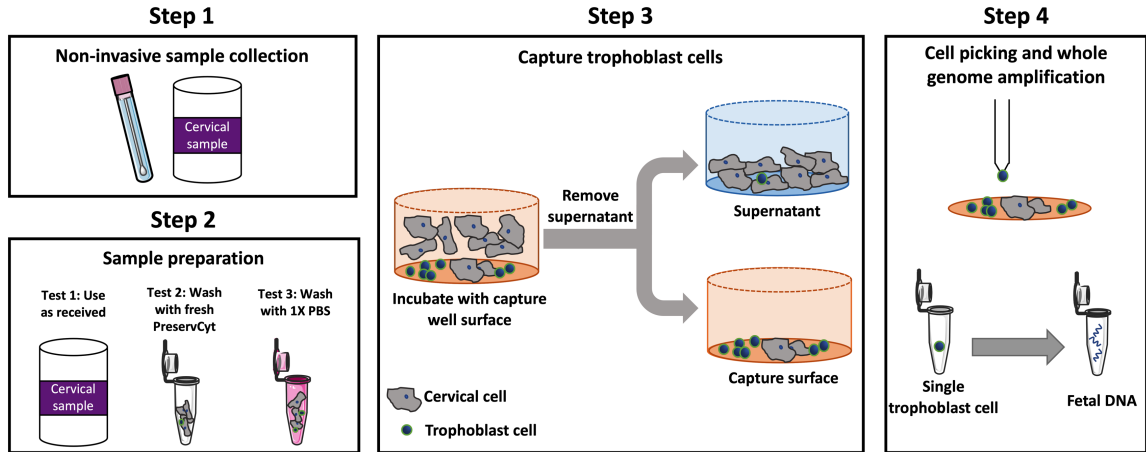


Figure 2.1: Workflow for trophoblast enrichment. Step 1 is the collection of trophoblast cells from the cervical canal using a cervical swab test method. Step 2 is the sample preparation by either using the sample as received from the clinic, washing with fresh PreservCyt®, or washing with 1× PBS. Step 3 is the enrichment of the cells using the workflow developed in this study. Step 4 is acquiring the fetal information by single cell picking and whole genome amplification.

2.3 Materials and methods

2.3.1 Patient selection

Approval for enrolling patients for non-invasive prenatal sample acquisition, including endocervical swabs, was given by the Biomedical Research Alliance of New York Institutional Review Board (BRANY IRB) (File # 14-02-450-408). Written informed consent was obtained from the participating women and all personal information was removed from the specimen prior to receiving. Women in their 5th to 20th week of pregnancy were selected for sampling. All studies were performed in accordance with relevant guidelines and regulations.

2.3.2 Endocervical sampling

Retrieval of trophoblast cells from the endometrial canal was performed using a Cytobrush and following standard Pap test protocol. Cells were rinsed from the cytology brush into 20 mL of ThinPrep® PreservCyt® (Hologic, Marlborough, MA) fixative solution immediately after removal from the cervix. The material was sent to the lab for experiment use within 24 h of collecting. 15 samples were used as received unless noted otherwise.

2.3.3 Trophoblast cell culture

JEG-3 (ATCC, Manassas, VA), TCL-1 (kindly donated by Women & Infants Hospital Kilguss Research Institute, Providence, RI), and HTR-8 (kindly donated by Women & Infants Hospital Kilguss Research Institute, Providence, RI) trophoblast cell lines were cultured in Rosewell Park Memorial Institute medium 1640 (Gibco, Waltham, MA) (RPMI supplemented with 10% (v/v) fetal bovine serum (FBS) (Corning, Corning, NY) and 1% (v/v) penicillin streptomycin) at 37°C under 5% CO₂. During cell passage, cells were put in PreservCyt® solution at a concentration of 100,000 cells/mL and stored at 4°C for use up to 2 months. JEG-3, TCL-1, or HTR-8 cells were added into a cervical specimen at a known concentration to investigate optimized enrichment.

2.3.4 Microscopy

2.3.4.1 Confocal microscopy

Confocal microscopy (Nikon Instruments A1 Confocal Laser Microscope with NIS Elements software) was used to examine geometries of cervical and JEG-3 cells. Cells in PreservCyt® were dried on glass slides and rinsed 3× with 1× phosphate buffered saline

(PBS). 0.1% triton X-100 in 1× PBS was incubated for 3-5 min and subsequently rinsed 3× with 1× PBS. 10% normal goat serum was incubated for 30 min at room temperature for blocking. 2.5% Alexa Fluor 532 phalloidin (Life Technologies, Carlsbad, CA) in 1% normal goat serum was incubated on the slides for 20 min at room temperature. A final wash with 1× PBS was performed and the slides were mounted with Vectashield antifade mounting media with 4',6-diamidino-2-phenylindole (DAPI) (Vector laboratories, Burlingame, CA). Images were processed using National Institutes of Health Image J software (version 2.0.0-rc-65/1.52b).

2.3.4.2 Epifluorescence microscopy

Adhered cells and the supernatant of cells were imaged using a Nikon Ti-E inverted fluorescent microscope with NIS Elements software. At least 25 images of the adhered cells were obtained to quantify an average number of adhered trophoblast and cervical cells. Trophoblast amounts less than 250 cells were counted while imaging.

2.3.5 Optimization of in vitro trophoblast cell line enrichment from cervical sample

The cell enrichment process was carried out using a polystyrene 24 well plate (non-tissue culture treated, surface area of 1.9 cm²/well) as the capture well surface. Cells and controls were introduced to these capture wells and allowed to settle for differing times to determine the timescale allowing optimal separation of cervical and fetal cells. JEG-3 cells were used to optimize this process. Prior to introducing cells to the capture wells, JEG-3 cells stored in PreservCyt® at a stock concentration of 100,000 cells/mL were centrifuged using a Labnet Prism microcentrifuge at 10,000 × g for 5 min. The supernatant was removed, and

the cells were incubated with DAPI for 5 min. Controls containing 1,000 DAPI stained JEG-3 cells in 600 μ L PreservCyt[®] were examined for cell settling for 0, 4, 20, and 60 min. To determine the capture well incubation time allowing optimal cell separation, DAPI stained JEG-3 cells were mixed with cervical samples and incubated in the capture wells for 0, 0.5, 1, 2, 4, 8, 16, or 60 min at a constant JEG-3 density of 1,000 cells per well. The JEG-3 cells (10 μ L in PreservCyt[®]) were added to either 600 μ L of cervical sample or 600 μ L of cervical sample diluted with 2.4 mL of PreservCyt[®] (yielding a final volume of ~3 mL). The different volumes lead to different liquid heights; at 600 μ L the well is filled to a 5 mm height, while at 3 mL the well is filled to a 20 mm height. After the capture time was completed, the supernatant was immediately removed and placed into a new well for imaging. Once the optimized separation time and height of settling were determined, JEG-3 densities of 15, 100, 250, 500, and 1,000 cells per well were studied.

The effect of washing the cells with fresh PreservCyt[®] or 1 $\times$ PBS was also examined, as it was hypothesized that these washes would remove mucus content. Cervical and JEG-3 cells were centrifuged at 2,000 rpm for 5 min, the supernatant was removed, and the cell pellet was re-suspended in fresh PreservCyt[®]. This wash procedure was performed a total of 3 times. JEG-3 cells were then incubated with DAPI prior to enrichment with the capture surface. The optimized settling time and 1,000 cells per well seeding density was used. The effect of solution density was investigated with JEG-3 cell settling at the optimized time point in Ficoll Type 400, 20% in H₂O solution. The JEG-3 cells (10 μ L in PreservCyt[®]) were added to 600 μ L of Ficoll and allowed to settle.

HTR-8 and TCL-1 cells stored in PreservCyt[®] at a stock concentration of 100,000 cells/mL were centrifuged and DAPI stained as previously performed with JEG-3 cells.

The optimized time determined for JEG-3 separation from the cervical cells was used with an HTR-8 or TCL-1 density of 1,000 cells per well (10 μ L in PreservCyt[®]) in 600 μ L of cervical sample. After the capture time was completed, the supernatant was removed and placed into a new well for imaging.

2.3.6 CyteFinder for trophoblast enrichment analysis

RareCyte CyteFinder (RareCyte, Seattle WA) was used to analyze JEG-3 capture and pick individual cells. A density of 100 JEG-3 cells per well with a cervical sample concentration of $\sim$ 100,000 cells/mL was used for performing cell settling at the optimized time condition identified for JEG-3 cell separation as described above in *Optimization of in vitro trophoblast cell line enrichment from cervical sample*. 600 μ L of sample was allowed to settle. Additionally, conditions with cervical cell washes and incubations were performed. The cervical sample was centrifuged at 2,000 rpm and washed 3 $\times$ in fresh PreservCyt[®] before spiking with JEG-3 cells for settling. The cervical sample and JEG-3 cells were also allowed to incubate for at least 1 h at room temperature prior to settling.

In order to perform imaging and picking on the CyteFinder, the captured cells required transfer from the 24 well polystyrene plate to a slide surface compatible with the instrument. After settling, the capture surface cells were immediately rehydrated with 600 μ L of fresh PreservCyt[®] and vigorously pipetted to remove cells from the bottom of the polystyrene well plate. Cells were transferred to a Shandon[™] coated cytoslide (ThermoFisher Scientific, Waltham, MA) and dried at 50°C. Purified mouse anti-human HLA-G denatured (BD Biosciences, San Jose, CA) at 1:100 in antibody diluent (Dako, Agilent Technologies, Inc., Santa Clara, CA) was incubated on the slides for 30 min at

room temperature. The slides were rinsed with 1× PBS and Alexa Fluor 488-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) at 1:250 in antibody diluent was incubated for 15 min at room temperature. The slides were then rinsed with 1× PBS and DAPI was incubated for 5 min. A final rinse was performed with 1× PBS and then the slides were mounted with CyteMount Mounting Media (RareCyte, Seattle, WA).

2.3.6.1 CytePicker for individual trophoblast cell picking

RareCyte CytePicker (RareCyte, Seattle, WA) was used to pick JEG-3 and potential real trophoblasts found during the CyteFinder process. Coverslips were soaked off of the slides in warmed PBS at 37°C. A CytePicker needle was used to remove the trophoblast cells of interest. The cell was then removed from the slide surface and dispensed into 5 µL of PCR grade water.

2.3.7 Whole genome amplification and polymerase chain reaction

Whole genome amplification (WGA) (SMARTer PicoPLEX WGA kit, Takara Bio Inc., Kusatsu, Shiga Prefecture, Japan) was performed by adding 5 µL of freshly-prepared extraction cocktail to the 5 µL single cell picked sample. The sample was incubated in a thermal cycler at 75°C for 10 min, then 95°C for 4 min, and 12°C on hold. The pre-amplification cocktail components were then combined and mixed well with 24 µL of pre-amplification buffer and 1 µL of pre-amplification enzyme. 5 µL of this cocktail was added to each cell and incubated in a thermal cycler with one 2 min cycle at 95°C, 12 cycles with 15 s at 95°C, 50 s at 15°C, 50 s at 25°C, 30 s at 35°C, 40 s at 65°C, and 40 s at 75°C. A

final cycle at 4°C was put on hold. The amplification cocktail was then mixed. 60 µL of the amplification cocktail was added to 15 µL of pre-amplification product and mixed. The sample was then amplified with one 2 min cycle at 95°C, and 14-16 cycles with 15 s at 95°C, 1 min at 65°C, and 1 min at 75°C. The WGA product was stored at -20°C.

Quantitative real-time polymerase chain reaction (PCR) was performed with TaqMan Fast Universal PCR Mix (ABI-4401631), Vic labeled RNaseP 20X Control Primers (ABI-4401631), FAM labeled SRY 20X Primer Mix (ABI-4400291), and DYS14 primers and probe with F: GAGCAGGCGTGGGTACTATTG, R: GTCTGCTGCTCGGCATCAC, P: /ROX/CCTGCATGCGGCAGAGAAACCC/IBRQ/. The DYS14 primer mix was made with 2 µM of each primer and probe. The PCR mix was made with 10 µL of SRY primers, 10 µL RNase primers, 5 µL DYS14 primers and 65 µL deionized water. BioRad CFX96 real time PCR was run with cycling at 95°C for 5 min, 95°C for 15 s, 60°C for 1 min. 50 cycles were performed for each run.

2.3.8 Statistical analysis

All studies were performed in triplicate at minimum. Results are shown as average $\pm$ standard deviation. All results are from at least n=3. Where applicable, a two-tailed t-test or two-way analysis of variance (ANOVA) with Tukey's post-hoc analysis and 95% confidence interval was performed where *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

2.4 Results and Discussion

2.4.1 JEG-3 and cervical cell geometry

In this work, we developed a label-free technique for trophoblast cell enrichment. Prior to investigating cell separation, we examined physical differences with cervical and trophoblast cells. To characterize the geometry of each cell type, the actin cytoskeleton and nuclei of JEG-3 and cervical cells were stained with phalloidin and DAPI, respectively. We observed that JEG-3 cells (Figure 2.2a) exhibited a spherical shape while individual cervical cells and cervical cell aggregates (Figure 2.2b) exhibited a more pancake-like morphology. The JEG-3 cells were significantly smaller (dimensions of $13 \pm 1 \mu\text{m} \times 11 \pm 1 \mu\text{m} \times 8 \pm 1.5 \mu\text{m}$) than single cervical cells (dimension of $66 \pm 2 \mu\text{m} \times 58 \pm 1.5 \mu\text{m} \times 6 \pm 1 \mu\text{m}$).

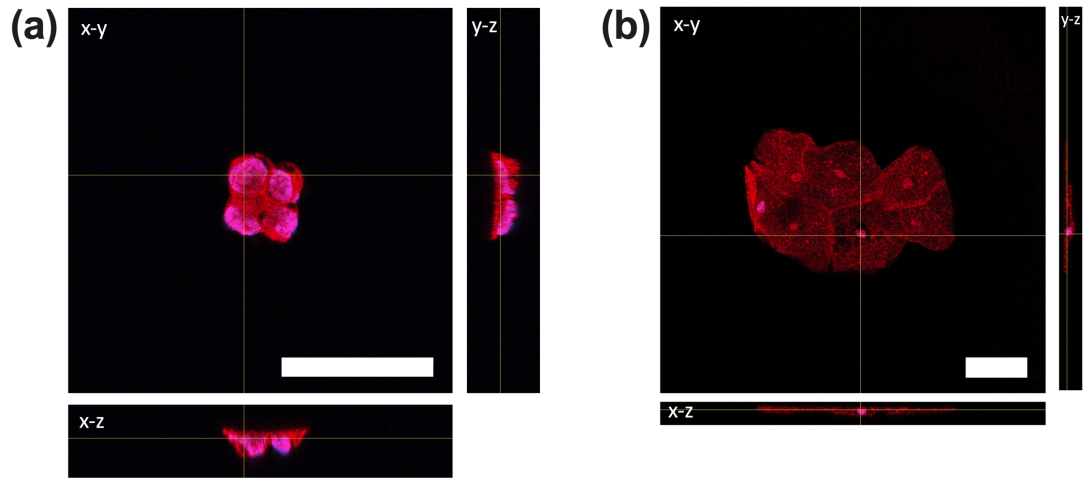


Figure 2.2: Confocal images of (a) JEG-3 and (b) cervical cells. Cells were stained with phalloidin (red) and DAPI (blue). Orthogonal views are shown with X-Y, Y-Z, and X-Z planes. The lines represent the view point. Scale bar = $50 \mu\text{m}$.

The size of the JEG-3 cell nuclei relative to the cell body was considerably larger than the cervical cells. The diameter of the JEG-3 cell nucleus was found to be 10.8 ± 1

μm while the diameter of the cervical cell nucleus was $8.7 \pm 1.5 \mu\text{m}$. The percentage of the cell nuclei was then taken relative to the X dimension of the cell body, resulting in approximately 82% for JEG-3 and 13% for the cervical cells. Previous investigations demonstrate cell type affects settling velocity.^{123–125} Based on the cell differences observed we hypothesize that the physical properties of cervical and JEG-3 cells may influence their gravitational settling in solution.

2.4.2 Optimization of JEG-3 cell enrichment using cell settling

Cervical samples can have a variable number of trophoblast cells, resulting in patient to patient variability. To optimize workflow parameters, cervical samples were first spiked with JEG-3 cells. By doing this, the JEG-3 cells experienced the mucus content present in the cervical sample from women during pregnancy.

2.4.2.1 Effect of time in capture well on cell settling

Our workflow, described in Figure 2.1 Step 3, requires a set amount of time in a capture well before the supernatant is removed. First, a control study was performed with 1,000 JEG-3 cells settling in only PreservCyt® (Figure 2.3a). We observed that on average 80% of JEG-3 cells settled in 4 min with an average velocity of 1.25 mm/min. As the cell suspension was very dilute, we assumed no hydrodynamic interactions between cells. The observed 10-20% unsettled cell population can be attributed to cell adsorption onto the walls of the wells utilized during capture. It appears that the cell interaction with the polystyrene surface plays a significant role in the JEG-3 surface capture. After 4 min there was no significant change in JEG-3 settling at longer time points. Next, cell settling was

performed with 1,000 JEG-3 cells in 600 μ L of cervical sample. To determine the optimal settling time, cervical samples with 1,000 JEG-3 cells were incubated for 0, 0.5, 1, 2, 4, 8, 16, and 60 min in the capture well before removing the supernatant (Figure 2.3b). At 4 min we observed the largest number of JEG-3 cells remaining on the surface, an average of 713 ± 103 cells from the original 1,000 cells incubated.

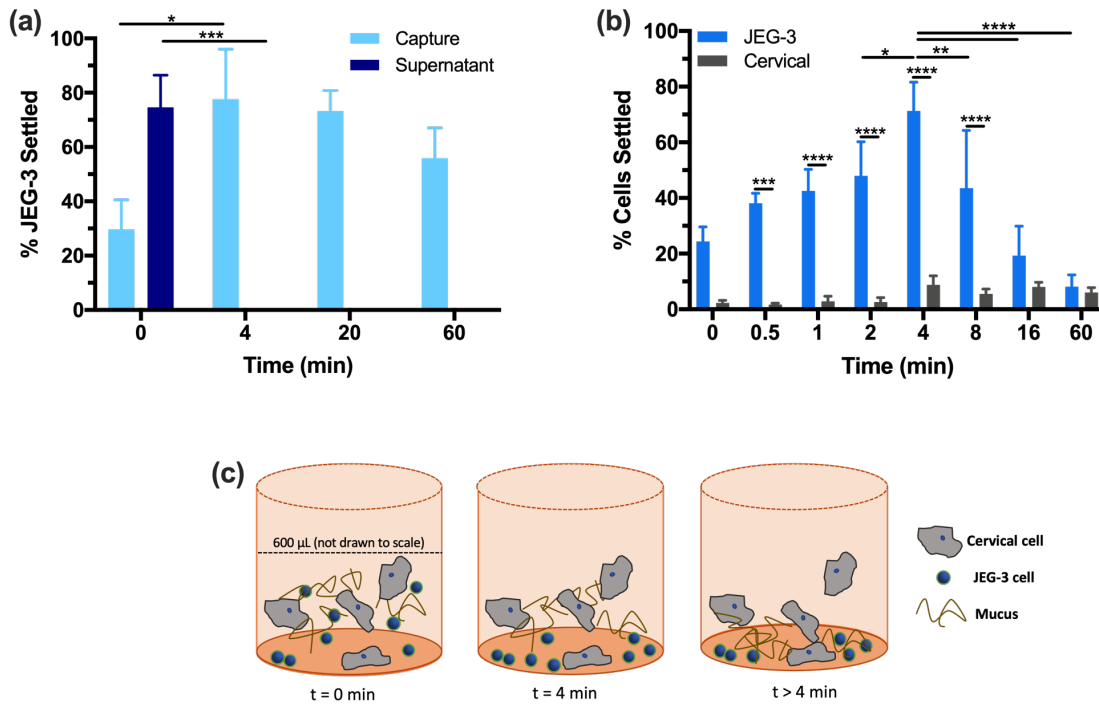


Figure 2.3: JEG-3 cell settling. (a) 1,000 JEG-3 cells settling in PreservCyt[®] for 0, 4, 20, and 60 min. (b) 1,000 JEG-3 cells in 600 μ L cervical sample for 0, 0.5, 1, 2, 4, 8, 16, and 60 min. (c) Schematic of the hypothesized settling of JEG-3 cells, cervical cells, and mucus over time. Two-way ANOVA with Tukey post-hoc analysis and a 95% confidence interval was performed. $n \geq 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

We also observed after 4 min, the number of JEG-3 cells captured at the well surface was significantly decreased at 16 and 60 min. At these time points no significant difference was observed between the percentage of cervical cells and the percentage of JEG-3 cells adhered to the capture surface. Our control experiment with JEG-3 cells

showed a plateau after 4 min of cell settling, so the observed decrease was due to partial recovery of JEG-3 cells in the presence of the cervical sample content. We hypothesize the decrease in JEG-3 capture following 4 min is due to the highly viscous and “sticky” nature of the mucus in the cervical sample, which itself continues to settle over time, as depicted in Figure 2.3c. Once a critical amount of mucus accumulates on the surface of the capture well, it obscures further settling or detection of cells on the surface. As the JEG-3 cells and cervical squamous cells exhibited different geometries, their settling behavior is expected to differ. The ability for JEG-3 cells to settle before the cervical cells may be due to a combination of Stoke-dependent settling,^{125,126} capture well polystyrene surface, and the trophoblast cell invasive nature.^{127–130}

Overall, we observed an optimized separation time of 4 min. The workflow was able to remove an average of $91 \pm 3\%$ of the cervical squamous cells from the sample, while capturing an average of $71 \pm 10\%$ of the JEG-3 cells. For this time point, the initial JEG-3 cell population was 1,000 cells while the initial cervical cell population was $\sim 72,000$ cells. After settling, 713 ± 103 JEG-3 cells were recovered with $6,320 \pm 2,390$ cervical cells, resulting in an unprecedented JEG-3 enrichment of $707 \pm 330\%$.

2.4.2.2 Effect of volume on cell settling

Having established JEG-3 separation behavior at a volume of 600 μL (~ 5 mm height), we next studied how JEG-3 settling changes with an increased volume of 3 mL (~ 20 mm height). We performed settling of 1,000 JEG-3 cells in cervical samples for 0, 4, 20, and 60 min (Figure 2.4a). Our results again indicated an optimal settling time of 4 min with $70 \pm 25\%$ JEG-3 settling and $8.4 \pm 3.6\%$ cervical cells remaining on the capture surface. These

results are similar to the optimal capture observed with 600 μ L, where the volume of settling did not result in significant differences in JEG-3 capture at 4 min (Figure 2.4b). Once again, settled mucus concentrates after 4 min, which disallowed or obscured further settling or detection of cells. With the 3 mL volume we still observed a trend of decreased JEG-3 cell settling after 4 min, but less pronounced compared to the 600 μ L volume. With the 3 mL volume, 20 and 60 min settling resulted in a significant difference between the JEG-3 and cervical cell settling, which was not observed for the 600 μ L volume. Therefore, if it is necessary to extend the time for optimal settling increasing the volume was shown to help. For the current study, because there was no significant difference in JEG-3 capture and cervical cell elimination at 4 min, we continued our optimization with 600 μ L.

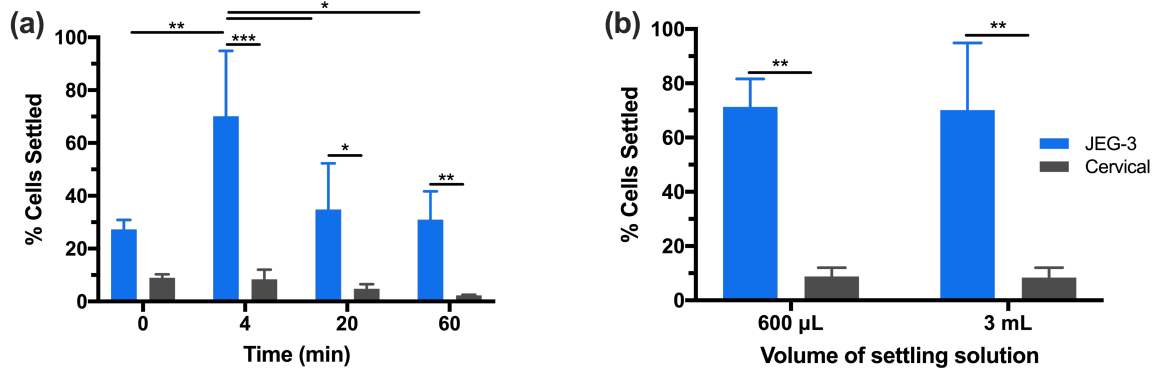


Figure 2.4: Effect of volume on cell settling. (a) JEG-3 and cervical cell settling over time with a total volume of 3 mL and height of 20 mm in the well. (b) 4 min time of settling for 600 μ L and 3 mL of settling volume. Two-way ANOVA with Tukey post-hoc analysis and a 95% confidence interval was performed. $n \geq 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

2.4.2.3 Effect of cell number on cell settling

Next, it was important to test if there was a dependence on the initial number of JEG-3 cells in the cervical sample. We studied 15, 100, 250, 500, and 1,000 JEG-3 cells in a

cervical sample with 4 min settling. Figure 2.5a and 2.5b show a representative image for the study performed with 1,000 JEG-3 cells per well. The JEG-3 cells remaining on the capture surface (Figure 2.5a) are observed with the blue DAPI stain. While cervical cells still remain on the capture surface, a large number of cervical cells are removed in the supernatant (Figure 2.5b). Figure 2.5c shows no significant difference in the percentage of JEG-3 cells settled to the capture surface at the tested cell numbers.

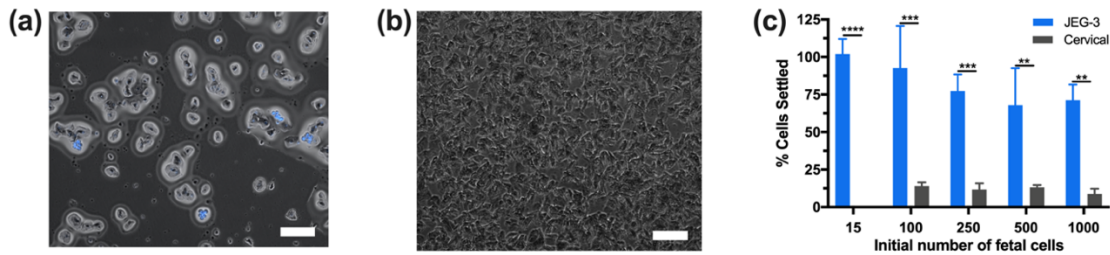


Figure 2.5: Capture and supernatant well images and effect of initial number of JEG-3 cells. (a) Capture well JEG-3 cells are DAPI stained and can be easily distinguished from cervical cells. (b) Population of cervical squamous cells removed with the supernatant. Scale bar = 200 μ m. (c) Effect of number of JEG-3 cells with cervical sample settling for 4 min. Two-way ANOVA with Tukey post-hoc analysis and a 95% confidence interval was performed. $n \geq 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

2.4.2.4 Effect of washing cells on cell settling

Previous studies have first removed mucus from cervical samples by PBS washing before performing isolation techniques.¹¹¹ Next, we washed the cervical sample with fresh PreservCyt[®] or 1 $\times$ PBS before cell settling to determine the role of mucus (as depicted in Figure 2.1 Step 2).

When washed with PreservCyt[®], only $34 \pm 4\%$ of JEG-3 cells settled (Figure 2.6) compared to the $71 \pm 10\%$ of JEG-3 settled when not washed (Figure 2.3b). With the PreservCyt[®] wash no significance was observed between JEG-3 and cervical cell settling. The percentage of cervical cells on the capture surface was not significant between the

washed ($13 \pm 3\%$, Figure 2.6) and unwashed ($9 \pm 3\%$, Figure 2.3b) conditions. When washed with PBS, the difference in the percentage of settling for JEG-3 cells and cervical cells was not statistically significant. The $13 \pm 12\%$ of JEG-3 settling after PBS washing was significantly lower than the $34 \pm 4\%$ of JEG-3 settling after PreservCyt[®] washing.

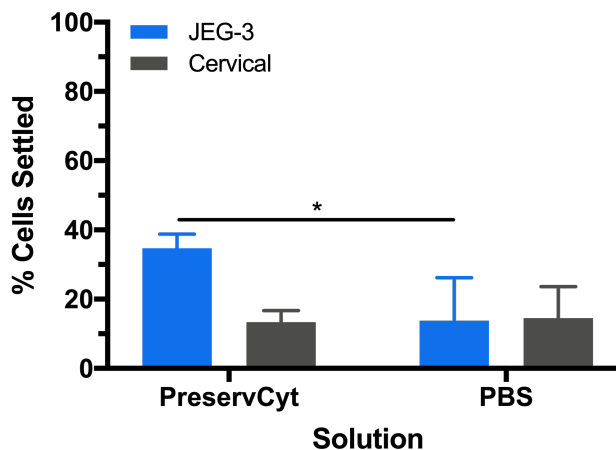


Figure 2.6: Cervical sample rinsed with PreservCyt[®] or PBS before 4 min cell capture study. Two-way ANOVA with Tukey post-hoc analysis and a 95% confidence interval was performed. $n \geq 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

These results point to the presence of mucus having a significant effect during the enrichment workflow. Previous work has indicated that cervical mucus impacts the diffusion of macromolecules and particles.^{131,132} These studies have focused on delivery across the cervical mucus barrier. It was noted that while some particles, such as the human papilloma virus and globular proteins diffuse as rapidly in the mucus as in saline, other particles such as the herpes simplex virus can colocalize with the mucus.¹³¹ Differences in surface modification of particles, such as polymer molecular weight, has also been shown to influence their interaction with cervical mucus.¹³² While the cervical and JEG-3 cells are significantly larger than these previously studied particles, differences in the cell

structure (e.g. geometry, surface proteins) can influence cervical mucus interactions. Based on our results we suggest that the mucus content either aids in the JEG-3 cell settling and/or restricts cervical cell settling. For our workflow, it is optimal to use the sample as received and not perform any washes or solution change in order to obtain maximal JEG-3 capture and enrichment.

2.4.2.5 Effect of solution density on cell settling

JEG-3 settling was then studied for 4 min in a Ficoll solution. Ficoll has a density of 1.055-1.075 g/mL compared to methanol with a density of 0.791 g/mL, which comprises 30-60% of PreservCyt®. Compared to the JEG-3 control with 4 min of settling in PreservCyt®, which captures $78 \pm 18\%$ JEG-3 cells, 4 min of settling in Ficoll resulted in $7.8 \pm 1.5\%$ of JEG-3 cell capture (Figure 2.7). Thus, the solution density of settling plays a role in the percentage of JEG-3 capture on the surface.

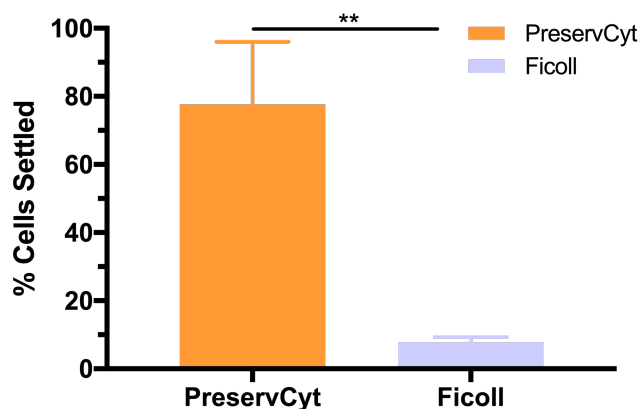


Figure 2.7: Percentage of JEG-3 cells settled after 4 min in PreservCyt® and Ficoll. Two-tailed t-test with 95% confidence interval was performed. $n \geq 3$; $*p < 0.05$.

Ficoll is a highly branched polymer formed by copolymerization of sucrose and pichlorohydrin.¹³³ It is possible Ficoll may act similarly to mucus content in solution as

both Ficoll and mucin are gel-like substances.^{133,134} An amount of Ficoll is adsorbed to the capture surface from the start of JEG-3 settling. This is dissimilar to the cervical sample where mucus is suspended in the PreservCyt[®] solution. Due to the concentrated Ficoll solution, we expected JEG-3 capture with Ficoll to be similar to JEG-3 capture after mucus settling. From our previous experiments, this occurs after 8 min (Figure 2.3b). The resulting JEG-3 cell capture in Ficoll is similar to the $8.2 \pm 4.2\%$ JEG-3 settling at 60 min in the cervical sample (Figure 2.3b), when mucus content at the capture surface is greatest. This further suggests presence of gel-like substances (Ficoll or mucus) at the capture surface restricts JEG-3 retrieval.

2.4.2.6 Effect of trophoblast cell line on cell settling

Using 4 min capture time, HTR-8 and TCL-1 trophoblast cell lines were used to investigate cell separation dependence on cell line (Figure 2.8). HTR-8 and TCL-1 trophoblast cell lines are representative of first and third trimester trophoblasts, respectively.^{135,136} Compared to the $71 \pm 10\%$ settling of the JEG-3 cells to the capture surface, the HTR-8 and TCL-1 resulted in $16 \pm 2\%$ and $27 \pm 4\%$, respectively. The JEG-3 capture was significantly different from the HTR-8 and TCL-1 cells. As was observed with the JEG-3 cells, a significant number of cervical cells was still removed from the population with the HTR-8 and TCL-1 spiked samples. It is possible that the ideal 4 min capture time may change for these additional cell lines.

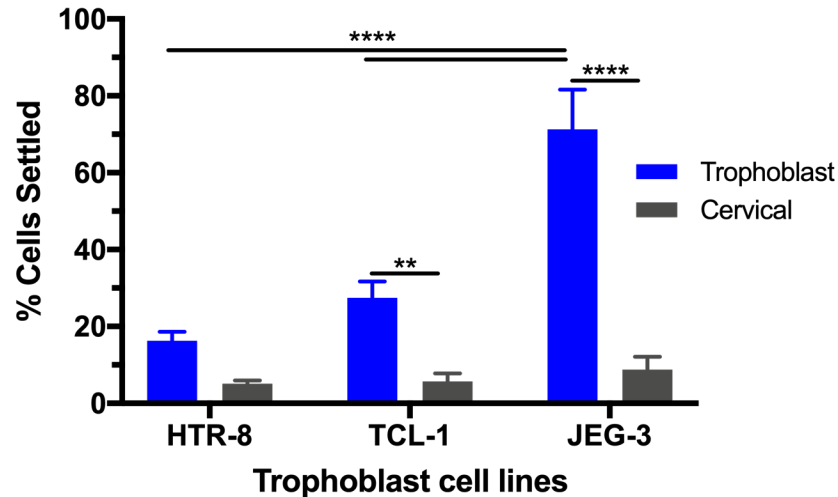


Figure 2.8: Comparison of trophoblast cell lines settling for 4 min. Two-way ANOVA with Tukey post-hoc analysis and a 95% confidence interval was performed. $n \geq 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

JEG-3 cells have commonly been used as a model EVT cell line.¹³⁷ Previous investigations have studied the villous versus extravillous nature of HTR-8 and TCL-1 cells. HTR-8 cells originated from first trimester villous explants¹³⁶ while TCL-1 cells were established from term placenta.¹³⁵ Investigations have looked at the EVT signature of these cell lines, and have found TCL-1 cells to have stronger EVT expression compared to HTR-8 cells.¹³⁸ This previous literature and our results suggest that the degree of EVT features on the cells may influence their settling in solution.

2.4.3 Enrichment of JEG-3 and trophoblasts for single cell picking and whole genome amplification

A hundred JEG-3 cells were spiked into a cervical sample with a concentration of ~100,000 cells/mL in order to more closely represent trophoblast concentration in cervical samples. First, we investigated settling of the JEG-3 cells at 4 min. After settling, the captured cells were transferred onto a coated slide. HLA-G staining was performed and

analyzed by the CyteFinder (Figure 2.9a and 2.9b). Images from the CyteFinder showed HLA-G labelled JEG-3 cells with single JEG-3 cells and cell clumps observed. Cervical cells that lack HLA-G are also observed in Figure 2.9b as DAPI stained nuclei only. We observed a JEG-3 capture of $73 \pm 36\%$ (Figure 2.9c), which is comparable to our previous optimization results. The role of incubating the JEG-3 and cervical cells together for a maximum of 1 h was also investigated for detection of captured JEG-3 cells using the CyteFinder (Figure SI 2.1).

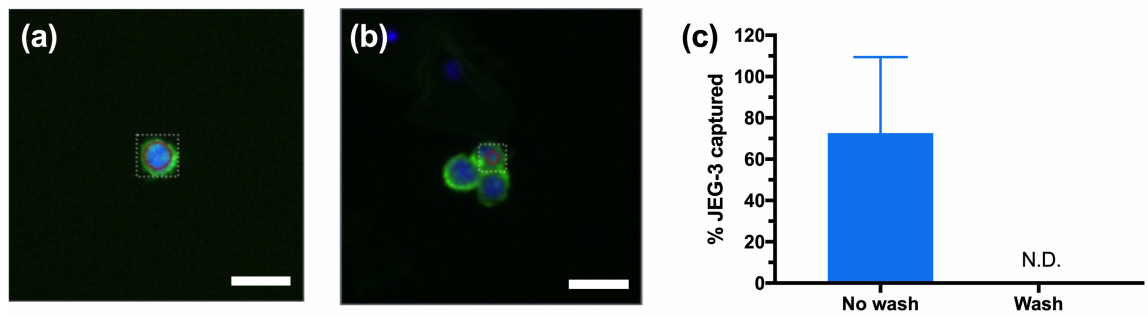


Figure 2.9: CyteFinder (a) Representative JEG-3 cell. (b) Representative JEG-3 cells. (c) Percentage of JEG-3 captured with and without PreservCyt® wash. The wash condition had no detectable (N.D.) cells found by the CyteFinder instrument. n=3; Scale bar = 20 μm .

The cervical sample was then washed 3 times with fresh PreservCyt® before settling with JEG-3 cells. We expected that these rinses would result in a reduced amount of cervical mucus present in the sample, similar to results from section *Effect of washing cells on cell settling*. After the wash, the CyteFinder instrument could not detect (N.D.) enough cells on the slide surface to produce a report. This result corresponds with our previous results and discussion on the role of mucus.

2.4.3.1 Cell picking

JEG-3 and potential real trophoblast cells of interest found during the CyteFinder were then picked from the slide surface (as depicted in Figure 2.1 Step 4). A control JEG-3 cell (Figure 2.10a) and two trophoblast cells (Figure 2.10b and 2.10c) were chosen for this proof-of-concept. Observed in each of these images was the ability to pick only these single cells with no cervical contamination in the surrounding area. Figures presented here were magnified using Image J software. Original images from the CyteFinder are presented in Supplementary Figure SI 2.2, which further demonstrate the boundary area free of cervical cells.

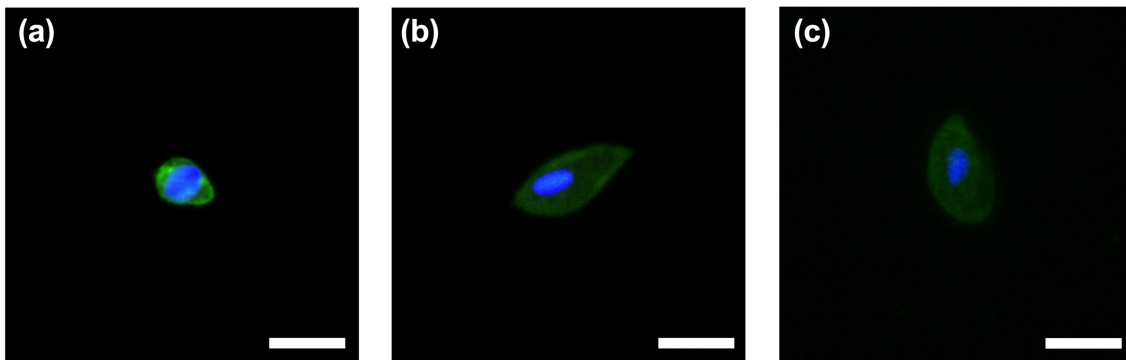


Figure 2.10: Three cells of interest isolated using CytePicker. JEG-3 cell control. (b) Potential real trophoblast. (c) Potential real trophoblast. Scale bar = 20 μm.

2.4.3.2 Whole genome amplification and polymerase chain reaction

For proof-of-concept testing, WGA with PicoPLEX was performed for a trophoblast cell and a JEG-3 cell picked using the CyteFinder (as depicted in Figure 2.1 Step 4). Following genome amplification, PCR was performed to identify presence of the Y chromosome. The Ribonuclease P (RNase P) signal demonstrated DNA presence in both the trophoblast and JEG-3 cell (Table 2.1). In this particular test case, the DYS14 and SRY Y chromosome markers, were not present for the putative native trophoblast cell as observed for the JEG-

3 control. Although this test cannot confirm the picked trophoblast as fetal in origin due to the lack of Y chromosome markers as this suggests a female fetus, this proof-of-concept shows the ability of our enrichment method to be applied to obtaining the genetic material from a single cell obtained from the cervical sample. Further, the strong Y chromosome signal observed for the JEG-3 cell demonstrates that cervical content is not masking the detection.

Table 2.1: Trophoblast and JEG-3 PCR.

Cell	Cycle Threshold		
	RNAse P	DYS14	SRY
Trophoblast	30	Fail	Fail
JEG-3	20.54	16.13	21.26

2.5 Conclusions

In this study we provide an optimized workflow for enriching trophoblast cells from a heterogeneous cervical cell population. Our enrichment method is inexpensive and label-free. Using JEG-3 cells, we conclude that enrichment is possible with removing at least 90% of squamous cervical cells while capturing at least 70% of JEG-3 cells at an optimal 4 min settling time. This results in a $707 \pm 330\%$ enrichment of trophoblast cells from the heterogenous population. This method is not dependent on initial trophoblast cell number and this process can be further scaled to larger volumes. We found that using the cervical sample as collected and sent to the lab was the most advantageous for enriching JEG-3 cells using our method, which makes this a streamlined process. As the cervical cell settling was observed to be similar in different solutions tested, we hypothesize that the polystyrene surface has a significant role restricting the cervical cell settling. However, as JEG-3 cell

settling changes with PreservCyt[®] and PBS washes and with Ficoll, we hypothesize the mucus content in the PreservCyt[®] solution is significant for trophoblast capture.

These results were further confirmed with our proof-of-concept studies using CyteFinder and CytePicker. We isolated single JEG-3 and native trophoblast cells found during the CyteFinder analysis and analyzed the DNA content through WGA and PCR. Ultimately, these results show that our workflow can make new isolation techniques, such as cell picking, more efficient and effective.

2.6 Author Contributions

C.M.B.H. contributed to the project aims, experimental planning, data collection and analysis, main manuscript text, and figure preparation. S.S. collected data towards Figs 3–6. M.K. and R.J. contributed cervical samples, and discussion and aims of the project. A.S. contributed to data analysis and manuscript revision. A.T. contributed to the project aims, experimental planning, data analysis, and manuscript revision. All authors reviewed the manuscript.

2.7 Acknowledgements

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Research Institute, Providence, RI) for kindly providing HTR-8 and TCL-1 cells and Dr. Christian Frank (University of Wisconsin, Madison) and graduate student Mark Scimone (Brown University) for assistance with confocal microscopy.

2.8 Supplemental Information

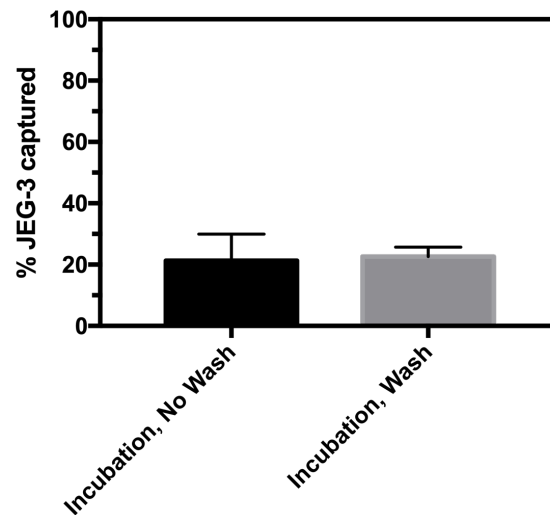


Figure SI 2.1: JEG-3 and cervical sample were allowed to incubate together for up to 1 h maximum time prior to performing the separation. This resulted in reduced percentage of JEG-3 recovery from the initial spiked in JEG-3 cells. JEG-3 cells were also allowed to incubate with the cervical sample for a period of time prior to settling after the cervical sample was washed 3x with fresh PreservCyt®.

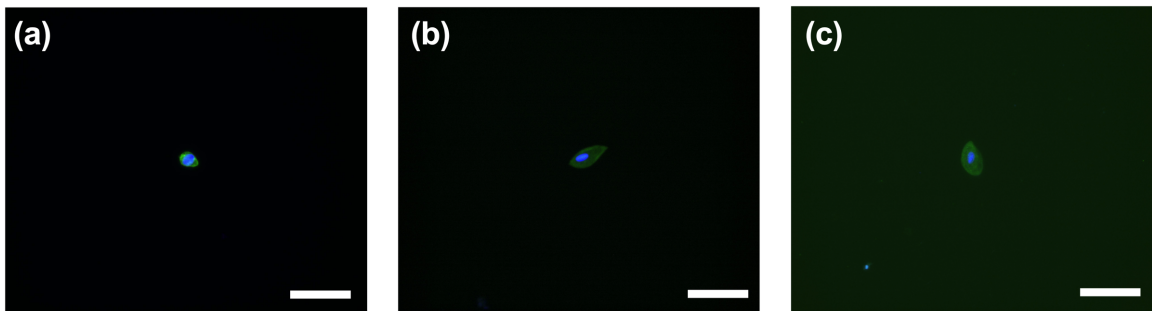


Figure SI 2.2: Images from the CyteFinder with no Image J alterations. Three cells of interest that were picked using CyteFinder from the slide surface as a proof-of-concept

trophoblast isolation. All cells were from the no incubation and no wash condition (a) JEG-3 cell control. (b) Potential real trophoblast. (c) Potential real trophoblast. Scale bar = 50 μm .

Chapter 3

Enrichment of JEG-3 Trophoblast Cells from Clinical Cervical Samples using Intrinsic Surface Adhesion Properties on an Inclined Plane

Chapter 3 is in preparation for submission as an original research article.

Bailey-Hytholt, C.M.,* Sayeed, S.,* Shukla, A., Tripathi, A., *Enrichment of JEG-3 trophoblast cells from cervical samples using inclined plane flow*, in preparation. *Co-first authors

3.1 Abstract

Objective: To develop a method and device for trophoblast enrichment from clinical cervical samples. The device must be capable of providing an increase in purity of trophoblast cells from the cervical cells and mucus and is user friendly to be used by non-specialized personal.

Design: Experimental laboratory study.

Setting: University and academic setting for biomedical engineering.

Patient(s)/Animal(s): Women in their 5th to 20th week of pregnancy were selected for endometrial canal sampling performed using a Cytobrush and following standard Papanicolaou smear protocol.

Intervention(s): Inexpensive, quick, and label free method for JEG-3 trophoblast enrichment from cervical samples using inclined gravitational flow on a surface.

Main Outcome Measure(s): We studied differential transport of cells on a flow channel device capable of offering varied angle of incline, surface material, incubation time on surface, volume, and channel width. These parameters allow for optimization of cell enrichment.

Result(s): Our method provides a maximum JEG-3 enrichment using polystyrene surfaces at a 50° incline with a 5 min incubation prior to inclined flow. This resulted in a $396 \pm 52\%$ increase in purity of JEG-3 cells from the clinical cervical samples. JEG-3 cell adhesion on the surface was confirmed with HLA-G fluorescence.

Conclusion(s): Our method is inexpensive, quick, and has the potential for direct integration into fetal cell isolation platforms. This design is developed on a slide surface, which can then be inserted onto an automatic cell picker instrument.

3.1.1 Keywords

trophoblast, cell enrichment, gravitational inclined flow, non-invasive prenatal diagnosis

3.2 Introduction

Prenatal testing is an important technology utilized during pregnancy. However, current methods are invasive, carrying risk for infection and fetal loss.^{139,140} Collection of cell-free fetal DNA provides a non-invasive technique,¹⁴¹ but tests are limited to certain chromosomal abnormalities and cannot confirm diagnosis.¹⁴² The ability to collect a whole fetal trophoblast cell would make new innovations in prenatal diagnostic technologies a possibility by providing the entire fetal genome. Previous work has determined that trophoblast cells are present in cervical swab samples; however, they are overwhelmed by the population of maternal cervical cells.^{107,111–114,139,143} Because of the complexity of the cervical matrix,^{116,131,144,145} the irregularity in cell size due to clumping,¹⁴⁶ and each sample's mucus and debris content,^{41,139} traditional cell separation methods, such as fluorescence activated cell sorting, are not suitable. Thus, there is a need to develop a method for trophoblast cell enrichment.

Cell rolling has been previously studied for understanding cell mechanics. Leukocyte cell rolling on a variety of surfaces has been widely studied in relation to recruitment to sites of inflammation,^{147–149} as well as the effect of rolling on the recruitment of hematopoietic stem cells to bone marrow niches, lymphocytes to secondary lymphoid organs, and platelets to sites of hemorrhage.¹⁵⁰ Presently, cell rolling methods have not yet been applied with gravitational rolling using inclined surfaces for the purpose of cell enrichment. The flow and transport on inclined plane offers the advantage of differences

in cell adhesion and proteins tethering as cells interact with surfaces.¹⁵⁰ By changing the incline of the surface, we can optimize how the cells experience gravitational rolling and interact with the surface.

During the initial implantation of a blastocyst, trophoblasts play a key role in the adhesion and invasion of the placenta into the maternal endometrium. While previous studies have investigated adhesion proteins and the invasiveness of trophoblast cells, these intrinsic properties have not been exploited for their use in isolation techniques. By using flow and transport on inclined planes with the unique surface and mechanical properties of trophoblast cells, we hypothesize the ability to provide an enrichment from cervical cells.

In this manuscript, we present a new method and a device (Figure 3.1) for controlling cell adhesion to a surface vs. flow/rolling down the surface into a collection area. During experimentation, the number of cells adhered to the slide or collected in the well or chamber can be imaged with microscopy or counted with a hemocytometer. Here we use JEG-3 trophoblast cells and cervical swab samples to present the effect of different channel designs, slide surfaces, angle of incline, and volume on the enrichment and capture of JEG-3 cells.

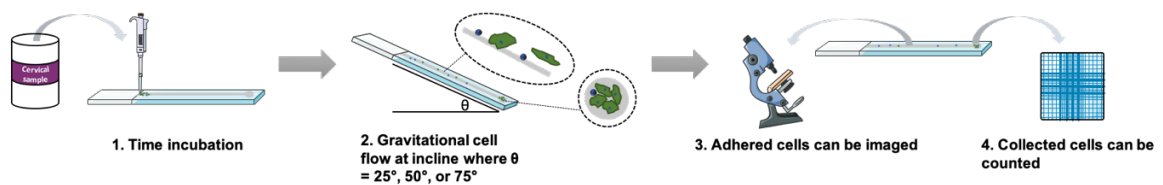


Figure 3.1: Enrichment by inclined cell flow schematic.

3.3 Materials and Methods

3.3.1 Patient Selection

Approval for enrolling patients for non-invasive prenatal sample acquisition, including endocervical swabs, was given by the Biomedical Research Alliance of New York Institutional Review Board (BRANY IRB) (File # 14-02-450-408). Written informed consent was obtained from the participating women and all personal information was removed from the specimen prior to receiving. Women in their 5th to 20th week of pregnancy were selected for sampling. All studies were performed in accordance with relevant guidelines and regulations.

3.3.2 Endocervical sampling

Retrieval of trophoblast cells from the endometrial canal was performed using a Cytobrush and following standard Papanicolaou smear protocol. Cells were rinsed from the cytological brush into 20 mL ThinPrep[®] PreservCyt[®] (Hologic, Marlborough, MA) fixative solution immediately after removal from the cervix. The material was sent to the lab for processing within 24 h of collecting. Samples were used as received unless noted otherwise.

3.3.3 JEG-3 trophoblast cell culture

The JEG-3 (ATCC, Manassas, VA) trophoblast cell line were cultured in Rosewell Park Memorial Institute medium 1640 (Gibco, Waltham, MA) (RPMI supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Corning, Corning, NY) and 1% (v/v) penicillin

streptomycin) at 37°C under 5% CO₂. Upon passaging, JEG-3 cells at a concentration of 100,000 cells/mL were stored in PreservCyt® at 4°C and used for up to 2 months.

3.3.4 Channel device development

On one side of a 1/8"-thick (0.317 cm) acrylic material (Rhode Island School of Design 3D store, Providence, RI), a double-sided adhesive (3M, Maplewood, MN) was applied. The adhesive was smoothed with a paint roller. The acrylic with the adhesive was then laser cut to form the cell flow devices designed on Adobe Illustrator CC. Two devices were developed: One with an open input (Figure 3.2a) and another with a closed input (Figure 3.2b). The open channel device design consists of a 0.2 cm width channel, 4.6 cm length channel, and 1 cm diameter collection chamber. The closed channel device design consists of a 0.2 cm width channel, 4.4 cm length channel, and 1 cm diameter collection chamber. After the device was cut, the adhesive backing was removed, and the adhesive side was stuck onto the slide surface. To ensure that the channel device was securely on the slide, pressure was applied to the device.

In order to vary the inclined plane surface, platforms for the slides at 25°, 50°, and 75° were designed on Solidworks™ 2016 and 3D-printed using a Makerbot Replicator (Makerbot, Brooklyn, NY) with Hatchbox polylactic acid filament (Megalowmart Holdings LLC, Pomona, CA).

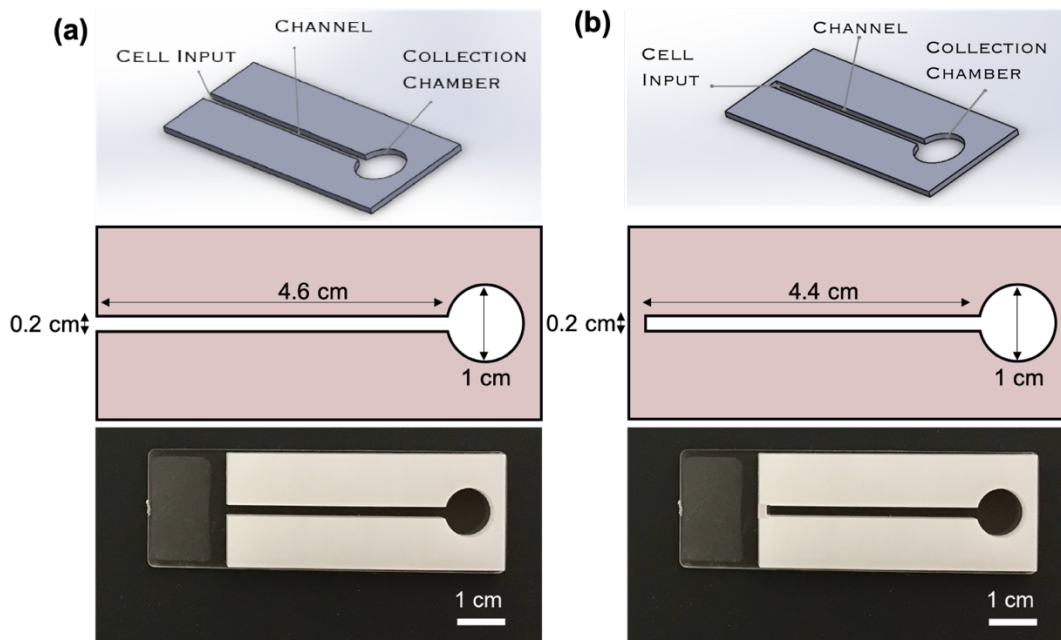


Figure 3.2: (a) open and (b) closed channel devices.

3.3.5 JEG-3 and cervical adhesion optimization

3.3.5.1 Open channel design testing with polystyrene

With the open channel device, polystyrene slides were tested for cell flow and adhesion at 25°, 50°, and 75° inclines. The polystyrene slides were cleaned with 70% ethanol and dried with high pressure nitrogen gas before adhering the 0.2 cm width channel device. 50 μ L of PreservCyt[®] containing either 1,000 JEG-3 cells or 50 μ L of the cervical sample in PreservCyt[®] containing approximately 5,000 cervical cells were administered to either an inclined surface directly or allowed to incubate on a flat horizontal surface for 5 min prior to inclining to the desired angle. Supernatants were counted using a hemocytometer, and adhered cells were counted using a Nikon Eclipse TS100 phase contrast inverted microscope.

The liquid flow rate in the open channel liquid flow is described by:

$$Q = \frac{\rho g \sin \theta}{3\mu} H^3, \quad (1)$$

where $\rho = 1 \text{ g/cm}^3$ is the density of the liquid, $\theta = 25^\circ, 50^\circ, \text{ or } 75^\circ$ is the angle of inclination, g is the gravity, $\mu = 0.01 \text{ P}$ is the viscosity of the liquid and H is the thickness of the liquid layer at time t . The initial liquid layer heights for 30, 50, and 70 μL were estimated to be 0.032, 0.054, and 0.076 cm, respectively. Under quasi-steady state assumption, the thickness of the liquid can be approximated using:

$$\left(\frac{1}{H^2} - \frac{1}{H_o^2} \right) = \frac{2\rho g \sin \theta}{3\mu A} t \quad (2)$$

where A is the area of the microchannel surface and H_o is the initial thickness of the liquid. Figure 3.3a shows the thickness of the liquid with time for various angles of inclination at a 50 μL initial volume. While little impact for the incline angle is observed for influencing thickness of the liquid layer, the initial volume of the solution does influence the rate of thickness layer decrease. Figure 3.3c shows the thickness of the liquid with time for various initial volumes at a 50° incline. JEG-3 cells are approximately 11 μm in diameter and cervical cells are disc shaped with an average diameter of approximately 60 μm .

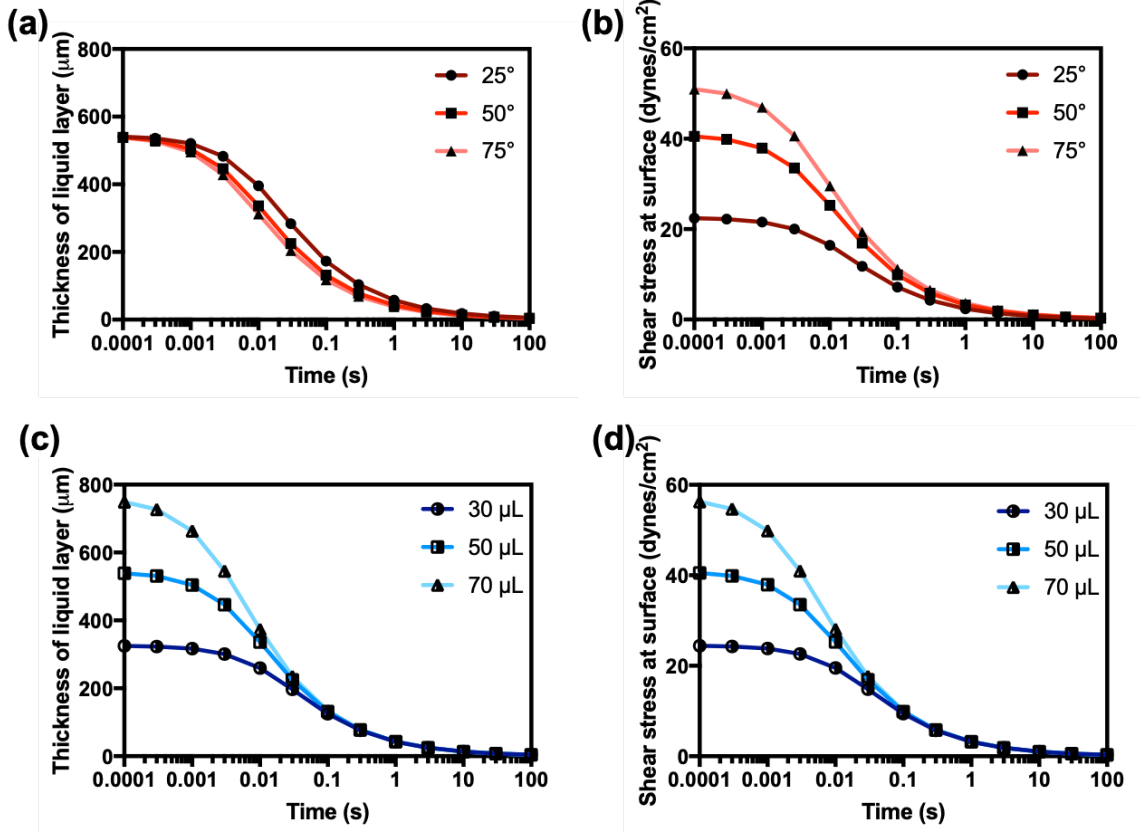


Figure 3.3: Thickness and shear stress changes of liquid based on incline angle and volume. (a) Thickness of the 50 μL liquid with time for 25°, 50°, and 75° inclines (b) Shear stress observed for cell during flows of 50 μL at 25°, 50°, and 75° inclines. (c) Thickness of the liquid with time for a 50° incline with 30, 50, and 70 μL liquid layer. (d) Shear stress observed for cell during flows at a 50° incline with 30, 50, and 70 μL liquid layer.

The average shear stress at the channel surface is then describe by:

$$\sigma = \mu\gamma = \rho g h \sin\theta \quad (3)$$

Figure 3.3b describes the approximate shear stress observed by cells during the flow. This predicts that the cells at lower inclines will observe less shear stress compared to cells at larger incline angles. Figure 3.3d describes the approximate shear stress observed by cells during flow at various initial volumes at a fixed 50° angle of incline. This predicts that

initial decreased layer height will observe less shear stress compared to larger initial layer heights.

3.3.5.2 Open and closed device design testing on glass

Glass slides were cleaned with 70% ethanol and dried with high pressure nitrogen (Corp Brothers, Providence, RI) before adhering the channel device (channel width of 0.2 cm). Open and closed channels were tested on glass slides at 25°, 50°, and 75° inclines. 50 µL of PreservCyt® containing either 1,000 JEG-3 cells or 50 µL of the cervical sample in PreservCyt® containing approximately 5,000 cervical cells were administered to either an inclined surface directly or allowed to incubate on a flat horizontal surface for 5 min prior to inclining to the desired angle. Supernatants were counted using a hemocytometer, and adhered cells were counted using a Nikon Eclipse TS100 phase contrast inverted microscope.

3.3.6 Purity change of JEG-3 cells incubated with cervical cells

1,000 JEG-3 cells and 5,000 cervical cells were mixed together and inclined gravitational flow was performed using the best conditions. Purified mouse anti-human HLA-G denatured (BD Biosciences, San Jose, CA) at 1:100 in antibody diluent (Dako, Agilent Technologies, Inc., Santa Clara, CA) was incubated on the slides for 30 min at room temperature. The slides were rinsed with 1× PBS and Alexa Fluor 488-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) at 1:250 in antibody diluent was incubated for 15 min at room temperature. The slides were then rinsed with 1× PBS and DAPI was incubated for 5 min. A final rinse was performed with 1× PBS and then the slides were imaged using a Nikon Ti-E inverted fluorescent

microscope with NIS Elements software. Using the best condition from this experiment, 10 JEG-3 cells and 5,000 cervical cells were mixed together, and inclined gravitational flow was performed and analyzed.

3.3.7 Optimizing flow solution volume and channel width

With the ideal enrichment incline and/or incubation conditions on both glass and polystyrene surfaces, the volume of PreservCyt[®] solution the cells were suspended in was varied. 30, 50, or 70 μ L of PreservCyt[®] containing either 1,000 JEG-3 cells or 30, 50, or 70 μ L of the cervical sample in PreservCyt[®] containing approximately 5,000 cervical cells were administered to either an inclined surface directly or allowed to incubate on a flat horizontal surface for 5 min prior to inclining to the desired angle.

The width of the channel was also investigated using the ideal enrichment incline and/or incubation condition on both glass and polystyrene surfaces. 50 μ L of PreservCyt[®] containing either 1,000 JEG-3 cells or 50 μ L of the cervical sample in PreservCyt[®] containing approximately 5,000 cervical cells were administered to either an inclined surface directly or allowed to incubate on a flat horizontal surface for 5 min prior to inclining to the desired angle with channel widths of 0.2, 0.3, or 0.4 cm.

3.3.8 Statistical analysis

All studies were performed in triplicate at minimum. Results are shown as average $\pm$ standard deviation. One-way or two-way analysis of variance (ANOVA) with Tukey's post-hoc analysis and 95% confidence interval was performed where * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001.

3.4 Results and Discussion

3.4.1 Polystyrene surface for inclined channel flow

We first performed the inclined flow experiments with polystyrene slide surfaces. These studies were performed at 25°, 50°, and 75° inclines with and without a 5 min incubation prior to inclining the surface for channel flow. Without the 5 min incubation, JEG-3 cells or cervical cells were administered to the channel at the incline of interest, and we observed a significant difference in JEG-3 adhesion due to the angle of incline (Figure 3.4a). Without the incubation, there was a 2.2× frequency increase at 25° of JEG-3 cells compared to the cervical cells for adhesion to the polystyrene surface. At an incline of 75°, we observed a 7.4× frequency increase of JEG-3 cells. We suggest that the greater rate of fluid flow at 75° compared to the 25° may cause cells within the layers of fluid that usually do not contact the surface to move fast enough to tumble to the surface, allowing for the cells to have more surface contact, and resulting in the increased JEG-3 adhesion at 75°.

Next, we added the 5 min time incubation before inclining the surface to the desired angles. Here, we observed a significant capture of JEG-3 cells on the channel surface while cervical cells flowed into the collection chamber well. At a 25° incline, $76 \pm 3\%$ of JEG-3 cells were captured on the polystyrene surface. At this same incline, only $5 \pm 3\%$ of cervical cells were captured on the polystyrene surface (Figure 3.4b). After a 5 min incubation and inclining to 50°, we observed a 6× frequency increase of JEG-3 cells remaining on the surface compared to cervical cells (Figure 3.4b). With the 50° incline, we observed an approximately 50% JEG-3 adhesion with less than 8% cervical cell adhesion. Thus, we suggest that recycling the supernatant on the same or new slide may yield a significant

enrichment while capturing a higher percentage of JEG-3 cells. Once JEG-3 cells are accumulated on the surface, shear stress affects the remaining JEG-3 cells as predicted from Figure 3.3b, indicating that the interaction is shear-stress-mediated.

From our observations, we can conclude the following mechanism: After JEG-3 cells are given an amount of time to reach the surface, JEG-3 cells respond to a flow and attach to the polystyrene substrate. We have previously studied this mechanism of differential trophoblast cell, and determined that approximately 4 min is an optimal time for JEG-3 capture.¹⁵¹ This was further studied here and results are observed in Figure 3.5. Once contact is made, the cells then remain adhered or roll with a specific speed that is proportional to the applied shear stress. The incubation is necessary for JEG-3 binding to the surface. Due to both cell geometries and material properties, we observe that cervical cells settle more slowly to the surface, and once the slide is inclined, the cervical cells cannot withstand the Stokes motion force that exceeds the binding strength to the surface, leading to cell removal.

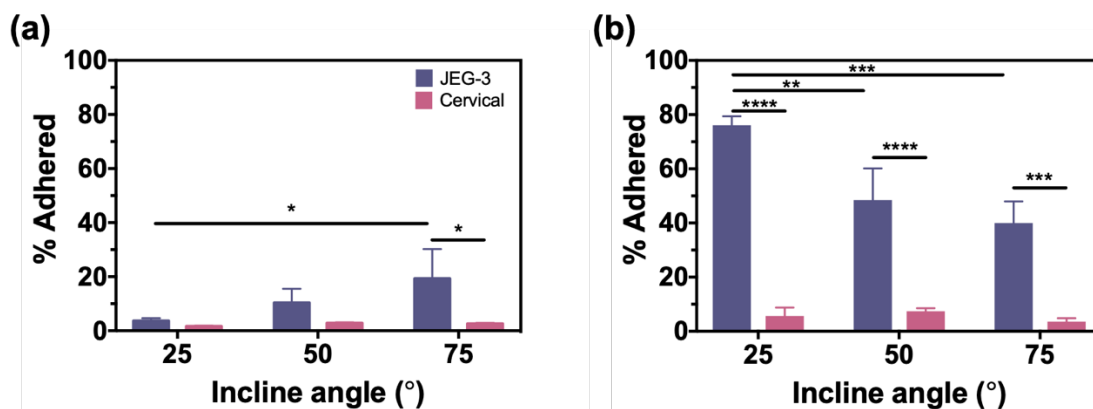


Figure 3.4: JEG-3 (blue) and cervical (pink) cell adhesion to polystyrene slide surface. (a) Open channel device design with no prior incubation. (b) Open channel device design with 5 min horizontal incubation before inclined flow. Statistics were performed with $n=3$, Two-way ANOVA with Tukey's post-hoc analysis, $\alpha = 0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

With the 50° incline, we continued investigating the incubation time prior to cell flow (Figure 3.5). A 10 min incubation prior to 50° inclined flow was performed. At the 10 min incubation time we observe a stagnating JEG-3 cell adhesion while the cervical cell adhesion to the surface increased significantly. From Figure 3.5 we can observe that the 5 min incubation time is the optimized time for maximizing JEG-3 adhesion while minimizing cervical cell adhesion. This suggests that the JEG-3 cells can adhere to the polystyrene surface with a strong enough force to withstand the shear stress faster than the cervical cells can.

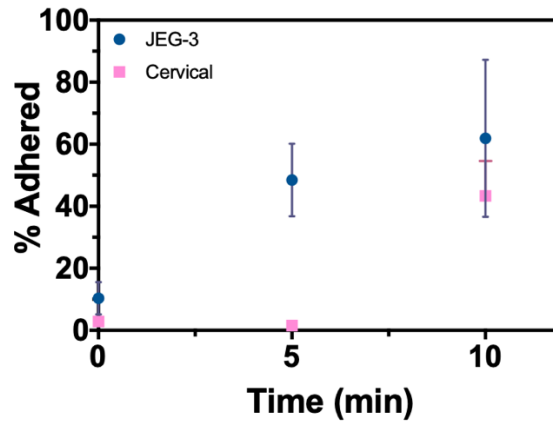


Figure 3.5: Effect of incubation time on JEG-3 and cervical adhesion during inclined flow at 50°.

3.4.2 Open and closed channel device on glass slide surfaces

To investigate the importance of surface material, cells were then flowed down glass slides at 25°, 50°, and 75° inclines with or without a prior 5 min horizontal incubation on the open channel device. Without incubation, we observed little JEG-3 or cervical cell adsorption to the surface, with $13 \pm 3\%$ JEG-3 adhesion and $3 \pm 2\%$ cervical adhesion at a 50° (Figure 3.6a). However, for all angles, the percent of cell adhesion was not significant between JEG-3 and cervical cells. Comparing this to incubating the cells on the surface for

5 min prior, we observed an increase in JEG-3 and cervical adhesion. At a 25° incline we observed a significant difference between the JEG-3 and cervical cell adhesion, with $50 \pm 18\%$ JEG-3 adhesion and $14 \pm 4\%$ cervical cell adhesion (Figure 3.6b). However, these results still yield a less significant frequency change of 3.6× compared to the JEG-3 frequency increases observed on polystyrene.

We hypothesized that increasing the surface tension of the fluid during the inclined flow may increase the capture of cells on the surface. To test this, we developed a closed channel device (Figure 3.2b). However, we observed similar results to the open channel device and no significance between any JEG-3 and cervical cell captures. We observed less than 30% JEG-3 capture and a maximum of 2.7× frequency increase of JEG-3 cells for all conditions tested on the closed device (Figures 3.6c and 3.6d). Ultimately, these results indicate the importance of the polystyrene surface for optimal JEG-3 capture and minimization of cervical cell capture using our method. These results also indicate the importance of surface material on providing an optimal enrichment.

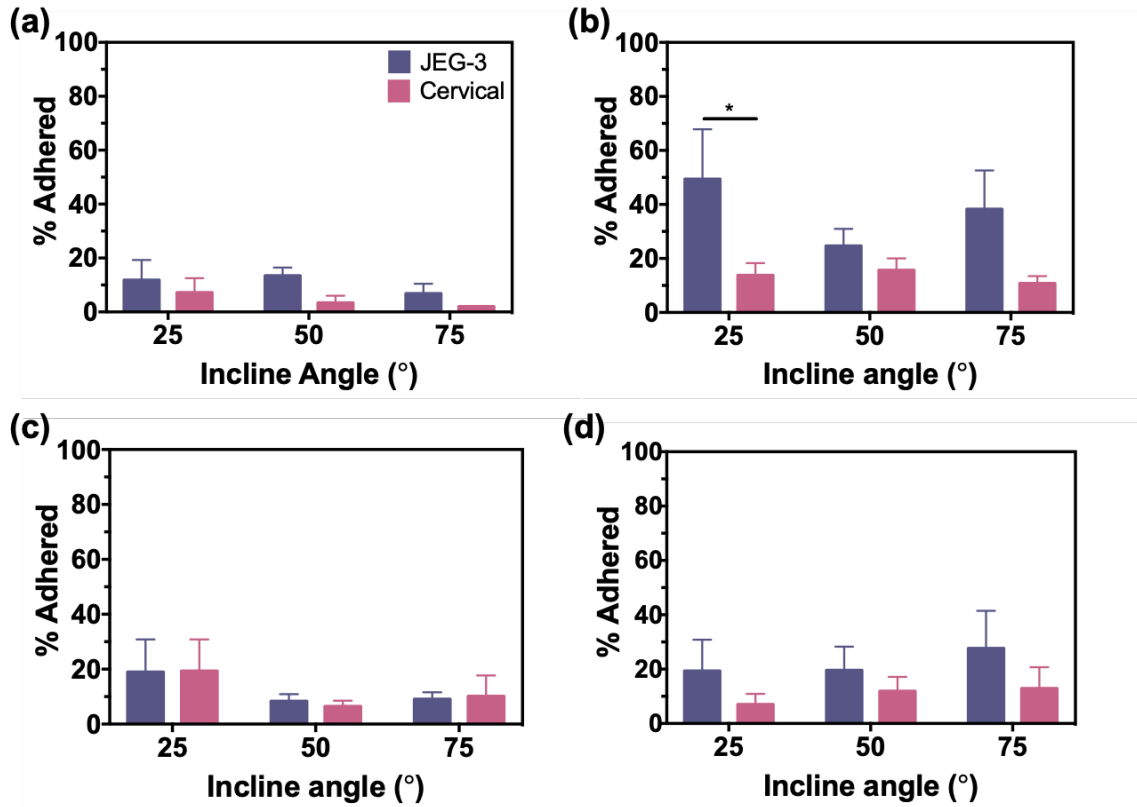


Figure 3.6: JEG-3 (blue) and cervical (pink) cell adhesion to glass slide surface. (a) Open channel device design with no prior incubation. (b) Open channel device design with 5 min horizontal incubation before inclined flow. (c) Closed channel device design with no prior incubation. (d) Closed channel device design with 5 min horizontal incubation before inclined flow. Statistics were performed with $n=3$, Two-way ANOVA with Tukey's post-hoc analysis, $\alpha = 0.05$, $*p<0.05$.

Trophoblast and cervical cells have differences in their surface adhesion and cellular proteins. Some examples of these similarities and differences are notes in Table 3.1. The mechanism of bond strengthening remains unclear, but one might speculate on catch bond theory¹⁵² or additional transport mechanisms¹⁵³ known from leukocyte rolling that may have analogs in trophoblast adhesion and invasion in the highly vascularized placenta-endometrium barrier. Our results indicate that JEG-3 surface receptors, such as HLA-G, might be a prerequisite for adhesion to polystyrene compared to cervical cells.

Table 3.1: Properties of Trophoblast and Cervical Cells.

	Extravillous trophoblast	Cervical
Approx. cell diameter (μm)	11	60
Height of liquid layer at 50° incline (cm)	0.054	0.054
Number of trophoblast and cervical cells tested	1,000	5,000
Examples of common surface adhesion proteins	Platelet-endothelial cell adhesion molecule-1 (PECAM-1), melanoma cell adhesion molecule (Mel-CAM), VE-cadherin (VE-cad), ephrin-B1 and -B2, neural cell adhesion molecule (NCAM) ¹⁵⁴	Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM), platelet-endothelial cell adhesion molecule (PECAM), E-selectin ¹⁵⁵
Examples of additional common proteins	Human leukocyte antigen-C (HLA-C), human leukocyte antigen-G (HLA-G) ¹⁵⁶	Mucin 16 (MUC16), Secretory leukocyte peptidase inhibitor (SLPI), WAP four-disulfide core domain 2 (WFDC2), Serpin family B member 3 (SERPINB3), Ankyrin repeat domain 35 (ANKRD35), estrogen receptor 1 (ESR1), transglutaminase 2 (TGM2), melanogenesis associated transcription factor (MITF) ^{157–159}

3.4.3 Cell flow after 5 min incubation on incline with cervical and JEG-3 cell heterogeneous solution

The JEG-3 and cervical cells were flowed together at the best enrichment conditions (polystyrene 25° and 50° with 5 min incubation). The JEG-3 cells were distinguished from cervical cells by their spherical morphology and HLA-G staining (Figure 3.7a). Although the polystyrene surfaces generated autofluorescence, the intensity of the HLA-G was great enough to distinguish the JEG-3 cells.

The 25° incline resulted in a $290 \pm 7\%$ enrichment of JEG-3 cells from cervical cells, capturing $36 \pm 5\%$ of JEG-3 cells and eliminating 95% of cervical cells (Figure 3.7b). The 50° incline resulted in a $396 \pm 52\%$ enrichment of JEG-3 cells from cervical cells, capturing $39 \pm 24\%$ of JEG-3 cells and eliminating 97% of cervical cells. While this results in a significant purity increase of JEG-3 cells for the ultimate goal of JEG-3 isolation, we do experience a decreased JEG-3 capture with this heterogeneous mixture of cells compared to our optimization condition with JEG-3 only. We hypothesize that the cervical mucus within the sample affects the JEG-3 adhesion during the inclined flow. We hypothesize that this mucus content also affects the shear stress the cells experience, which results in the 50° incline yielding the better enrichment. Thus, we continued with the 50° incline on polystyrene slides after a 5 min incubation to test if we could optimize any additional parameters.

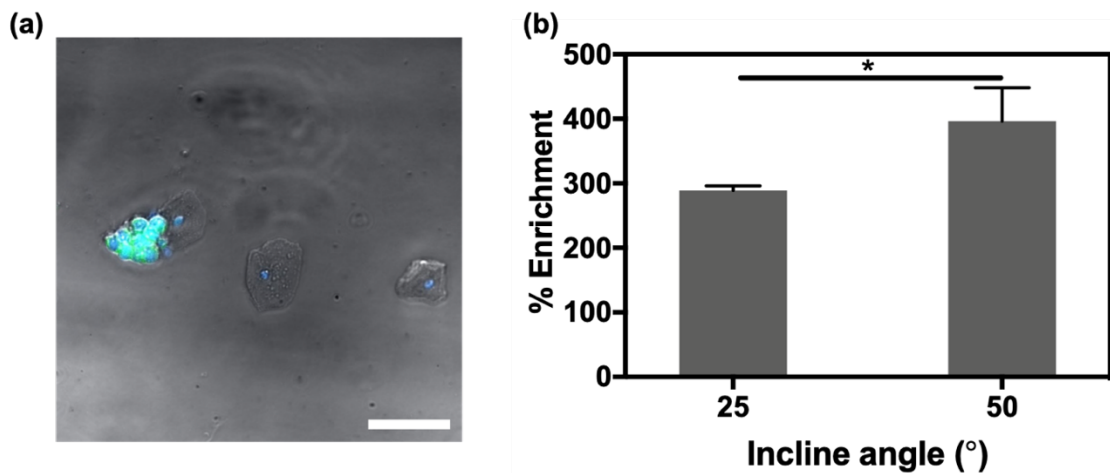


Figure 3.7: JEG-3 enrichment from heterogeneous cervical samples using polystyrene surface inclined plane. (a) JEG-3 (green HLA-G stain) and cervical cells on polystyrene capture channel surface, scale bar = 200 μm (b) Percentage of increased enrichment of JEG-3 cells from cervical cells after 5 min horizontal incubation followed by gravitational flow at 25° and 50° inclines. Statistics were performed with $n=3$, One-way ANOVA with Tukey's post-hoc analysis, $\alpha = 0.05$, $*p < 0.05$.

We also wanted to ensure that this enrichment could be achieved with a lower quantity of JEG-3 cells present in the cervical sample. Thus, we incubated 10 or less JEG-3 cells with 50 μ L of cervical sample and performed the 5 min incubation and flow at 50° incline. Here we observed 100% JEG-3 recovery and a purity enrichment of 50% (Figure SI 3.1). We did observe an increase in cervical adhesion during this testing condition.

3.4.4 Effect of volume and channel width on cell channel capture

Continuing with the best conditions on glass (50° with no incubation) and polystyrene (50° with incubation), the effect of the volume and channel width on cell capture was tested. First, we varied volume from the original 50 μ L to 30 μ L and 70 μ L of JEG-3 in PreservCyt® or of cervical sample. With the glass surface, we observed that lowering the volume led to increased JEG-3 adhesion but also a similar percentage of cervical adhesion (Figure 3.8a), as all cells became situated closer to the slide surface. Increasing the volume led to overall decreased cell capture due to increased distance from the slide surface. With the 5 min incubation and flow at a 50° incline on polystyrene surface, we observed that the 50 μ L was ideal for both the largest amount of JEG-3 capture and highest frequency increase of JEG-3 cells compared to cervical (Figure 3.8b). A 30 μ L volume led to increased cervical adhesion to the channel surface, as cervical cells obtained better access to the surface, and a 70 μ L volume led to similar percentages of JEG-3 and cervical cell capture, as cells were now further away from the surface. As the volume is increased for glass and with it, the velocity at which the fluid flows, the adhesion decreases (Figure 3.8a), which can be due to the fact that on glass, the cell's settling (a static phenomenon) is

compromised by the faster fluid flow. On the other hand, on polystyrene, greater volume increases the distance at which electrostatic and hydrophobic interactions can act and decreases adhesion, while smaller volumes result in smaller fluid height and dominance of cervical cell adhesion on the available slide surface, leading to the optimal 50° condition with incubation (Figure 3.8b).

Investigating the impact of the channel width, we performed experiments with a channel width of 0.3 and 0.4 cm, in addition to our original 0.2 cm channel. On glass surfaces, we observed no significant change due to channel width (Figure 3.8c). On the polystyrene surfaces, we also observed no statistical significance between the JEG-3 adhesion based on increasing the channel width (Figure 3.8d). No significant difference due to channel width is ideal for scale up of this device. Ultimately, we observed here that 50 μ L is an ideal volume for performing a JEG-3 capture and cervical cell removal from solution and that this process could be scaled up in the future by altering the channel width.

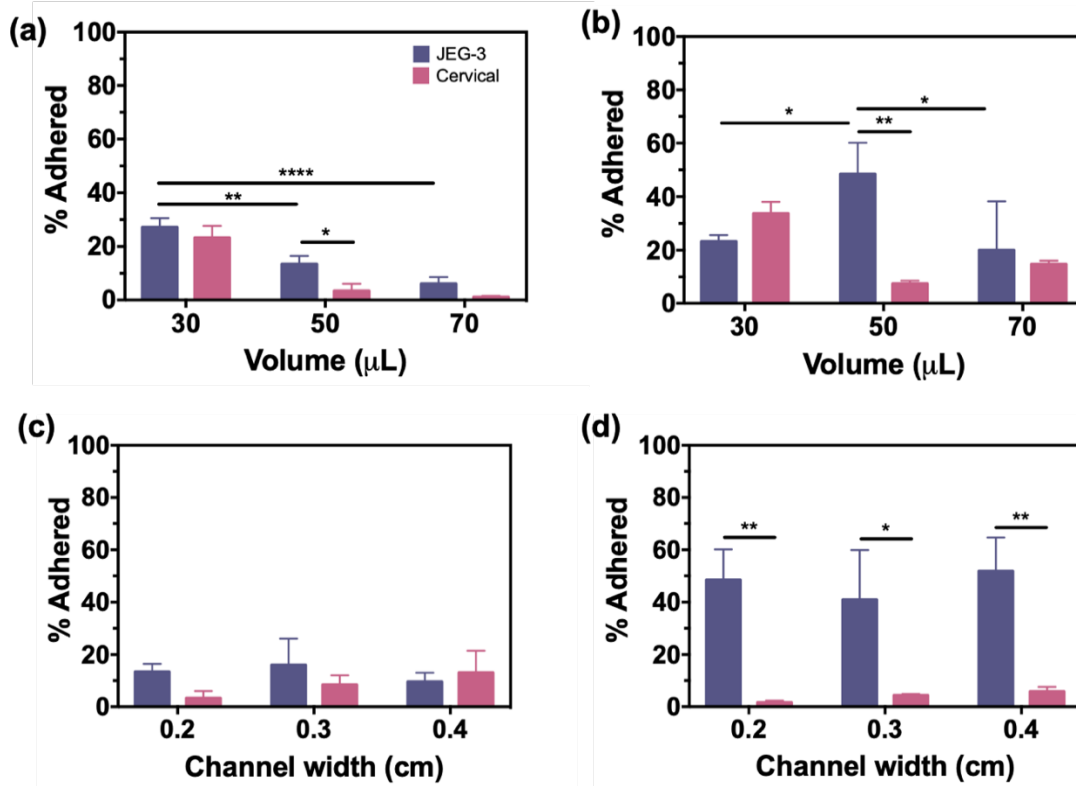


Figure 3.8: Impact of volume and channel width on JEG-3 (blue) and cervical (pink) cell capture. (a) JEG-3 and cervical cells in 30, 50, and 70 μL volumes captured on glass surface channel with flow down 50° incline with no prior incubation. (b) JEG-3 and cervical cells in 30, 50, and 70 μL volumes captured on polystyrene surface with flow down 50° incline after 5 min incubation on horizontal surface. (c) JEG-3 and cervical cells captured on glass surface channels of 0.2, 0.3, and 0.4 cm widths with flow down 50° incline with no prior incubation. (d) JEG-3 and cervical cells captured on polystyrene surface channels of 0.2, 0.3, or 0.4 cm widths with flow down 50° incline after 5 min incubation on horizontal surface. Statistical analysis was performed with $n=3$, Two-way ANOVA with Tukey's post hoc analysis, $\alpha = 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5 Conclusions

In this work, we investigated a new method for the collection of rare trophoblast cells in a heterogeneous population of cervical cells through the movement of cells down an inclined plane. Due to both surface properties and shear stress at the surface, we determined optimal JEG-3 capture and purity enrichments by incubating the cells for 5 min on a horizontal

polystyrene surface with our channel device, followed by inclining for gravitational cell flow. In this study we showed our optimization of open and closed channel device designs, investigated glass and polystyrene slide surfaces, and determined the optimal volume and channel width to provide JEG-3 enrichment. We performed studies with JEG-3 and cervical cells alone for optimization and used the ideal conditions to investigate purity changes when the cells were mixed together. In this heterogeneous mixture we determined our method increases the purity of the sample by $396 \pm 52\%$.

This approach to enriching JEG-3 cells paves the way for label-free cell enrichment techniques that can allow for cells to be separated based on their interactions within a dynamic fluid medium above varied surface materials. This is especially useful when cells, such as the extravillous trophoblasts, are found in small numbers amidst other types of cells and mucus in outnumbering quantity. Using this method allows for samples to be tested without changing sample chemical characteristics (ie. by adding nanoparticles or reagents) that could impact downstream fetal genome analysis and can be suitably used by untrained personnel. Ultimately, this study is a first step to a quick and inexpensive method to enrich fetal cells.

3.6 Author Contributions

This chapter was written through contributions of all authors. C.M.B.H. and S.S. contributed to the project aims, experimental planning, data collection and analysis, main manuscript text, and figure preparation equally. A.S. contributed to data analysis and manuscript revision. A.T. contributed to the project aims, experimental planning, data analysis, and manuscript revision. All authors reviewed the manuscript.

3.7 Acknowledgements

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3.8 Supplemental Information

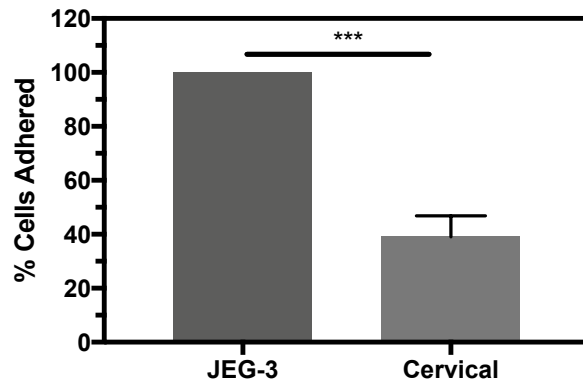


Figure SI 3.1: JEG-3 and cervical cell enrichment with 10 or less JEG-3 cells incubated with 50 μ L containing approximately 5,000 cells of the cervical sample. One-way ANOVA with Tukey's post-hoc analysis, $\alpha = 0.05$, *** $p < 0.001$.

Chapter 4

Effects of Flow and Bulk Vesicle Concentration on Supported Lipid Bilayer Formation

Chapter 4 has been published as an original research article.

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4.1 Abstract

Supported lipid bilayers (SLBs) have been used extensively in a variety of biotechnology applications and fundamental studies exploring lipid behavior. Despite their widespread use, various physicochemical parameters have yet to be thoroughly investigated for their impact on SLB formation. In this work, we have studied the importance of flow in inducing the rupture of surface adsorbed chicken egg derived L- α -phosphatidylcholine (egg PC) vesicles on silica and gold surfaces via quartz crystal microbalance with dissipation monitoring (QCM-D). On silica at 25°C, egg PC vesicles were found to adsorb in a flattened configuration (~13 nm thick compared to bulk vesicle diameters of ~165 nm) but only undergo a transition to a stable SLB under flow conditions. In the absence of flow, an increase in system temperature to 37°C was able to promote vesicle rupture and SLB formation on silica with a 10 times lower rupture time compared to rupture under continuous flow (175 μ L/min flow rate). Gold surfaces, with their increased hydrophobicity, led to less vesicle flattening once adsorbed (~60 nm thick structures), and did not support vesicle rupture or SLB formation even at flow rates of up to 650 μ L/min. We also showed that under continuous flow conditions, vesicle adsorption rates on silica surfaces follow Langmuir kinetics, with an inverse dependence on bulk vesicle concentration, while an empirical power law dependence of vesicle rupture time on bulk vesicle concentration was observed. Ultimately, this work elicits fundamental insight into the importance of flow and bulk vesicle concentration in the adsorbed vesicle rupture process during SLB formation using QCM-D.

4.2 Introduction

Supported lipid bilayers (SLBs) can serve as models of the phospholipid cell membrane commonly used in biotechnology applications.^{75–77} SLBs have been used to study molecular interactions of lipid bilayers with various macromolecules and small molecules.^{78–81} Previous work has investigated SLB formation with varying lipid compositions,⁸² incorporation of lipid proteins in SLBs,^{83–85} and utilizing polymer cushions¹⁶⁰ to yield SLBs that mimic specific aspects of cell membrane function. The great potential of lipid bilayers for biomedical applications has been demonstrated with investigations spanning molecular and nanoparticle transport,^{78,79} protein assembly and function,^{83–85} peptide arrangement and insertion,^{80,81} drug screening,⁸⁷ and microfluidic platforms.⁸⁸ In order to optimally utilize SLBs in these and other advanced applications, it is necessary to fully understand the bilayer self-assembly process.

Previous studies have investigated the formation of SLBs on various surfaces such as silica,⁷⁵ mica,¹⁶¹ gold,¹⁶² and titanium.¹⁶³ Properties of the substrate material can influence whether the vesicles remain adsorbed to the surface or rupture, leading to eventual formation of a planar bilayer structure. For example, silica and mica are often used to induce vesicle rupture, while more hydrophobic surfaces, such as gold, have been utilized to maintain intact adsorbed vesicles.⁸⁹ Additionally, studies have examined the impact of varying lipid compositions,¹⁶⁴ osmotic effects,¹⁶⁵ the presence of divalent ions,^{166,167} gel-liquid domain effects,¹⁶⁸ and lipid transition temperatures⁷⁶ on vesicle adsorption and rupture abilities. While these studies have led to several significant advances in the SLB field, more work needs to be done to enhance understanding of factors

contributing to the different stages of vesicle rupture and SLB formation at a fundamental level.

Ensemble techniques, such as quartz crystal microbalance with dissipation monitoring (QCM-D), which is used extensively in the formation and monitoring of SLBs, typically indicate vesicle rupture once a critical vesicle coverage (CVC) has been reached.⁷⁵ Using techniques such as atomic force microscopy, isolated rupture of single vesicles into bilayer islands has been observed.^{169,170} The role of vesicle fusion and coalescence has also been discussed, where two or more neighboring vesicles merge in order to develop a complete SLB.^{94,95} The goal of the current study was to investigate the role of flow, temperature, and substrate properties in the SLB formation process, along with examining the dependence of vesicle adsorption and rupture times on bulk vesicle concentration.

Here we utilized QCM-D to precisely control physical and chemical parameters during phospholipid vesicle adsorption to provide further insight into the driving forces governing SLB formation. QCM-D measures frequency and dissipation changes occurring in real-time on a substrate consisting of a coated piezoelectric quartz sensor. This is achieved by applying a voltage over the QCM-D substrate inducing resonance; frequency and dissipation are monitored at odd intervals of the fundamental frequency.⁹⁵ Briefly, to monitor the vesicle adsorption to SLB formation process via QCM-D, vesicles are administered to an enclosed QCM-D module (Figure 4.1a), where they are allowed to interact with the substrate and the change in frequency and dissipation are continually monitored. In this study, we investigate the importance of flow in promoting QCM-D SLB formation. We demonstrate that in the absence of flow, increasing thermal energy can

promote SLB formation. To the best of our knowledge, the specific effects of flow on SLB formation in a QCM-D system have not yet been reported. By using a range of lipid concentrations, we also investigated the impact of bulk vesicle concentration on vesicle adsorption and rupture kinetics.

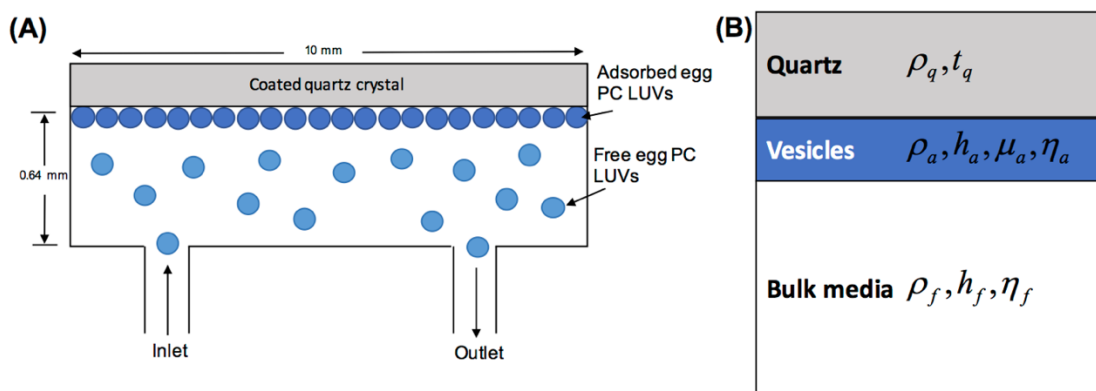


Figure 4.1: Vesicle adsorption step during supported lipid bilayer formation using quartz crystal microbalance with dissipation monitoring (QCM-D). (A) Cross section of QCM-D module during egg PC large unilamellar vesicle (LUV) adsorption. The adsorbed LUV configuration shown is arbitrary. (B) Spatial schematic of quartz surface (grey), adsorbed vesicle layer (blue), and bulk media (white), where ρ_q is the density of the quartz, t_q is the thickness of the quartz, ρ_a is the density of the adsorbed vesicles, h_a is the film thickness of the vesicle layer, μ_a is the elastic modulus of the vesicle film, η_a is the shear viscosity of the vesicle film, ρ_f is the density of the bulk fluid, h_f is the thickness of the bulk fluid, and η_f is the shear viscosity of the bulk fluid.

4.3 Materials and Methods

4.3.1 Lipid vesicle formation, vesicle characterization, and surface characterization

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. MilliQ water (18.2 M Ω , EMD Millipore, Taunton, MA) was used in all experiments. L- α -phosphatidylcholine (Egg, Chicken) (egg PC) (Avanti Polar Lipids, Inc., Alabaster, AL) in chloroform was stored at -20°C and used to form lipid vesicles, as previously reported.^{171,172} Briefly, stock egg PC was dried under ultra-high purity nitrogen gas (Corp Brothers, Inc., Providence, RI) in a chemical fume hood and kept under vacuum for at least

4 hours to remove excess chloroform. Dried egg PC was rehydrated in Tris sodium chloride (NaCl) buffer containing 10 mM Trizma base and 100 mM NaCl, yielding a final concentration of 2.5 mg/mL (3.2 mM) egg PC in Tris NaCl (pH 7.8). The rehydrated egg PC was subsequently subjected to 5 freeze-thaw cycles, freezing at -78°C with dry ice and thawing at 45°C on a heat block with vortexing between each cycle, yielding multilamellar vesicles (MLVs). To form large unilamellar vesicles (LUVs), this egg PC MLV suspension was extruded 21 times (Avanti Polar Lipids Mini Extruder, Alabaster, AL) through a 100 nm pore size polycarbonate membrane (Nuclepore Track-Etch Membrane Filtration Products, Whatman, Maidstone, United Kingdom). LUVs were stored under nitrogen gas at 4°C and used within one week of extrusion. Prior to experimental use, egg PC LUVs were diluted to the desired concentration using Tris NaCl.

Dynamic light scattering (DLS) using a Malvern Zetasizer ZS90 with Zetasizer 7.01 software was used to examine the hydrodynamic size and diffusivity of the MLVs and LUVs in Tris NaCl at 25°C at concentrations of 0.01, 0.1, and 1 mg/mL. Cryo-transmission electron microscopy (TEM) was also used to examine the egg PC LUVs for 1 mg/mL samples. Vesicles were deposited on a specially prepared lacey carbon electron microscopy grid, transferred under liquid nitrogen to the cooled tip of a cryo-transfer stage (CT3500J; Oxford Instruments, Concord, MA), and imaged using a JEOL 1200 TEM as previously reported.¹⁷³

A goniometer (Ramé-Hart, Succasunna, NJ) was used to measure the static contact angle of the egg PC LUVs (0.1 mg/mL in Tris NaCl), Tris NaCl, and water on silica-coated and gold-coated substrates (Biolin Scientific, Västra Frölunda, Sweden). Silica surfaces were cleaned with a water, 2% w/v sodium dodecyl sulfate, and water rinse sequence,

followed by drying with N₂ and UV/ozone treatment with a UV/ozone ProCleaner (Bioforce Nanosciences, Salt Lake City, UT) prior to contact angle measurements. Gold surfaces were cleaned with a 3:1 (v/v) ammonium hydroxide: hydrogen peroxide mixture (i.e., TL1 cleaning), rinsed with MilliQ water, dried with N₂ and UV/ozone treated prior to contact angle measurements. DROPimage software (Ramé-Hart, Succasunna, NJ) was used to analyze the static contact angle after deposition of 2 μ L of liquid on the surface.

4.3.2 Quartz crystal microbalance with dissipation monitoring (QCM-D) measurements

A QCM-D E4 system (Biolin Scientific, Västra Frölunda, Sweden) was used to monitor *in situ* lipid vesicle adsorption and bilayer formation by measuring frequency changes (ΔF) and dissipation changes (ΔD) occurring on quartz QCM-D substrates over time. Figure 4.1 provides a schematic of the QCM-D chamber geometry and shows how the QCM-D substrate interacts with vesicles introduced into the QCM-D chamber. Changes in frequency correspond to the oscillation of the piezoelectric quartz crystal (with fundamental frequency of 5 MHz) sandwiched between gold electrodes over which an alternating voltage is applied. Silica- and gold-coated quartz crystals (Biolin Scientific) were utilized in this work. Prior to use in QCM-D experiments, the substrates were cleaned in the same way as they were cleaned for contact angle measurements (see section *Lipid vesicle formation, vesicle characterization, and surface characterization*).

Change in mass adsorbed on the crystal, including bound water, can be obtained using Sauerbrey^{174,175} or Voigt^{92,176} modeling analysis of these frequency changes, depending on whether the adsorbed film is more rigid or viscoelastic, respectively. Dissipation changes are measured when the applied voltage is turned off and the

dampening of the acoustic waves is monitored over time. This energy dissipation is related to the viscoelasticity of the sensor-adsorbed material. For all vesicle adsorption and bilayer formation studies, a baseline frequency and dissipation measurement was first established in Tris NaCl for a period of 10 min. Egg PC LUVs were then introduced into the QCM-D flow chamber and the frequency and dissipation measurements were continued.

4.3.3 QCM-D measurements under flow conditions

QCM-D monitoring of egg PC LUV behavior under flow conditions was carried out at 25°C on both gold- and silica-coated QCM-D substrates. For experiments with gold substrates, a single egg PC concentration (c_o) of 0.1 mg/mL in Tris NaCl was examined at a pre-adsorption flow rate (Q_o) of 175 μ L/min. Following vesicle adsorption, Tris NaCl was flowed through the system at an increased flow rate (Q) of 650 μ L/min. For experiments utilizing silica-coated substrates, six separate egg PC LUV c_o values of 0.01, 0.0167, 0.05, 0.1, 0.3 and 0.5 mg/mL, with a constant flow rate (Q_o and Q) of 175 μ L/min, were examined.

4.3.4 QCM-D measurements without flow

The impact of flow on vesicle behavior was further analyzed by introducing a no flow experimental condition. No flow QCM-D studies were conducted at two temperatures, 25°C and 37°C, using silica-coated substrates with a c_o of 0.1 mg/mL and Q_o of 175 μ L/min. For the elevated temperature studies, all solutions were heated to 37°C in a heating block prior to flow through the QCM-D modules. Vesicle flow was stopped once a small amount ($\Delta F \sim -4$ Hz) of egg PC LUVs were introduced into the QCM-D chamber (not

corresponding to complete substrate coverage) and ΔF and ΔD were continuously monitored. An additional delayed flow introduction condition was examined at 25°C, in which the QCM-D system was allowed to incubate for a period of at least 15 hours once flow was stopped, following which, flow was introduced with Tris NaCl flowing at a Q of 175 $\mu\text{L}/\text{min}$. ΔF and ΔD were continuously monitored.

4.3.5 Statistical analysis and mathematical modeling

All experiments were conducted in triplicate at minimum. Representative results for the 3rd overtone are shown in figures for all QCM-D ΔF and ΔD measurements unless otherwise specified. Results are given as mean $\pm$ standard deviation and statistical significance was calculated using one-way analysis of variance (ANOVA; $\alpha = 0.05$ and $p < 0.05$ was considered statistically significant). QTools 3 software (Biolin Scientific, Västra Frölunda, Sweden) was used to determine the mass and thickness of the adsorbed egg PC structures at different time points using the ΔF and ΔD QCM-D data gathered. A custom MATLAB (MathWorks, Natick, MA) algorithm was also used to further analyze the transient raw frequency and dissipation data obtained from QCM-D measurements.

4.4 Results and Discussion

4.4.1 Characterization of egg PC lipid vesicles and substrate surface properties

Lipid vesicles composed of egg PC were developed using the dry lipid thin film technique.¹⁷¹ Prior to utilizing these vesicles in QCM-D studies, the successful formation of vesicles was characterized using cryo-TEM imaging and DLS. Cryo-TEM imaging confirmed the unilamellar structure of the extruded LUVs (Figure 4.2a) and suggested a

vesicle diameter of approximately 100 to 150 nm. In order to confirm the decrease in the hydrodynamic diameter of egg PC LUVs compared to egg PC MLVs, DLS was performed on both the MLVs obtained prior to extrusion and the LUVs obtained after extrusion (Figure 4.2b). DLS demonstrated a z-average of $d = 165 \text{ nm} \pm 10 \text{ nm}$ and $d = 2552 \text{ nm} \pm 1617 \text{ nm}$ for LUVs and MLVs, respectively. The average standard deviation of the size distribution within one sample based on an average of three measurements was $\pm 57 \text{ nm}$ and $\pm 345 \text{ nm}$ for LUVs and MLVs, respectively. The LUV polydispersity index (PDI) was 0.08 ± 0.03 , while the MLV PDI was 0.31 ± 0.11 . No significant difference in the z-average size range was observed for DLS conducted at different vesicle concentrations (data not shown).

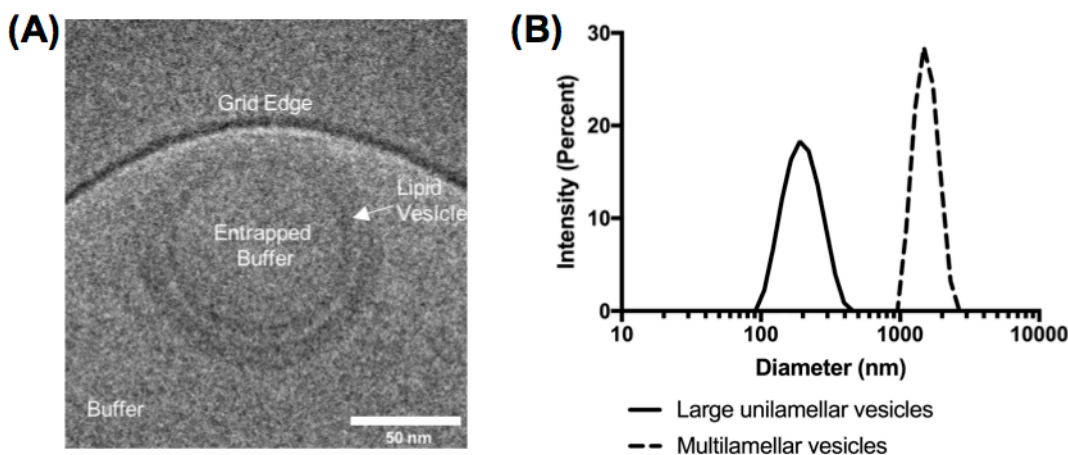


Figure 4.2: Characterization of egg PC lipid vesicles. (A) Representative cryo-transmission electron microscopy image of large unilamellar vesicles (LUVs). Scale bar = 50 nm. (B) Size distribution of multilamellar vesicles (MLVs) prior to extrusion and LUVs post extrusion using dynamic light scattering.

The diffusivity, D_v , of egg PC LUVs in Tris NaCl reported by the DLS was found to be $2.94 \times 10^{-8} \text{ cm}^2/\text{s}$. We also estimated this diffusivity using the Stokes-Einstein equation for a sphere,

$$D = \frac{k_B T}{3\pi\eta d} \quad (4.1)$$

Here, k_B is the Boltzmann constant, T is the temperature, η is the viscosity, and d is the vesicle diameter. Using Equation (4.1), for $T = 25^\circ\text{C}$, η estimated as the viscosity of water at 25°C ($\eta = 8.90 \times 10^{-4} \text{ Pa} \cdot \text{s}$), and $d = 165 \text{ nm}$, we obtained a D of approximately $2.64 \times 10^{-8} \text{ cm}^2/\text{s}$, which is in close agreement with the D_v reported by the DLS.

To better understand vesicle adsorption and rupture on the QCM-D substrates of interest, we also investigated the wetting properties of silica- and gold-coated QCM-D substrates. The static contact angle of water, Tris NaCl buffer, and egg PC LUVs on these substrates was measured and is shown in Table 4.1. As expected, the silica substrates were significantly more hydrophilic than gold in each of these test conditions ($p < 0.0001$ using one-way ANOVA with $\alpha = 0.05$). The addition of egg PC LUVs to Tris NaCl did not appreciably alter wettability of the substrates. The water contact angle on silica was not measurable due to the rapid spread of water on the silica substrate.

Table 4.1: Contact Angles on QCM-D Substrates.

QCM-D substrate coating	Solution	Contact angle ($^\circ$)
Silica	Water	Not measurable
	Tris NaCl buffer	13.4 ± 2
	Egg PC LUVs (0.1 mg/mL)	17 ± 4
Gold	Water	74 ± 5
	Tris NaCl buffer	64 ± 4
	Egg PC LUVs (0.1 mg/mL)	64 ± 4

4.4.2 Effect of bulk vesicle concentration on adsorption and rupture of lipid vesicles

Using QCM-D, an egg PC lipid bilayer was observed to form under continuous flow of $Q_o = 175 \mu\text{L}/\text{min}$ on silica-coated quartz substrates. We investigated the impact of varying bulk vesicle concentration, c_o , from 0.01, 0.0167, 0.05, 0.1, 0.3 and 0.5 mg/mL on both the vesicle adsorption and rupture processes, key to eventual bilayer formation.^{75,161} Figure 4.3a shows both the QCM-D frequency change (ΔF) during the vesicle introduction to bilayer formation process as well as dissipation change (ΔD) for odd overtones (n), 3, 5, 7, 9, and 11 for a c_o of 0.1 mg/mL. Frequency changes relate to mass and thickness of the adsorbed material.⁹² Dissipation relates to the viscoelastic properties of the adsorbed material,¹⁷⁷ and is defined as:

$$D = \frac{E_l}{2\pi E_s} \quad (4.2)$$

where E_l is the energy lost during an oscillation cycle and E_s is the total stored energy.

Figure 4.3a shows that at $c_o = 0.1 \text{ mg/mL}$, lipid vesicles adsorb without rupturing on the silica surface until they reach a critical vesicle coverage (CVC) at $\Delta F = -45 \pm 4 \text{ Hz}$ for $n = 3$. CVC has previously been described as the amount of vesicles on a surface required to trigger vesicle rupture and the start to bilayer formation.^{93,178} During this process, ΔD increases from 0 to $5.3 \times 10^{-6} \pm 0.4 \times 10^{-6}$ for $n = 3$ due to adsorption of the softer vesicles on the rigid silica substrate. After CVC is achieved over an adsorption time period of $t_a = 4.5 \pm 1 \text{ min}$, the ΔF increases to $-26 \pm 1 \text{ Hz}$ over a $3 \pm 1 \text{ min}$ time period (for $n = 3$), which we will refer to as the rupture time period, t_r . During this time, ΔD decreases to $0.4 \times 10^{-6} \pm 0.2 \times 10^{-6}$, indicating vesicle rupture into a SLB, a process accompanied by

the release of entrapped water and excess lipids.^{179,180} The bilayer is significantly more rigid than the non-ruptured vesicles and this low dissipation value is indicative of the bilayer being closely coupled to the substrate. The decrease in separation of ΔF traces over time for the different overtones following the rupture period also confirms the increased rigidity of the SLB as compared to the adsorbed vesicles.¹⁸¹

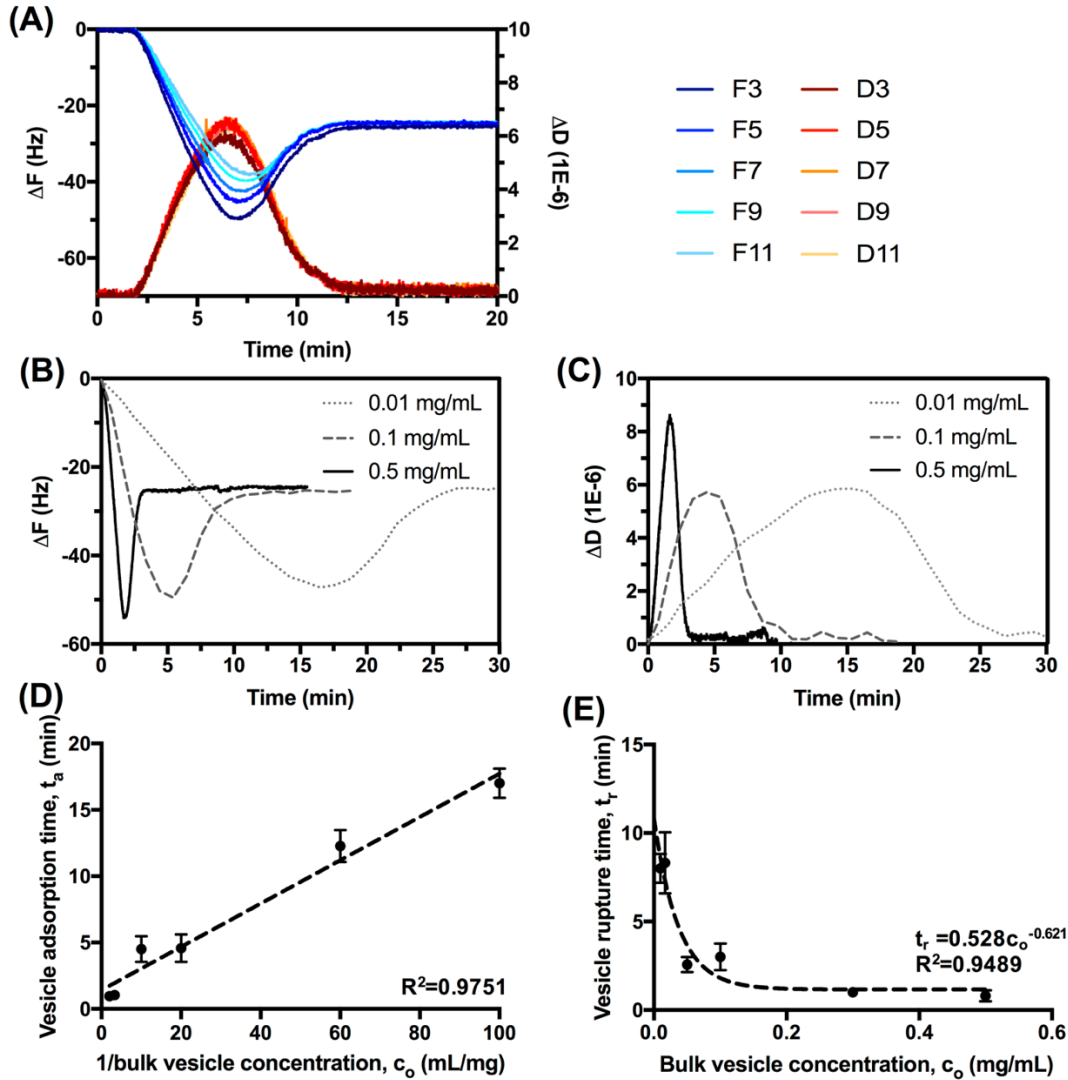


Figure 4.3: Egg PC supported lipid bilayer (SLB) formation under continuous flow conditions ($Q_o = 175 \mu\text{L/min}$) on silica at 25°C . (A) Frequency change (ΔF) and dissipation change (ΔD) over time during SLB formation. Color gradient represents odd overtones 3, 5, 7, 9, and 11. (B) ΔF versus time during egg PC SLB formation for varying bulk vesicle concentration, $c_o = 0.01$ (dotted line), 0.1 (dashed line), and 0.5 mg/mL (solid line). Data

is shown for the 3rd overtone. (C) ΔD versus time during egg PC SLB formation for varying bulk vesicle concentration, $c_o = 0.01$ (dotted line), 0.1 (dashed line), and 0.5 mg/mL (solid line). Data is shown for the 3rd overtone. (D) Vesicle adsorption time, t_a , versus inverse of bulk vesicle concentration demonstrates a linear relationship ($R^2 = 0.9751$). (E) Vesicle rupture time, t_r , versus bulk vesicle concentration demonstrates a power law relationship ($R^2 = 0.9489$). A-C shows data representative of at least 5 experimental trials.

Figure 4.3b and 4.3c show a comparison of the ΔF and ΔD QCM-D data obtained for $c_o = 0.01$, 0.1, and 0.5 mg/mL on silica-coated quartz substrates, for $n = 3$. ΔF and ΔD QCM-D data obtained for all concentrations examined ($c_o = 0.01$, 0.0167, 0.05, 0.1, 0.3, and 0.5 mg/mL) on silica-coated quartz substrates, for $n = 3$ can be found in Figure SI 4.1. Compared to a t_a of 4.5 ± 1 min for c_o of 0.1 mg/mL, a c_o of 0.01 mg/mL, 0.0167, 0.3 and 0.5 mg/mL led to t_a of 17 ± 1 min, 12 ± 1 min, 1 ± 0.2 min, and 0.9 ± 0.4 min, respectively. These adsorption times are much greater than the diffusion times of these vesicles to the surface, given by Equation (4.3).

$$t_d \sim 2 \left(\frac{M_w N_{agg}}{c_o N_A} \right)^{2/3} / D_v \quad (4.3)$$

Here, D_v was obtained from the DLS (2.94×10^{-8} cm²/s), M_w is the molecular weight of egg PC (768 g/mol), N_A is Avagadro's number, and N_{agg} is the average number of lipid molecules per LUV, which was estimated to be 2.7×10^5 using an egg PC lipid molecule area of ~ 0.64 nm².¹⁸² With these values, we estimate $t_d \sim 1.5$ s. Thus, vesicle adsorption onto the surface is the rate-limiting step in the overall process leading to SLB formation.

Although the adsorption of vesicles onto a surface is a complex process, we find that Langmuir kinetics with a negligible vesicle desorption rate can accurately describe the relationship between adsorption time, t_a , and bulk vesicle concentration, c_o . Assuming Langmuir kinetics, the vesicle adsorption rate is proportional to the number of vacant

adsorption sites ($\Gamma_\infty - \Gamma$) and the bulk vesicle concentration, c_o , in the buffer. Here Γ_∞ is the total concentration of vesicle adsorption sites on the surface [mg/cm²] and Γ is the surface concentration of vesicle-bound sites [mg/cm²]. Hence, the rate of vesicle adsorption can be written as:

$$\frac{d\Gamma}{dt} = k_1 c_o (\Gamma_\infty - \Gamma) \quad (4.4)$$

Here, k_1 is the vesicle adsorption rate constant. We have assumed c_o is in excess compared to the adsorbed vesicle concentration. Equation (4.4) can be integrated to obtain:

$$t_a = \frac{1}{k_1 c_o} \ln \left(\frac{\Gamma_\infty}{\Gamma_\infty - \Gamma} \right) \quad (4.5)$$

Equation (4.5) suggests that t_a is inversely proportional to c_o . Figure 4.3d confirms that indeed the adsorption time is inversely proportional to the bulk vesicle concentration in the concentration range examined in this work ($R^2 = 0.9751$).

Rupture times, t_r , have been shown to depend on vesicle size, initial vesicle geometry, and the chosen material substrate.^{89,161,162} We found that t_r also depends on the vesicle concentration when flow rate and surface properties are fixed, where $t_r = 8 \pm 1$, 8 ± 2 , 2.7 ± 0.5 , 3 ± 1 , 1 ± 0.1 , and 0.8 ± 0.3 min, for $c_o = 0.01$, 0.0167 , 0.05 , 0.1 , 0.3 , and 0.5 mg/mL, respectively. An empirical correlation of t_r versus c_o is shown in Figure 4.3e, indicating a power law relationship between t_r and c_o ($R^2 = 0.9489$):

$$t_r = 0.528 c_o^{-0.621} \quad (4.6)$$

Due to the rigidity of the SLB, we can determine the change in adsorbed mass (Δm_f) on the oscillating surface using a normalized Sauerbrey Equation:^{177,183}

$$\Delta m_f = -C \Delta F \quad (4.7)$$

Here, $C = 17.8 \text{ ng cm}^{-2} \text{ Hz}^{-1}$, which is the Sauerbrey mass sensitivity constant arising from the quartz crystal fundamental frequency (5 MHz). From Equation (4.7), using the final ΔF data point obtained upon SLB formation, we determined that mass of the SLB formulated for all bulk vesicle concentrations examined is $460 \pm 14 \text{ ng/cm}^2$. As expected, there is no dependence of SLB mass adsorbed on c_o , as all bulk vesicle concentration values lead to a similar SLB ΔF , despite the dependence of the adsorption and rupture times on c_o . This mass includes the hydration layer sandwiched between the silica-coated QCM-D sensor and the SLB. For SLBs formulated using 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), this hydration layer was previously reported to have an areal mass of $\sim 102 \text{ ng/cm}^2$.¹⁸⁴ Using this value as an estimate for the mass of the hydration layer of the egg PC SLBs formulated in this work, we estimated the areal mass of the egg PC SLB without the hydration layer as $\sim 358 \text{ ng/cm}^2$. We used these adsorbed masses to obtain an estimate of the SLB thickness with and without the hydration layer, using Equation (4.8):

$$d_{eff} = \frac{\Delta m_f}{\rho_{eff}} \quad (4.8)$$

Here, d_{eff} is the estimated SLB thickness and ρ_{eff} is the effective film density. We used the same ρ_{eff} utilized by Rodahl et al. ($800 \frac{\text{kg}}{\text{m}^3}$) in their analysis of lipid vesicle adsorption for egg PC vesicles.¹⁸⁵ Using Equation (8), we estimated the thickness of the SLB to be $\sim 5.8 \text{ nm}$ and $\sim 4.5 \text{ nm}$ with and without the hydration layer, respectively. Thus, the entrapped water is $\sim 1.3 \text{ nm}$ in thickness, similar to what has previously been reported.^{186,187} The egg PC SLB bilayer thickness we estimated is in agreement with previous reports for SLBs formulated from 4:1 (v/v) DOPC:1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) ($\sim 4.1 \text{ nm}$ thickness)⁷⁵ and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (3.5 nm thickness).¹⁸⁸

4.4.3 Effect of flow on lipid vesicle rupture

To elicit mechanistic insight into the significance of flow in the vesicle adsorption and rupture process, we performed QCM-D experiments at 25°C with a c_o of 0.1 mg/mL using silica-coated quartz substrates, in which flow was stopped following the introduction of vesicles into the QCM-D chamber (corresponding to $\Delta F \sim -4$ Hz). Figure 4.4a shows the frequency and dissipation changes for odd overtones, $n = 3, 5, 7, 9$, and 11. The vesicles were incubated within the QCM-D chamber over a period of at least 15 hours in the absence of flow, following which flow was introduced at $Q = 175 \mu\text{L}/\text{min}$. During the no flow incubation, vesicles adsorbed on the surface reaching a plateau $\Delta F = -55 \pm 2$ Hz for $n = 3$ at approximately 10 hours. From Figure 4.3a, in a continuous flow system we have seen that once a CVC corresponding to a ΔF of -45 ± 4 Hz is obtained (for $n = 3$), vesicle rupture follows, leading to SLB formation. However, without flow, we observe the vesicles to remain on the surface as a monolayer of non-ruptured vesicles even after exceeding the CVC noted for the continuous flow system and reaching the plateau ΔF . The dissipation changes occurring in Figure 4.4a are similar to the

continuous flow system where we observed an increase in dissipation during the vesicle adsorption phase, here reaching $\Delta D = 8 \times 10^{-6} \pm 1$ (for $n = 3$).

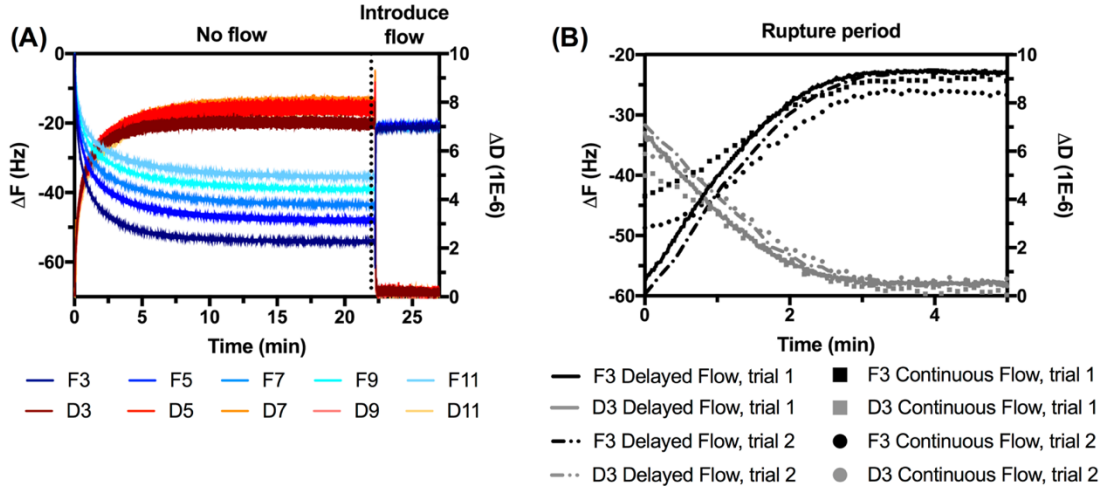


Figure 4.4: Adsorbed lipid vesicle behavior in the absence of flow on silica at 25°C with a bulk vesicle concentration of 0.1 mg/mL. (A) Frequency change (ΔF) and dissipation change (ΔD) over time before and after flow is introduced ($Q = 175 \mu\text{L/min}$). Color gradient represents odd overtones 3, 5, 7, 9, and 11. An intact monolayer of vesicles adsorbs to the surface and is stable until flow is introduced. (B) Overlay of ΔF and ΔD versus time during vesicle rupture period in continuous flow and delayed flow introduction experiments. Two replicates are shown for each scenario. Time = 0 is where rupture begins. Data is shown for the 3rd overtone. Data representative of 5 experimental trials is shown.

When flow is introduced following the no-flow incubation period, a rupture of the vesicles occurs leading to SLB formation, as indicated by the rise in ΔF to approximately -26 ± 1 Hz and decrease in ΔD to $0.4 \times 10^{-6} \pm 0.2 \times 10^{-6}$ for $n = 3$ in Figure 4.4a. Figure 4.4b provides an overlay of ΔF and ΔD data obtained during the continuous flow experiments (i.e, the same experimental conditions whose representative data is shown in Figure 4.3a) and the delayed flow introduction experiments (i.e., the same experimental conditions shown in Figure 4.4a) following the commencement of the rupture process. The rupture process begins once the CVC is reached for the continuous flow condition and once

flow is introduced after the no-flow incubation period for the delayed flow introduction experiments (set to time = 0 in Figure 4.4b). From Figure 4.4b, we see that the rupture to SLB formation process takes the same amount of time in both the stop flow and continuous flow conditions and occurs at similar rates.

4.4.4 Estimating adsorbed vesicle layer thickness

The dynamics of the viscoelastic adsorbed vesicle layer prior to rupture, with a thickness, $h_a(t)$, can be modeled using the Voigt model to fit the QCM-D ΔF and ΔD output.^{176,183} The important properties of the adsorbed vesicles and the semi-infinite bulk Newtonian liquid utilized in this modeling shown in Figure 4.1b are the density, ρ_a , shear viscosity, η_a , and elastic modulus, μ_a , of the adsorbed vesicles, and the density, ρ_f , and viscosity, η_f , of the bulk fluid. The observed change in frequency and dissipation energy due to the presence of the viscoelastic film can be derived using the Voigt model as follows:^{176,185}

$$\Delta F = \frac{Im(\beta)}{2\pi\rho_q t_q}, \quad (4.9)$$

and

$$\Delta D = -\frac{2Re(\beta)}{\omega\rho_q t_q}, \quad (4.10)$$

where

$$\beta = \kappa_a \xi_a \frac{1 - A \exp(2\xi_a h_a)}{1 + A \exp(2\xi_a h_a)}, \quad (4.11)$$

$$\kappa_a = \eta_a - \frac{i\mu_a}{\omega} \text{ and } \xi_a = \sqrt{-\frac{\omega^2 \rho_a}{\mu_a + i\omega\eta_a}} \quad (4.12 \text{ and } 4.13)$$

$$A = \frac{\xi_a \kappa_a + \xi_f \kappa_f}{\xi_a \kappa_a - \xi_f \kappa_f} \text{ and } \xi_f = \sqrt{i \frac{\omega \rho_f}{\eta_f}} \quad (4.14 \text{ and } 4.15)$$

Note, ρ_q is the quartz density (2648 kg/m³) and t_q is the thickness of the quartz substrate (0.33 mm). The QCM-D sensor oscillates at resonant frequency of $\omega = 2\pi f_0 = 10\pi$ MHz. The Newtonian liquid properties are based on water properties at 25°C as $\eta_f = 8.9 \times 10^{-4}$ Pa·s and $\rho_f = 1000$ kg/m³. The sensitivity of $h_a(t)$ on ρ_a , η_a and μ_a of the adsorbed vesicle layer was examined using a custom MATLAB algorithm utilizing Equations (4.9-4.15). Figure 4.5a and 4.5b show changes in ΔF and ΔD versus h_a , for various ρ_a , in the range of 800 to 1200 kg/m³. As expected, h_a depends strongly on ρ_a . However, ΔD is relatively insensitive to changes in ρ_a . Figure 4.5c and 4.5d show changes in ΔF and ΔD versus h_a for various μ_a , in the range of 4×10^3 to 4×10^6 N/m². We see that h_a is most sensitive to changes in μ_a in the range 4×10^4 N/m² to 4×10^5 N/m². ΔD , however, is a strong function of elastic modulus and changes non-linearly with h_a over the entire range of μ_a examined. Figure 4.5e and 4.5f show changes in ΔF and ΔD versus h_a for various η_a in the range of 1×10^{-3} to 1.05×10^{-2} Pa·s. ΔF and h_a are almost insensitive to changes in η_a . The energy dissipation of the adsorbed layer decreases with its viscosity.

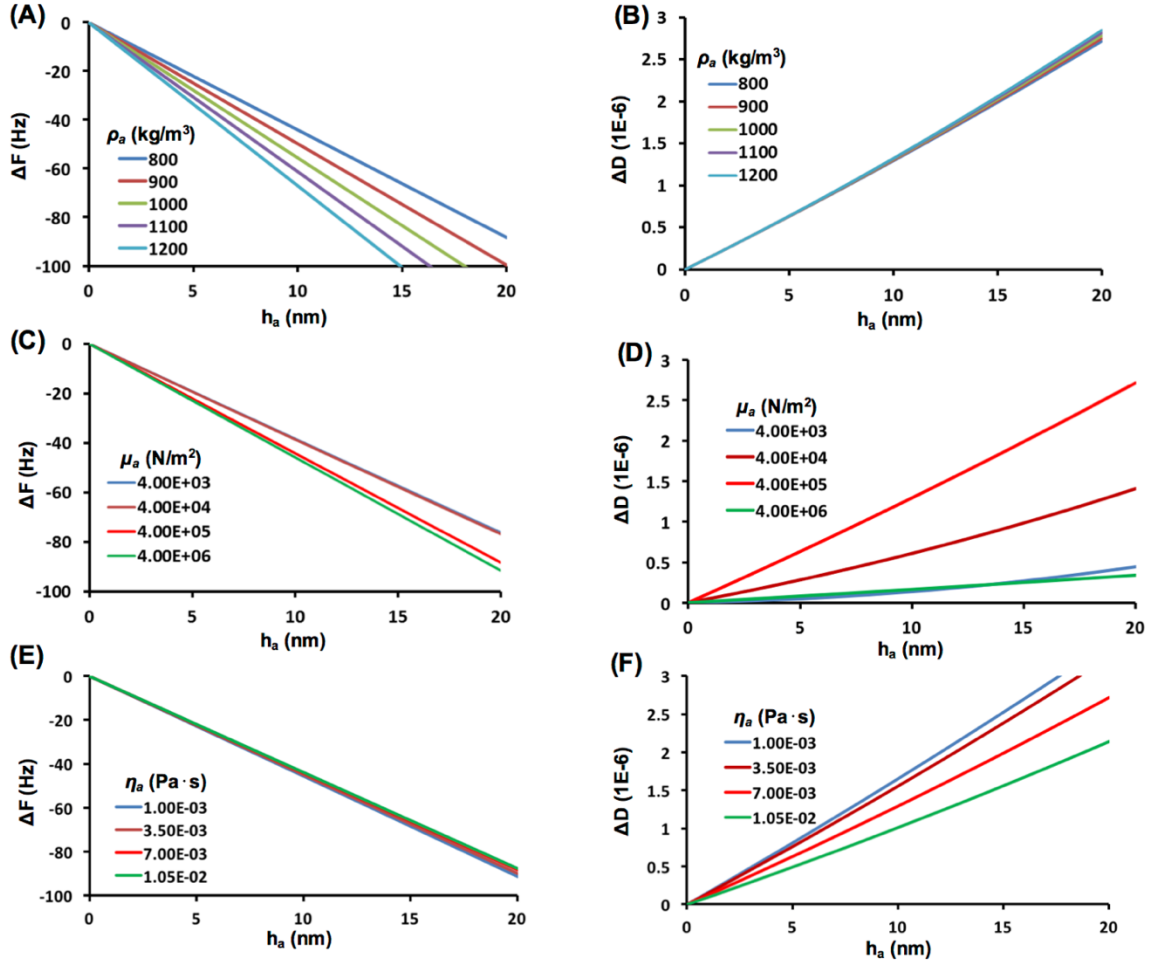


Figure 4.5: Voigt model derived relations between frequency change (ΔF) and dissipation change (ΔD) versus adsorbed vesicle layer thickness (h_a) for varying (A and B) adsorbed vesicle layer density (ρ_a), (C and D) adsorbed vesicle layer elastic modulus (μ_a), and (E and F) adsorbed vesicle layer shear viscosity (η_a).

The experimental $\Delta F(t)$ and $\Delta D(t)$ data obtained during the no-flow incubation period in the delayed flow introduction experiments, in which vesicles are adsorbing to the QCM-D substrate, were fit using the Voigt model. Figure 4.6a shows the comparison between theoretical and experimental ΔF and ΔD using the best-fit values of $\rho_a = 800$ kg/m³, $\eta_a = 0.007$ Pa·s, and $\mu_a = 4 \times 10^4$ N/m². Figure 4.6b shows the Voigt model estimate of h_a versus time during the no-flow incubation. The plateau thickness obtained from Voigt

modeling for the adsorbed vesicle layer during the no-flow incubation was 13 ± 3 nm. Compared to the original diameter of the vesicles (~ 165 nm), this thickness value suggests that the vesicles are arranged in flattened pancake-like structures on the surface.

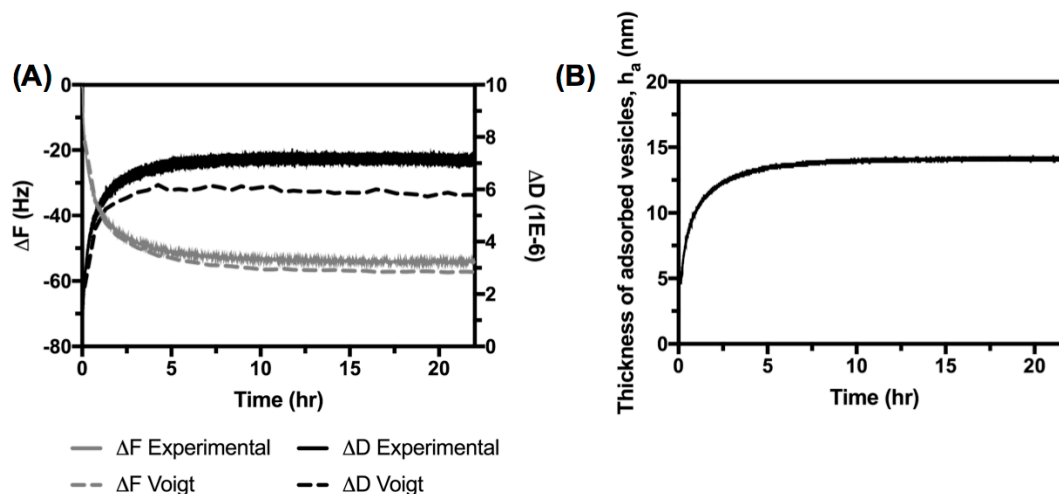


Figure 4.6: Theoretical modeling of experimentally obtained ΔF and ΔD versus time data in the absence of flow on silica at 25°C with a bulk vesicle concentration of 0.1 mg/mL . (A) Theoretical fit of the 3rd overtone for ΔF and ΔD versus time during vesicle adsorption period using the Voigt model (with $\rho_a = 800 \text{ kg/m}^3$, $\mu_a = 4 \times 10^4 \text{ N/m}^2$, and $\eta_a = 0.007 \text{ Pa}\cdot\text{s}$) compared to experimental values. (B) Modeled thickness of adsorbed vesicle layer, h_a , during vesicle adsorption period using the Voigt model. Representative modeling results using QTools 3 software is shown for data obtained from 5 experimental trials.

Lind et al. previously suggested that a structure consisting of vesicles adsorbed onto an already formed SLB may not be distinguishable from an adsorbed layer of only vesicles based on QCM-D ΔF and ΔD data.⁷⁶ In our studies, if this SLB plus vesicle structure was a major contributor to the CVC layer prior to the start of flow, then flow would serve to simply remove the additional vesicles, revealing the characteristic SLB ΔF and ΔD traces. However, these structures are prominent when experiments are conducted below the lipid melting temperature (T_m). Egg PC has a T_m below 0°C ,^{189,190} and all experiments in this

work were performed well above this temperature. Thus, it is unlikely that these structures are contributing significantly to our pre-flow surface.

4.4.5 Thermodynamics of egg PC SLB formation

It has previously been shown that flattened adsorbed lipid vesicles may either fuse with other flattened vesicles or remain as single flattened vesicles on the surface. These vesicle structures can eventually rupture forming single-bilayer patches that can coalesce and lead to SLB formation.^{169,191} Energetically, the bound vesicles will rupture if the free energy associated with adsorbed vesicles, $F_{vesicle}$, is greater than the free energy of the bilayer, $F_{bilayer}$. These free energies are defined as:¹⁶⁹

$$F_{vesicle} = -2\pi W R_v^2 + 2\pi g(2kW)^{\frac{1}{2}} R_v \quad (4.16)$$

$$F_{bilayer} = -4\pi W R_v^2 + 4\pi \Sigma R_v \quad (4.17)$$

where W is the effective interaction potential for silica ($W \sim 2 \text{ mJ/m}^2$), g is a numerical constant ($g = 2.8$), k is the bending rigidity of the bilayer ($k = 3.9 \times 10^{-20} \text{ J}$), R_v is the initial vesicle radius ($R_v = 82.5 \text{ nm}$), and Σ is the line tension along the circumference of the bilayer ($\Sigma = 1.3 \times 10^{-7} \text{ mJ/m}$).^{92,169,192–194} Note that $F_{bilayer}$ is defined as the energy required for the onset of vesicle rupture, which can result in bilayer patches or disks that can then coalesce into a uniform bilayer on the surface. $F_{bilayer}$ does not account for the energetics of the coalescence process.

Performing these free energy calculations for the egg PC vesicles and silica substrates examined in our studies, $F_{vesicle} = 8 \times 10^{-13} \text{ mJ}$ while $F_{bilayer} = 2 \times 10^{-12} \text{ mJ}$. Thus, the adsorbed vesicles have a lower free energy as compared to the ruptured structures, requiring an additional energy input greater than $F_{bilayer} - F_{vesicle}$ in order to induce rupture.

Our experimental observations from the delayed flow introduction studies, which required flow to induce vesicle rupture, suggest that flow in the system following vesicle adsorption provides the energy needed to promote the adsorbed vesicle to bilayer transition. We can obtain a conservative estimate of the energy input associated with flow in the QCM-D module using the following equation, which represents both the convection and viscous dissipation energy contributions:

$$E_{flow} \sim \frac{1}{2} \rho V u^2 + \mu \dot{\gamma} V \quad (4.18)$$

Here, ρ is the fluid density (approximated as that of water at 25°C, 1000 kg/m³), μ is the fluid viscosity (approximated as that of water at 25°C, 8.90 × 10⁻⁴ Pa·s), V is the QCM-D chamber volume (0.72 mm³), u is the average fluid velocity, and $\dot{\gamma}$ is the shear rate. We obtained u by dividing the fluid volumetric flow rate by the cross-sectional area of the region above the crystal in the QCM-D chamber. We approximated this region to have a constant rectangular cross-section (7.68 mm²), yielding u of 0.73 mm/s. The shear rate is calculated by $2u/h$, where h is the height above the crystal sensor (0.64 mm). Using Equation (4.18), E_{flow} is approximately 1.6 × 10⁻¹² mJ, which exceeds the difference between the bilayer and vesicle free energy (1.2 × 10⁻¹² mJ) needed for bilayer formation.

Previous studies have shown that the CVC can be affected by lipid charge and lipid composition.^{75,195} For this reason we used a zwitterionic egg PC lipid for our studies that reproducibly forms SLBs under flow. While achieving CVC is a necessary step in bilayer formation, our study suggests that achieving the CVC alone does not ensure SLB formation. Leonenko et al. used atomic force microscopy to investigate DOPC SLB formation on mica and modified mica substrates, showing that SLB formation occurs primarily via fusion of neighboring adsorbed vesicle structures rather than migration of

lipid bilayer disks.¹⁹⁶ Our studies suggest that although the CVC may be reached, an additional energy input (in the form of flow for the delayed flow experiments) is required to enable interaction of the stable adsorbed egg PC vesicles on the surface. Without flow, a stable adsorbed vesicles layer with a thickness greater than the SLB is observed (13 ± 3 nm versus ~ 6 nm, respectively), until additional energy is supplied to promote neighboring vesicle interaction and lead to SLB formation.

4.4.6 Effect of temperature on vesicle rupture without flow

We investigated whether increasing thermal energy input could induce vesicle rupture on silica without flow. In these studies, egg PC LUVs were again introduced into the system corresponding to a $\Delta F \sim -4$ Hz and flow was halted; the system temperature was either held at 25°C or 37°C. Representative data for $n = 3$ is shown in Figure 4.7. At 37°C, vesicles continued to adsorb to the surface with $t_a = 12 \pm 1.7$ min, reaching a ΔF of 43.9 ± 2.6 Hz, agreeing with the CVC observed in our continuous flow experiments. Following this adsorption period, vesicle rupture was observed despite the absence of flow with $t_r = 30 \pm 2.5$ min. The resultant SLB was formed on the surface with a final $\Delta F = -28 \pm 0.5$ Hz and $\Delta D = 0.9 \pm 0.6 \times 10^{-6}$, similar to final ΔF and ΔD values in continuous flow experiments. At 25°C, vesicles continued to adsorb on the surface, exceeding the continuous flow ΔF CVC value, without any rupture as we had previously observed prior to flow introduction following a no-flow incubation period (Figure 4.4).

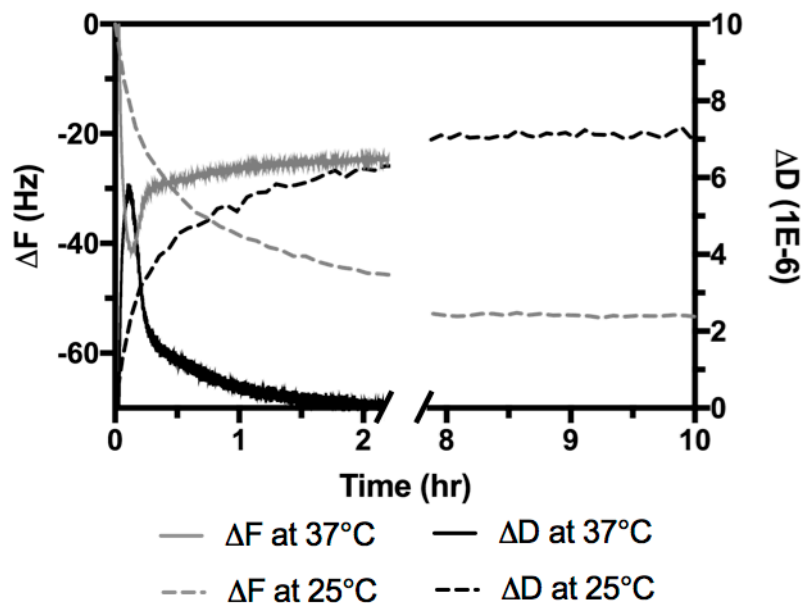


Figure 4.7: Effect of temperature on adsorbed lipid vesicle behavior in the absence of flow on silica with a bulk vesicle concentration of 0.1 mg/mL. Frequency change (ΔF) and dissipation change (ΔD) over time indicates that a stable vesicle monolayer is adsorbed at 25°C while a supported lipid bilayer forms at an elevated temperature of 37°C in the absence of flow. Data representative of 5 experimental trials is shown.

Using the Voigt model, we obtained the thickness for the adsorbed vesicle layer, h_a , once the CVC ΔF was reached at 37°C compared with 25°C in the absence of flow. Using the properties of water at the appropriate temperatures to approximate buffer density and viscosity, and utilizing a layer density, $\rho_a = 800 \text{ kg/m}^3$, h_a was determined to be unchanged between 37°C and 25°C and the delayed flow introduction study plateau thickness ($11 \pm 1 \text{ nm}$, $12 \pm 1 \text{ nm}$, and $13 \pm 3 \text{ nm}$ at 37°C, 25°C, and in the delayed flow introduction studies, respectively). These h_a values all suggest a similar flattened vesicle configuration for these conditions. Using Equations (4.7) and (4.8), the final SLB thickness including the hydration layer for the 37°C study was estimated to be $\sim 5.5 \text{ nm}$, equivalent to the SLB thickness formed under continuous flow at 25°C.

This is the first study to our knowledge examining the effects of elevated temperature on SLB formation in a QCM-D chamber in the absence of flow. Our experiments demonstrate that an increase in temperature is able to supply enough energy to induce vesicle rupture, and lead to an identical SLB compared to what is formed in a continuous flow scenario using egg PC on silica in a QCM-D chamber, confirmed by examining ΔF , ΔD , and h_a . Both vesicle adsorption and rupture occur at a slower rate with this no flow elevated temperature condition as compared to the 25°C continuous flow condition, with a longer t_a (12 ± 1.7 min versus 4.5 ± 1 min, respectively) and t_r (30 ± 2.5 min versus 3 ± 1 min, respectively).

4.4.7 Effect of substrate properties on SLB formation

While vesicle rupture readily occurs on a silica surface, this process is not as likely to occur on other more hydrophobic surfaces, such as gold.¹⁹⁷ As shown in Table 4.1, the contact angle of egg PC LUVs on gold is greater than their contact angle on silica. Using a gold-coated quartz QCM-D substrate, we examined vesicle behavior by monitoring ΔF and ΔD over time, with $c_o = 0.1$ mg/mL under continuous flow conditions. Figure 4.8 shows the representative data obtained starting with vesicles introduced for 15 min at $Q_o = 175$ $\mu\text{L}/\text{min}$ for odd overtones, $n = 3, 5, 7, 9$, and 11. In this time, the vesicles adsorbed and remained stable on the surface, reaching a plateau $\Delta F = -230 \pm 30$ Hz and $\Delta D = 27 \times 10^{-6} \pm 3 \times 10^{-6}$ for $n = 3$.

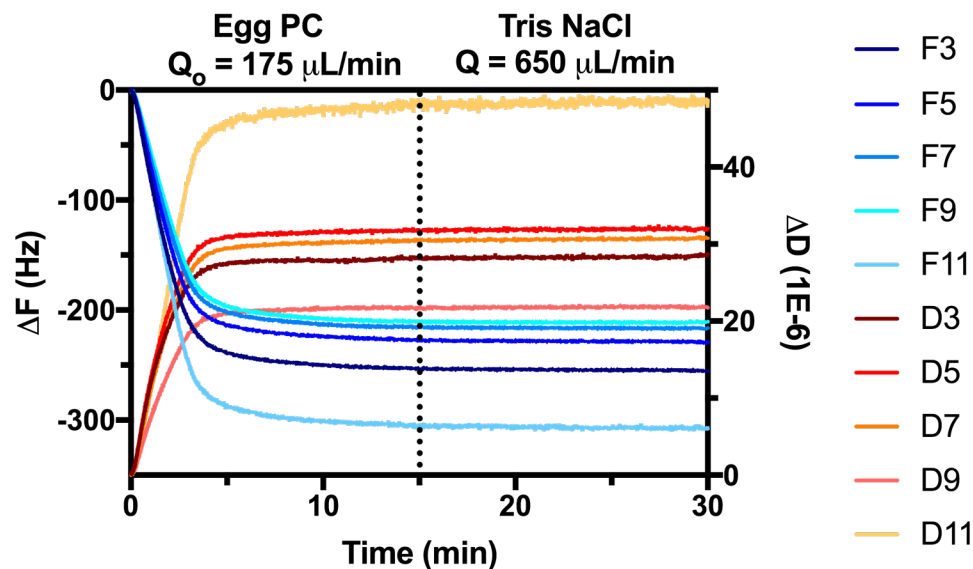


Figure 4.8: Vesicle adsorption on gold substrate at 25°C with a bulk vesicle concentration of 0.1 mg/mL. Frequency change (ΔF) and dissipation change (ΔD) over time. Color gradient represents odd overtones 3, 5, 7, 9, and 11. Egg PC vesicles are first introduced with a flow rate of $Q_0 = 175 \mu\text{L/min}$. Vesicles adsorb forming a stable monolayer. At 15 min, Tris NaCl buffer without vesicles is introduced at an increased flow rate of $Q = 650 \mu\text{L/min}$. Vesicles remain adsorbed stable at this elevated flow rate condition. Data representative of 5 experimental trials is shown.

This large $|\Delta F|$ and ΔD compared to the silica surface during the adsorption period, represents the deposition of a greater amount of intact LUVs adsorbed to the gold surface compared to silica. Table 4.2 summarizes the adsorbed vesicle layer thickness, h_a , at time corresponding to the largest vesicle adsorption on the surface (i.e., largest $|\Delta F|$) for all the experimental conditions we have reported in this study. For a gold surface, the monolayer of vesicles is stable at a thickness of $60 \pm 9 \text{ nm}$. The thickness of vesicles adsorbed to gold was significantly greater than the thickness of vesicles adsorbed on silica under all conditions examined ($p < 0.0001$ using one-way ANOVA with $\alpha = 0.05$). As shown in Figure 4.8, even when the flow rate was increased to $Q = 650 \mu\text{L/min}$, the vesicles

continued to remain stable on gold and there was no significant change in h_a ($p > 0.05$ using one-way ANOVA).

Table 4.2: Adsorbed Egg PC Vesicle Layer Thickness for Various Flow Conditions and Substrate Coatings.

QCM-D Substrate Coating	Temperature (°C)	Flow condition	ΔF corresponding to greatest vesicle adsorption (Hz)	Adsorbed vesicle layer thickness, h_a (nm)
Silica	25	Flow ($Q_o = 175 \mu\text{L}/\text{min}$)	-45 ± 4	12 ± 1
Silica	25	No flow	-55 ± 2	13 ± 3
Silica	37	No flow	-44 ± 3	11 ± 1
Gold	25	Flow ($Q_o = 175 \mu\text{L}/\text{min}$)	-229 ± 30	60 ± 9
Gold	25	Flow ($Q = 650 \mu\text{L}/\text{min}$)	-233 ± 33	61 ± 10

The effective interaction potential of vesicles adsorbed to the gold substrate is greater compared to the silica surface. Thus, the vesicles remain intact on the gold surface in a configuration with adsorbed vesicle thickness closer to their original diameter of the LUVs in the bulk fluid. It has previously been observed that PC vesicles are unable to rupture on oxidized gold surfaces.⁹⁴ Flow, in the range examined, does not appear to impact vesicle rupture on gold as it does for the case of silica-adsorbed vesicles. Recent work has demonstrated the successful rupture of vesicles on gold surfaces using the addition of α -helical peptides that can destabilize the outer leaflet of the vesicle, leading to SLB formation.¹⁹⁸ Similar to what we have observed, this study found that POPC vesicles adsorbed to gold surfaces were unable to rupture under flow conditions.¹⁹⁸ The α -helical peptide works as a “catalyst,” where the peptide-vesicle electrostatic interaction provides

the energy needed to overcome the energy barrier associated with vesicle to bilayer transformation.¹⁹⁸ This is similar to our use of flow to overcome this energy barrier for vesicles adsorbed on silica surfaces. In the case of gold surfaces, the energy barrier for vesicle to SLB transformation is greater than on silica. Flow alone is unable to provide enough energy to overcome this barrier, and the addition of the α -helical peptide aids in overcoming this barrier.¹⁹⁸

Richter et al. demonstrated that DOPC/DOPS (4:1 v/v) vesicles remained stable on a silica surface using AFM.⁷⁵ Occasionally, the AFM tip would induce vesicle rupture, which would then cause rupture of neighboring vesicles.⁷⁵ Flow in our studies may play a similar role as the AFM tip in these past studies. On the hydrophilic silica, the egg PC vesicles adopt a highly flattened configuration, which we hypothesize enhances interaction between neighboring vesicles once flow initiates the rupture process. On the more hydrophobic gold, the adsorbed vesicle layer is nearly six times thicker than the vesicle layer on silica. This configuration likely does not promote neighboring vesicle interaction even in the presence of flow, allowing the vesicles to remain stable on the gold surface.

4.5 Conclusions

In this study, we have demonstrated that bulk vesicle concentration, flow, and temperature can each influence egg PC SLB formation using QCM-D. We conclude that achieving CVC, while an important step in the bilayer rupture process, alone is not enough to cause vesicle rupture. At 25°C on silica, we demonstrated the Langmuir dependence of vesicle adsorption rate on bulk vesicle concentration and derived an empirical relationship between bulk vesicle concentration and vesicle rupture time. We estimated that the free energy of

the adsorbed vesicle state is less than that of the SLB, and thus, energy must be supplied to induce vesicle rupture and promote SLB formation. Both flow at 25°C and elevated temperature (37°C) conditions in the absence of flow caused vesicle rupture and eventual SLB formation on silica, which was not observed in the absence of flow at 25°C. Finally, we also showed that these findings are substrate dependent. On gold surfaces, which are significantly more hydrophobic than silica, vesicles adsorb without the level of flattening observed to occur on silica, as estimated using the Voigt model. Flow was unable to promote vesicle rupture of the adsorbed egg PC vesicles on gold. These findings suggest that a highly flattened adsorbed vesicle configuration, as observed to occur on silica, may be required to enable neighboring vesicle interaction and rupture propagation in the presence of flow. Overall, this work serves to enhance the fundamental understanding of SLB formation in response to specific physicochemical factors as we move forward towards realizing the next generation of SLB applications and uses.

4.6 Author Contributions

This work was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

4.7 Acknowledgements

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4.8 Supplemental Information

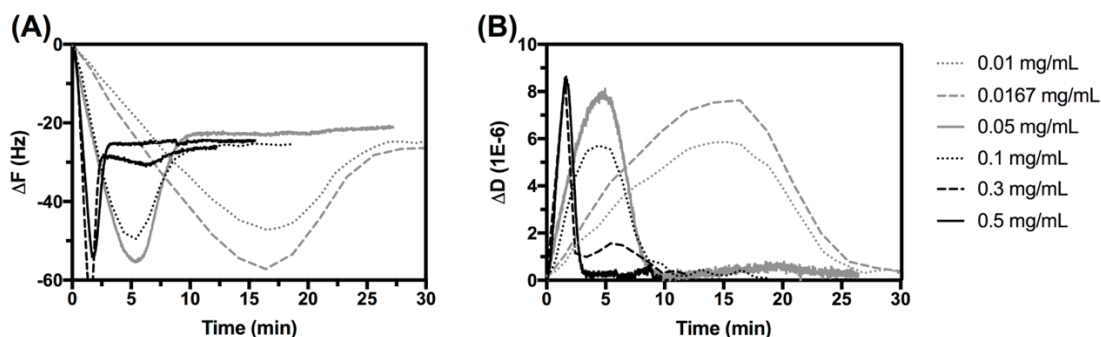


Figure SI 4.1: Egg PC supported lipid bilayer (SLB) formation under continuous flow conditions ($Q_o = 175 \mu\text{L}/\text{min}$) on silica at 25°C. (A) ΔF versus time during egg PC SLB formation for varying bulk vesicle concentration, $c_o = 0.01$ (grey dotted line), 0.0167 (grey dashed line), 0.05 (grey solid line), 0.1 (black dotted line), 0.3 (black dashed line), and 0.5 mg/mL (black solid line). Data is shown for the 3rd overtone. (C) ΔD versus time during egg PC SLB formation for varying bulk vesicle concentration, $c_o = 0.01$ (grey dotted line), 0.0167 (grey dashed line), 0.05 (grey solid line), 0.1 (black dotted line), 0.3 (black dashed line), and 0.5 mg/mL (black solid line). Data is shown for the 3rd overtone. Data representative of at least 5 experimental trials.

Chapter 5

Investigating the Impact of Phthalate Environmental Toxicants with Lipid Structures

Chapter 5 has been submitted as an original research article.

Bailey-Hytholt, C.M., Puranik, T., Tripathi, A., Shukla, A., Investigating the Impact of Phthalate Environmental Toxicants with Lipid Structures, in review.

5.1 Abstract

Di(2-ethylhexyl) phthalate (DEHP) is one of the most abundant plasticizers found in common household products. It leaches from materials, resulting in environmental and human exposure. In the body, DEHP is commonly metabolized to mono(2-ethylhexyl) phthalate (MEHP), which is associated with detrimental health effects. Here, we present the first direct study investigating how these phthalates interact with and permeate lipid structures, namely vesicles and planar bilayers. We observed significant changes in the size and polydispersity of L- α -phosphatidylcholine (egg PC) vesicles when incubated with DEHP but not MEHP at the same concentrations (100 and 200 μ M). These effects could be mitigated by pre-treatment with chitosan nanoparticles which adsorb the phthalates. Using quartz crystal microbalance with dissipation monitoring (QCM-D), we observed a concentration dependence on the interaction of DEHP with egg PC supported lipid bilayers (SLBs). QCM-D results suggested lipid removal for 5 and 100 μ M DEHP, and adsorption and potential embedment in the bilayer at 50 and 200 μ M DEHP. SLB mass decrease was observed for all concentrations of MEHP (5, 50, 100, and 200 μ M), suggesting lipid removal. We also investigated the permeability of DEHP and MEHP as well as several small molecules across a 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) suspended lipid bilayer. We found that DEHP and MEHP both had low permeabilities, but only DEHP remained associated with the bilayer. Exposure to DEHP and MEHP influenced how several common small molecules interacted with DOPC bilayers. Ultimately, this work provides insight into mechanisms of phthalate interactions with lipid structures, having implications for human health.

5.1.1 Keywords

Lipid vesicles, supported lipid bilayers, quartz crystal microbalance with dissipation, parallel artificial membrane permeability assay, di(2-ethylhexyl) phthalate, mono(2-ethylhexyl) phthalate

5.2 Introduction

Di(2-ethylhexyl) phthalate (DEHP) (Figure SI 5.1a) is a common plasticizer present in many household products. It readily leaches from materials, posing health concerns over potential environmental exposure. In the presence of lipases in the body, DEHP is metabolized into monoesters such as mono(2-ethylhexyl) phthalate (MEHP) (Figure SI 5.1b).^{199,200} Phthalates are known endocrine disruptors,^{201,202} and epidemiological studies have suggested that phthalates can influence human development, impacting fetuses, newborns, and children.²⁰³ A recent study investigated the urinary phthalate metabolite concentration of 350 pregnant women and observed positive association of DEHP with major gestational diabetes risk factors.⁶² DEHP concentration has also been observed to inversely correlate with oocyte yield, clinical pregnancy, and live birth.^{60,61} Toxicology studies have observed changes in the structure and migration of A549 lung carcinoma cells,²⁰⁴ production of reactive oxygen species and changes to key signaling pathways in primary mouse cortical neurons and glial cells,²⁰⁵ and reduced thyroid hormones in rats due to DEHP exposure.²⁰⁶ DEHP and MEHP have also been reported to increase risk of allergic diseases including asthma.^{207,208} Methods for removing phthalates from the environment are being explored to combat these toxic effects. For example, DEHP, among

other phthalate esters, can adsorb to chitosan particles via hydrophobic interactions for removal from aqueous environmental solutions.²⁰⁹

Daily phthalate acquisition ranges from $\sim 5 - 400 \mu\text{M}$, where higher concentrations are typically found in medical settings.²¹⁰ For example, DEHP in blood stored in plastic bags for blood transfusions has been shown to contain upwards of $\sim 1000 \mu\text{M}$ DEHP, due to DEHP leaching from the plastic.²¹¹ DEHP also commonly leaches from kidney dialysis tubing and respirators, increasing patient exposure.²¹² Although several studies have focused on quantifying urinary and blood serum levels of phthalates and investigating cell toxicity upon exposure, there have been limited studies investigating the physical interaction of DEHP and MEHP with lipid structures. Lipid structures are ubiquitous in biology and play a critical role in the human body, from forming a large component of the cell membrane to controlling the function of membrane-associated proteins^{213,214} and encapsulating organelles and intracellular and extracellular vesicles. The influence of plasticizers, including DEHP, on multilamellar dipalmitoyl phosphatidylcholine (DPPC) vesicles has been investigated;^{215,216} changes in the transition temperature (ΔH) and shape and width of the transition peak as measured by differential scanning calorimetry (DSC) were observed even at low phthalate concentrations (1% w/w with respect to DPPC).²¹⁶ Previous work has also examined nanoparticle,⁷⁹ peptide,^{80,81} polymer,²¹⁷ and small molecule^{218,219} interactions with lipid bilayers to elucidate possible toxicity associated with these substances. The goal of this study was to thoroughly explore the effects of DEHP and MEHP on adsorption and permeability of lipid vesicles and bilayers and investigate the impact this may have on small molecule interactions with these structures.

To the best of our knowledge this is the first study to directly investigate physical interactions of phthalate esters with unilamellar lipid vesicles and lipid bilayers. To investigate changes to lipid vesicles, we observed size and polydispersity upon exposure to DEHP and MEHP. Phthalate effects on lipid bilayers were investigated by monitoring frequency and dissipation changes using quartz crystal microbalance with dissipation monitoring (QCM-D) and permeability using parallel artificial membrane permeability assays (PAMPA). We observed an increase in vesicle size upon introduction of DEHP at all concentrations tested, while adsorption or lipid removal from lipid bilayers was DEHP concentration dependent. Both DEHP and MEHP were found to have low permeabilities in suspended lipid bilayers. However significant differences between level of interaction between these phthalates and the bilayers was observed, which influenced how small molecules, including common pharmaceutical agents, caffeine, and folic acid, interacted with the lipid bilayer. Given previous findings showing the interaction of DEHP and chitosan particles,^{209,220,221} we found that these nanoparticles are able to eliminate any effect of DEHP on vesicle size, demonstrating that facile strategies can be utilized to reduce phthalate interactions with lipid structures.

5.3 Materials and Methods

5.3.1 Lipid vesicle formation and characterization

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Ultra-pure water (18.2 M Ω , Milli-Q, EMD Millipore, Taunton, MA) was used in all experiments. L- α -phosphatidylcholine (egg, chicken) (egg PC) (Avanti Polar Lipids, Inc., Alabaster, AL) vesicles were developed as previously described.^{172,222} Briefly, egg

PC dissolved in chloroform was dried under ultrahigh-purity nitrogen gas (Tech Air, Providence, RI) to form a thin lipid film and kept under vacuum at 25°C for at least 4 h. Dried egg PC was rehydrated in Tris sodium chloride (NaCl) buffer (10 mM Trizma base and 100 mM NaCl). Tris NaCl was filtered for all experiments through a 0.2 μm polyvinylidene fluoride syringe filter. The rehydrated egg PC was subjected to five freeze-thaw-vortex cycles, followed by extrusion through a 100 nm pore size polycarbonate membrane (Nuclepore Track-Etch Membrane Filtration Products, Whatman, Maidstone, U.K.) 21 times using a mini extruder (Avanti Polar Lipids Mini Extruder, Alabaster, AL) to yield unilamellar egg PC vesicles suspended in Tris NaCl. These vesicles were stored at 4°C prior to use in subsequent experiments. Egg PC vesicle hydrodynamic diameter and polydispersity index (PDI) were determined using dynamic light scattering (DLS) using a Malvern Zetasizer ZS90 with Zetasizer 7.01 software.

5.3.2 DEHP and MEHP preparation and aggregate and vesicle interaction characterization

Di(2-ethylhexyl)phthalate (PESTANAL[®], analytical standard) and phthalic acid mono-2-ethylhexyl ester were used for interaction studies. DEHP and MEHP were dissolved in Tris NaCl with 1% v/v dimethyl sulfoxide (DMSO) prior to experimental use.^{204,223} DLS was used to observe the sizes of DEHP and MEHP aggregates at concentrations of 5, 50, 100, and 200 μM in Tris NaCl with 1% v/v DMSO. The effect of 5, 50, 100, and 200 μM DEHP and MEHP on egg PC vesicle size and polydispersity was also investigated using DLS. Egg PC vesicles (0.1 mg/mL in Tris NaCl) were incubated with DEHP and MEHP at a final concentration of 5, 50, 100, or 200 μM in 1% v/v DMSO at 25°C for ~10 min prior to measurement by DLS.

5.3.2.1 Investigation of DEHP interaction with chitosan nanoparticles

Chitosan nanoparticles (80-100 nm, purity > 99%) were purchased from Nanoshel LLC (Wilmington, DE). These beads were suspended in DMSO at a concentration of 20 mg/mL. For experimental use, these were further diluted in Tris NaCl to a concentration of 0.2 mg/mL, yielding 1% v/v DMSO in Tris NaCl. A QCM-D E4 system (Biolin Scientific, Västra Frölunda, Sweden) was used to investigate DEHP adsorption on the chitosan particles at 25°C and a constant flow rate of 175 μ L/min. Frequency changes (ΔF) and dissipation changes (ΔD) occurring on silica-coated quartz QCM-D substrates (Biolin Scientific, Västra Frölunda, Sweden) over time were studied as previously described.^{91,94,161,224} Prior to use in QCM-D experiments, the substrates were cleaned with water, 2% w/v sodium dodecyl sulfate, water, and air at 400 μ L/min in the QCM-D chambers. The substrates were then removed from the chambers, dried with N₂, and UV/ozone treated with a UV/ozone ProCleaner (Bioforce Nanosciences, Salt Lake City, UT). After a Tris NaCl buffer flow across silica-coated quartz crystals, 1% v/v DMSO in Tris NaCl was flowed to mimic the bead suspension solution viscosity. The chitosan particles were flowed through the modules and adsorbed to the silica-coated surface, followed by 200 μ M DEHP in 1% v/v DMSO in Tris NaCl. The hydrodynamic diameter of the chitosan beads as prepared (0.2 mg/mL in 1% v/v DMSO in Tris NaCl) and egg PC lipid vesicles incubated with 200 μ M DEHP that had first been incubated with 0.2 mg/mL of the chitosan particles for 5 min at 25°C in 1% v/v DMSO in Tris NaCl were observed using DLS.

5.3.3 Supported lipid bilayer formation and measurements using quartz crystal microbalance with dissipation monitoring (QCM-D)

QCM-D (E4, Biolin Scientific, Västra Frölunda, Sweden) was used to monitor lipid vesicle adsorption, bilayer formation, and DEHP and MEHP interactions with these bilayers *in situ*. QCM-D silica-coated quartz substrates were cleaned as described in *section 2.2.1*. These QCM-D experiments were performed at 25°C and at a flow rate of 175 $\mu\text{L}/\text{min}$ unless noted otherwise. Baseline ΔF and ΔD values were collected with Tris NaCl before introducing egg PC vesicles. Vesicles in Tris NaCl (0.1 mg/mL) were then introduced onto the silica-coated surface for adsorption and rupture as previously investigated.^{161,224} Once the bilayer was formed, a Tris NaCl rinse was performed for at least 6 min, followed by flow of 1% v/v DMSO in Tris NaCl for at least 8 min. 1% v/v DMSO was introduced to account for ΔF and ΔD changes due to viscosity differences between Tris NaCl and DMSO prior to introduction of DEHP and MEHP (soluble in 1% v/v DMSO in Tris NaCl). DEHP or MEHP (5, 50, 100, and 200 μM) was then introduced into the QCM-D chamber and allowed to interact with the egg PC bilayer for at least 10 min. If a plateau was not reached within this time, the flow was stopped to allow incubation of the phthalate for an additional 10 min. Following DEHP or MEHP interaction, 1% v/v DMSO was again introduced followed by Tris NaCl for at least 6 min.

5.3.4 Suspended lipid bilayer formation and measurements using a parallel artificial membrane permeability assay (PAMPA)

Polyvinylidene difluoride (PVDF) 96-well multiscreen filter plates (0.45 μm) and transport receiver plates (Millipore-Sigma, Burlington, MA) were used to investigate DEHP and MEHP diffusion from a donor compartment, through the porous membrane treated with

lipid, to an acceptor compartment. Donor and acceptor compartments were filled with 1× phosphate buffered saline (PBS) (200 μL acceptor, 300 μL donor). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, Inc., Alabaster, AL) was solubilized in dodecane at 20 mg/mL.^{99,225} DOPC (5 μL) was added to the porous PVDF membrane and immediately submerged in the donor and acceptor 1× PBS solutions to form a suspended lipid bilayer. Controls of filters introduced to dodecane only (5 μL) and treated similarly were included along with untreated filters also exposed to the donor and acceptor 1× PBS. The donor compartment was then filled with 200 μL of filtered 1× PBS with 1% v/v DMSO with 100 μM or 200 μM DEHP or MEHP. The acceptor compartment was filled with 300 μL of filtered 1× PBS with 1% v/v DMSO. The plate was rocked for 16 h at 25°C prior to collecting 100 μL of solution from the donor and acceptor compartments. The absorbance of DEHP or MEHP in this solution was measured at 280 nm using a plate reader (Cytation 3, BioTek, Winooski, VT). The experimental apparent permeability (P_{app}) of DEHP and MEHP through the DOPC bilayer was calculated using equations (5.1) and (5.2)^{226,227}.

$$P_{app} = C \cdot -\ln \left(1 - \frac{[small\ molecule]_{acceptor}}{[small\ molecule]_{initial}} \right) \quad (5.1)$$

$$\text{Where } C = \frac{V_D \cdot V_A}{(V_D + V_A)A \cdot t} \quad (5.2)$$

In equation (1), $[small\ molecule]_{acceptor}$ is the concentration of the analyte (e.g., DEHP) in the acceptor compartment and $[small\ molecule]_{initial}$ is the initial concentration of analyte. In equation (5.2), V_D is the donor compartment volume, V_A is the acceptor compartment volume, A is the membrane area, and t is the time.

The permeability of various small molecules through the DOPC bilayer following exposure to DEHP and MEHP was also investigated. The small molecules of interest (caffeine, carbamazepine, warfarin, furosemide, folic acid, and methotrexate) were solubilized in 5% v/v DMSO in 1× PBS at a final concentration of 500 μM. Absorbance maximums for each small molecule were determined using a plate reader (Cytation 3, BioTek, Winooski, VT). DEHP or MEHP were introduced at 200 μM to the DOPC bilayers for 16 h at 25°C as done in the DEHP/MEHP-DOPC interaction studies. Donor compartments were then rinsed with 1× PBS and the acceptor compartments were replaced with a fresh multiscreen acceptor plate containing 300 μL of 1× PBS with 5% v/v DMSO. Solubilized small molecules (200 μL) were added to the donor compartment and allowed to permeate for 2 h gently rocking at 25°C. Following this time, 100 μL of the donor and acceptor compartment solutions were collected, and the absorbance of the small molecule was measured at the absorbance maximum previously identified using a plate reader (Cytation 3, BioTek, Winooski, VT). P_{app} was determined for each small molecule using equations (1) and (2). Controls of filters introduced to dodecane only (5 μL) and treated similarly were included along with untreated filters also exposed to the donor and acceptor solutions. Permeability of the small molecules was calculated using equations (5.1) and (5.2). The amount of small molecule remaining associated with the lipid bilayer was also determined by comparing the concentration of small molecule in the acceptor and donor compartments at 2 hours with the initial drug concentration in the donor compartment.

5.3.5 Statistical analysis

All experiments were conducted in triplicate at a minimum. Representative results for the third overtone are shown in figures for all QCM-D ΔF and ΔD measurements, unless otherwise specified. Results are given as mean $\pm$ standard deviation where appropriate. Statistical significance was calculated using Prism 7 (GraphPad) with student's t-test, one-way analysis of variance (ANOVA), or two-way ANOVA where applicable. Tukey's post hoc analysis was performed for ANOVA tests ($\alpha = 0.05$; $p < 0.05$ was considered statistically significant).

5.4 Results and Discussion

5.4.1 Effect of DEHP and MEHP on egg PC vesicle size and polydispersity

In this work we formulated several lipid structures, including unilamellar vesicles, SLBs, and suspended lipid bilayers and investigated their interactions with DEHP and MEHP. Specifically, we examined the effect of lipid vesicle size, adsorption to and potential lipid removal from supported bilayers, and permeability of suspended bilayers in the presence of these phthalates. First, we investigated the size and polydispersity of DEHP and MEHP suspensions at varying concentrations (5, 50, 100, and 200 μM in 1% v/v DMSO in Tris NaCl). These concentrations were selected as they are within the typical daily human acquisition range of DEHP and MEHP. The DLS hydrodynamic diameter distribution histograms for these suspensions are shown in Figure SI 5.2. A minor peak (~ 5 μm hydrodynamic diameter) appeared for DEHP concentrations of 50, 100, and 200 μM , but was not observed for 5 μM or any of the MEHP concentrations examined. Figure 5.1 shows

the major aggregate peak hydrodynamic diameter and PDI for DEHP and MEHP at all concentrations. For DEHP, there was a significant difference between the hydrodynamic diameter of the major peak between 5 μM DEHP (~ 250 nm) and 200 μM DEHP tested (~ 450 nm). In contrast, MEHP aggregate size did not depend on MEHP concentration and remained consistent around ~ 200 nm. The hydrolysis of DEHP to MEHP results in a free carboxyl group in the MEHP structure. This carboxyl can participate in hydrogen bonding, making MEHP less hydrophobic than DEHP, which is supported by the reported octanol:water partition coefficients of DEHP (logP of 7.6)²²⁸ and MEHP (logP of 4.66)²²⁹. The greater hydrophobicity of the DEHP results in larger aggregates than those seen in MEHP suspensions. The PDI of all suspensions were similar (~ 0.1 - 0.2), with the exception of the 100 μM condition, at which DEHP had a statistically significant increase in PDI compared to MEHP suspensions (0.205 versus 0.0512, respectively).

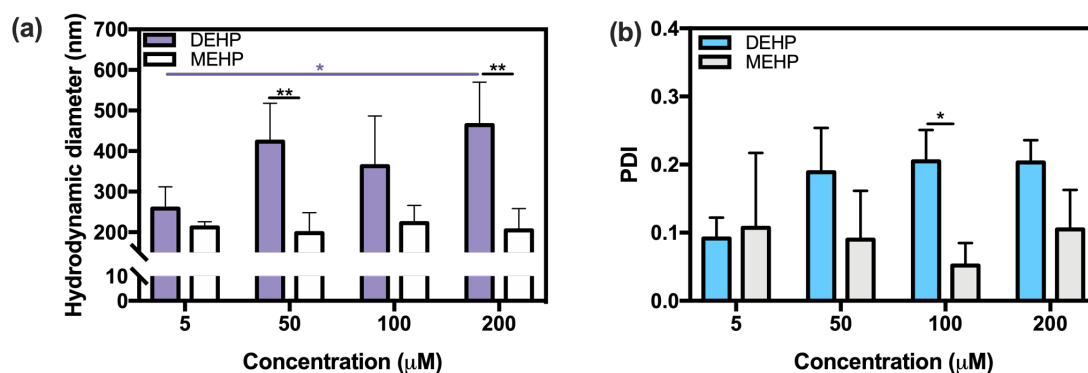


Figure 5.1: Size and polydispersity of DEHP and MEHP aggregates. Suspensions were formulated in Tris NaCl with 1% v/v DMSO at 5, 50, 100, and 200 μM DEHP or MEHP. **(a)** DEHP and MEHP average hydrodynamic diameter for major DLS peak. **(b)** DEHP and MEHP PDI. Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc analysis; $\alpha = 0.05$; $n \geq 3$; * $p < 0.05$; ** $p < 0.01$.

DEHP and MEHP were then incubated with unilamellar egg PC vesicles in order to study vesicle size change due to the phthalate exposure. Figure SI 5.3 shows the DLS hydrodynamic diameter distribution histograms for these mixtures. As with DEHP and MEHP alone, a minor peak with large diameter ($> \sim 1 \mu\text{m}$) appeared for vesicles incubated with 50, 100, and 200 μM DEHP, and not for 5 μM DEHP and all MEHP incubated egg PC vesicles. In general, distinct phthalate aggregate peaks similar to those observed in phthalate alone incubation studies (Figure 5.1) and egg PC vesicle peaks ($\sim 100 \text{ nm}$) were not observed in any of the incubation solutions, indicating an effect of incubating vesicles with the DEHP and MEHP on aggregation of these molecules. Egg PC vesicles in Tris NaCl and 1% DMSO in Tris NaCl had similar peak hydrodynamic diameters and PDIs as egg PC vesicles incubated with 5 μM DEHP and MEHP at any of the concentrations investigated in 1% DMSO conditions (Figure 5.2). Increasing DEHP concentration beyond 5 μM led to a significant increase in vesicle major peak hydrodynamic diameter and polydispersity. Overall, the hydrodynamic diameters observed for the major peak of egg PC vesicles incubated with DEHP were much lower than the diameters observed for DEHP only suspensions. We hypothesize that when incubated with egg PC vesicles the DEHP interacts directly with the egg PC vesicles instead of only self-aggregating, leading to overall diameters closer to that of the egg PC vesicles alone. Although less dramatic, the hydrodynamic diameter of the major peak of egg PC vesicles incubated with MEHP were also lower than the diameters observed for MEHP only suspensions. Due to the zwitterionic properties of the egg PC vesicles, MEHP which is negatively charged at the conditions tested is less likely to interact with the vesicles as compared to the DEHP, which may interact more readily through hydrophobic interactions.

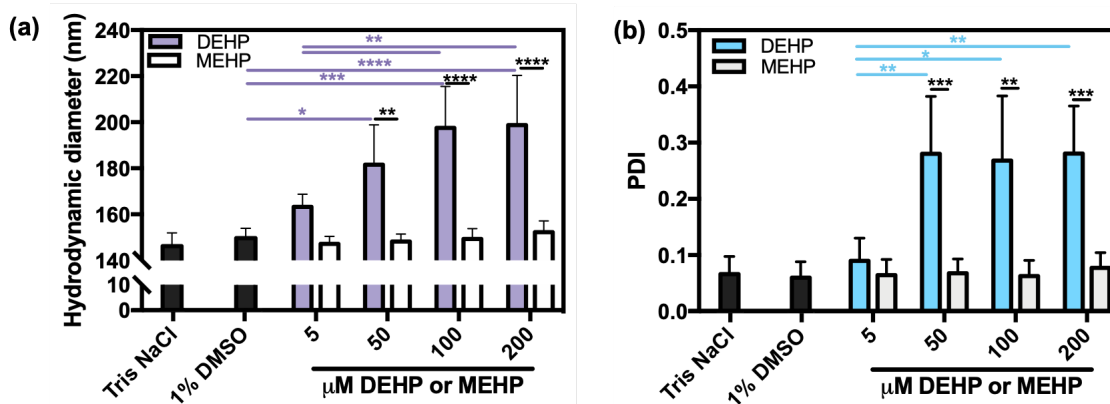


Figure 5.2: Size and polydispersity of DLS major peak for egg PC vesicle sizes incubated with 5, 50, 100, and 200 μM DEHP and MEHP in Tris NaCl with 1% DMSO. **(a)** Hydrodynamic diameter of vesicle major peak with different DEHP and MEHP concentrations. **(b)** Vesicle PDI at different DEHP and MEHP concentrations. Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc analysis $\alpha = 0.05$; $n \geq 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

5.4.1.1 Mitigating DEHP interaction with lipid vesicles using chitosan nanoparticles

Given the interaction of DEHP and MEHP with lipid vesicles, which may influence potential biological function, there is an interest in developing methods that may reduce these interactions. Previous studies have investigated using chitosan particles to facilitate DEHP adsorption for the removal of DEHP from environmental substances.^{209,230} Chitosan has previously been used to remove organic and inorganic substances from wastewater following the Freundlich adsorption isotherm.²³¹ Interactions between phthalate esters and chitosan microparticles have been shown to be driven by hydrophobic interactions.²³⁰ Here we investigated whether pre-incubation of DEHP with chitosan particles could mitigate interactions between egg PC vesicles and DEHP. First, we confirmed chitosan-DEHP interaction using QCM-D. At the conditions examined the chitosan nanoparticles were found to aggregate, yielding larger micron-scale structures with diameters of $2.26 \pm 0.80 \mu\text{m}$ (PDI of 0.84 ± 0.21) as confirmed by DLS. As shown in Figure 5.3a, when these

suspensions were introduced via flow over a silica-coated QCM-D sensor, a decrease in ΔF and plateauing was observed over ~ 8 minutes, indicating adsorption of these particles on the substrate. After introducing 200 μM DEHP and following rinsing with 1% DMSO in Tris NaCl, we observed an overall ΔF decrease and ΔD increase, indicating adsorption of DEHP to the chitosan particle layer as expected.

Having confirmed the chitosan particle-DEHP interaction, we next investigated the impact of this interaction on subsequent DEHP interactions with egg PC unilamellar vesicles. First, 200 μM DEHP was incubated with chitosan nanoparticles (0.2 mg/mL) for 5 min before adding this mixture to egg PC vesicles. The DLS size distribution for this suspension is shown in Figure SI 5.4 compared with controls of egg PC vesicles in 1% DMSO, the chitosan particles alone, and chitosan particles incubated with DEHP. Peak diameters for chitosan particles and egg PC vesicles were distinct in these size distributions. Figure 5.3b compares the peak hydrodynamic diameter of egg PC vesicles exposed to 1% DMSO, 200 μM DEHP, or 200 μM DEHP and chitosan nanoparticle mixtures. No significant change in vesicle hydrodynamic diameter was observed for particles incubated with the DEHP-chitosan mixture compared with those in 1% DMSO, which was significantly smaller than vesicles exposed to 200 μM DEHP, as previously discussed. Thus, pre-incubation with chitosan particles eliminated DEHP interactions with the vesicles, presumably by rapidly adsorbing all of the molecule before it has an opportunity to interact with the vesicles and preventing subsequent interactions with these vesicles. These particles are a promising option for use in mitigation of DEHP interactions with lipid structures in future applications. The potential for chitosan particles to have a similar effect for other lipid compositions can be extensively explored in future studies.

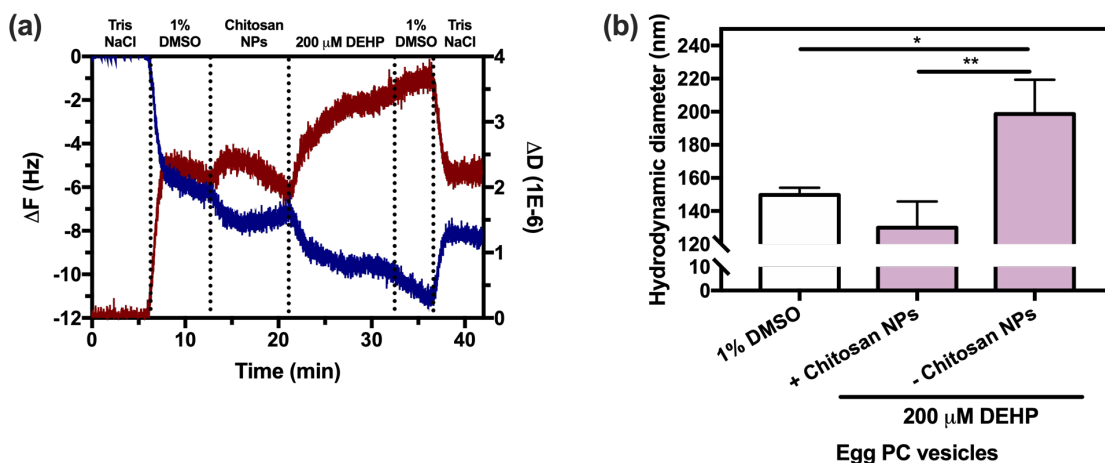


Figure 5.3: Effects of chitosan nanoparticle incubation with 200 μ M DEHP. **(a)** QCM-D of 0.2 mg/mL chitosan particles and 200 μ M DEHP adsorption. Frequency changes (ΔF) and dissipation changes (ΔD) are shown in blue and red, respectively for the 3rd overtone. **(b)** Hydrodynamic peak diameter of egg PC vesicles with 1% DMSO only, 200 μ M DEHP mixed with 0.2 mg/mL chitosan nanoparticles prior to incubation with egg PC vesicles, and 200 μ M DEHP only. Results for (b) are shown as mean $\pm$ standard deviation. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc analysis; $\alpha = 0.05$; $n \geq 3$; * $p < 0.05$; ** $p < 0.01$.

5.4.2 Effect of DEHP and MEHP on egg PC SLB structure

QCM-D was used to investigate DEHP and MEHP interactions with egg PC SLBs. Egg PC SLBs have previously been used to model biological membranes.^{79,224,232–234} Although there are no previous reports of phthalate-SLB interactions, possible mechanisms of interaction can be hypothesized based on existing studies of peptide-lipid bilayer interactions.^{81,235,236} Peptides are proposed to have multiple modes of interaction with bilayers, including adsorption,²³⁵ embedment within adjacent lipid head groups,²³⁵ insertion resulting in pore formation,²³⁵ or formation of peptide-lipid aggregates that detach from the bilayer causing membrane disintegration.²³⁶ Among these, two main models of interaction include: the barrel-stave model and the carpet model. The barrel-stave model results when peptides insert into the lipid core in an orientation perpendicular to the bilayer

with the hydrophobic portions of the peptide interacting with the lipid and hydrophilic portions forming the interior of the pore.²³⁶ The carpet model of interaction results when peptides reach the membrane surface and once a concentration threshold is reached the membrane is permeated mimicking surfactant-like activity, leading to membrane disintegration.²³⁶ We discuss our pthlatate-egg PC SLB interaction results in relationship to these two widely recognized models of peptide-membrane interactions.

Upon forming egg PC SLBs on silica-coated QCM-D sensors as previously reported,^{79,94,224} DEHP was flowed over the bilayer at 5, 50, 100, and 200 μM . Representative QCM-D results for these studies are shown in Figure 5.4a-d, starting with the initial egg PC vesicle adsorption and rupture leading to SLB formation within the first 10 minutes. We observed concentration dependent changes in DEHP interaction with these SLBs, indicated by changes in ΔF and ΔD . ΔF and ΔD represent changes in mass and rigidity, respectively, and can allow us to hypothesize whether interactions similar to the barrel-stave or carpet model are at play. Figures 5.4e and 5.4f show ΔF and ΔD upon DEHP exposure evaluated from the Tris NaCl buffer rinse after egg PC bilayer formation to the Tris NaCl buffer rinse after DEHP flow. All ΔF and ΔD changes observed for 5 and 100 μM due to DEHP were statistically significant ($p < 0.05$), with the exception of ΔF changes following exposure to 100 μM DEHP. A ΔF increase of 6.3 ± 2.6 Hz and a ΔD of -1.37×10^{-6} , indicating a mass loss from the SLB and a more rigid SLB, respectively, were observed after DEHP flow at 5 μM . Although no significant change in ΔF was observed with 100 μM DEHP, a change in ΔD of -0.86×10^{-6} indicated a more rigid surface following DEHP introduction at these conditions. For the 5 and 100 μM DEHP concentrations, we hypothesize that DEHP interaction with egg PC SLBs follows the carpet model, leading to

some membrane disintegration as lipids are removed following the establishment of a threshold DEHP concentration on the bilayer surface. To better understand this potential mechanism, we can examine the 5 and 100 μM DEHP QCM-D flow step over time. Specifically, for 5 μM DEHP, upon initial introduction a decrease in ΔF from ~ -27 Hz to ~ -36 Hz and ΔD increase from $\sim 2 \times 10^{-6}$ to $\sim 6 \times 10^{-6}$ was observed (from 19 to 21 min in Figure 5.4a), suggesting increase in mass and decrease in surface rigidity. The ΔF then increased and plateaued at ~ -23 Hz and the ΔD decreased to $\sim 1 \times 10^{-6}$ over the entire DEHP incubation time, suggesting a mass loss and more rigid surface formation. These changes are suggestive of the carpet model where DEHP first adsorbs on the SLB surface and once a threshold surface concentration is achieved, lipid removal begins.

For 200 μM DEHP, we observed a monotonic ΔF decrease and ΔD increase over a flow time of at least 1 hour (Figure SI 5.5), indicating a continuous mass increase upon DEHP exposure to the egg PC SLB at these conditions. These results are in contrast to the plateau in ΔF observed for 5 and 100 μM DEHP within ~ 10 minutes, suggesting that DEHP at 200 μM interacts with egg PC SLBs via a different mechanism than 5 and 100 μM DEHP. We found that when 50 and 200 μM DEHP was not continuously replenished and allowed to incubate with the SLB following a 10 minute flow step (Figures 5.4b and 5.4d), a rapid plateau in ΔF and ΔD was reached. At these conditions, a -8.6 ± 8.2 Hz and -17.4 ± 8.0 Hz change in ΔF and a ΔD increase by $1.8 \times 10^{-6} \pm 1.7 \times 10^{-6}$ and $5.7 \times 10^{-6} \pm 2.0 \times 10^{-6}$ was observed for 50 and 200 μM DEHP, respectively. All ΔF and ΔD changes comparing before and after DEHP introduction for 50 and 200 μM DEHP were statistically significant ($p < 0.05$). These observations indicate DEHP adsorption to the SLB. The increase in surface fluidity (indicated by increasing ΔD) may suggest potential insertion in

the membrane, potentially implying DEHP interaction may follow the barrel-stave model at these DEHP concentrations.

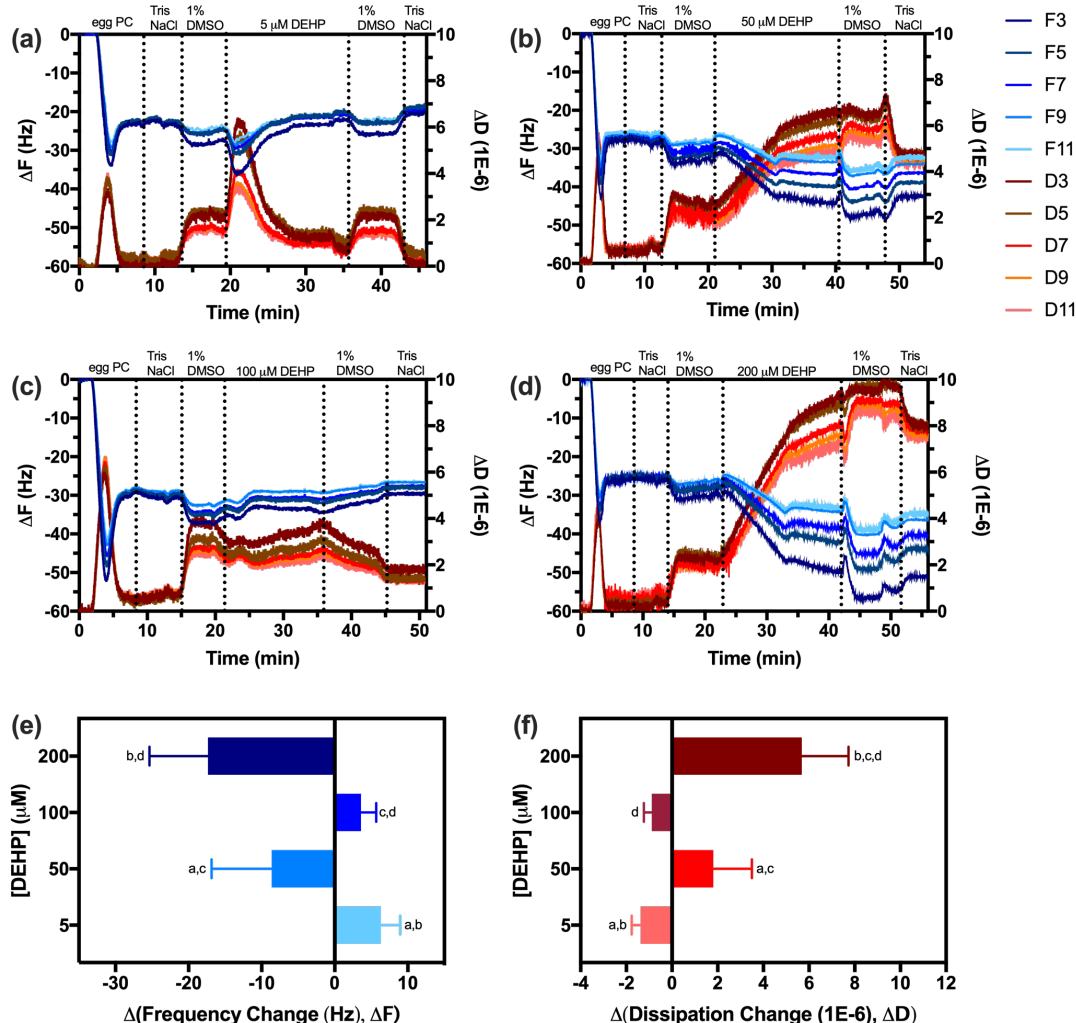


Figure 5.4: DEHP interaction with an egg PC SLB examined via QCM-D. Frequency changes (ΔF) and dissipation changes (ΔD) are shown in blue and red, respectively for overtones 3, 5, 7, 9, and 11 for (a) 5 μM , (b) 50 μM , (c) 100 μM , (d) 200 μM DEHP. Representative plots are shown from $n \geq 3$. Change in (e) ΔF and (f) ΔD from the initial Tris NaCl buffer rinse after forming egg PC bilayer to the final Tris NaCl buffer rinse after DEHP flow for 5, 50, 100, and 200 μM DEHP. Results for (e) and (f) are shown as mean $\pm$ standard deviation. Statistical analyses were performed for each ΔF and ΔD using a two-tailed t-test with $n \geq 3$; $\alpha = 0.05$. All changes were significant with the exception of 100 μM ΔF . Statistical analysis comparing the changes in ΔF and ΔD for different DEHP concentrations was performed using one-way ANOVA with Tukey's post hoc analysis, $n \geq 3$; $\alpha = 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Matching letters indicate significance with (e) *a; ***b; *c; ***d. (f) *a; ****b; ***c; ****d.

Next, we investigated the interaction of MEHP with egg PC SLBs using QCM-D (Figure 5.5a-d). Similar to DEHP, MEHP concentrations of 5, 50, 100, and 200 μM were flowed across the SLB. Unlike DEHP, we observed a similar increase in ΔF for all MEHP concentrations (Figure 5.5e). No significant difference in ΔD was observed before and after MEHP flow for any of the concentrations examined (Figure 5.5f) (note, differences in ΔF and ΔD were calculated from the Tris NaCl buffer rinse after egg PC bilayer formation to the Tris NaCl buffer rinse after MEHP flow). These findings suggest that although some lipid mass or mass from the hydrated layer between the SLB and silica surface was removed upon MEHP exposure at all tested concentrations, the rigidity of the bilayer remained unchanged.

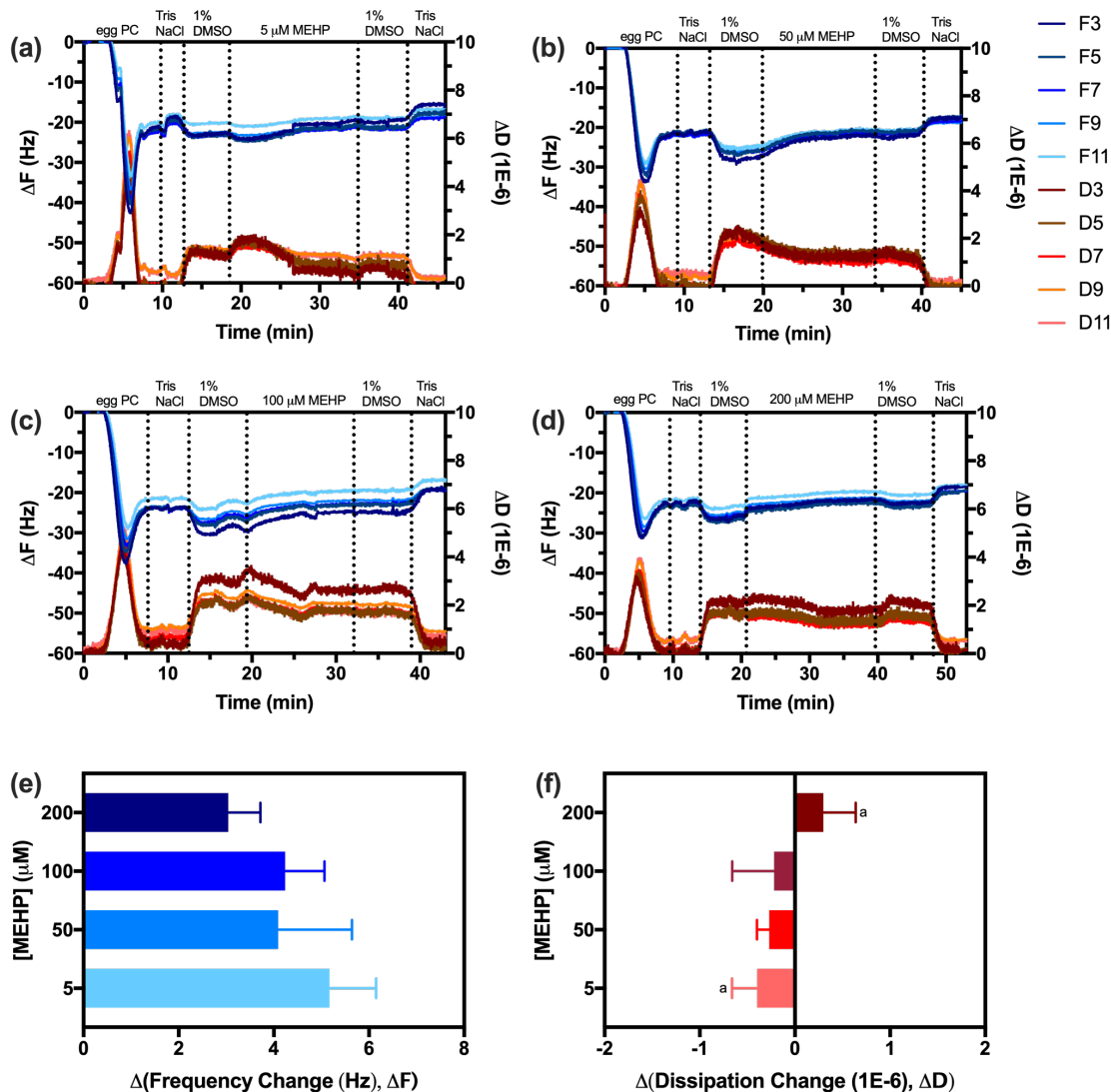


Figure 5.5: MEHP interaction with an egg PC SLB examined via QCM-D. Frequency changes (ΔF) and dissipation changes (ΔD) are shown in blue and red, respectively for overtones 3, 5, 7, 9, and 11 for (a) 5 μM , (b) 50 μM , (c) 100 μM , (d) 200 μM . Representative plots are shown from $n \geq 3$. Change in (e) ΔF and (f) ΔD from the initial Tris NaCl buffer rinse after forming egg PC bilayer to the final Tris NaCl buffer rinse after MEHP flow for 5, 50, 100, and 200 μM MEHP. Results for (e) and (f) are shown as mean $\pm$ standard deviation. Statistical analyses were performed for each ΔF and ΔD using a two-tailed t-test with $n \geq 3$; $\alpha = 0.05$. All ΔF changes were significant while all ΔD changes were not significant. Statistical analysis comparing the changes in ΔF and ΔD for different MEHP concentrations was performed using one-way ANOVA with Tukey's post hoc analysis, $n \geq 3$; $\alpha = 0.05$; * $p < 0.05$ Matching letters indicate significance with (f) *a.

Relating the results of DEHP and MEHP aggregation in solution with the QCM-D studies of egg PC SLBs incubated with these phthalates suggests that the size of phthalate aggregates may influence their interactions with SLBs. Both 50 and 200 μM DEHP aggregates were significantly larger in hydrodynamic diameter compared to MEHP aggregates. We observed stark differences in DEHP versus MEHP-egg PC SLB interactions, with 50 and 200 μM DEHP causing adsorption to the egg PC bilayer while all concentrations of MEHP resulted in a mass loss. For the 5 and 100 μM DEHP and all concentrations of MEHP, phthalate-lipid aggregates may develop and detach from the bilayer, resulting in the ΔF increases observed. This observation is supported by previous studies of nanoparticle (NP)-SLB interactions observing that NP size and hydrophobicity dictate these interactions.^{237,238}

5.4.3 Permeability of DEHP and MEHP across DOPC suspended lipid bilayers

With differences noted between DEHP and MEHP interactions with egg PC SLBs, we next investigated the permeability of DEHP and MEHP across a suspended lipid bilayer using PAMPA. This assay has been used for investigating permeability of compounds across phospholipid membranes^{239–241} but has not previously been used to examine phthalate interactions with bilayers. Examining transport across the bilayer can provide further information on potential mechanisms of phthalate interaction with lipid bilayers. Specifically, we examined DEHP and MEHP transport across a DOPC bilayer formed across a PVDF porous polymer support when introduced to the donor compartment at concentrations of 100 and 200 μM . For these assays, DOPC was selected due to its solubility in dodecane enabling formation of suspended lipid bilayers across hydrophobic

porous materials as previously studied.^{225,241,242} We report our results normalized to DEHP and MEHP permeability across a blank PVDF porous support. Figure SI 5.6 shows DEHP and MEHP transport properties across controls of PVDF porous supports without any bilayer, PVDF supports treated with dodecane, and DOPC bilayers formed on PVDF supports. In general, we observed negligible interaction of DEHP and MEHP with the porous support without dodecane or DOPC bilayer treatment. After 16 h, we observed significant differences between DEHP and MEHP in the percentage of phthalate within the donor and acceptor compartments of the PAMPA setup (Figure 5.6). For DEHP, we observed that ~75% of the phthalate remained interacting with the DOPC bilayer and was not transported across the membrane. In contrast, with MEHP we observed that most of the phthalate was in the donor solution (~85-100%). Both DEHP and MEHP had low permeability across the suspended lipid bilayer, resulting in $P_{app} \sim 5 \times 10^{-11} - 10 \times 10^{-11}$ cm/s. Our egg PC SLB interaction studies had indicated that DEHP was likely adsorbing and embedding itself within the lipid bilayers, which supports the PAMPA results observed. On the other hand, MEHP resulted in smaller ΔF and ΔD changes compared to DEHP, indicating less SLB interaction, which is also consistent with the PAMPA findings.

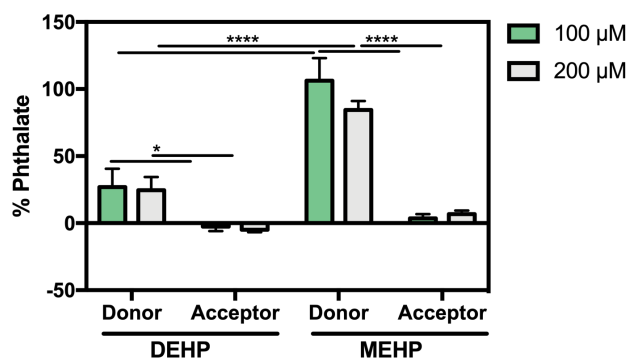


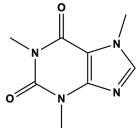
Figure 5.6: Transport of DEHP and MEHP across DOPC suspended lipid bilayers. (a) Percentage of DEHP and MEHP in donor and acceptor compartments of PAMPAs with DOPC membranes assembled on PVDF porous supports in dodecane introduced to the

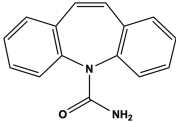
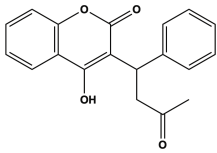
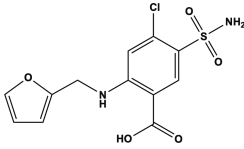
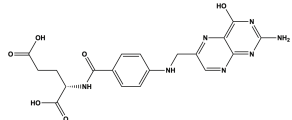
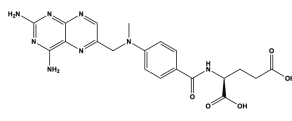
donor compartment at 100 and 200 μM . Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc analysis; $\alpha = 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

5.4.3.1 Impact of DEHP and MEHP pre-treatment on small molecule permeability across DOPC suspended lipid bilayers

The interactions of DEHP and MEHP with lipid bilayers may potentially influence how these bilayers then interact with other molecules they are exposed to, which may have implications for human health. In this study, we were interested in exploring the transport of various small molecules across lipid bilayers upon exposure to DEHP and MEHP, ranging from commonly ingested caffeine and folic acid to pharmaceuticals, methotrexate, furosemide, carbamazepine, and warfarin (Table 5.1). We specifically chose these pharmaceutical agents as ones that have been reported to have toxic biological effects (e.g., considered not safe to take during pregnancy by the U.S. Food and Drug Administration). After exposing DOPC bilayers to 200 μM DEHP or 200 μM MEHP for 16 h in a PAMPA setup, we investigated diffusion of the small molecules across the DOPC bilayer. We hypothesized that properties such as molecular weight and hydrophobicity of these molecules would influence transport across the membrane and that pre-treatment with phthalates could affect this transport.

Table 5.1: Small molecule properties

Molecule	Mol. weight (g/mol)	Water solubility (mg/mL)	logP	Max. abs. (nm)_d	Structure	Use
Caffeine	194.19	21.6 ^b	- 0.07 ^a	270		Central nervous system stimulant

Carbamazepine	236.27	0.017 ^b	2.45 ^a	280		Anticonvulsant and anti-epileptic
Warfarin	308.33	0.017 ^b	2.7 ^a	310		Blood thinner to treat and prevent blood clots
Furosemide	330.75	0.073 ^b	2.03 ^a	280		Diuretic used to treat fluid retention and swelling
Folic acid	441.4	Almost insoluble ^c	-2.5 ^b	280		B vitamin
Methotrexate	454.44	2.6 ^b	-1.85 ^a	300		Immunosuppressive and chemotherapeutic

^aUnited States Environmental Protection Agency (EPA), Distributed Structure-Searchable Toxicity (DSSTox) Database

^bDrugbank.ca

^cNational Toxicology Program, Institute of Environmental Health Sciences, National Institutes of Health

^dExperimentally determined

While there are many different properties of these compounds that could affect their permeability and interaction with the lipid bilayers, we examined general trends observed based on molecule molecular weight and hydrophobicity differences. We observed that lower molecular weight and higher logP values (indicating increased hydrophobicity) both generally resulted in increased permeability of the molecule across the membrane

following both DEHP and MEHP pre-treatment (Figure 5.7a and 5.7b, respectively), with P_{app} values all several orders of magnitude greater than P_{app} observed for the phthalates alone. Interestingly, caffeine appeared to be an outlier in the trend in increased permeabilities observed for lower molecular weight species, underlying the complexity of the interactions that may occur which may not be entirely captured by considering molecular weight or hydrophobicity alone.

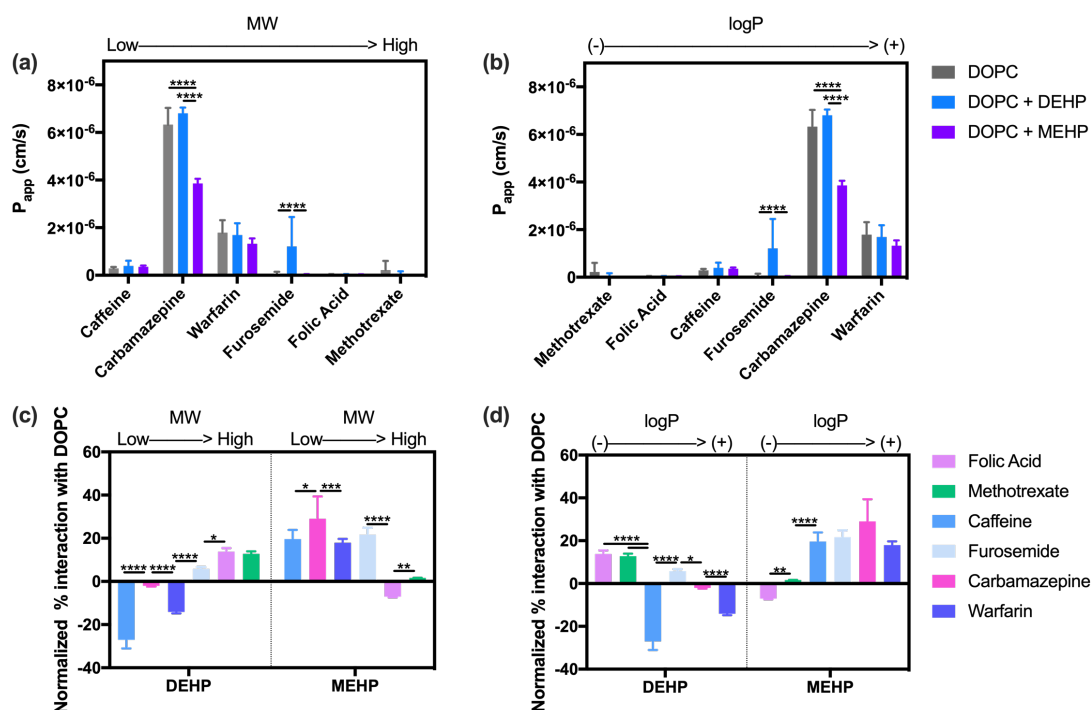


Figure 5.7: Small molecule transport across DOPC suspended lipid bilayers following pre-treatment with DEHP or MEHP. **(a)** Permeability (P_{app}) of small molecules with increasing molecular weight across DOPC bilayers without and with pre-treatment with 200 μ M DEHP or MEHP for 16 h. **(b)** Permeability (P_{app}) of small molecules with increasing hydrophobicity (measured via logP) across DOPC bilayers without and with pre-treatment with 200 μ M DEHP or MEHP for 16 h. **(c)** Percentgate of small molecule interaction with DOPC bilayer due to pre-treatment of DEHP or MEHP normalized to DOPC without phthalate pre-treatment plotted from low to high MW. **(d)** Percentage of small molecule interaction with DOPC bilayer due to DEHP or MEHP normalized to DOPC without phthalate pre-treatment plotted from negative to positive logP. Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc analysis; $\alpha = 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

In addition to molecule permeability, we examined potential differences observed in the small molecule interaction with the lipid bilayer with and without pre-treatment with either DEHP or MEHP. This interaction may consist of adsorption to the bilayer and/or embedding. Figure 5.7c and 5.7d show the percentage of drug interacting with the DOPC suspended lipid bilayers after either DEHP or MEHP pre-treatment normalized to how the drug interacts with DOPC bilayers without DEHP or MEHP pre-treatment. Overall, we observed opposing trends with how these small molecules interact after DEHP versus MEHP pre-treatment. For example, after DEHP pre-treatment, higher molecular weight compounds showed a greater interaction with the lipid bilayer compared with lower molecular weight molecules. However, after MEHP pre-treatment, higher molecular weight compounds interacted less with the lipid bilayer compared with lower molecular weight compounds. This same opposing trend is also observed when evaluating bilayer interactions with respect to compound hydrophobicity. For example, after DEHP pre-treatment, molecules with a more negative logP (i.e., more hydrophilic) were found to interact more with the lipid bilayer compared with molecules with higher logP (i.e., more hydrophobic). However, after MEHP pre-treatment, compounds with a more negative logP interacted less with the lipid bilayer compared with molecules with higher logP. The opposing interaction trends observed may be a result of the differing bilayer interaction mechanisms that occur upon DEHP and MEHP exposure as we had noted in our egg PC SLB QCM-D studies. In these studies, we observed that DEHP adsorbs to egg PC SLBs when introduced at 200 μ M, while MEHP at the same concentration leads to a decrease in mass, suggesting lipid removal (PAMPA studies looking at the phthalate transport also confirmed these observations). Lipid removal due to MEHP exposure may result in smaller

molecular weight molecules now being able to interact more readily with the structure than larger molecules and more hydrophobic molecules being able to interact with the bilayer even at potential defect sites. DEHP interaction and potential embedment within the SLB may enable more hydrophilic molecules to interact more readily with the bilayer via adsorption to the surface. Overall, our results indicate that the mechanism of phthalate interaction can further influence small molecule interactions with lipid structures. These interactions can pose further health concerns due to phthalates potentially interfering with the mechanism of action or uptake of certain small molecules in the body.

5.5 Conclusions

This is the first study to directly investigate the effect of DEHP and MEHP on lipid structures. In this study we have noted differences in how DEHP and MEHP interact with lipid vesicles, supported lipid bilayers, and suspended lipid bilayers. Our results have shown conditions in which phthalate adsorption is observed, possible phthalate-lipid aggregates resulting in lipid removal from the bilayer, and potential phthalate embedment in the lipid bilayer. We also observed differences in how small molecules then interact with the lipid bilayer pre and post-exposure to DEHP and MEHP, with large effects observed for certain molecules. The types of interactions that have been observed may impact the interactions of phthalates with cell membranes or biological lipid vesicles important for human health, may affect the interactions of other small molecules with these structures, and may provide insight into the mechanisms of phthalate exposure associated health conditions. We have also provided preliminary studies guiding future work on mitigation of phthalate-lipid interactions by utilizing chitosan nanoparticles. Ultimately, the

significance of this study spans increasing fundamental knowledge associated with molecular interactions with lipid structures to potential future impact on biological systems.

5.6 Author Contributions

This chapter was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

5.7 Acknowledgements

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5.8 Supplemental Information

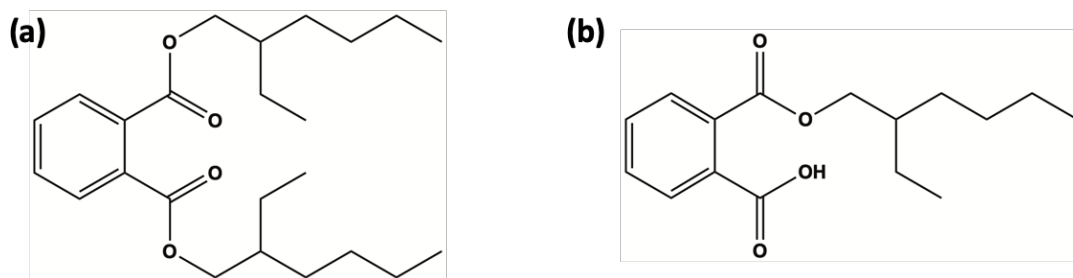


Figure SI 5.1: Molecular structure of phthalate esters (a) DEHP and (b) MEHP. DEHP is found in many household products and is known to leach from these materials. MEHP is a DEHP metabolite.

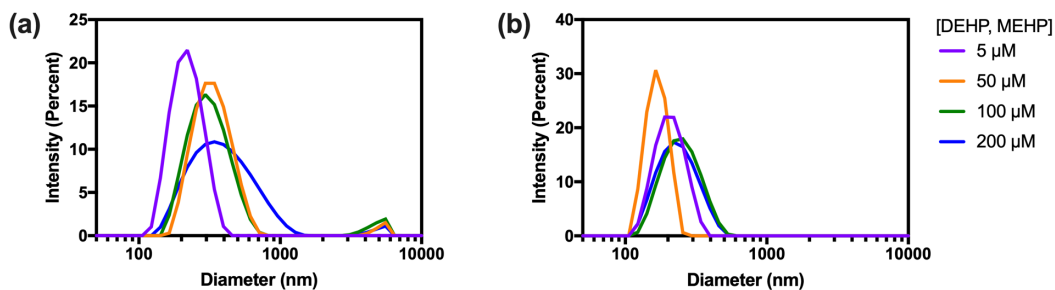


Figure SI 5.2: Size distributions for (a) DEHP and (b) MEHP for 5, 50, 100, and 200 μM

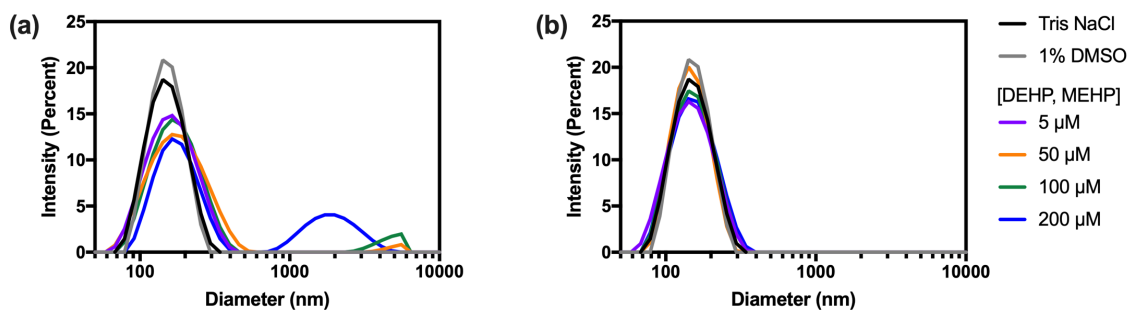


Figure SI 5.3: Size distributions for (a) DEHP incubated with egg PC vesicles and (b) MEHP incubated with egg PC vesicles for 5, 50, 100, and 200 μM .

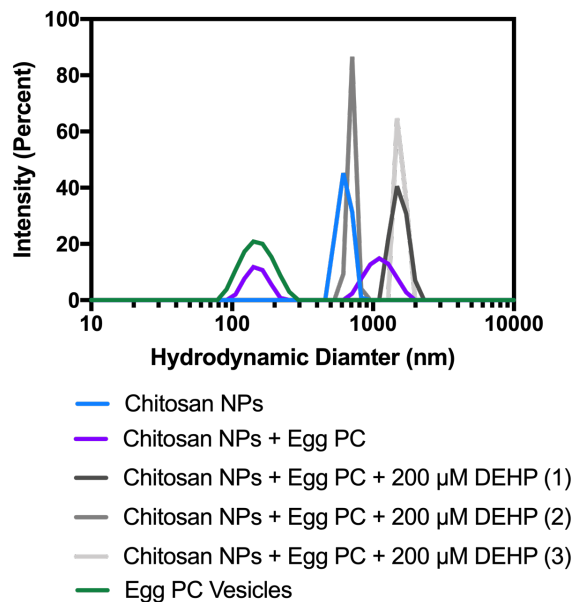


Figure SI 5.4: Size distributions of chitosan NPs, chitosan NPs with egg PC vesicles, and three repeats of chitosan NPs with egg PC vesicles and 200 μM DEHP.

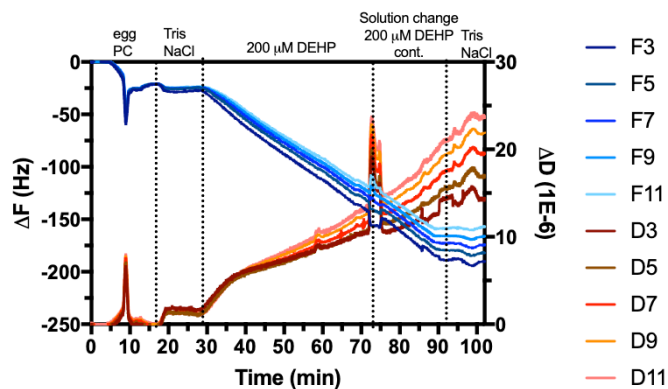


Figure SI 5.5: Continued adsorption of 200 μM DEHP on an egg PC lipid bilayer. During DEHP solution change, a spike in dissipation occurred.

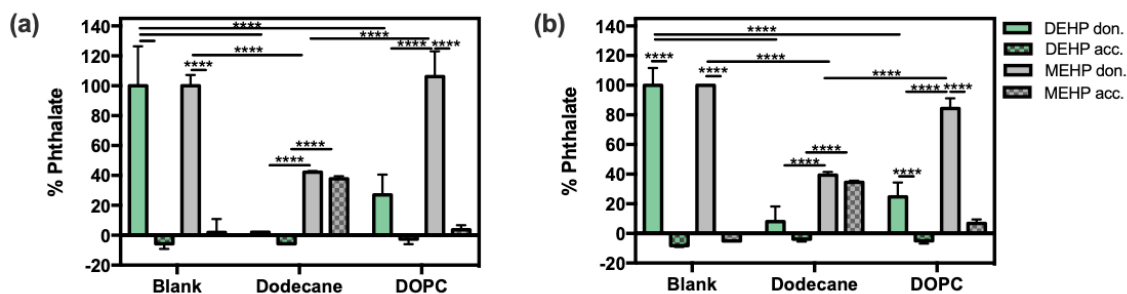


Figure SI 5.6: DEHP and MEHP parallel artificial membrane permeability assay for blank, dodecane, and DOPC conditions. (a) Percentage of 100 μ M DEHP and MEHP in donor and acceptor compartments of blank PVDF, dodecane, and DOPC membranes. (b) Percentage of 200 μ M DEHP and MEHP in donor and acceptor compartments of blank PVDF, dodecane, and DOPC membranes. The percentage of phthalate is normalized to the blank. Statistics were performed using two-way ANOVA with Tukey post-hoc analysis; $\alpha = 0.05$; ****p<0.0001.

Chapter 6

Development and Application of a Placental Trophoblast Inspired Lipid Bilayer for Cell-Free Molecular Interaction Studies

Chapter 6 is in preparation for submission as an original research article.

Bailey-Hytholt, C.M., Shen, T.L., Nie, B., Tripathi, A., Shukla, A., *Development and application of a placental trophoblast inspired lipid bilayer for cell-free molecular interaction studies*, in preparation.

6.1 Abstract

Here we develop lipid vesicles and bilayers mimetic of the placental trophoblast composition. We have quantified the lipid composition of HTR-8, TCL-1, and primary trophoblast cells, representative of first, third, and term placenta, respectively. Using this composition, we develop synthetic vesicles, supported lipid bilayers, and suspended lipid bilayers. Vesicles were developed using the dry lipid film and extrusion technique and were quantified to have a hydrodynamic diameter of ~ 100 nm with low polydispersity and varying degrees of negative charge. We then used quartz crystal microbalance with dissipation monitoring (QCM-D) in order to rupture the vesicles on a silica substrate using a previously described α -helical (AH) peptide to induce vesicle rupture. Next, we developed suspended trophoblast bilayers in order to perform transport studies and compare these results with transport across trophoblast cells. The goal of this work was to both characterize and develop these structures, as well as test the application of them. Test molecules including di(2-ethylhexyl) phthalate (DEHP), a common plasticizer and environmental toxicant, amphotericin B, a commonly used antifungal, and AmBisome, the liposomal formulation of amphotericin B, were investigated for their interaction with the trophoblast bilayers. Interestingly, we observed that AmBisome interacts less with both the supported and suspended trophoblast bilayers in comparison to amphotericin B. Ultimately, these lipid bilayers can serve as a platform for quicker initial screenings of how molecules may interact with the maternal-fetal interface to better determine next steps for testing.

6.1.1 Keywords

placenta, trophoblast, lipid bilayer, lipid vesicle, parallel artificial membrane permeability assay, quartz crystal microbalance with dissipation, di(2-ethylhexyl) phthalate, amphotericin B, AmBisome

6.2 Introduction

Humans are continuously exposed to exogenous small molecules. This exposure is often most carefully considered during pregnancy. Exposure to various small molecules, particularly pharmaceutical agents and environmental toxicants, have been linked with birth defects, loss of pregnancy, prematurity, developmental disabilities, and infant death.¹ Currently, the risk of exposure to a developing fetus is understood for less than 10% of pharmaceutical agents.¹ Meanwhile 90% of women in the United States take at least one medication throughout pregnancy.¹ Many chronic conditions, such as epilepsy,^{243,244} high blood pressure,²⁴⁵ asthma,^{246,247} and depression,^{248,249} often require medications that are difficult to discontinue during pregnancy. Furthermore, conditions such as gestational diabetes^{250,251} and preeclampsia²⁵² can develop during pregnancy requiring pharmaceutical intervention. The Centers for Disease Control and Prevention (CDC)'s Treating for Two program emphasizes the need for more information to better inform treatments during pregnancy.¹ Similarly, although exposure to environmental toxicants has been linked with decreased oocyte yield^{60,61} and increased risk for gestational diabetes⁶² and miscarriage,⁵⁹ the exact mechanisms are not entirely understood. Improving understanding of small molecule interactions with the placenta, which is the primary maternal-fetal interface, can provide this information.

The placenta has one of the most important roles in controlling fetal development during pregnancy, yet it remains one of the least understood organs.^{5,253,254} Placental dysfunction has been associated with increased perinatal and neonatal mortality and lifelong health complications for the mother and child.⁵ However, techniques available for assessing placental function are generally qualitative.²⁵⁵ There are highly limited human placenta-representative *in vitro* and *in vivo* models, making it difficult to readily study this dynamic organ. Animal models, most commonly mouse models, for placental studies are controversial,¹⁶ as the placenta is a highly species specific organ.^{64,65} Recent studies have begun development of *in vitro* cell-based placenta models, including microfluidic “placenta-on-a-chip” devices^{256,257} and three-dimensional placental microtissue models.^{64,74} However, currently there is no cell-free model of the placenta; such models can enable rapid, cost-effective screening of small molecules and their potential impact to placental structure, which can influence prenatal and maternal health.

The overall goal of this work was to develop cell-free *in vitro* lipid bilayer models that mimic the lipid composition of placental trophoblast cells at different time points during pregnancy, which were then used to explore small molecule interactions. Lipid models have previously been developed for biological interfaces including the blood-brain barrier,^{239,258} buccal mucosa,²⁵⁹ skin,^{260–262} and intestinal tract²⁶³.¹⁰¹ Synthetic lipid membranes are used commercially to screen for intestinal drug absorption, and studies have compared these models to the Caco2 cell line.^{226,227,241} Lipid bilayer models of placental trophoblast cells have not previously been reported. Given the important functional roles of lipids in biological tissues and cells (e.g., structural and barrier properties, energy storage, signaling, protein aggregation, etc.),²⁶⁴ we hypothesize that a lipid-based placental

model will successfully serve as a screening tool for molecular interactions representing interactions with the placenta.

To formulate our lipid bilayer models, we utilized HTR-8 and TCL-1 trophoblast cell lines and primary trophoblast cells, representing different time points during pregnancy, where HTR-8 are representative of first trimester trophoblasts, TCL-1 are representative of third trimester trophoblasts, and the primary cells are representative of term placenta.²⁶⁵ The lipid profile of these cell types had not previously been characterized.¹³⁸ Thus, we first investigated the lipid composition of these cell types. Based on these compositions, we formulated synthetic lipid bilayers mimicking the composition of the major lipid classes identified for each trophoblast cell type.

To demonstrate the utility of these placenta mimicking lipid bilayers for small molecule interaction studies, we focused on two molecules of interest, di(2-ethylhexyl) phthalate (DEHP) and amphotericin B. DEHP is a common plasticizer used in many household products, including shoes, toys, medical tubing, and food packaging.^{266,267} It readily leaches from plastics making it an environmental toxicant,²⁶⁸ and recent studies have demonstrated that acquiring higher concentrations of phthalates, such as DEHP, increases risk for miscarriage.⁵⁹ Amphotericin B is a highly effective antifungal drug. During pregnancy, there is an increased vulnerability to fungal infections. For example, vulvo-vaginal candidiasis occurs in up to 20% of pregnant women and systemic fungal infections (which can have mortality rates ranging from 46-75-%²⁶⁹) are more severe during pregnancy. The potential fetal toxicity of antifungals used in treatment of such infections, including amphotericin B, is concerning.¹⁰⁶ By improving these understandings, risk to a

developing fetus can be minimized, treatment plans can be better informed, and new therapies can be screened.

Here we formed our trophoblast inspired lipid bilayers both supported on a surface using quartz crystal microbalance with dissipation monitoring (QCM-D) and suspended on a porous insert. QCM-D enabled monitoring of successful bilayer formation for our three different trophoblast cells, and subsequent monitoring of small molecule interactions with these bilayers (e.g., lipid removal, adsorption, and embedment in the membrane). Suspended bilayers were utilized to monitor interactions via a parallel artificial membrane permeability assay (PAMPA), which also enabled investigation of transport across bilayers and direct comparison with HTR-8 and TCL-1 cell monolayers on transwells. Overall, we observed significant differences between trophoblast lipid composition based on representative gestational time point, which impacted the observed interactions with amphotericin B. We observed the adsorption of DEHP to all trophoblast lipid bilayers, which can impact the integrity of the structures. Finally, we observed in both the supported and suspended lipid bilayers that AmBisome, the liposomal formulation of amphotericin B, interacts less with the trophoblast bilayers compared to free drug, which demonstrates implications for considering drug delivery design for treatment during pregnancy.

6.3 Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water (Milli-Q, 18.2 M Ω , EMD Millipore, Taunton, MA) was used for all experiments requiring water.

6.3.1 TCL-1, HTR-8, and primary cell culture

TCL-1 and HTR-8 trophoblast cell lines were kindly donated by Women & Infants Hospital Kilguss Research Institute (Providence, RI). TCL-1 and HTR-8 cells were cultured in Rosewell Park Memorial Institute medium 1640 (Gibco, Waltham, MA) (RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS) (Corning, Corning, NY) and 100 U/mL penicillin and 100 μ g/ml streptomycin (1% (v/v) penicillin-streptomycin) (Gibco, Waltham, MA)) at 37°C under 5% CO₂. Human primary trophoblasts (purchased from ScienCell Research Laboratories, Carlsbad, CA) were cultured following this same protocol.

6.3.2 Lipid extraction and characterization from HTR-8, TCL-1, and primary trophoblast cells

Lipids were extracted separately from the TCL-1, HTR-8, and primary trophoblast cells following the Bligh-Dyer procedure.²⁷⁰ Briefly, upon achieving 80% confluence, cells were detached via a 5 min incubation in trypsin-ethylenediaminetetraacetic acid at 37°C. Cells were then centrifuged at 1,000 rpm for 5 min. Cell pellets were resuspended in 1 mL of water. A 1:2 (v/v) mixture of chloroform:methanol (3.75 mL) was added to the suspended cell pellet. The solution was vortexed for 15 min before adding 1.25 mL of chloroform and mixing. An additional 1.25 mL of water was added and mixed for 1 min prior to centrifugation at 1000 g for 10 min. The bottom layer of liquid where the lipids are present in the organic phase was collected and the organic solvent was removed by drying under an ultrapure nitrogen gas stream and lipids were then quantified using liquid chromatography with mass spectrometry (LC-MS).

LC-MS was used to identify the extracted lipid composition for each cell type (extracts from three separate cultures per cell type were analyzed). LC-MS experiments employed a Thermo Surveyor MS pump and Thermo Micro AS sampler, and Waters Sunfire C18, 5 mm, 2.1 x 50 mm high performance liquid chromatography (HPLC) column. Mobile phases were prepared as: (A) 60:40 (v/v) acetonitrile:water with 10 mM ammonium formate, (B) 90:10 (v/v) isopropanol:acetonitrile with 10 mM ammonium formate. The gradient started at 35% (v/v) B and increased to 95% (v/v) B in 60 min. The HPLC system was coupled to Thermo LXQ linear ion trap mass spectrometer, equipped with an electrospray ionization source. The HPLC effluent was detected in negative ionization mode, with consecutive full scan MS and tandem MS/MS experiments. Identification of the individual phospholipid molecular species was based on their mass-to-charge (m/z) ratios, and analysis of the product ions spectra produced from collision induced dissociation fragmentation. Once the m/z ratios of the major lipid species were identified, extracted ion chromatograms were obtained and the area under the curve was integrated to determine the abundance of the lipid ion. Results of phospholipids using full scan LC-MS were expressed as relative abundance in percentage. A differential ion mobility system suitability Lipidomix kit (Avanti Polar Lipids, Alabaster, AL) was used to determine the composition of the total major lipid classes within the samples.

6.3.3 Trophoblast lipid composition-inspired synthetic vesicle preparation

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), L- α -phosphatidylinositol (soy) (Soy PI), and sphingomyelin (egg, chicken)

(SPH) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and stored in chloroform at -20°C. POPC, POPE, POPS, Soy PI, and SPH were dried under ultra-high purity nitrogen gas (TechAir, Providence, RI) at the desired ratios obtained from the trophoblast cell lipid extractions. The dried lipid film was kept under vacuum for at least 4 h to remove excess chloroform. Dried lipids were rehydrated in a Tris sodium chloride (NaCl) buffer containing 10 mM Trizma base and 100 mM NaCl, yielding a final concentration of 2.5 mg/mL lipids in the form of multilamellar vesicles in Tris NaCl (pH 7.8). These vesicles were then subjected to five freeze-thaw cycles and extruded 10 times (LF-50 Extruder, Avestin, Ottawa, Canada) through a 100 nm pore size polycarbonate membrane (Nuclepore Track-Etch Membrane Filtration Products, Whatman, Maidstone, U.K.) to form large unilamellar vesicles (LUVs) as previously described.²²⁴ LUVs were stored under nitrogen gas at 4°C. LUVs were termed HTR-8, TCL-1, or primary vesicles based on which lipid extract composition they utilized, and subsequent lipid bilayers were similarly named.

Vesicles were examined for their hydrodynamic size distribution and polydispersity using dynamic light scattering (DLS) (Malvern Zetasizer ZS90 with Zetasizer 7.01 software). ζ (zeta) potential measurements were also performed using the Zetasizer and a folded capillary zeta cell (Malvern Panalytical, Westborough, MA). The lipid composition of the vesicles was confirmed by lyophilizing (FreeZone 4.5 L, Labconco, Kansas City, MO) the LUV sample and performing LC-MS measurements as described in *Lipid extraction and characterization from trophoblast cells*.

6.3.4 QCM-D measurements of lipid bilayer formation and molecular interactions

A QCM-D E4 system (Biolin Scientific, Västra Frölunda, Sweden) was used to monitor lipid vesicle adsorption and bilayer formation as we have previously described.²²⁴ Briefly, QCM-D monitors frequency changes (ΔF) and dissipation changes (ΔD) on quartz QCM-D substrates in real-time. Changes in frequency are inversely related to mass changes, while dissipation changes are related to viscoelasticity. Silica-coated substrates used in all experiments were cleaned with a water, 2% (w/v) sodium dodecyl sulfate, and water rinse sequence, followed by drying with N₂ and UV/ozone treatment (UV/ozone ProCleaner, Bioforce Nanosciences, Salt Lake City, UT) prior to experimental use. All studies were performed at 25°C at a flow rate of 175 μ L/min. A baseline ΔF and ΔD was first established in Tris NaCl for a period of at least 5 min. HTR-8, TCL-1, or primary vesicles were then introduced into the QCM-D flow chamber.

6.3.4.1 Trophoblast lipid composition-inspired supported lipid bilayer formation

We observed that the trophoblast vesicles were able to adsorb to a silica surface without spontaneous rupture. An α -helical (AH) peptide previously demonstrated to induce bilayer rupture for multicomponent vesicles (peptide sequence: H-Ser-Gly-Ser-Trp-Leu-Arg-Asp-Val-Trp-Asp-Trp-Ile-Cys-Thr-Val-Leu-Thr-Asp-Phe-Lys-Thr-Trp-Leu-Gln-Ser-Lys-Leu-Asp-Tyr-Lys-Asp-NH₂)^{198,271–274} was synthesized using a PS3 Peptide Synthesizer (Gyros Protein Technologies, Tucson, AZ). The target molecular weight (3820 Da) of the synthesis products was verified using LC-MS. For QCM-D experiments, after vesicles were adsorbed to the silica surface, 13 μ M AH peptide (in 1% DMSO (v/v)) was introduced in the chamber and incubated with the adsorbed vesicles for 10 min followed by rinsing

with Tris NaCl, leading to successful vesicle rupture and formation of a lipid bilayer. The removal of the AH peptide from the bilayer after vesicle rupture was confirmed by removing the silica crystal from the QCM-D chamber and rinsing with 2 mL of 2% SDS to solubilize the adsorbed components. This rinse solutions were collected and examined using absorbance readings at 280 nm and a Pierce quantitative colorimetric peptide assay (ThermoFisher, Waltham, MA) and comparing with AH peptide standards of known concentration.

6.3.4.2 Interaction studies

The interactions of DEHP, amphotericin B, and AmBisome (obtained from Brown University pharmacy (Providence, RI)) (a liposomal formulation of amphotericin B) were investigated via QCM-D with HTR-8, TCL-1, and primary supported lipid bilayers. For DEHP interaction studies, DEHP was flowed over the bilayers at 200 μ M (within the typical daily acquisition range)²¹⁰ in 1% (v/v) DMSO. Amphotericin B was flowed over the bilayers at 0.1 M (i.e., 0.1 mg/mL, which is a typical clinically utilized infusion concentration),^{275,276} in 1% v/v DMSO. For both DEHP and amphotericin B interaction studies, 1% (v/v) DMSO was introduced before and after the DEHP for 5 min to account for ΔF and ΔD changes due to viscosity changes between Tris NaCl and 1% (v/v) DMSO. Finally, for AmBisome interaction studies, AmBisome was flowed over the bilayers at an equivalent amphotericin B concentration of 0.1 M (i.e., 26.5 mg/mL AmBisome in 67.8% (w/w) sucrose and 2% (w/w) succinic acid in water, the clinically utilized suspension buffer). A buffer solution with equivalent amounts of sucrose and succinic acid in water was also flowed before and after AmBisome for 5 min to account for ΔF and ΔD changes

due to viscosity changes between Tris NaCl and the sucrose with succinic acid buffer solution. ΔF and ΔD were monitored throughout each flow step.

6.3.5 Suspended lipid bilayer interactions using parallel artificial membrane permeability assay (PAMPA)

A parallel artificial membrane permeability assay (PAMPA) was developed for suspended lipid bilayers using polyvinylidene difluoride (PVDF) 96-well multiscreen filter plates (0.45 μm pore size) and transport receiver plates (Millipore-Sigma, Burlington, MA) to investigate DEHP, amphotericin B, and AmBisome diffusion from a donor compartment, through the porous membrane treated with lipid, to an acceptor compartment. To form suspended lipid bilayers, first 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), Soy PI, and SPH were solubilized in dodecane at a concentration of 20 mg/mL. The lipid classes were then mixed at the same ratio as quantified from the HTR-8, TCL-1, and primary trophoblast lipid extractions. Lipid mixtures (5 μL) were added to the porous PVDF membrane and immediately submerged in 1 $\times$ phosphate buffered saline (PBS) in the donor and acceptor compartments (200 μL and 300 μL , respectively) forming a suspended lipid bilayer. All 1 $\times$ PBS was filtered through a 0.22 μm sterile filter prior to use. Controls of filters introduced to dodecane only (5 μL) and untreated filters also exposed to the donor and acceptor solutions were included. The donor compartments were then filled with 200 μM DEHP, 0.1 M amphotericin B, and AmBisome (26.5 mg/mL, i.e., 0.1 M amphotericin B) in a 200 μL volume of 1 $\times$ PBS. The acceptor compartment was filled with 300 μL of 1 $\times$ PBS. For DEHP and amphotericin B, a 1% (v/v) DMSO in 1 $\times$

PBS was utilized instead in both donor and acceptor compartments to promote small molecule solubility. The plate was rocked for 2 h at 25°C prior to collecting 150 µL of solution from the donor and acceptor compartments. The absorbance of DEHP, amphotericin B, and AmBisome in the solutions was measured at 280 nm, 340 nm, and 320 nm, respectively (i.e., the measured absorbance maximums), using a plate reader (Cytation 3, BioTek, Winooski, VT) and compared with standards with known DEHP, amphotericin B, and AmBisome concentration. Permeability was calculated on a logarithmic scale for comparison studies using the following equations:

$$\log P_{app} = \log\left\{C \cdot -\ln\left(1 - \frac{[small\ molecule]_{acceptor}}{[small\ molecule]_{initial}}\right)\right\} \quad (1)$$

$$\text{Where } C = \frac{V_D \cdot V_A}{(V_D + V_A)A \cdot t} \quad (2)$$

In equation (1), $[small\ molecule]_{acceptor}$ is the concentration of the analyte (e.g., DEHP, amphotericin B, and AmBisome) in the acceptor compartment and $[small\ molecule]_{initial}$ is the initial concentration of analyte. In equation (2), A is the membrane area, t is the time, V_D is the donor compartment volume, and V_A is the acceptor compartment volume.

6.3.6 Interaction studies with trophoblast cells

Transwell inserts (24 well plate, Corning 3470, Corning, NY) were prepared for trophoblast culture as previously described.²⁷⁷ Briefly, human placental collagen coating material was formed by dissolving placental collagen in 0.1% (v/v) acetic acid, which was stored at 4°C. Before immediate use, the stock of placental collagen was diluted 1:3 v/v in 70% (v/v) ethanol. Plates were sterilized for 20 min under UV prior to use. In a 24 well plate insert, 21 µL of the placental collagen solution was added to each well and dried for

2 h at 25°C. The wells were then hydrated in 1× PBS for ~30 min at 25°C. HTR-8 and TCL-1 cells were plated on the transwell inserts (30,000 cells/mL). The confluence of the cells on the transwells was visualized by first fixing the cells using 4% paraformaldehyde for 15 min at 25°C and then staining with Alexa Fluor 594 phalloidin (Life technologies, Carlsbad, CA) to label F-actin and 4',6-diamidino-2-phenylindole (DAPI) for nuclei. Fluorescence imaging was performed using a Nikon Ti-E inverted fluorescent microscope with NIS Elements software (version 4.20.02). Once confluent, transport studies with DEHP, amphotericin B, and AmBisome were performed similar to *Suspended lipid bilayer interactions using parallel artificial membrane permeability assay (PAMPA)*. For these transwell plates, the donor and acceptor compartment was filled with 200 μ L of 1× PBS containing the small molecule of interest and the acceptor compartment was filled with 500 μ L of 1× PBS (both donor and acceptor compartments contained 1% (v/v) DMSO for DEHP and amphotericin B transport studies). The plates were incubated for 2 h at 37°C prior to absorbance measurements of the donor and acceptor compartment solutions for DEHP, amphotericin B, and AmBisome at 280 nm, 340 nm, and 320 nm, respectively, using a plate reader (Cytation 3, BioTek, Winooski, VT). Permeability was calculated as previously described in *Suspended lipid bilayer interactions using parallel artificial membrane permeability assay (PAMPA)*.

6.3.7 Statistical analysis

All experiments were conducted in triplicate at a minimum. Representative results for the third overtone are shown in figures for all QCM-D ΔF and ΔD measurements, unless otherwise specified. Results are given as mean $\pm$ standard deviation whenever appropriate.

Statistical significance was calculated using Prism 7 (GraphPad) with one-way analysis of variance (ANOVA) or two-way ANOVA where applicable. Tukey's post hoc analysis was performed for ANOVA tests ($\alpha = 0.05$; $p < 0.05$ was considered statistically significant).

6.4 Results and Discussion

6.4.1 Characterization of trophoblast lipids

Extracted lipids from HTR-8, TCL-1, and primary trophoblast cells were isolated and analyzed using LC-MS. These three cell types are indicative of different time points during pregnancy where HTR-8, TCL-1, and primary cells are representative of first, third, and term placenta, respectively.²⁷⁸ We observed differences in the fatty acid distributions within each lipid class (Figure SI 6.1). Figure 6.1 shows the composition of the major lipid classes relative to one another. We observe significant differences in phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) composition between HTR-8, TCL-1, and primary bilayers. Interestingly, the TCL-1 composition has an increased amount of negatively charged lipids PI and PS, with a decreased amount of zwitterionic PC compared to the HTR-8 and primary compositions.

Previous studies have investigated the lipid composition of the placenta.^{279–281} Here we indicate for the first time the lipid composition of specifically trophoblast cells. While trophoblast cells are the main cell type comprising the placenta, other cell types are present including mesenchymal cells, mesenchymal derived macrophages, fibroblasts, and fetal vascular cells (including vascular smooth muscle cells, pericytes, and endothelial cells).²⁸² Trophoblast cells, particularly villous trophoblast cells, are responsible for the nutrient and waste transport across the placenta. By mimicking this composition, we are able to focus

on the lipids responsible for transport, which is the goal of this platform. Trophoblast cells are dynamic and undergo changes throughout the course of pregnancy. More studies are required in order to understand the cause for the different lipid compositions presented here. One possible reason includes changes in protein expression throughout pregnancy, where lipid composition is an important backbone structure for the proteins present in the lipid bilayer.²⁸³ Another possible reason is to vary the electrostatics of the placenta²⁸⁴ in order to control transport requirements at the given stages of pregnancy (both nutrients and unwanted substances).

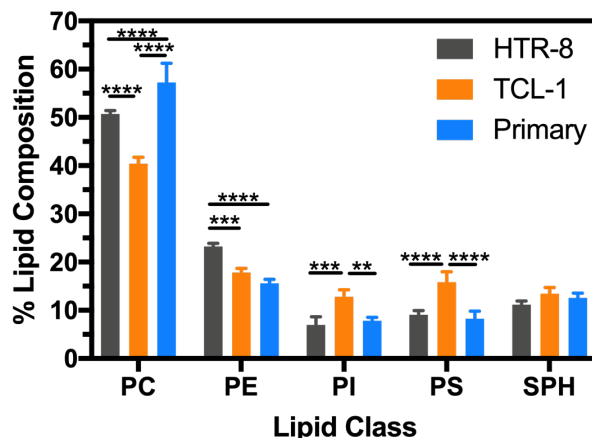


Figure 6.1: Composition of major lipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SPH) for HTR-8, TCL-1, and primary cell extracts. Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc analysis; $n=3$; $\alpha=0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

6.4.2 Development and characterization of synthetic placental vesicles

Using the lipid compositions from Figure 6.1, next we developed lipid vesicles using synthetic lipids with the above composition. Vesicles were developed using the dry lipid film technique and extrusion through a 100 nm polycarbonate membrane. Using DLS and

ζ potential measurements we identify the formation of uniform vesicles with a hydrodynamic diameter of ~ 100 nm for each composition, with low polydispersity for each vesicle (Figure 6.2a and 6.2b). The ζ potential differences between vesicle types were statistically significant with TCL-1 being the most negative and primary vesicles being the least negative (Figure 6.2c). We expected the TCL-1 vesicles to have the most negative charge due to the higher presence of PI and PS lipids within the composition. We also observe that primary trophoblast cells are the least negative vesicle, which was also anticipated due to this composition having the highest amount of PC.

We also investigated that the synthetic lipid vesicles have the expected compositions. Since these are complex vesicles with multiple lipids that can potentially form lipid rafts, we wanted to ensure that some lipids were not aggregating and remaining within the extruder and loading to different compositions. Figure SI 6.2 shows a comparison between our lipid composition of our vesicles and the cell extracts, demonstrating that the vesicles contain within 3% of the composition for all lipids and vesicle types.

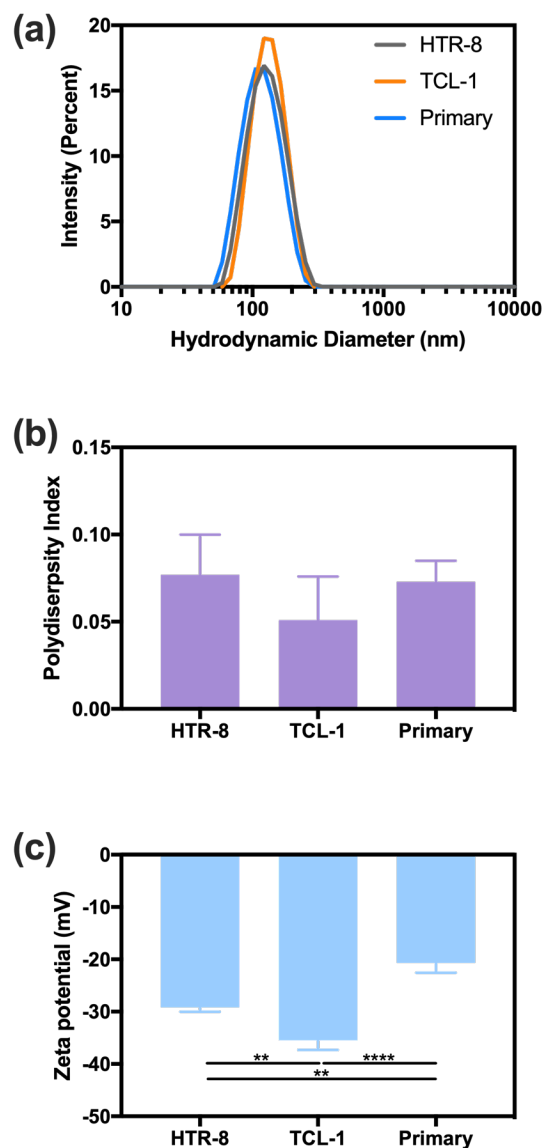


Figure 6.2: Synthetic placental vesicle characterization. (a) Hydrodynamic size distributions for vesicles formed using HTR-8, TCL-1, and primary lipid compositions. (b) Polydispersity of vesicles. (c) ζ potential for each vesicle composition. Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc analysis; $n=3$; $\alpha=0.05$; ** $p<0.01$; **** $p<0.0001$.

6.4.3 Supported placental bilayer formation using AH peptide with QCM-D

Next, our goal was to develop supported lipid bilayers with the representative placental compositions. Vesicles adsorb to the silica-coated substrates as observed by the initial ΔF decrease and ΔD increase within the first 30 min (Figure 6.3). Notably, we observe

differences in the amount of vesicle adsorption between the different compositions. TCL-1 vesicles adsorb the least, likely due to the electrostatics between the negatively charged vesicles and negative charge on the silica surface.

After a Tris NaCl rinse, AH peptide is incubated in order to rupture the vesicles into a SLB. This is observed at the final Tris NaCl rinse, where the ΔD for all bilayer types decreases. This indicates a more rigid structure, which is expected of bilayer formation.^{162,285} The ΔF resulting for HTR-8, TCL-1, and primary bilayers were -21.9 ± 2.6 Hz, -15.6 ± 0.67 Hz, and -30.0 ± 4.35 Hz, respectively. Previous studies have noted that ΔF can vary based on lipid composition of the structures.²⁸⁶

These results further indicate the usefulness of the investigated AH peptide to rupture biologically relevant vesicles into SLBs. AH peptide was derived from the hepatitis C virus's nonstructural protein 5A (NS5A).²⁸⁷ Previous studies have suggested that the peptide destabilizes the lipid vesicle and causes the vesicle to swell, which enhances neighboring vesicle interactions.²⁸⁸ Neighboring vesicle interactions have previously been reported for their importance in rupturing vesicles into SLBs.²²⁴ This peptide has been used to develop lipid bilayers on various substrate materials (e.g. gold)^{198,289} and with complex lipid compositions.^{273,290} Here we utilize it for our complex composition that would not spontaneously rupture without the AH peptide.

Previous studies have investigated the removal of the AH peptide during the buffer rinse. Previous work has used atomic force microscopy to ensure no thickness differences or pore formation caused by AH peptide.²⁸⁶ To confirm that the AH peptide was removed after rupture with the Tris NaCl rinse in our studies, we removed the QCM-D crystal from the chamber and rinsed with 2% SDS in order to remove and collect the lipid layer. We then

performed both absorbance measurements and a colorimetric peptide assay, which showed that no peptide remained embedded within or adsorbed to the lipid bilayer (Figure SI 6.3).

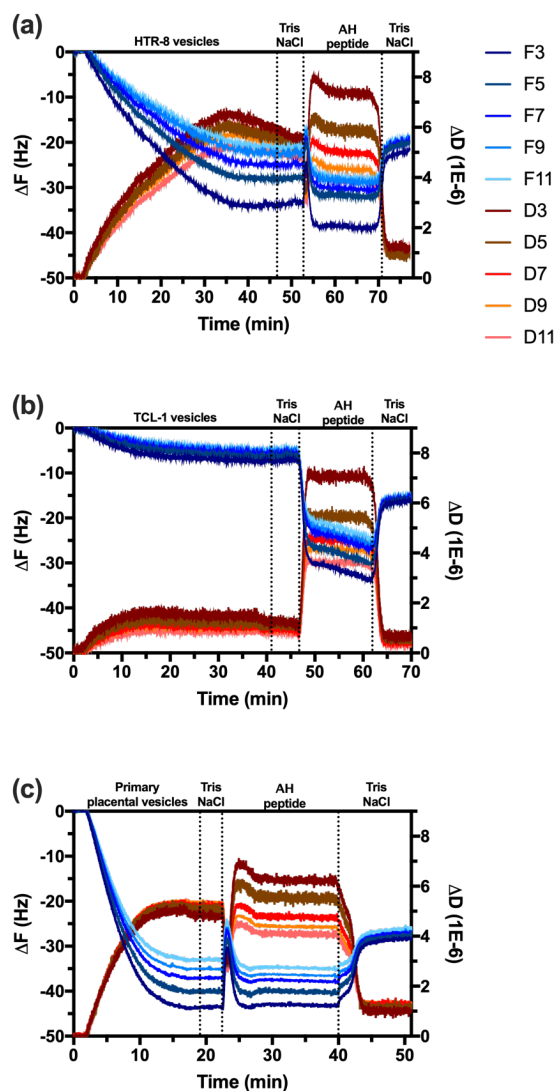


Figure 6.3: Supported lipid bilayer formations using QCM-D for (a) HTR-8, (b) TCL-1, and (c) primary bilayers. Frequency changes (ΔF) and dissipation changes (ΔD) are shown in blue and red, respectively for overtones 3, 5, 7, 9, and 11.

6.4.4 Trophoblast bilayer interaction studies using QCM-D

With the developed and characterized trophoblast lipid bilayers, we next sought to investigate some test molecules and their interactions with these structures. DEHP is a

commonly used plasticizer that easily leaches from many household products (Figure 6.4a). For a variation of compounds, we also investigated a common pharmaceutical. We chose amphotericin B, which is a commonly used antifungal (Figure 6.4b). AmBisome, the liposomal formulation of amphotericin B, is also commonly used. We chose to investigate this nanoparticle in order to observe how delivery carrier may impact interactions with the trophoblast bilayers.

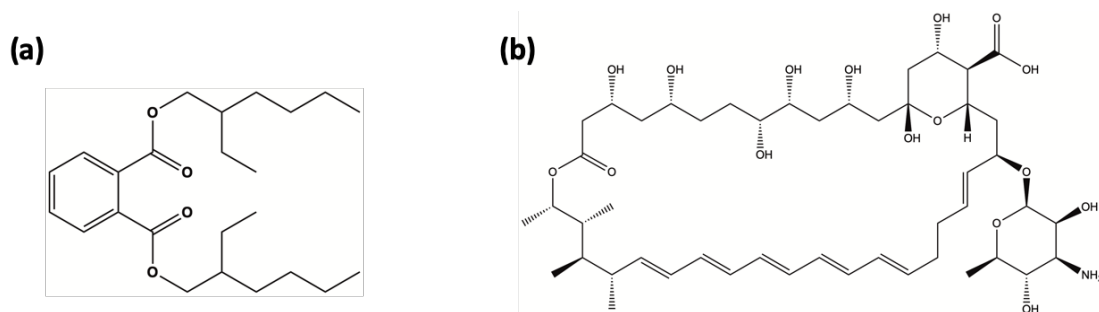


Figure 6.4: Molecular structures of (a) DEHP and (b) amphotericin B. DEHP is a potential environmental toxicant used as a plasticizer in many household products. Amphotericin B is an antifungal pharmaceutical agent. AmBisome is the liposomal formulation of amphotericin B.

After forming the trophoblast bilayers as previously discussed, we allowed 200 μM of DEHP to flow across the structures (Figure 6.5a-c). In all cases, we observed a ΔF decrease of -13.7 ± 4.7 , -16.4 ± 5.2 , and -9.4 ± 3.0 for HTR-8, TCL-1 and primary, respectively, indicating adsorption of DEHP to the trophoblast bilayers (Figure 6.5d). ΔD indicated a significant difference in rigidity of the bilayers after DEHP interaction between the TCL-1 ($5.2 \times 10^{-6} \pm 1.3 \times 10^{-6}$) and primary bilayers ($2.5 \times 10^{-6} \pm 0.9 \times 10^{-6}$) (Figure 6.5e). This suggests that there is dependence of the DEHP interaction based on different compositions. The TCL-1 bilayers were influenced the most due to DEHP exposure, resulting in increased softness and fluidity of the adsorbed layer.

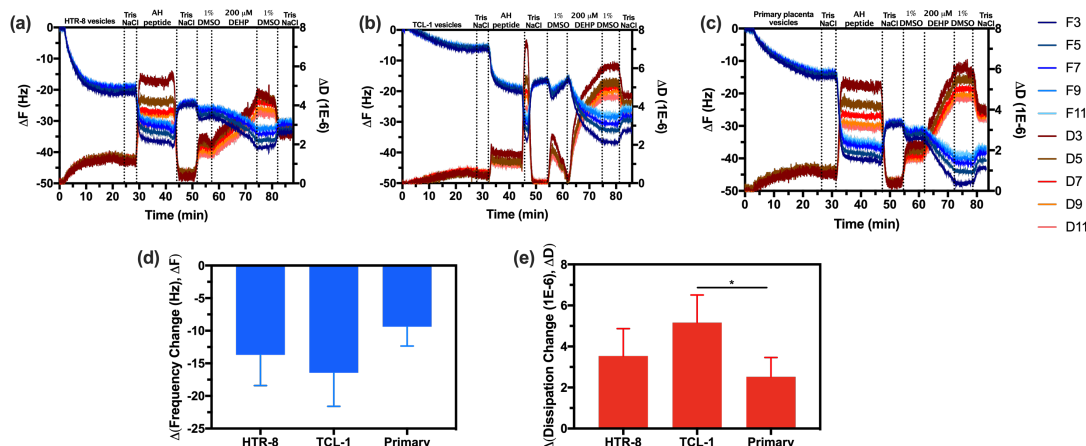


Figure 6.5: DEHP interaction with (a) HTR-8, (b) TCL-1, and (c) primary trophoblast lipid bilayers. Frequency changes (ΔF) and dissipation changes (ΔD) are shown in blue and red, respectively for overtones 3, 5, 7, 9, and 11. Change in (d) ΔF and (e) ΔD after DEHP flow for HTR-8, TCL-1, and primary bilayers. Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc analysis; $n=3$; $\alpha=0.05$; * $p<0.05$.

Next, we investigated how amphotericin B and AmBisome interacted with the HTR-8, TCL-1, and primary trophoblast bilayers. After forming these structures, we allowed either amphotericin B (Figure 6.6a) or AmBisome (Figure 6.6b) to flow across. Figure 6.6a and 6.6b are representative QCM-D plots for HTR-8 bilayers. QCM-D traces for all three bilayers with amphotericin B and AmBisome are in Figures SI 6.4 and SI 6.5. Focusing on the ΔF due to amphotericin B or AmBisome with the SLBs, we observed larger ΔF changes due to amphotericin B interaction compared to AmBisome (Figure 6.6c). For example, the amphotericin B resulted in a loss of $\sim 5\%$ of the HTR-8 lipid bilayer mass.

It is also interesting to note the difference in interaction with adsorption of amphotericin B to the TCL-1 bilayers in comparison to mass removal occurring due to amphotericin B with the HTR-8 and primary bilayers. Interestingly, the mechanism of amphotericin B involves interactions with lipid bilayers. Typically, within the fungal cell

amphotericin B interacts with ergosterol present in the cell membrane.²⁹¹ Several mechanisms are debated, but two common mechanisms for amphotericin B involve pore formation and surface adsorption.²⁹¹ Amphotericin B molecules are able to aggregate and form pores within the bilayer.²⁹¹ These pores are often associated with a removal of lipids, resulting in ion leakage.²⁹¹ This mechanism may be occurring with the amphotericin B interactions with HTR-8 and primary trophoblast bilayers, where mass removal is observed. Alternatively, amphotericin B can adsorb to lipid bilayer surfaces, where in fungi the amphotericin B would extract ergosterol, which is a key component for the integrity of the fungal cell.²⁹¹ In the case of our TCL-1 trophoblast bilayers, we observe adsorption of the amphotericin B molecule.

For the AmBisome formulation, we observe little interaction with all three trophoblast bilayers. Likely, these negatively charged 100 nm structures are being repelled by the negatively charged trophoblast bilayers. Ultimately, our trophoblast bilayers enable the detailed interactions of small molecules and can be used in the future to understand other molecular interactions.

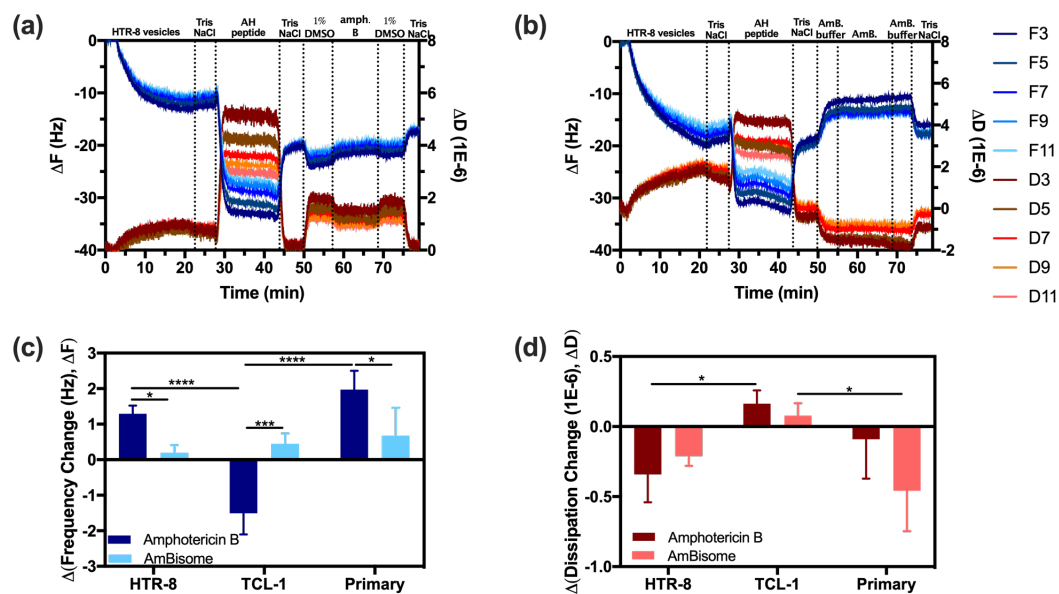


Figure 6.6: Interactions of (a) amphotericin B and (b) AmBisome with HTR-8 placental bilayer. Frequency changes (ΔF) and dissipation changes (ΔD) are shown in blue and red, respectively for overtones 3, 5, 7, 9, and 11. Change in (c) ΔF and (d) ΔD after amphotericin and AmBisome flow for HTR-8, TCL-1, and primary bilayers. Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc analysis; $n=3$; $\alpha=0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

6.4.5 Transport studies comparing suspended trophoblast lipid bilayers with HTR-8 and TCL-1 trophoblast cells

SLBs provide a useful tool to investigate adsorption or mass removal interactions occurring. In order to gain more information, we next developed suspended lipid bilayers across PVDF porous inserts. This developed a parallel artificial membrane permeability assay (PAMPA) to further investigate DEHP, amphotericin B, and AmBisome interactions with HTR-8, TCL-1, and primary lipid models.

HTR-8 and TCL-1 cells were then also cultured on transwell transport plates. This allowed for a comparison of permeability and embedment into the cell layer with the trophoblast lipid bilayers. HTR-8 and TCL-1 cells were cultured on transwell plates coated

with placental collagen. After four days, we observed a confluent layer as confirmed with phalloidin/DAPI imaging (Figure SI 6.6).

Figure 6.7 shows a comparison of the permeabilities of DEHP, amphotericin B, and AmBisome between our HTR-8 and TCL-1 trophoblast bilayers and cells cultured on transwell plates. DEHP results for TCL-1 are not shown because the permeability of DEHP across the TCL-1 bilayers was zero. The graph contains a linear line to indicate where points would be located if there was a direct correlation between our PAMPA model and the cell transport. Overall, we observe that the permeabilities are greater than this linear relationship with the cells, indicating the lipid bilayers may underestimate the amount of permeation. This is common for PAMPA models, because active transport mechanisms are not accounted for.²⁴¹ We do observe that the degree of underestimation is similar for all compounds tested.

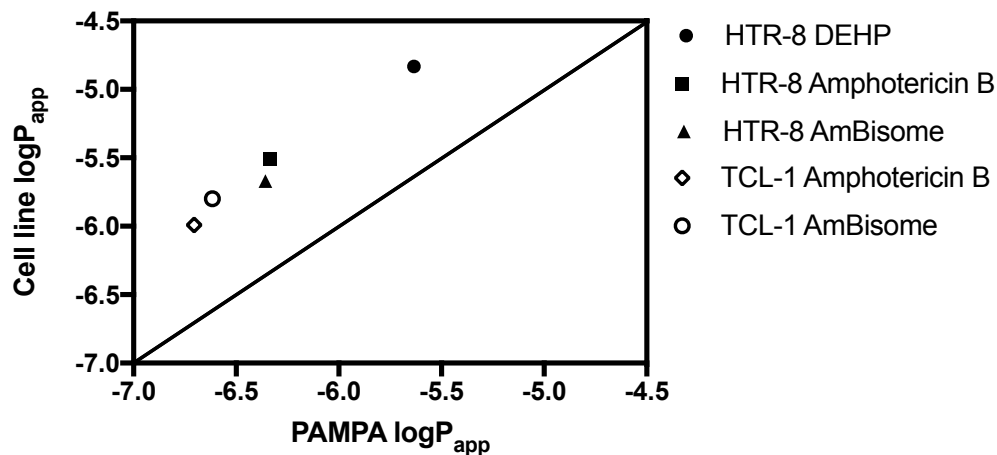


Figure 6.7: Comparison of DEHP, amphotericin B, and AmBisome permeabilities for HTR-8 and TCL-1 placental bilayer PAMPA and cultured cell monolayers. A linear line is drawn to visually show where points would lie if the exact permeability was observed between the two models.

In addition to permeabilities, these methods also allow for investigating how the compounds remain interacted and embedded within either the trophoblast bilayers or cell layers (Figure 6.8). The results observed here demonstrate similar trends in the percentage of pharmaceutical remaining interacted between the trophoblast bilayers and cell monolayers. Overall, we observed a large percentage of amphotericin B remaining within the lipid bilayer or cell layer compared to little interaction with the AmBisome. These results further support the QCM-D results where we observed significantly increased ΔF with the amphotericin B interacting with all trophoblast bilayers compared with little ΔF changes due to AmBisome exposure. Thus, the delivery vehicle does influence interactions with the trophoblast lipid bilayers. Our results suggest that liposomes may help to mitigate some interactions with this biological barrier.

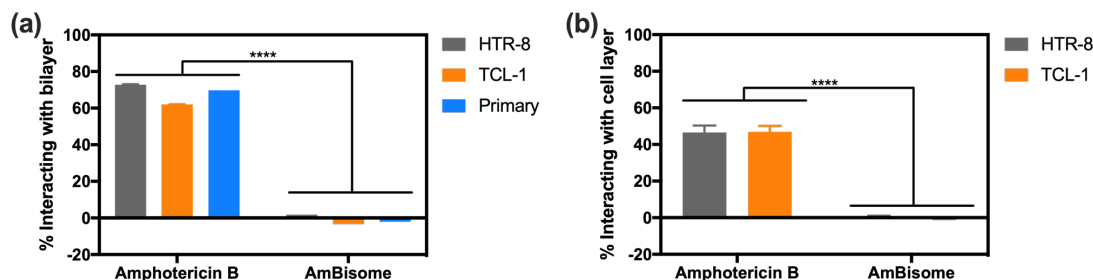


Figure 6.8: Comparison of the percentage of amphotericin B or AmBisome remaining interacted with either the (a) trophoblast lipid bilayers or (b) cell layer. Results are shown as mean $\pm$ standard deviation. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc analysis; n=3; $\alpha=0.05$; ****p<0.0001.

6.5 Conclusions

This work quantified the lipid composition of trophoblast cells at various time points during pregnancy. We observed significant differences in compositions comparing first, third, and term trophoblasts. Using these compositions, we next developed the first synthetic lipid

vesicles and bilayers with representative trophoblast lipid distributions. After characterization of the development of these structures, we then used these structures to investigate interactions of DEHP, amphotericin B, and AmBisome. We observed that DEHP adsorbs to all three of the bilayers, which can have potential to influence transport properties across the interface. Amphotericin B interacted more with both supported and suspended bilayers compared to AmBisome. This result is particularly interesting, suggesting that the delivery vehicle of a pharmaceutical should be considered for treatment needs during pregnancy and that this technique can be useful for these types of studies. We observed similar interaction trends between the bilayers and trophoblast monolayers, indicating correlation of our model. Ultimately, this work provides a platform that can now be used to test a variety of environmental toxicants and pharmaceuticals. While we provide a few examples of these interactions, this system can be useful for quantifying many other small molecules in a high-throughput manner. This will allow for a first screening tool to provide information on what next tests should be performed in order to fully understand risks during pregnancy.

6.6 Author Contributions

This chapter was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

6.7 Acknowledgements

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6.8 Supplemental Information

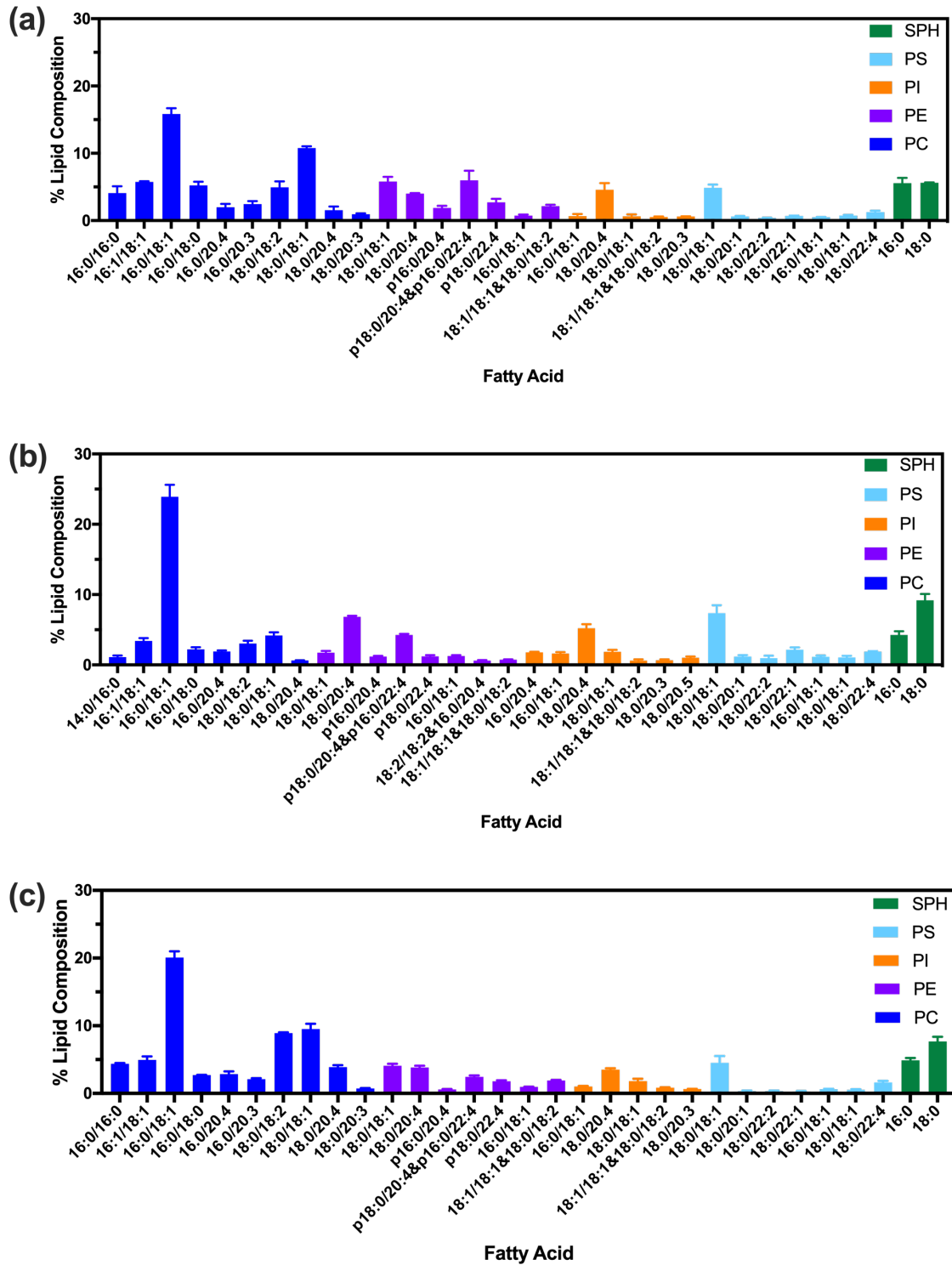


Figure SI 6.1: Lipid composition with all fatty acid distributions for (a) HTR-8, (b) TCL-1, and (c) primary cells.

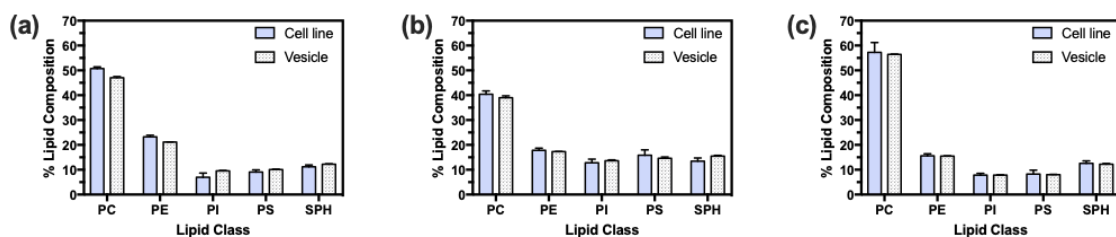


Figure SI 6.2: Lipid compositions quantified for (a) HTR-8, (b) TCL-1 and (c) primary cell lines compared to the synthetic vesicles developed using the dry lipid film technique and extrusion.

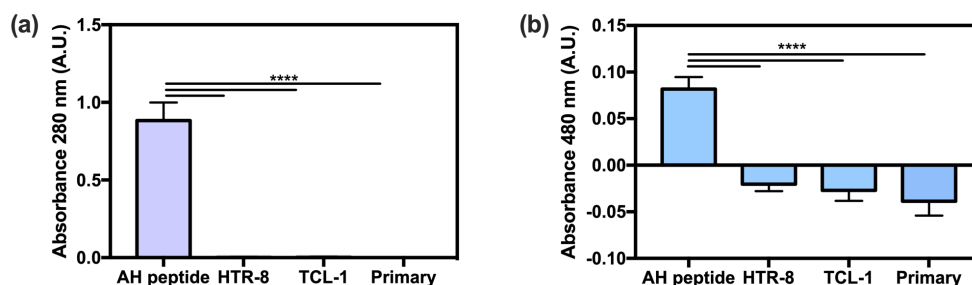


Figure SI 6.3: Confirmation of peptide removal after rupture of supported lipid bilayers. (a) Absorbance measurement for AH peptide at the experimental concentration. After HTR-8, TCL-1, and primary bilayer formation, QCM-D silica coated quartz crystals were rinsed with 2% SDS to remove the adsorbed layer. (b) Absorbance measurements for AH peptide using a pierce quantitative colorimetric peptide assay. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc analysis; $n=4$; $\alpha=0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

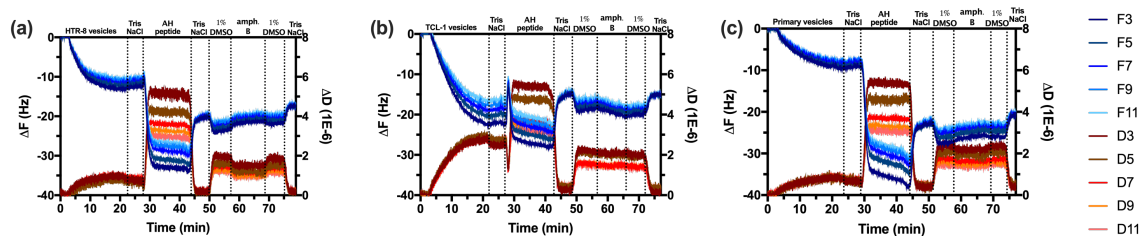


Figure SI 6.4: Amphotericin B interactions with (a) HTR-8, (b) TCL-1, and (c) primary trophoblast bilayers via QCM-D. Frequency changes (ΔF) and dissipation changes (ΔD) are shown in blue and red, respectively for overtones 3, 5, 7, 9, and 11.

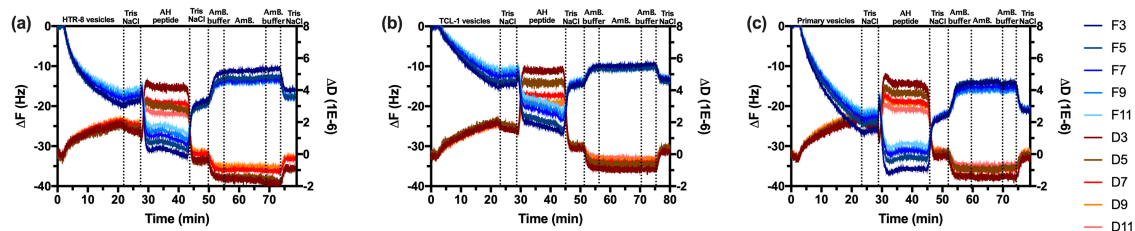


Figure SI 6.5: AmBisome interactions with (a) HTR-8, (b) TCL-1, and (c) primary trophoblast bilayers via QCM-D. Frequency changes (ΔF) and dissipation changes (ΔD) are shown in blue and red, respectively for overtones 3, 5, 7, 9, and 11.

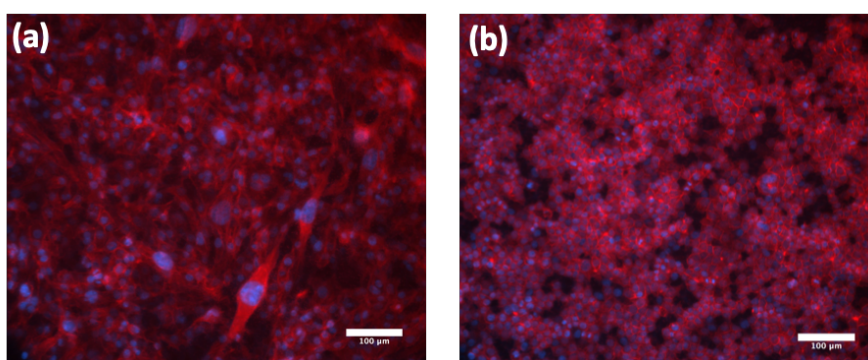


Figure SI 6.6: Trophoblast cell lines (a) HTR-8 and (b) TCL-1 after 4 days of growth on transwell inserts. Cells were stained for actin cytoskeleton in red and nuclei in blue.

Chapter 7

Anidulafungin Nanoparticles Exhibit Antifungal Activity Against Planktonic and Biofilm *Candida albicans*

Chapter 7 has been submitted as an original research article.

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7.1 Abstract

Fungal infections can cause significant patient morbidity and mortality. Nanoparticle therapeutics have the potential to improve treatment of these infections. Here we report the development of liposomal nanoparticles incorporating anidulafungin, a potent antifungal, with the goal of increasing its solubility and aiding in localization to fungi. Liposomes were fabricated with three concentrations of anidulafungin yielding monodisperse ~100 nm unilamellar vesicles. All three formulations inhibited planktonic *Candida albicans* growth at a minimum inhibitory concentration equivalent to free drug. All three formulations also disrupted preformed *C. albicans* biofilms, reducing fungal burden by as much as 99%, exhibiting superior biofilm disruption compared with free drug. Liposome formulations tested *in vivo* in *C. albicans* infected *Galleria mellonella* wax moth larvae demonstrated increased survival compared to free drug equivalents, leading to a survival of 33 to 67% of larvae over 7 days depending on the liposome utilized compared with only 25% survival of larvae administered free drug. Liposomal formulations along with free anidulafungin did not cause red blood cell lysis. Ultimately, the liposome formulations reported here increased anidulafungin solubility, displayed promising efficacy against planktonic and biofilm *C. albicans*, and improved the survival of *C. albicans* infected *G. mellonella* compared to free anidulafungin.

7.1.1 Keywords

Anidulafungin, liposomal nanoparticles, *Candida albicans*, *Galleria mellonella*, fungal infection

7.2 Introduction

Candida albicans is an opportunistic fungus that exists primarily in the gastrointestinal and genitourinary tracts of healthy individuals.²⁹² Although typically harmless, *C. albicans* can cause candidiasis as a result of gastrointestinal mucosal disruption or overgrowth due to the use of broad-spectrum antimicrobial agents.²⁹³ This fungal infection may be localized in one part of the body or spread throughout, resulting in ailments such as thrush, vaginal yeast infection, esophagitis, and candidemia. Candidiasis has a high mortality rate of 33 to 54%,²⁹⁴ which amounts to 2,800 to 11,200 deaths annually in the United States.²⁹⁵ In addition, treatment imposes a large burden on the health care system, costing an estimated 7.2 billion USD annually.²⁹⁶ *Candida* typically exist in biofilms,²⁹⁷ serving as strongholds for infection in which the microorganisms embed themselves within a protective matrix of secreted extracellular polymeric substances.²⁹⁸ This matrix, combined with quiescent persister cells, impart biofilm *Candida* with a high level of drug resistance, making them significantly harder to treat.²⁹⁹

Despite growing antifungal resistance rates, the antifungal drug arsenal remains highly limited in comparison to antibiotics, with only ~10 antifungal therapeutics used clinically for systemic fungal infections.^{300,301} Additionally, most antifungal drugs exhibit significant host toxicity and are hydrophobic, necessitating low-dose administration. Compounded, these antifungal therapy complications can lead to incomplete eradication of the infection, causing recurrent infections sometimes over years,^{302–304} and increasing the occurrence of resistance. Encapsulation of antifungals into delivery vehicles has the potential to improve drug solubility, reduce toxicity, and potentially mitigate growing resistance. Liposomal nanoparticles are particularly promising for these purposes.

Several liposomal formulations are currently utilized clinically to treat conditions including cancer, macular degeneration, influenza, and fungal infections.³⁰⁵ Liposomes have the ability to encapsulate both hydrophobic and hydrophilic molecules consisting of a lipid bilayer particle with an aqueous inner space.³⁰⁶ Liposomes have been shown to improve drug circulation time³⁰⁷ and can change drug biodistribution, leading to increased accumulation at a diseased site (e.g., tumor tissue³⁰⁸). Specifically, for fungal infections, liposomal amphotericin B, a polyene therapeutic (marketed as AmBisome®) was the first, and to date, the most successful commercial nanoparticle formulation of an antifungal drug used systemically. AmBisome has been shown to decrease host toxicity of amphotericin B³⁰⁹ and is the most common treatment for systemic fungal infections caused by *Candida* and *Aspergillus*.³¹⁰ Other antifungal liposome formulations have recently been reported that can enhance skin penetration and increase the stability of antifungals used topically.³¹¹ For example, ultra-deformable amphotericin B liposomes incorporating a surfactant displayed enhanced penetration through the epidermis by a factor of 40 when compared to liposomes lacking the surfactant.³¹² Antifungal liposomes also have the potential to better penetrate fungal biofilms when compared to free antifungal drugs.^{313,314} In this work, we report the development of a new liposomal nanoparticle formulation of anidulafungin, the latest echinocandin drug to receive U.S. Food and Drug Administration (FDA) approval (2006).

The echinocandins, introduced in 2001,³¹⁵ are the newest class of antifungal drugs. These antifungals exhibit excellent fungicidal activity against susceptible *Candida* spp. and are starting to be used as a first line of defense for many fungal infections.^{316–318} The echinocandins function by inhibiting the production of the major fungal cell wall

component, β -1,3 glucan, by binding primarily to the Fks1 transmembrane protein, which serves as the catalytic subunit of the (1,3)- β -D-glucan synthase enzyme complex, causing loss of cell wall integrity.^{317,319,320} While hepatotoxicity and drug interactions are less common with echinocandins than with the earlier developed polyenes and azoles,³²¹ recent findings indicate that echinocandins have increased cardiac toxicity and are associated with decreased left ventricular contractility possibly due to mitochondrial damage.^{322,323} Of the echinocandins, anidulafungin is considered the least toxic.³²⁴ Resistance to this drug class usually occurs due to amino acid mutations in Fks1, but can also involve other complex compensatory mechanisms utilized in conserving cell wall integrity (e.g., the upregulation of chitin).^{325,326} Between 2004 and 2014, the number of echinocandin resistant *C. albicans* strains doubled.³²⁷ Resistance to anidulafungin, as the newest echinocandin, is still relatively rare, but will inevitably arise with increased usage.^{327,328} Anidulafungin is highly water insoluble, and is currently administered intravenously at a low concentration (~0.8 mg/mL) over lengthy repeat infusions (1.4 mL/min for 180 min on the first day, followed by daily administration for at least 14 days), limiting the practical use of this potent drug.³²⁹ The liposomal anidulafungin formulations we have developed in this work seek to address this limitation, while still enabling excellent antifungal therapy.

Our liposome formulations successfully encapsulated varying concentrations of anidulafungin with a drug loading of at least ~3% (w/w), remaining stable and effective against *C. albicans* upon storage for over 140 days. All formulations were effective in inhibiting planktonic *C. albicans* growth *in vitro* with comparable activity to equivalent concentrations of free anidulafungin. We found that liposome formulations exhibited improved efficacy in disrupting pre-formed *C. albicans* biofilms compared with equivalent

concentrations of free drug. In prophylactic treatment of *Galleria mellonella* infected with *C. albicans*, these liposomal anidulafungin nanoparticles increased larvae survival beyond the increased survival noted with free drug treatment. Anidulafungin has previously been incorporated into poly(methyl methacrylate) bone cement³³⁰ and covalently tethered to glass and silicon surface coatings³³¹ to prevent fungal attachment and infections. To the best of our knowledge, this is the first report of a nanoparticle encapsulating anidulafungin for future use in antifungal drug delivery.

7.3 Materials and Methods

7.3.1 Materials

All lipids, hydrogenated (soy) L- α -phosphatidylcholine, 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) and cholesterol, were purchased from Avanti Polar Lipids, Inc. (Alabaster, Al). Liposomes were formed using ultrapure deionized water (18.2 M Ω .cm, Milli-Q, EMD Millipore, Taunton, MA). Anhydrous chloroform, methanol, α -tocopherol, Triton X-100, crystal violet, sodium chloride, trifluoroacetic acid, Roswell Park Memorial Institute (RPMI) 1640 (without sodium bicarbonate and phenol red, with L-glutamine) medium, yeast nitrogen base with ammonium sulfate (YNB-AS), yeast peptone dextrose (YPD) broth, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Dulbecco's phosphate-buffered saline without calcium or magnesium (PBS), and Hamilton® syringes were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile, glacial acetic acid, and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Hampton, NH).

CyQUANT 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) cell viability assay was purchased from Thermo Fisher Scientific (Waltham, MA). Bovine red blood cells (bRBCs) (10% v/v) were purchased from Innovative Research (Novi, MI). *C. albicans* 10231 and *C. albicans* SC5314 were obtained from American Type Culture Collection (Manassas, VA). Anidulafungin was purchased from AdooQ BioScience (Irvine, CA). AmBisome® (Gilead Sciences, Inc.) was obtained from the Brown University Pharmacy (Providence, RI). *G. mellonella* larvae were obtained from the Invertebrate Breeding Laboratory at the Institute of Science and Technology (ICT/UNESP, São José dos Campos, São Paulo, Brazil).

7.3.2 Fabrication of anidulafungin liposomes

Three different anidulafungin liposome formulations were developed (Table 7.1). All liposome components other than anidulafungin were stored at -20°C in chloroform at a concentration of 10 mg/mL prior to use, except for α -tocopherol which was stored at a concentration of 0.95 mg/mL. For all formulations, anidulafungin was first dissolved in a 50/50 (v/v) water/acetonitrile solution at a concentration of 1 mg/mL. To form 10 mL of 1× anidulafungin liposomes, a dry lipid film technique was used.³³² Briefly, 78 μ L of this solution was added to a 20 mL borosilicate glass scintillation vial, followed by removal of the acetonitrile using a rotary evaporator (Rotavapor R-300, Buchi, New Castle, DE). The volume of anidulafungin solution was adjusted to meet the desired concentration for each formulation. The sample was reduced to a pressure of 20 mbar and submerged in a 35°C water bath, rotating at 120 rpm for approximately 15 minutes. Subsequently, the remaining water/anidulafungin solution was placed in dry ice until frozen. The sample was then

lyophilized (FreeZone 4.5 L, Labconco, Kansas City, MO) for 30 minutes at a pressure of 0.014 mbar to sublime any remaining water, resulting in a dry film of anidulafungin.

The remaining liposome components (hydrogenated soy phosphatidylcholine (HSPC), phosphatidylglycerol (PG), cholesterol, and α -tocopherol) in chloroform were added to the dry anidulafungin film to achieve the desired lipid and drug ratios. Chloroform was removed using a rotary evaporator (Rotavapor R-300, Buchi, New Castle, DE). The sample was reduced to a pressure of 20 mbar and submerged in a 35°C water bath, rotating at 120 rpm for approximately 15 minutes or until the chloroform was no longer visible. The resulting dry lipid film containing all liposome components in the correct ratios was placed on high vacuum for 4 h to remove any residual chloroform and stored overnight inside a vacuum desiccator. The dry lipid film was rehydrated at 65°C in 10 mL of ultrapure water to achieve a final concentration of 2.5 mg/mL. Subsequently, the rehydrated sample was sonicated at 65°C for 30 minutes to allow the multilayer lipid film to self-assemble into multilamellar vesicles (MLVs). To attain a solution of unilamellar liposomes, the MLVs were extruded through a 100 nm polycarbonate membrane using a Liposofast LF-50 extruder (Avestin, Ottawa, Canada) with ultrapure nitrogen at 300-400 psi (TechAir, Providence, RI) and a temperature of 65°C. The extrusion was conducted at this temperature to allow transition from the gel phase to the crystal liquid phase of HSPC. The liposome solution was extruded a total of ten times.

Table 7.1: Anidulafungin liposome fabrication composition.

Liposome components	1× Formulation (% w/w)	5× Formulation (% w/w)	10× Formulation (% w/w)
Hydrogenated Soy Phosphatidylcholine (HSPC)	60.73	59.97	59.03
Phosphatidylglycerol (PG)	23.95	23.65	23.28
Cholesterol	14.83	14.65	14.42
α -tocopherol	0.18	0.18	0.17
Anidulafungin	0.31	1.55	3.10

7.3.3 Characterization of liposome physical properties

Characterization of liposome hydrodynamic diameter and polydispersity index (PDI) was performed using dynamic light scattering (DLS) on a Zetasizer Nano ZS90 (Malvern Panalytical, Westborough, MA). A sample of the liposome solution was added to a micro volume, UV transparent, disposable cuvette (Sarstedt, Nümbrecht, Germany). Three measurements were obtained per sample. Both freshly prepared liposomes and those stored at 4°C for 140 days in ultrapure water were measured using DLS to determine any size distribution changes. The Zetasizer Nano ZS90 was also used to obtain zeta (ζ) potential measurements of the liposome formulations using a folded capillary zeta cell (Malvern Panalytical, Westborough, MA).

Cryogenic transmission electron microscopy (cryo-TEM) was also used to examine the 1×, 5×, and 10× anidulafungin liposomes. The liposomes were vitrified in liquid ethane. The vitrified grids were imaged by cryo-TEM with a JEOL JEM 2100 operated at 100 kV, equipped with an AMT XR401 sCMOS camera.

7.3.4 Determining anidulafungin encapsulation efficiency and loading in liposomes

Anidulafungin encapsulation efficiency (EE) in liposomes was determined using equation (7.1).

$$EE\% = \frac{\text{Encapsulated drug (mg)}}{\text{Total drug added (mg)}} \times 100 \quad (7.1)$$

Here, total drug added was the mass of drug added during liposome fabrication. The amount of encapsulated drug was determined by lysing liposomes using 1% Triton X-100 in ultrapure water and diluting 1:1 (v/v) in acetonitrile to fully dissolve released drug. These samples were added to black bottom 96-well plates and fluorescence of each well was recorded at an excitation wavelength of 250 nm and emission wavelength of 450 nm (i.e., anidulafungin max excitation and emission wavelengths). The measured emission was converted to a drug concentration utilizing a fluorescence emission standard curve of free anidulafungin prepared in a solution of 1:1 (v/v) ultrapure water:acetonitrile with a final Triton X-100 concentration of 0.5% (v/v).

Drug loading (DL) was determined using equation (7.2).

$$DL\% = \frac{\text{Encapsulated drug (mg)}}{\text{Liposome dry mass (mg)}} \times 100 \quad (7.2)$$

Here the liposome dry mass was determined by lyophilizing 1 mL of a liposome suspension in water and obtaining the liposome dry weight. The encapsulated drug mass in this sample was determined in the same way in which it was for calculating EE.

7.3.5 Evaluating anidulafungin liposome localization to *C. albicans*

In order to investigate interactions with *C. albicans* cells, anidulafungin liposomes (1×) were reformulated using lissamine rhodamine B functionalized phosphoethanolamine (Liss

Rhod PE) at 0.1% (w/w) of the total liposome composition, rendering the liposomes fluorescent (excitation: 560 nm, emission: 583 nm). All other liposome components were kept at the same ratio as the 1× formulation described in Table 7.1. To quantify localization of the liposomes to *C. albicans*, flow cytometry was utilized. First, *C. albicans* 10231 at a concentration of 10⁶ CFU/mL in 2 mL of saline was centrifuged at 10,000 rpm for 10 minutes, pelleting the cells. The *Candida* was resuspended in 1 mL of YNB-AS media and 1 mL of 2.5 mg/mL 1× anidulafungin liposomes with Liss Rhod PE. The solution was incubated for two hours at 37°C while shaking at 120 rpm. Fungi-liposome samples were then centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed, and the *Candida*-liposome pellet was resuspended in 1 mL of 1× PBS. This solution was subsequently analyzed using a 4-laser, 19-parameter BD PACSAria Illu flow cytometer (Franklin Lakes, NJ) using a 561 nm (yellow/green) laser.

7.3.6 Investigating *in vitro* activity of liposome formulations against planktonic *C. albicans*

Broth microdilution assays were used to determine the minimum inhibitory concentration (MIC) of all anidulafungin liposomes against planktonic *C. albicans* 10231. MIC is defined as the lowest therapeutic concentration at which a prominent decrease in turbidity (i.e., > 50% inhibition of growth as compared to the growth control) is observed after 24 hours of incubation with the fungi.³³³ To determine the MIC of the different liposome formulations, the vesicles were serially diluted (starting at 50 µg/mL) and added to fungi at a final concentration of 1 × 10⁴ to 5 × 10⁴ CFU/mL in RPMI 1640 medium and cultured at 35°C while shaking at 120 rpm.³³⁴ Negative controls of media with no fungi and positive controls of RPMI 1640 with fungi in the absence of liposomes were also included. After 24 hours

the optical density (OD) of these samples was examined at 595 nm (Cytation 3, BioTek, Winooski, VT) and the normalized fungi density was calculated using equation (3).

$$\text{Normalized fungi density} = \frac{OD_{595\text{ nm}}(\text{Sample}) - OD_{595\text{ nm}}(\text{Negative Control})}{OD_{595\text{ nm}}(\text{Positive Control}) - OD_{595\text{ nm}}(\text{Negative Control})}$$

(7.3)

7.3.7 Investigating *in vitro* activity of liposome formulations against biofilm *C. albicans*

Fungal biofilm disruption in the presence of anidulafungin liposomes and free anidulafungin was tested using *C. albicans* 10231. Initially, 1 mL of 10⁷ CFU/mL of *C. albicans* in RPMI 1640 buffered with 10 mM HEPES (pH 7.4) was added to non-tissue culture treated 4-well glass bottom chamber slides and left to incubate for 90 minutes to allow for the initial adhesion of cells. The supernatant was then aspirated, and the wells washed with saline to remove non-adherent cells. Then 1 mL of fresh RPMI media was added and the biofilms were left to mature for 24 hours at 37°C while gently shaking at 75 rpm. At this point the media was aspirated and the wells gently washed with saline to remove planktonic cells. The therapeutic in ultrapure water (500 µL) was mixed with fresh RPMI (500 µL) to achieve a final liposome (1×, 5×, and 10×) concentration of 1.25 mg/mL and equivalent free drug concentration, which was then introduced to the biofilms. After 24 more hours of culture at 37°C with gentle shaking at 75 rpm, the biofilms were either imaged with differential interference contrast microscopy (Nikon Eclipse Ti-A1R equipped with NIS Elements software) or were disrupted by vigorous pipetting, and the colony forming units (CFU) were quantified by plating dilutions of each sample on YPD agar (2% w/v). To better assess fungal cell viability, a metabolic assay using XTT was also conducted. Briefly, 70 µL of a 1:6 (v/v) mixture of electron coupling reagent and XTT

reagent, respectively, were added to 100 µL of the culture solution and allowed to incubate in the dark for 4 hours at 37°C while gently shaking at 75 rpm. Negative controls with only media and no fungi were also included in these studies. After incubation the absorbance of the samples was read at 450 nm and 660 nm (Cytation 3, BioTek, Winooski, VT). The relative metabolic activity (RMA) of each sample was determined using equation (7.4).

$$RMA\% = \frac{[Abs_{450\text{ nm}}(Sample) - Abs_{450\text{ nm}}(Negative\ Control)] - Abs_{660\text{ nm}}(Sample)}{[Abs_{450\text{ nm}}(Positive\ Control) - Abs_{450\text{ nm}}(Negative\ Control)] - Abs_{660\text{ nm}}(Positive\ Control)} \times 100$$

(7.4)

7.3.8 Investigating *in vivo* activity of liposome formulations against *C. albicans* infected *G. mellonella*

To investigate the ability of anidulafungin liposomes and free drug to improve survival of *C. albicans* SC5314 infected *G. mellonella* wax moth larvae, the methodology described by Barros et al. was used with some modifications.³³⁵ Twelve randomly chosen *G. mellonella* larvae of similar weight and size (250–350 mg) were used per group in all assays. Two control groups were included, one group that was injected with sterile 10 mM HEPES and one group that received no injection to monitor general *Galleria* viability. We initially determined the sub-lethal inoculum concentration of *C. albicans* SC5314 in *Galleria* larvae. For this purpose, serial dilutions of *C. albicans* SC5314 (1×10^5 to 1×10^8 CFU/larva) were inoculated into *G. mellonella* via the last left proleg (10 µL volume) using a 26s gauge Hamilton® syringe (10 µL x 50 mm); a HEPES control group was inoculated similarly. Based on this study (data not shown), we chose the sub-lethal inoculum concentration of 10^6 CFU/larva to evaluate the effects of anidulafungin liposome formulations and free drug. First, *G. mellonella* was prophylactically injected (10 µL

volume) at the last left proleg with either 1× and 10× anidulafungin liposome formulations or equivalent free anidulafungin concentration in 10 mM HEPES at either 1 h, 24 h, or 48 h pre-infection. The larvae were then infected with 10⁶ CFU/larva of *C. albicans* SC5314 suspended in HEPES at the last right proleg (10 µL volume). Survival of *Galleria* was monitored by counting dead larvae daily for seven consecutive days; larvae that showed no movement to touch were considered dead.

7.3.9 Evaluating anidulafungin liposome hemocompatibility

Hemolytic activity of the anidulafungin liposome and free drug was examined as previously described.^{336,337} Briefly, bRBCs (10% (v/v) suspension in PBS) were incubated with 1×, 5×, and 10× anidulafungin liposomes (10% v/v), equivalent free drug concentrations, and a 1% DMSO control in a 96 well plate for 1 h at 37°C with gentle rocking. The 1% DMSO control was included as free anidulafungin was solubilized in 1% DMSO. Negative controls of bRBCs only and positive controls of bRBCs with 0.2% (v/v) Triton X-100 were included. After incubation, the plate was centrifuged for 5 min at 1000 × g. Next, 125 µL of the supernatant from each well was sampled and absorbance was read at 540 nm (Cytation 3, BioTek, Winooski, VT). Normalized % hemolysis was calculated using equation (7.5):

$$\text{Normalized Hemolysis (\%)} = \frac{\text{Abs}_{540}(\text{Sample}) - \text{Abs}_{540}(\text{Negative Control})}{\text{Abs}_{540}(\text{Positive Control}) - \text{Abs}_{540}(\text{Negative Control})} \times 100$$

(7.5)

7.3.10 Statistical analysis

All experiments were conducted in triplicate at minimum. Results are given as mean $\pm$ standard deviation. Statistical significance was analyzed using Prism 7 (GraphPad) with student t-test, one-way analysis of variance (ANOVA), two-way ANOVA, or log rank test where applicable ($\alpha = 0.05$; $p < 0.05$ was considered statistically significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

7.4 Results

7.4.1 Characterization of liposome physical properties

In this work we formulated several unilamellar liposome drug delivery formulations encapsulating anidulafungin, one of the latest antifungal drugs to receive FDA approval. We developed formulations with increased loading of anidulafungin, namely 1 $\times$, 5 $\times$, and 10 $\times$ (i.e., 7.8, 39, and 78 $\mu\text{g/mL}$ anidulafungin, respectively). Cryo-TEM imaging confirmed the formation of uniform unilamellar structures approximately 100 nm in diameter (Figure 7.1a). DLS was utilized to examine the liposome hydrodynamic diameter distribution. All formulations resulted in a uniform size distribution with an average hydrodynamic diameter of ~ 100 nm and PDI of ~ 0.1 (Figure 7.1b). ζ potential

measurements suggested that these liposomes had a similar negative surface charge (Figure 7.1c).

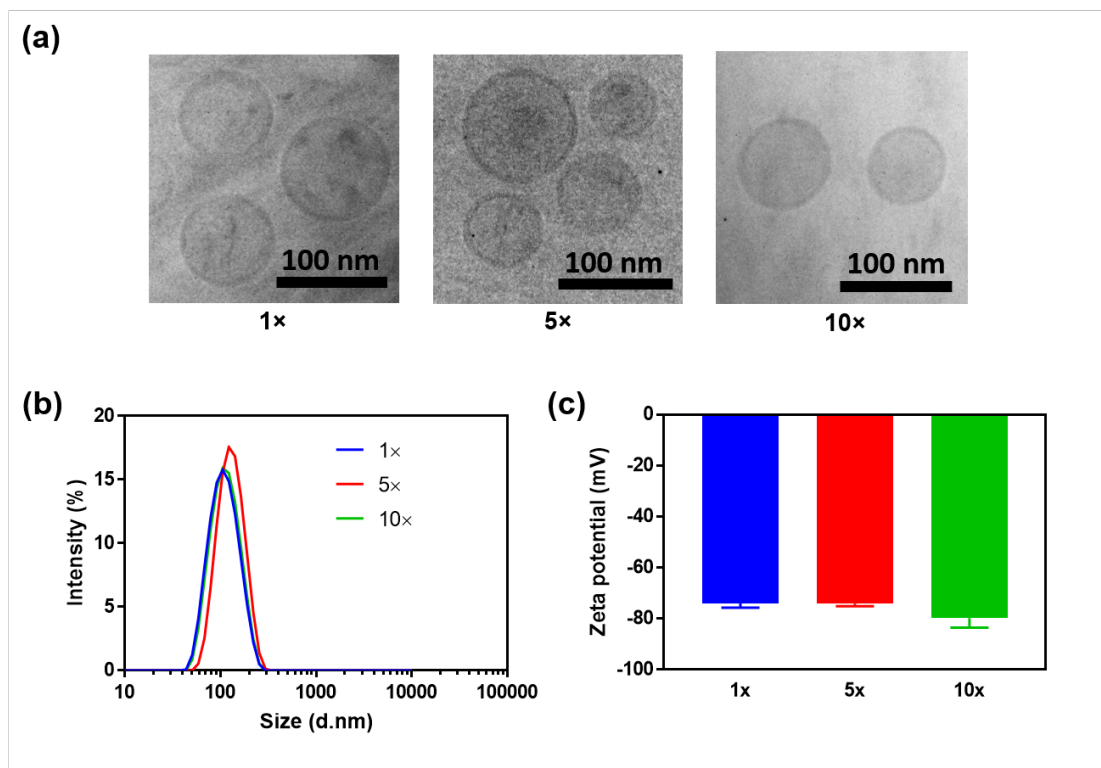


Figure 7.1: Characterization of 1×, 5×, and 10× anidulafungin loaded liposomes. (a) Cryo-TEM of liposomes. (b) Liposome hydrodynamic diameter distribution as determined by DLS. (c) ζ potential of anidulafungin loaded liposomes in water ($p > 0.05$, not significant; one-way ANOVA with Tukey’s post-hoc analysis, $n=3$).

Next, we confirmed that anidulafungin was successfully encapsulated into these liposomes. The encapsulation efficiency for 1×, 5×, and 10× anidulafungin liposomes was $94.6 \pm 1.2\%$, $97.2 \pm 0.4\%$, and $84.3 \pm 16.4\%$, respectively (Figure 7.2a), with no statistically significant difference in encapsulation efficiency between all three formulations. Anidulafungin loading increased significantly with increasing anidulafungin fabrication concentration as expected, at $0.32 \pm 0.01\%$ (w/w), $1.59 \pm 0.05\%$ (w/w), and $2.83 \pm 0.53\%$ (w/w) for 1×, 5×, and 10× liposomes, respectively (Figure 7.2b). This drug

loading was similar to the theoretical maximum drug loading (w/w) indicated in Table 7.1.

Table 7.2 summarizes the hydrodynamic diameter, ζ potential, encapsulation efficiency, and anidulafungin loading of all formulations.

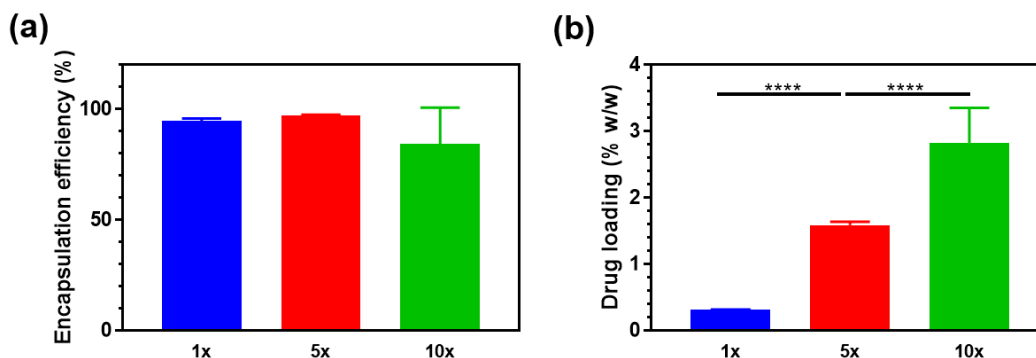


Figure 7.2: Successful encapsulation of anidulafungin in 1 $\times$, 5 $\times$, and 10 $\times$ liposomes. (a) Anidulafungin liposome encapsulation efficiency ($p > 0.05$, not significant; one-way ANOVA with Tukey's post-hoc analysis, $n = 6$). (b) Anidulafungin liposome drug loading (**** $p < 0.0001$, one-way ANOVA with Tukey's post-hoc analysis, $n = 6$).

Table 7.2: Summary of anidulafungin liposome physical and drug loading properties.

Formulation	Diameter (nm)	PDI	Zeta (ζ) potential (mV)	EE (%)	DL (% w/w)
1 $\times$	102.6 $\pm$ 1.8	0.10 $\pm$ 0.01	-74.0 $\pm$ 2.0	94.6 $\pm$ 1.2	0.32 $\pm$ 0.01
5 $\times$	103.5 $\pm$ 1.6	0.01 $\pm$ 0.02	-74.1 $\pm$ 1.1	97.2 $\pm$ 0.4	1.59 $\pm$ 0.05
10 $\times$	104.4 $\pm$ 1.6	0.08 $\pm$ 0.02	-79.8 $\pm$ 3.7	84.3 $\pm$ 16.4	2.83 $\pm$ 0.53

7.4.2 Liposome localization to and antifungal activity against planktonic *C. albicans*

Having confirmed liposome fabrication and drug loading capability, we examined the behavior of these liposomes with planktonic *C. albicans* 10231. First, 1 $\times$ liposomes were reformulated to add a small amount of lissamine rhodamine B functionalized phospholipid, rendering the liposomes fluorescent with an excitation and emission maximum of 560 and

583 nm, respectively. Following 2 hours of incubation of these fluorescent 1× liposomes with *C. albicans*, flow cytometry results indicated that approximately 40% of *C. albicans* cells exhibited fluorescence compared to minimal fluorescence of cells that were not incubated with these liposomes, indicating liposome localization to fungi (Figure 7.3a). All formulations were then examined for their MICs against planktonic *C. albicans* utilizing broth microdilution assays. Growth inhibition was noted at 12.50 µg/mL, 6.25 µg/mL, and 1.56 µg/mL for 1×, 5×, and 10× liposome formulations, respectively (Figure 7.3b). These concentrations correspond to an anidulafungin MIC of ≤ 0.039 , 0.098, and 0.049 µg/mL for 1×, 5×, and 10× liposomes, respectively. Non-liposome encapsulated anidulafungin was found to exhibit an MIC of < 0.06 µg/mL against *C. albicans* 10231 (data not shown), consistent with reported MIC values of 0.01 µg/mL.³³⁸

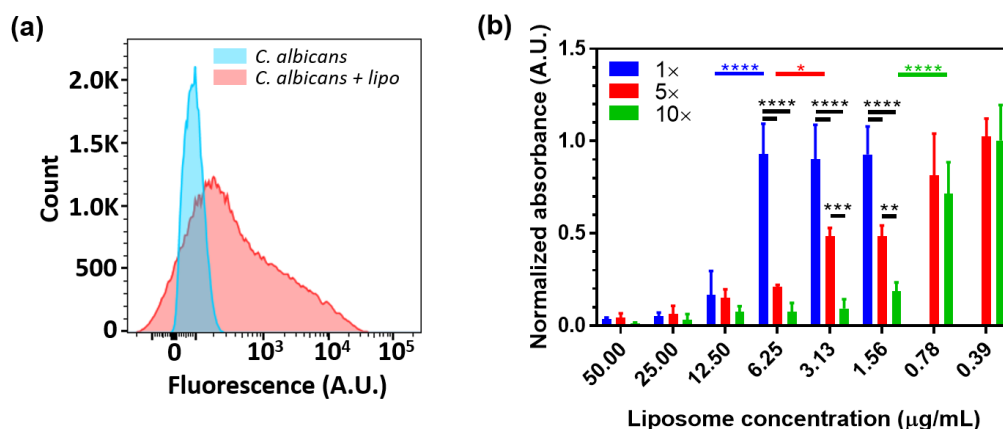


Figure 7.3: Anidulafungin liposomes localize to and prevent growth of planktonic *C. albicans*. (a) Flow cytometry histogram overlay of *C. albicans* untreated and treated with fluorescent 1× anidulafungin loaded liposomes for 2 hours at 37°C. Approximately 40% of cells were found to exhibit fluorescence in the liposome treated condition indicating liposome localization to fungal cells. (b) Microdilution assay of 1×, 5×, and 10× anidulafungin loaded liposomes against *C. albicans* 10231 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Tukey's post-hoc analysis, $n = 3$).

7.4.3 Liposome activity against *C. albicans* biofilms

The anidulafungin liposomes were also examined for their ability to disrupt mature *C. albicans* 10231 biofilms. Biofilms were allowed to mature for 24 hours followed by incubation with 1×, 5×, and 10× liposome formulations (1.25 mg/mL; selected to ensure minimal liposome aggregation), and free anidulafungin at equivalent concentrations for 24 additional hours. Optical microscopy was used to image biofilms after incubation with liposome formulations or free drug (Figure 7.4a) and cell metabolic activity and CFU counts were quantified upon treatment. All anidulafungin liposome formulations outperformed equivalent samples of free anidulafungin in reducing *C. albicans* metabolic activity (measured by XTT reduction) and cell number upon incubation with biofilms. Compared to untreated controls, biofilms incubated with 1×, 5×, and 10× liposomes displayed metabolic activities of $46.59 \pm 5.69\%$, $53.15 \pm 3.83\%$, and $61.51 \pm 4.56\%$, respectively, while those treated with free drug at equivalent concentrations showed metabolic activities similar to the untreated control biofilm (Figure 7.4b). When CFU were quantified, approximately a two-log reduction was observed between the untreated biofilms ($3.6 \times 10^8 \pm 1.8 \times 10^8$ CFU/mL) and those treated with anidulafungin liposomes (Figure 7.4c). Free anidulafungin also reduced the CFU compared to untreated biofilms but was not as effective as liposome formulations. More specifically, 1× liposomes reduced fungal burden to $5.8 \times 10^6 \pm 0.5 \times 10^6$ CFU/mL compared to free drug at an equivalent concentration which reduced fungal burden to $9.0 \times 10^6 \pm 6.9 \times 10^6$ CFU/mL after 24 hours. 5× and 10× liposomes reduced the fungal burden to $5.3 \times 10^6 \pm 0.3 \times 10^6$ CFU/mL and $2.4 \times 10^6 \pm 0.3 \times 10^6$ CFU/mL, respectively, compared to free drug at 5× and 10× equivalent

concentrations which reduced fungal burden to $9.7 \times 10^6 \pm 2.3 \times 10^6$ CFU/mL and $4.8 \times 10^6 \pm 2.4 \times 10^6$ CFU/mL, respectively.

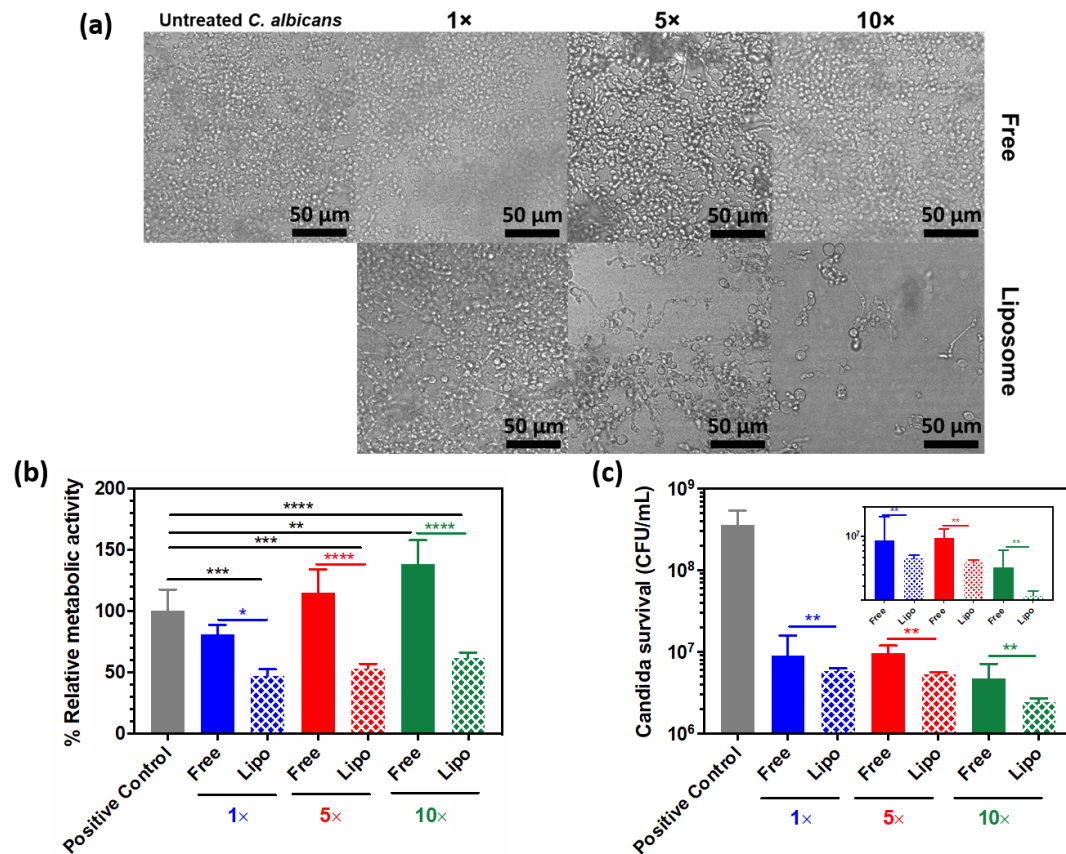


Figure 7.4: Anidulafungin liposomes demonstrate increased activity against mature *C. albicans* biofilms compared to free drug. (a) Representative differential interference contrast microscopy images of *C. albicans* 10231 biofilms after 24 hours of treatment with 1x, 5x, and 10x anidulafungin loaded liposomes or equivalent free drug. (b) Relative metabolic activity of *C. albicans* 10231 biofilm cells 24 hours after treatment with 1x, 5x, and 10x anidulafungin loaded liposomes or equivalent free drug (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Tukey's post hoc analysis, n = 4). (c) *C. albicans* 10231 biofilm CFU 24 hours after treatment with 1x, 5x, and 10x anidulafungin loaded liposomes or equivalent free drug (**p < 0.01, unpaired two-tailed t-test, n = 4). Positive controls of untreated cells are shown. Negative controls lacking fungi were included in each study.

7.4.4 Investigating liposome storage stability

The stability of the 1× anidulafungin liposomes stored in ultrapure water at 4°C was observed over a period of 140 days. Compared to the size distribution of liposomes after extrusion, the size distribution of 140 day stored liposomes remained unchanged with an average diameter of 101.6 ± 0.7 nm (PDI: 0.08 ± 0.01) (Figure 7.5a). The drug loading was determined for these stored liposomes and found to be slightly less than freshly extruded 1× liposomes (Figure 7.5b). No significant difference in *C. albicans* 10231 growth inhibition was observed between freshly extruded 1× anidulafungin liposomes and the liposomes stored for 140 days (Figure 7.5c), with inhibition observed at 12.50 µg/mL, corresponding to an anidulafungin MIC of ≤ 0.039 µg/mL.

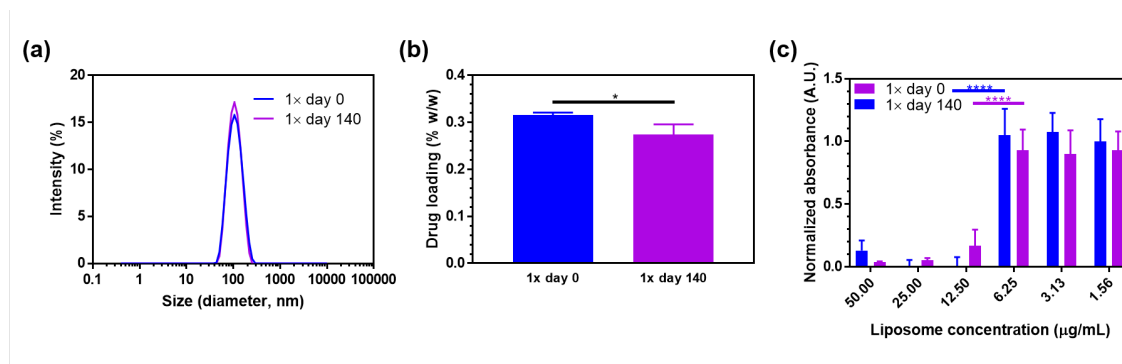


Figure 7.5: Stability of 1× anidulafungin liposomes after 140 days of aqueous storage at 4°C. (a) Liposome hydrodynamic diameter distribution of freshly made 1× anidulafungin liposomes compared to 1× anidulafungin liposomes stored at 4°C in water for 140 days determined by DLS. (b) Drug loading of freshly made 1× anidulafungin liposomes compared to 1× anidulafungin liposomes stored at 4°C in water for 140 days (* $p < 0.05$, unpaired two-tailed t-test, $n = 3$). (c) Microdilution assay of freshly made 1× and 1× anidulafungin loaded liposomes stored at 4°C in water for 140 days against *C. albicans* 10231 (**** $p < 0.0001$, two-way ANOVA with Tukey's post-hoc analysis, $n = 3$).

7.4.5 Liposome activity in *C. albicans* infected *G. mellonella*

Having established the therapeutic efficacy of anidulafungin liposome formulations *in vitro*, we next investigated their activity in *G. mellonella* infected with *C. albicans* SC5314,

a pathogenic strain widely used for experimental studies in the *G. mellonella* model.^{339–341} Survival of *G. mellonella* with prophylactic administration of liposomes and free drug was investigated. By injecting 10 μ L of the liposome formulations at a concentration of 2.5 mg/mL and equivalent free drug we achieved a drug dose of 2.60 μ g/g (10 $\times$) and 0.26 μ g/g (1 $\times$) in *Galleria*, which is similar to the anidulafungin dose utilized in humans (1.5 μ g/g³⁴²). Treatments were administered at 1, 24, or 48 h prior to infecting *G. mellonella* with *C. albicans* (Figure 7.6). In each of these cases, the 10 $\times$ liposome formulation resulted in the largest increase in *Galleria* survival over the 7 day study compared with untreated *Candida* infected *Galleria*. For the 1 h pre-infection prophylactic administration of 10 $\times$ liposomes, an initial decrease in survival to 67% was noted at 1 day following *Candida* infection. However, following this initial drop, survival at 67% was sustained over 7 days. In contrast, prophylactic administration at 1 h pre-infection with the equivalent dose of free anidulafungin led to a drop in *Galleria* survival to 33% by 3 days. The time of administration pre-infection also resulted in changes in *Galleria* survival. For example, in the case of administration of 10 $\times$ liposomes 48 h prior to infection, *Galleria* survival dropped to 67% over 4 days instead of 1 day observed with liposome administration 1 h pre-infection. For 1 $\times$ liposomes, administration 48 h pre-infection improved therapeutic

efficacy with a 33% survival of *Galleria* at 7 days post-infection compared to a 1 h pre-infection administration, resulting in 17% *Galleria* survival at 7 days.

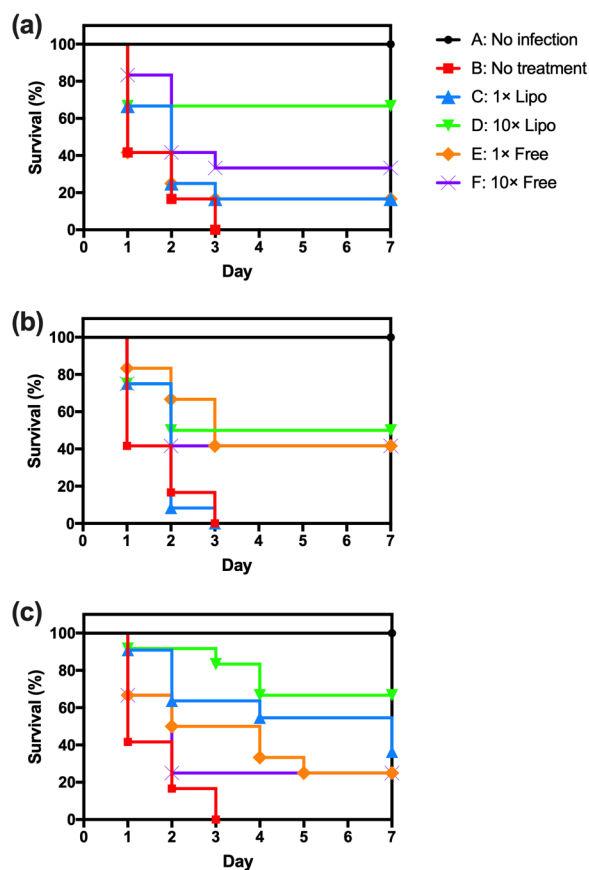


Figure 7.6: *Galleria mellonella* survival once infected with *C. albicans* following (a) 1 h, (b) 24 h, and (c) 48 h prophylactic administration of 1× and 10× anidulafungin liposomes or free drug at an equivalent concentration. Statistical significance was determined using a log-rank test (n = 12 larvae per group); *p*-values comparing groups for: (a) C×D: *p* = 0.0415; B×D: *p* = 0.0015; B×F: *p* = 0.0168, (b) C×D: *p* = 0.0312; C×E: *p* = 0.0066; B×D: *p* = 0.0079; B×E: *p* = 0.0033; B×F: *p* = 0.0174, and (c) D×F: *p* = 0.0181; B×C: *p* = 0.0007; B×D: *p* < 0.0001; B×E: *p* = 0.0125.

7.4.6 Evaluating anidulafungin liposome hemocompatibility

As a preliminary investigation of the cytocompatibility of the anidulafungin liposomes developed in this work, the hemolytic activity of these nanoparticles against bRBCs was examined and compared with free anidulafungin. Liposomes and free drug at equivalent concentrations were incubated with bRBCs and the release of hemoglobin from bRBCs

was quantified, where release of hemoglobin is associated with cell damage. No hemolytic activity was observed for both free anidulafungin and all liposomal formulations compared to the Triton-X 100 control, which is highly toxic to the bRBCs (Figure 7.7).

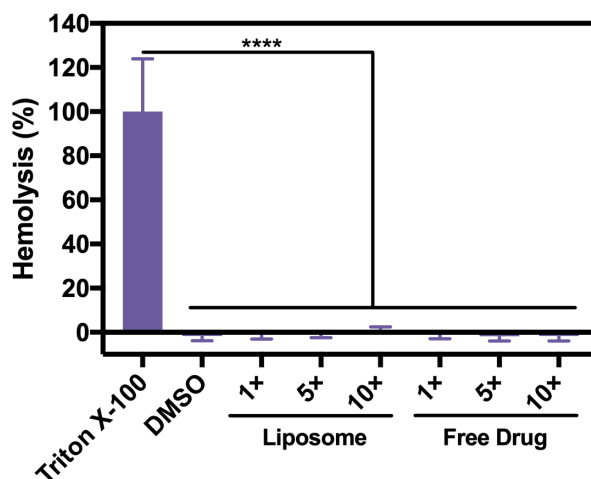


Figure 7.7: Bovine red blood cell hemolysis activity upon treatment with 1×, 5×, and 10× anidulafungin liposomes and equivalent free drug concentrations. Controls of 1% (v/v) DMSO, and 0.2% (v/v) Triton X-100 were included (**** $p < 0.0001$, two-way ANOVA with Tukey's post-hoc analysis, $n = 3$).

7.5. Discussion

In this work, we have presented the first study to encapsulate anidulafungin into nanoparticles. Currently, liposomal amphotericin B is the only commercially available liposomal antifungal therapeutic in the United States; this formulation has both improved the therapeutic efficacy and reduced the toxicity associated with amphotericin B.^{343,344} As one of the latest antifungal therapeutics available for clinical use, it is highly desirable to investigate new formulations of anidulafungin, which have the potential to increase solubility, improve antifungal efficacy, reduce toxicity, and possibly lower susceptibility to resistance. With a rise in invasive fungal infections³⁴⁵, these new therapeutic formulations have the potential to improve treatment outcomes for patients facing these ailments. Our liposome composition (1×) was inspired by the reported formulation of

clinically utilized liposomal amphotericin B (AmBisome).³⁴⁶ Due to the hydrophobicity and lipopeptide structure of anidulafungin (insoluble in water)^{324,347}, we hypothesize that the drug is incorporated into the lipid bilayer portion of the liposome during fabrication. Similar to AmBisome, we fabricated ~100 nm particles, which displayed negative ζ potential (Figure 7.1, Table 7.2). Three different formulations were developed with increasing drug loading, termed the 1 $\times$, 5 $\times$, and 10 $\times$ formulations based on the theoretical loading and 100% encapsulation efficiency. A drug loading comparable to the theoretical loading was noted (Figure 7.2b, Table 7.2). We expected any free anidulafungin in solution to aggregate and be removed during the extrusion process. However, with the high encapsulation efficiency (greater than ~84%, Figure 7.2a, Table 7.2), we expected little free anidulafungin remaining during the liposome fabrication process.

Prior to investigating the antifungal activity of our formulations, we first examined whether these liposomes were capable of localizing to fungal cells. *Candida* yeast was selected because it is the most common fungal agent causing life-threatening invasive fungal infections in humans, of which *C. albicans* is the most prevalent.³⁴⁸ It has previously been observed that AmBisome localizes to fungi *in vitro* and *in vivo* in as little as one hour, and it has recently been suggested that this localization is due to the attraction of the amphotericin B itself to fungal cell membrane ergosterol.^{346,349} We found that our 1 $\times$ liposomes, when modified to display a fluorophore, were able to localize to *C. albicans* cells with 40% of cells displaying fluorescence after 2 hours of incubation with these liposomes (Figure 7.3a). The exact mechanism of localization (e.g., attachment, translocation, etc.) was not confirmed; however, these results suggest that although the

encapsulated anidulafungin therapeutic does not function by sequestering ergosterol, these liposomes have a propensity to associate with *C. albicans*.

In this study we examined the anidulafungin liposome efficacy against both planktonic and biofilm *C. albicans*. Using a broth microdilution assay the MIC values of all 3 liposome formulations examined against planktonic *C. albicans* when converted to equivalent anidulafungin concentration based on drug loading values for each formulation, remained constant (0.039-0.098 $\mu\text{g/mL}$) and unchanged from free anidulafungin MIC values against *C. albicans* 10231.³³⁸ As expected, the corresponding liposome concentration required to inhibit *Candida* growth decreased with increasing drug loading. As drug efficacy and encapsulation efficiency were not reduced with increased drug loading up to 2.8% (w/w) (i.e., the 10 $\times$ formulation), future studies may explore further increasing the anidulafungin loading. In general, both the physicochemical and functional properties of the liposomes are expected to vary with composition.^{350,351}

Fungal biofilms are much more difficult to treat than planktonic fungi, requiring significantly higher concentrations of free drug; biofilms are often the cause of device-associated infections and recurrent infections.^{352,353} We first compared the activity of 1 $\times$ anidulafungin liposome formulations, blank liposomes, and AmBisome against preformed *C. albicans* biofilms to compare the biofilm disruption potential of these different treatments. Using crystal violet staining we found that 1 $\times$ anidulafungin liposomes at 2.5 mg/mL (i.e., 7.4 $\mu\text{g/mL}$ anidulafungin) were as effective as clinically utilized AmBisome (at a concentration of 4 mg/mL) in reducing fungal burden five-fold over 24 hours of incubation with biofilms (Figure SI 7.1). Treatment with blank liposomes did not exhibit any statistically significant difference in crystal violet staining from untreated biofilm

controls (Figure SI 7.1). Having established that the 1× anidulafungin liposome formulation exhibited *in vitro* antibiofilm activity comparable to a clinical gold standard, we compared the activity of free anidulafungin and all three anidulafungin liposome formulations developed against *C. albicans* biofilms. We observed that our liposome formulations at 1.25 mg/mL and equivalent free anidulafungin concentrations were effective at disrupting *C. albicans* biofilms, by lowering CFU count by approximately 2 log compared with untreated biofilms grown for 24 hours (Figure 7.4c). Liposomal formulations of amphotericin B have previously been shown to penetrate fungal biofilms more effectively than free drug due to electrostatic interactions between the liposome phospholipids and the charged polysaccharide extracellular matrix secreted by the biofilm fungi.³⁵⁴ Fungal biofilms are rich in hexosamines³⁵⁵, positively charged sugars that may interact with negatively charged liposomes, such as the vesicles we have fabricated here, potentially improving nanoparticle penetration and transport through the biofilm matrix. We also observed that the *Candida* metabolic activity in biofilms was reduced in the case of liposomal anidulafungin treatment only and not free drug treatment compared to untreated controls (Figure 7.4b). Interestingly, we noted an increase in metabolic activity of *Candida* cells with increasing free anidulafungin concentration, with a metabolic activity greater than the untreated biofilm control observed for the highest free drug concentration tested (i.e., 39 µg/mL anidulafungin), accompanying the ~2 log reduction in CFU. *Candida* exposed to sub-lethal levels of echinocandins have previously been shown to upregulate genes associated with cell wall integrity and stress response.³⁵⁶ In *C. albicans* exposed to sub-lethal levels of another echinocandin, caspofungin, there is a significant increase in RNA polymerase II binding to genes important in tolerating cell wall stress in

the G1 and G1/S phases of the cell cycle that are otherwise dormant in healthy cells.³⁵⁷ RNA polymerase II recruitment is a strong indicator of gene expression upregulation and increased metabolic activity.

G. mellonella have previously been used as an *in vivo* model to investigate antifungal therapeutic efficacy when administered before or after *Candida* infection.^{340,358} *G. mellonella* provide a good model for infection studies in higher organisms due to the similarity of their innate immune response with that of vertebrates.³⁵⁹ Prophylactic studies performed in this work with *G. mellonella* generally resulted in increased survival using anidulafungin loaded liposomes compared to equivalent concentrations of free drug (the only exception was observed with 1× free drug which resulted in increased survival compared to the 1× anidulafungin loaded liposomes when administered 24 hours pre-infection). For all prophylactic time points tested, 10× liposomes resulted in ~50-70% survival of larvae at 7 days. One previous prophylactic investigation that looked at *G. mellonella* survival with multiple antifungal therapeutics, including amphotericin B, observed less than 40% survival after 10 days with 5×10^5 CFU/larva^{340,360}, lower than our chosen inoculum dose of 10^6 CFU/larva. Prophylactic administration of free anidulafungin led to a less than ~40% survival of *Galleria* for all administration times investigated. While *G. mellonella* infections have been used to evaluate the activity of antifungal therapeutics,^{340,360} limited studies have been performed that investigate administration of nanoparticles in a *G. mellonella* infection model.³⁶¹ Here, we hypothesize that after infection, the liposomes improve localization to the *C. albicans* within the *G. mellonella* open circulation,³⁶² similar to fungal cell localization results observed via flow cytometry *in vitro*.

Future translation of the formulations reported in this work towards pre-clinical and clinical studies, requires ensuring the stability of these materials. We found that our 1× liposomes remained stable when stored for 140 days at 4°C suspended in ultrapure water. No significant change in vesicle size was noted and only a slight decrease (~13%) in anidulafungin loading was observed compared with a fresh preparation. Previous work has noted that negatively charged liposomes, regardless of ionic strength, maintain their size in aqueous storage conditions.³⁶³ The PG within our lipid composition lends the negative charge observed in the ζ potential measurements for all anidulafungin loadings. The negative charge of our liposomes is also useful to maintain repulsion within the colloidal solution in order to prevent liposome aggregation. High electric charges on the surface of nanoparticles are well known to prevent aggregation in solution because of the strong repellent electrostatic forces among the particles.^{364,365}

Additionally, when 140 day old liposomes were filtered (220 nm mesh size nylon filter) and tested in a broth microdilution assay against *C. albicans*, there was no statistical difference in MIC values when compared to liposomes tested at day 0. This supports that our liposomes not only retain their physical characteristics, but also remain effective. Previously, phosphoethanolamine and phosphocholine liposomes encapsulating the antimicrobial peptide LL-37 have been shown to remain stable in solution at 4°C for up to a year with no aggregation and minimal drug leakage.³⁶⁶ Amphotericin B liposomes fabricated using Pro-Lipo Neo showed no significant differences in MIC after storage in solution at 4°C after 11 weeks.³⁶⁷ The stability of our particles is superior to other antifungal liposomes, including nystatin loaded liposomes formulated with phosphocholine and cholesterol which lose approximately 50% of the total loaded drug

after just 60 days in solution at 4°C.³⁶⁸ We hypothesize that by maintaining our liposomes below the gel-to-liquid crystalline phase transition temperature of HSPC (53°C)³⁶⁹ passive drug release from the vesicles was minimized.

7.6 Conclusions

We have shown that the echinocandin antifungal drug, anidulafungin, can be incorporated into liposomes. We were able to control liposome drug loading by altering the drug to lipid ratio of the starting formulation, generating three different liposomes (1×, 5×, and 10×) and showed that these liposomes were effective at inhibiting planktonic *C. albicans* growth with an MIC equivalent to that of free anidulafungin. As with AmBisome, these anidulafungin liposomes were found to localize to the *C. albicans* cells in as little as two hours. Differences in activity between the liposomes and free drug were noted when these formulations were examined for their efficacy in disrupting mature *C. albicans* biofilms. We observed a two-log reduction in fungal burden after only 24 hours of biofilm treatment with liposomes, which was a greater decrease than what was observed with free drug treatment. These findings suggest that our liposome formulations may be able to better penetrate fungal biofilms compared with free anidulafungin. Major differences between free drug and anidulafungin liposomes were observed when these therapeutics were utilized prophylactically in *G. mellonella* that were subsequently infected with *C. albicans*. We found that 10× liposomes increased survivability to 67% when administered as early as two days prior to infection, performing much better than free drug at an equivalent concentration (25% survival). The liposomes did not cause lysis of bRBCs and were stable over at least 140 days, important considerations in future translation. These liposome

compositions could serve as a platform for the encapsulation of other antifungals, including those not yet approved by the FDA due to toxicity or solubility concerns. These drug formulations have the potential to improve drug administration and therapeutic outcomes for fungal diseases and may play a longer term role in reducing rising rates of antifungal resistance, by enabling more effective treatments.

7.7 Author Contributions

This chapter was written through contributions of all authors. C.M.B.H. and N.V.G. contributed to the project development and execution (e.g. forming liposomes, planning experiments, data analysis) equally together. N.V.G. and L.L. executed *Candida albicans* *in vitro* studies. C.M.B.H. executed hemolytic activity studies. F.C.R., E.L.S.S., and J.C.J. performed the *Candida* infected *Galleria* studies. A.S. contributed to the project aims, experimental planning, data analysis, and manuscript revision. All authors have given approval to the final version of the chapter.

7.8 Acknowledgements

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College of Engineering core facility partially funded by the National Science Foundation EPSCoR, Cooperative Agreement #OIA-1655221. We thank Irene Andreu (URI) for performing the cryo-TEM preparation and imaging. We thank Kevin Carlson and the Flow Cytometry and Sorting Facility at Brown University for assistance with flow cytometry experiments. We thank Professor Robert Hurt (Brown University) for use of his Zetasizer.

7.9 Supplemental Information

7.9.1 Investigating *in vitro* activity of liposome formulations including AmBisome against biofilm *Candida albicans* 10231 via crystal violet staining

C. albicans 10231 biofilm formation for this assay comparing 1× anidulafungin liposomes with AmBisome was performed as described in *Section 2.7: Investigating in vitro activity of liposome formulations against biofilm C. albicans*. The therapeutics were introduced following 24 hours of biofilm maturation at 2.5 mg/mL (blank liposomes formulated without anidulafungin and 1× anidulafungin liposomes) and at 4 mg/mL (AmBisome). After 24 hours of incubation at 37°C with gentle shaking at 75 rpm, the biofilms were fixed in ice cold methanol for 10 minutes and stained with 0.5% (w/v) crystal violet in 25% (v/v) methanol for 10 minutes. The biofilms were washed with ultrapure water five times. The biofilms were then air dried and the crystal violet stain was extracted from the cells using 33% (v/v) glacial acetic acid. This process was also performed on empty wells containing no cells (negative control), and on non-treated biofilms (positive control). Absorbance of extracted crystal violet was read at 595 nm (Cytation 3, BioTek, Winooski, VT). The normalized absorbance of each sample was determined using the following equation:

Normalized absorbance

$$= \frac{Abs_{595\text{ nm}}(\text{Sample}) - Abs_{595\text{ nm}}(\text{Negative control})}{Abs_{595\text{ nm}}(\text{Positive Control}) - Abs_{595\text{ nm}}(\text{Negative control})}$$

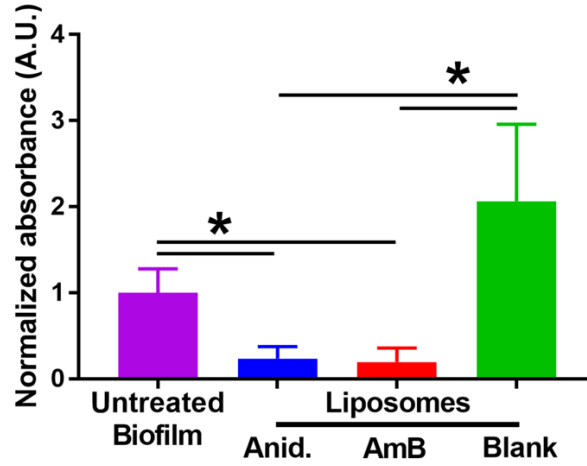


Figure SI 7.1: Biofilm eradication assay comparing crystal violet staining of *C. albicans* 10231 biofilms treated with 2.5 mg/mL 1× anidulafungin liposomes and 4 mg/mL AmBisome for 24 hours at 37°C (* $p < 0.05$, one-way ANOVA with Tukey's post-hoc analysis, n=3).

Chapter 8

Conclusions

This thesis focused on three main areas: non-invasive prenatal testing, placental models, and therapeutic design. Conclusions and future perspectives are given for each of these areas, as well as perspective on the field of biomedical engineering in women's health.

8.1 Non-invasive prenatal testing

8.1.1 Conclusions

In Chapters 2 and 3 we demonstrated two methods for enriching trophoblast cells from cervical samples. We observed the greatest enrichment of ~700% with differential cell settling of cervical samples in micro-well plates for 4 minutes prior to removing the supernatant. This enrichment was confirmed using an automated cell imager and the technique was further applied for single cell isolation using an automated cell picker.

In this work we observed that mucus plays a large role in the enrichment process. When the cervical samples were washed with either PBS or PreservCyt® to remove mucus, the enrichment of trophoblast cells using our cell settling method was significantly decreased. The mechanism for why this occurs is intriguing. We hypothesize that the mucus is creating a density gradient that the trophoblast cells can readily traverse through to the surface, resulting in the trophoblast capture and enrichment observed. As extravillous trophoblast cells are intrinsically invasive, they may have a preference towards invading the mucus gradient compared to cervical cells. We did observe a decreased enrichment with villous trophoblast cells in the cervical samples, which are not as invasive as extravillous trophoblasts, further supporting the proposed mechanism. By understanding

the mucus' role in the enrichment process, a synthetic material could be developed for experimental consistency. If trophoblast invasion is an underlying cause to our enrichment method, it could have further use to test for abnormalities, such as preeclampsia, which are dependent on shallow trophoblast invasion.

The impact of this work is providing a new method towards achieving non-invasive prenatal testing. This method provides a rapid and inexpensive enrichment method. The enrichment process takes place within 4 minutes and is label-free with no specialized equipment needed.

Future studies with more cervical samples are needed to confirm our initial efforts towards using this method in practice. Further studies should also focus on verifying the yield of cells necessary to expect a desired enrichment. The enrichment method identified through this thesis lends itself to automation, and future studies on streamlining the enrichment with single cell isolation and next generation sequencing will improve the clinical application.

8.1.2 Future Perspectives

In our studies we focused on trophoblast cells present in cervical samples. The field continues to understand the fetal cell type and the clinical sample (e.g. cervical vs. blood) that will lead to non-invasive prenatal diagnosis.

Two main fetal cell types have been studied to date: trophoblast cells and fetal nucleated red blood cells (fnRBCs). Trophoblast cells can be found in both cervical and blood samples, while fnRBCs have been investigated in blood samples. While we did not investigate fetal cell retrieval from blood samples in this thesis, it is an area for the field to

continue to consider. Both sample types have advantages and disadvantages that the field needs to further parse out while working towards new non-invasive prenatal testing options. More details and comparisons about the potential of both sample types is below (Table 8.1).

Table 8.1: Comparison between fetal cell types for non-invasive sampling

Attribute	Trophoblast from cervical sample	Trophoblast from blood sample	Fetal nucleated red blood cell from blood sample
Gestational age for retrieval (weeks)	5-20 ³⁷⁰	> 8 ³⁷⁰	7-28 ³⁷¹
Major cell contaminants	Cervical cells and mucus	Maternal plasma, red blood cells, and white blood cells	Maternal plasma, red blood cells, and white blood cells
Location derived	Placenta ⁴³	Placenta ⁴³	Directly from fetus ⁴³
Number of fetal cells per maternal cells	~1/2000 ⁴⁷	~1/1,000,000 ³⁷²	~1/1,000,000 ³⁷³
Sample volume	20 mL	10-30 mL	10-30 mL

Trophoblast cells in cervical samples are present in a higher quantity than within blood samples, where in cervical samples it is estimated that there is 1 trophoblast cell for every 2,000 cervical cells.⁴⁷ Although these trophoblast cells are still rare within the heterogeneous sample, this makes the likelihood of isolating a trophoblast cell within the sample greater for cervical compared to blood where there is only ~1 trophoblast or fnRBC per 1,000,000 cells.³⁷³ However, the major clinical limitation is the variability of samples. Within a cervical sample there was observed variability from patient to patient on number of cells and quality of the sample.

The concept of using circulating fetal cells in maternal blood originated decades ago with major hurdles limiting its success. Primarily studies focused on the isolation of fnRBC. In particular, the National Institute of Child Health and Human Development Fetal Cell Isolation Study (NIFTY trial) comprised of ten research centers using different methods to isolate fnRBCs from maternal samples with limited success due to variability in isolating cells from every pregnancy and a high false-positive rate of both sex and aneuploidy detection.³⁷⁴ Overall, this led to a decreased interest in fetal cell isolation (Figure 8.1 shows the decrease in publications in this area).³⁷⁴ However, recently new technology advances (e.g. in single-cell genetic analysis) has again created a spark towards the goal of non-invasive prenatal diagnosis.

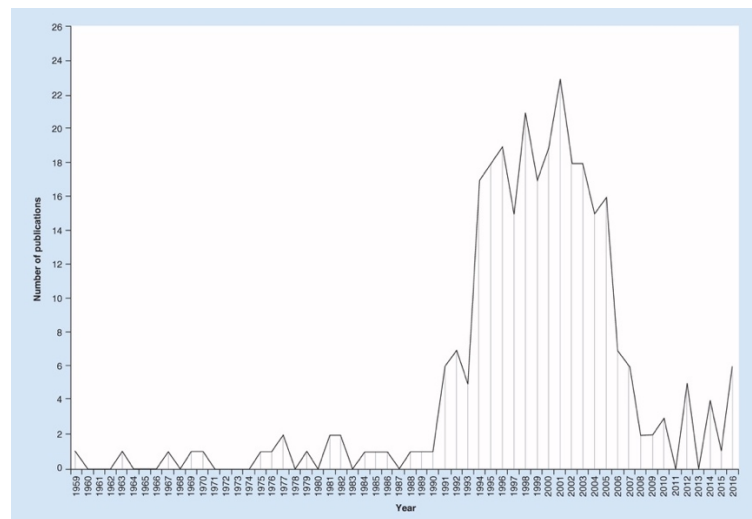


Figure 8.1: Number of publications relating to isolating fetal cells from blood samples generated from Pubmed data.³⁷⁴ Reproduced from Biomarker in Medicine (2017) 11(9), 705-710 with permission of Future Medicine Ltd.

We also note that the non-invasive prenatal testing field faces some disagreements and challenges to be considered. First, it is not clear the number of trophoblast cells present per sample. While some estimates have been discussed and reported in this thesis, we

observed sample to sample variations, which can make enrichment methods more challenging. The field would benefit from more studies on optimizing the collection method of cervical sample, which was not a focus of this thesis. Second, there is some dispute on the patient preferred sampling method. Cervical sampling does require an office visit compared to a blood sample, which is more routine, convenient, and can be collected by a technician. However, if the expecting mother is undergoing routine prenatal care, a cervical swab sample should not be considered an inconvenience and can be performed during one of the early visits to the obstetrics provider.

Overall, the field is looking towards both samples with using new technologies to isolate fetal cells. This thesis contributed to a new method for trophoblast isolation from cervical samples. Future studies comparing the two samples and different isolation techniques will continue to move the field forward.

8.2 Placental models

8.2.1 Conclusions

Chapters 4-6 demonstrated our work towards developing a placental lipid bilayer for cell-free interaction studies. We demonstrated important criteria for forming supported lipid bilayers, in particular the need for an energy input such as flow. We further demonstrated environmental toxicants, particularly phthalates, interact with lipid structures. Finally, we quantified lipid compositions from trophoblast cells representative of different time points throughout pregnancy. Using this composition, placental bilayers were developed, characterized, and tested for small molecule interactions.

One interesting finding through this work was noting that liposomes help to mitigate interactions with both our placental lipid bilayers and trophoblast cell layers. This decreased interaction can have potential to improve therapies for administration during pregnancy where transport across the maternal-fetal interface is of concern. In this work we utilized the AmBisome liposome formulation, which was determined to carry a strong negative charge. We hypothesize that both the larger size of AmBisome (100 nm vesicles) compared to free amphotericin B as well as repulsion of the negatively charged liposomes with the negatively charged lipid bilayers and trophoblast cell membranes resulted in this decreased interaction. To solidify this mechanism, future studies can be performed to investigate different liposome compositions with various lipids, sizes, and charges associated. The different properties will provide comparisons for how these nanomaterials interact with the trophoblast bilayers and cell layers.

The impact of this work is the development of an initial screening technique for interactions of pharmaceuticals, nanomaterials, and environmental toxicants with the placental barrier. This tool can be developed into a high-throughput technique to understand a large quantity of molecules.

Future work is needed in using the model for more small molecule interaction studies with comparisons to trophoblast cell lines and placental models (e.g. perfused placenta). Additional studies on developing the complexity of the lipid bilayers with incorporating proteins can be considered as the field of lipid bilayers starts to learn more about how to incorporate proteins into these structures. With the compositions from this study, computational modeling may also be performed to add complexity, such as proteins, while also incorporating the full lipid fatty acid distributions.

8.2.2 Future Perspectives

The placental bilayers have similarities to the lipid structures comprising the placenta; however, no *in vitro* or *in vivo* tool will completely recapitulate this dynamic organ. Limitations exist with these lipid models, such as that they model only passive transport. Thus, more information is needed to determine when it is appropriate to use each of the new models being developed. Additionally, there are current limitations in the techniques that can be used to confirm formation of lipid bilayers. In our work we focus on using QCM-D and PAMPA methods. While both of these methods have been highly reported on for forming lipid bilayers, neither method fully confirms the presence of the lipid composition expected, especially for multi-lipid compositions. Quantifying the lipid composition once the bilayer is formed is an important next step for the field to consider.

Having more tools available, including the contribution of our placental bilayers, will build a strategic workflow that can help improve pharmaceutical safety information. We suggest that the trophoblast lipid bilayers developed in this work can be used as a first-step screening technique to provide information to educate following steps in determining toxicity of a molecule during pregnancy.

8.3 Therapeutic design

8.3.1 Conclusions

Chapter 7 discussed the development of an antifungal liposomal formulation. In this chapter we encapsulated anidulafungin into liposomes and characterized their formation and drug loading ability. The liposomes improved survival of *Candida* infected *Galleria*

mellonella and improved eradication of difficult-to-treat *Candida albicans* biofilms compared to free drug.

With the improved efficacy observed using the liposomes, the mechanism for how the liposomes are interacting with the *Candida* cells is of interest. Specifically, we observed that liposomes improve treatment of *Candida* biofilms. Previous studies have demonstrated that liposomal formulations of amphotericin B were able to improve the penetration of fungal biofilms compared with free drug due to electrostatic interactions between the liposome phospholipids and the charged polysaccharide extracellular matrix secreted by the biofilm fungi.³⁵⁴ Fungal biofilms are rich in hexosamines³⁵⁵, positively charged sugars that may interact with the negatively charged liposomes we fabricated here. Future work on comparing different lipid compositions for *Candida* biofilm treatment may further elucidate this mechanism. Additionally, testing more strains of *Candida* that have varying degrees of secreted hexosamines may also be beneficial for understanding the liposome interactions.

The impact this work has is on developing a more effective treatment option for anidulafungin while also improving solubility of this hydrophobic drug for improved clinical administration. Furthermore, we demonstrated in chapter 6 that liposomal AmBisome interacts less with the trophoblast bilayers and cell layers compared to free amphotericin B. Thus, the anidulafungin liposomes may also help to mitigate interactions during pregnancy while not compromising on treatment efficacy.

Future work can be performed on altering the lipid composition of the liposomes to observe how these changes may impact efficacy. Anidulafungin, presented in this thesis, is an antifungal of the echinocandin drug class. It would be of interest to develop liposomal

formulations of antifungals from different drug classes including the polyene and azole drug classes and test their efficacy against planktonic and biofilm *Candida*.

8.3.2 Future Perspectives

Currently, expecting mothers are put in a tough situation: choosing between their health and the health of their developing child, which is already directly connected. One startling statistic from a 2006 study showed the choice of maintaining or discontinuing antidepressant medication during pregnancy, where 68% of women who discontinued medication relapsed into depression during pregnancy.³⁷⁵ Increased research into therapeutic design and using models, such as our placental bilayers, to test these therapeutics is needed.

Limited research within the pharmaceutical industry has been performed, which has been thought to be largely due to thalidomide.³⁷⁶ Thalidomide was used in the 1950s and 1960s for nausea treatment during pregnancy, but led to thousands of birth defects and has since been banned for use during pregnancy.³⁷⁷ Although this led to investigations on the molecular interactions of thalidomide with impact on understanding limb development and formation,³⁷⁷ it has made the field hesitant on pursuing direct studies on pregnancy medications and trials (Figure 8.2).³⁷⁶ Thus, this further demonstrates the need for placental models to study therapeutic design, and how the two advancements are closely linked. With new tools available, more confidence may be gained on developing pharmaceuticals for use during pregnancy.

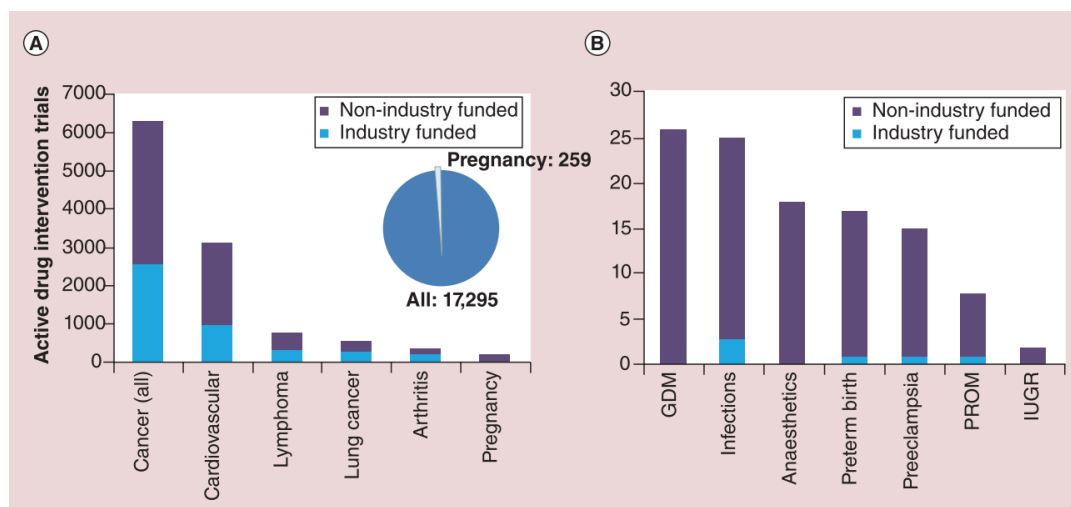


Figure 8.2: Inequality of funding for drug development during pregnancy. Reproduced from Keelan *et al.*, Nanomedicine, 2015 with permission.

8.4 Future research directions from this thesis

The conclusions from this thesis pave way for new studies to be performed. Each of these future directions are considered to be highly important next steps for the field to consider.

8.4.1 Mechanistic understanding of cervical mucus within a clinical sample

The mucus contained within the cervical sample is present in a fixative solution. Our investigation observed that this mucus plays a critical role in the enrichment process using the differential cell settling workflow. While we provided some hypotheses as to what might be occurring, future work to elucidate this mechanism is important. For example, mucus differential settling studies may be considered to observe the percentage of mucus that settles over time in solution. Additionally, trophoblast invasion across the cervical mucus could be performed in order to further understand this mechanism. Studies may be performed on porous transwell inserts coated with cervical mucus in the PreservCyt fixative solution and in PBS. Various trophoblast cell lines (including extravillous JEG-3

cells, and villous HTR-8 and TCL-1 cells) can be used to investigate degrees of invasion across the cervical mucus.

8.4.2 Using trophoblast lipid composition for drug delivery

This thesis provided the lipid composition representative of trophoblast cells at various gestational time points. As mentioned throughout the thesis, developing therapies for use during pregnancies is needed but current research is limited. Developing liposomes with the composition similar to trophoblast cells could help to facilitate delivery to the placenta for treatment of potential fetal abnormalities. The liposomes would have similarities to placental exosomes, which are vesicles secreted by cells to aid in communication. Previous work has indicated that the origin of exosomes impacts exosome cell targeting.³⁷⁸ We hypothesize that there might be an affinity for this composition towards placental cells, which can be tested in future work.

8.4.3 Addition of targeting moiety to antifungal liposomes

Now that we have demonstrated the ability to incorporate anidulafungin into liposomes and that they remain effective against *C. albicans*, moieties that have the ability to target *C. albicans* can be considered. Liposomes are an excellent delivery vehicle for the functionalization with moieties such as proteins and peptides to the surface. One example is the incorporation of a peptide P113Q2.10, which is a fragment of the Histatin 5 protein, and has been previously shown to localize to beta-glucans present on the *Candida* cell wall. Future studies can be focused on the incorporation of such targeting peptides.

8.5 Biomedical engineering in women's health

Women's health is comprised of categories including reproduction, fertility, maternal health, normal and abnormal pregnancy as well as diseases including cancer, pain, cardiac disease, and osteoporosis.³⁷⁹ This thesis has focused on a small sector of women's health, focusing on pregnancy and then furthermore on the role of the placenta.

During my Ph.D. studies, I have observed an increased interest in the biomedical engineering field within the area of women's health. While fundamental life science studies have been previously conducted, the engineering community is now seeing that this is a largely uncharted space that can benefit from biomedical engineering approaches. Engineering conferences, including the Biomedical Engineering Society annual meeting and Society for Biomaterials meetings now include sessions focused on bioengineering in women's and reproductive health. More recent examples of this include a 2019 special issue in the journal *Interface Focus* concentrated on bioengineering in women's health and the cover of MIT news magazine for November 2019 focusing on the science of pregnancy. While there is still a lot of work to accomplish, I am proud to be a part of this growing field and to have this thesis contribute to its growth.

8.6 Contributions to the field

Academically, the contributions to the field are summarized below.

- Understanding of trophoblast cell settling in cervical samples for non-invasive prenatal testing application

- Quantification of trophoblast lipids representative of 1st, 3rd, and term placenta and development of representative model placental trophoblast lipid bilayers
- Knowledge on phthalate interactions with both uni-lipid and placental lipid bilayers
- Knowledge on antifungal therapeutic interactions with placental bilayers
- Paradigm shift in supported lipid bilayer formation demonstrating critical vesicle coverage does not induce vesicle rupture alone and that energy input, such as flow, is necessary
- New antifungal nanoparticle encapsulating anidulafungin in liposomes demonstrating improved fungal treatment with the potential for mitigating interactions during pregnancy

This thesis also serves to demonstrate the benefit of biomedical engineering approaches for prenatal and women's health. It has led to a number of publications, conference presentations, and media posts. For example, the trophoblast enrichment work from chapter 2 was highlighted in Brown News (<https://www.brown.edu/news/2019-08-26/trophoblasts>). This kind of publicity not only serves for our specific study, but also brings to light the need for engineering approaches in this area. This thesis has identified a number of new steps for the field to consider, ultimately serving to advance prenatal and women's health.

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