DESIGNING Q-GRIFITHSIN-INCORPORATED RECTAL AND VAGINAL PRODUCTS FOR HIV PREVENTION

by

Xin Tong

Bachelor of Science, Sun Yat-Sen University, 2016

Master of Science, University of Pittsburgh, 2018

Submitted to the Graduate Faculty of the
School of Pharmacy in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

University of Pittsburgh

2022
This dissertation was presented

by

Xin Tong

It was defended on

March 4, 2022

and approved by

Donna M. Huryn, Ph.D., Professor
Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh
Chemical Diversity Center, University of Pittsburgh

Junmei Wang, Ph.D., Associate Professor
Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh
Computational Chemical Genomics Screening Center, University of Pittsburgh

Vinayak Sant, Ph.D., Assistant Professor
Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh

Joshua L. Fuqua, Ph.D., Assistant Professor
Department of Pharmacy and Toxicology, School of Medicine, University of Louisville

Dissertation Director: Lisa C. Rohan, Ph.D., Professor
Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh
Department of Obstetrics, Gynecology, and Reproductive Sciences, School of Medicine
Clinical and Translational Science Institute, University of Pittsburgh
Human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) remains a global health concern, with the majority of infections caused by sexual transmission. Due to biological, behavioral, and social factors, key populations including men who have sex with men (MSM), women, and adolescent girls are disproportionally at risk of HIV infection. The urgent need for novel approaches to prevent disease spread has prompted the research of topical microbicides, including both rectal and vaginal products. The work presented within this dissertation contributes to the development of dosage form options for rectal and vaginal application as topical pre-exposure prophylactics (PrEP) for HIV infection. Specifically, it focuses on developing novel formulations for the delivery of Q-Griffithsin (Q-GRFT) which can be utilized in these highly susceptible populations to prevent HIV infection.

We hypothesize that by manipulating excipients, Q-GRFT can remain stable in both liquid and solid dosage forms. We further hypothesize that the designed rectal/vaginal products can achieve physicochemical properties suitable as topical microbicides.

Pre-formulation assessments, including excipient screening and stability study in the manufacturing environment, were performed for Q-GRFT. Experimental and computational methods were explored to investigate strategies for preventing aggregation. Physicochemical properties, including appearance, pH, osmolality, flowability, crystallinity, water content, puncture
strength, and drug content, were characterized for different products respectively. The bioactivity (gp120 binding efficacy), permeability, and toxicity were also assessed for selected formulations.

Major findings from this dissertation indicate that: (1) Q-GRFT can remain stable with tenofovir (TFV), a nucleotide reverse transcriptase inhibitor (NRTI) that has synergetic effects against HIV infection; (2) protein-excipient interactions impact the stability of Q-GRFT; (3) formulation modifications can overcome the challenges during manufacturing processes.

Overall, this dissertation (1) explored the potential of combining Q-GRFT and TFV in liquid and solid dosage forms; (2) investigated the protein-excipient interactions and their impact on Q-GRFT stability; (3) developed stable Q-GRFT-incorporated formulations that meet critical quality attributes (CQAs) as topical PrEP options. Furthermore, the products generated from this work can be used as topical PrEP options that expand the current HIV prevention strategies for the key population at risk.
# Table of Contents

Preface ................................................................................................................................. xx

Dedication .............................................................................................................................. xxii

List of Abbreviations ........................................................................................................... xxiii

1.0 INTRODUCTION ............................................................................................................. 1

1.1 HIV INFECTION: A GLOBAL EPIDEMIC ................................................................. 1

1.1.1 Sexually Transmitted HIV Infection ....................................................................... 2

1.1.2 Key Population: Men Who Have Sex With Men (MSM) ........................................ 3

1.1.3 Key Population: Women and Adolescent Girls ..................................................... 4

1.2 HIV PREVENTION: PRE-EXPOSURE PROPHYLAXIS (PrEP) ............................ 5

1.2.1 Oral PrEP and Its Current Challenges ................................................................. 5

1.2.2 Topical Microbicides ............................................................................................ 7

1.2.2.1 Rectal Microbicides ....................................................................................... 8

1.2.2.2 Vaginal Microbicides .................................................................................... 9

1.3 Q-GRIFFITHSIN: A PROMISING CANDIDATE FOR HIV PREVENTION ...... 12

1.3.1 Manufacturing Process ...................................................................................... 13

1.3.2 The Potency and Safety Profile .......................................................................... 14

1.3.3 The Protein Structure ......................................................................................... 15

1.3.4 The Mechanism of HIV Prevention .................................................................... 16

1.3.5 Current Applications and Future Directions ..................................................... 16

1.4 GAP ANALYSIS OF INCORPORATING Q-GRFT IN DOSAGE FORMS .......... 18

1.4.1 The Challenges of Loading Proteins in Liquid Dosage Forms ......................... 18
1.4.2 The Challenges of Loading Proteins in Lyophilized Powders .................19
1.4.3 The Challenges of Loading Proteins in Polymeric Films ......................20

1.5 HYPOTHESIS AND SPECIFIC AIMS ................................................................. 21

2.0 Q-GRFT LIQUID FORMULATION DESIGN: ENEMA ......................................... 24

2.1 INTRODUCTION .................................................................................................. 25

2.1.1 Identification of Critical Quality Attributes (CQAs) ................................26
2.1.2 Development of the Manufacturing Process ..............................................26

2.2 MATERIALS AND METHODS ........................................................................ 27

2.2.1 Materials ........................................................................................................27

2.2.2 The Manufacturing Process .........................................................................28

2.2.2.1 The Process Development ................................................................. 28
2.2.2.2 The Final Manufacturing Procedure ................................................. 28
2.2.2.3 The Stability Study Procedure ....................................................... 29

2.2.3 Physicochemical Characterization of Enema Solutions .............................29

2.2.3.1 Appearance ......................................................................................... 29
2.2.3.2 pH ..................................................................................................... 30
2.2.3.3 Osmolality ......................................................................................... 30
2.2.3.4 TFV Content Determination by Ultra Performance Liquid Chromatography ................................................................. 30
2.2.3.5 Q-GRFT Content Determination by High-Performance Liquid Chromatography ................................................................. 31

2.2.4 Cell Culture and Cell Toxicity Studies ..................................................... 31
2.2.5 Permeability and Toxicity Assessment with the Caco-2 Monolayer Cell Model ..........................................................................................................................................................................................32
2.2.6 Permeability and Toxicity Assessment with the Ex Vivo Human Colon Tissue ..........................................................................................................................................................................................33
2.2.7 Bioactivity Determination using Enzyme-linked Immunosorbent Assay .....34
2.2.8 Statistical Analysis ..........................................................................................................................................................................................35
2.3 RESULTS.................................................................................................................................................................................................................35
2.3.1 Determination of Manufacturing Procedure..........................................................................................................................................................................................35
2.3.2 Characterizations of the TFV/Q-GRFT Combination Enema Solutions....37
2.3.3 Stability of the Two Combination Enema Formulations.....................................38
2.3.4 In Vitro Cell Toxicity Studies of the Enema Solutions.........................................41
2.3.5 Permeability Validation of the Different Enema Formulations on the Caco-2 Monolayer Cell Model ..........................................................................................................................................................................................46
2.3.6 Permeability of TFV Revealed by In Vitro and Ex Vivo Models .................48
2.3.7 The Permeability of Q-GRFT Evaluated by the Ex Vivo Colon Tissue Model ..........................................................................................................................................................................................49
2.3.8 Both Enema Solution Retained Bioactivity after 24 Months .......................51
2.4 DISCUSSION.................................................................................................................................................................................................................52
2.5 CONCLUSION.................................................................................................................................................................................................................56
3.0 Q-GRFT SOLID FORMULATION DESIGN: POWDER..........................................................................................................................................................................................58
3.1 INTRODUCTION.................................................................................................................................................................................................................59
3.1.1 Protein Instability Caused by Lyophilization........................................................59
3.1.1.1 Cryo-stress.................................................................................................................................................................................................................59
3.1.1.2 Dehydration ............................................................................................ 59
3.1.1.3 Concentration Effects .............................................................................. 60
3.1.1.4 pH Shifts .................................................................................................. 60
3.1.1.5 Other Theories ....................................................................................... 61
3.1.2 Cryoprotectants Prevent Protein Aggregation during Lyophilization .......61
3.1.3 Critical Quality Attributes for Q-GRFT Lyophilized Powder ...............63

3.2 MATERIALS AND METHODS ................................................................................. 64
3.2.1 Materials .............................................................................................................64
3.2.2 Lyophilization .....................................................................................................65
3.2.3 Physicochemical Characterization of the Lyophilized Powder ...............65
   3.2.3.1 Appearance ............................................................................................. 65
   3.2.3.2 Crystallinity Determination by Differential Scanning Calorimetry (DSC) .................................................................................................................. 66
   3.2.3.3 Crystallinity Determination by X-Ray Powder Diffraction (XRD) .. 66
   3.2.3.4 Flowability ............................................................................................. 66
   3.2.3.5 Size Distribution ..................................................................................... 67
   3.2.3.6 Water Content ......................................................................................... 67
3.2.4 Physicochemical Characterization of the Reconstituted Enema Solution ....67
   3.2.4.1 Appearance ............................................................................................. 67
   3.2.4.2 pH ............................................................................................................ 68
   3.2.4.3 Osmolality ............................................................................................... 68
3.2.5 Drug Content .....................................................................................................68
3.2.5.1 TFV Content Determination by Ultra Performance Liquid Chromatography ................................................................. 68
3.2.5.2 Q-GRFT Content Determination by High-Performance Liquid Chromatography ........................................................................................................ 69
3.2.5.3 Aggregation Determination by Size Exclusion Chromatography .... 69
3.2.6 Bioactivity Determination by Enzyme-linked Immunosorbent Assay ....... 69
3.2.7 Statistical Analysis .................................................................................................. 70
3.3 RESULTS ...................................................................................................................... 70
3.3.1 Cryoprotectant Selection Under Accelerated Condition ......................... 70
3.3.2 Maltitol Ratio Selections .................................................................................... 71
3.3.3 Short Term Stability Study for the Q-GRFT Lyophilized Powder ............... 74
3.3.4 Physicochemical Characterizations of the Q-GRFT Lyophilized Powder ... 76
3.3.5 Crystallization Detections by DSC ................................................................... 77
3.3.6 Crystallinity determination by XRD .................................................................. 78
3.3.7 LMP Retained gp120 Binding Bioactivity .......................................................... 79
3.3.8 Combination Powder: Short Term Stability Study for the Lyophilized TFV Powders ........................................................................................................ 80
3.3.9 Combination Powder: the Physically Combined Powder Strategy .......... 81
3.4 DISCUSSION ............................................................................................................ 83
3.5 CONCLUSION ........................................................................................................... 88
4.0 Q-GRFT SOLID FORMULATION DESIGN: FILMS ................................................. 90
4.1 INTRODUCTION ...................................................................................................... 91
4.1.1 Advantages of Polymeric Vaginal Films .......................................................... 91
4.1.2 FDA Approved Polymeric Vaginal Films .................................................................91
4.1.3 Film Manufacturing Methods ..................................................................................92
4.1.4 Excipients in the Polymeric Vaginal Film Formulations .......................................93
4.1.5 Critical Quality Attributes for the Polymeric Vaginal Film ...................................93
4.1.6 Challenges for Protein Drugs in the Polymeric Vaginal Film ...............................94

4.2 MATERIALS AND METHODS .................................................................................. 96
    4.2.1 Materials .............................................................................................................96
    4.2.2 The Q-GRFT Lyophilized Powder Stress-Testing under the Processing Condition ..................................................................................................................96
    4.2.3 Compatibility Study ..........................................................................................97
    4.2.4 The Q-GRFT SC Film Formulations and the Manufacturing Procedure ..........98
    4.2.5 The Q-GRFT HME Film Formulations and the Manufacturing Procedure ..........................................................................................................................101
    4.2.6 Drug Content Determination by High-Performance Liquid Chromatography ..........................................................................................................................103
    4.2.7 Aggregation Determination by Size Exclusion Chromatography ....................103
    4.2.8 Physicochemical Characterizations of Polymeric Films ...................................104
        4.2.8.1 Appearance ........................................................................................... 104
        4.2.8.2 Weight ................................................................................................... 104
        4.2.8.3 Thickness .............................................................................................. 104
        4.2.8.4 Water Content ...................................................................................... 104
        4.2.8.5 Puncture Strength ................................................................................ 105
    4.2.9 Bioactivity Determination by ELISA ................................................................105
4.2.10 Computational Study: Homology Modeling of Q-GRFT .........................106
4.2.11 Computational Study: Selections of the Aggregation Prone Regions ....106
4.2.12 Computational Study: Molecular Docking between Q-GRFT and Selected
Excipients ..............................................................................................................................107
4.2.13 Statistical Analysis .................................................................................................107
4.3 RESULTS ......................................................................................................................108
4.3.1 Stress Testings for the Q-GRFT Lyophilized Powder ..............................108
4.3.2 Compatibility Study of Q-GRFT with Common Excipients in Film
Formulations ........................................................................................................................110
4.3.3 Homology Modeling for Q-GRFT and Aggregation Prone Region
Identifications ......................................................................................................................111
4.3.4 Molecular Docking Study for Protein-Excipient Interactions ................116
4.3.5 Formulation Selection for the Q-GRFT SC Films .....................................117
4.3.6 Formulation Selection for the Q-GRFT HME Films ................................ 118
4.3.7 Physicochemical Characterizations of Q-GRFT Polymeric Films ..........119
4.3.8 Stability Studies for the Selected Q-GRFT HME Films .........................121
4.4 DISCUSSION .............................................................................................................121
4.5 CONCLUSION ..........................................................................................................127
5.0 DISCUSSION ............................................................................................................128
5.1 MAJOR FINDINGS ...................................................................................................129
5.1.1 Addressing the Adherence Issue with Patient-Centered Product Designs 129
5.1.1.1 Utilizing Pre-Existing Behaviors ..........................................................130
5.1.1.2 Advancing to Solid Dosage Forms .......................................................131
List of Tables

Table 2.1 The Final Stage (Stage C) Screening Process for Both Vehicle-based Enema Formulation Development................................................................. 36
Table 2.2 Physicochemical Characterizations of the Two Vehicle-based Combination (Combo) Enema. ............................................................................................................. 37
Table 2.3 A Composition Comparison of Similar Enema Formulations That Are in Clinical Trials or on the Market. ................................................................. 41
Table 2.4 A Full Comparison of Multiple Enema Formulations with Different Osmolality using the Caco-2 Monolayer Cell Model................................................................. 47
Table 2.5 A Comparison of the Permeability of TFV in Three Hypotonic Enema Formulations using the Caco-2 Monolayer Cell Model. (n=3–6). ......................... 48
Table 2.6 A Comparison of the Q-GRFT Permeability among Four Formulations. ....... 50
Table 3.1 A Comparison of Q-GRFT Only Lyophilized Powder and LMP for the Physiochemical Characterizations. ................................................................. 76
Table 4.1 Stress Testing Conditions (Mimicking HME Processing) for LMP. ............... 96
Table 4.2 A List of Excipients Used in the Compatibility Study. ................................. 97
Table 4.3 Formulation-1 (F-1) for the Q-GRFT SC Polymeric Films. ......................... 99
Table 4.4 Formulation-2 (F-2) for the Q-GRFT SC Polymeric Films. ......................... 99
Table 4.5 Formulation-3 (F-3) for the Q-GRFT SC Polymeric Films. ......................... 100
Table 4.6 A Comparison of Excipient Amounts in Three Q-GRFT SC Polymeric Film Formulations. ................................................................................................. 101
Table 4.7 Formulation-4 (F-4) for the Q-GRFT HME Polymeric Films......................... 102
Table 4.8 Formulation-5 (F-5) for the Q-GRFT HME Polymeric Films............................ 102
Table 4.9 A Summary Table for the Docking Scores. ........................................................... 116
Table 4.10 Physicochemical Characterizations of Q-GRFT-incorporated Polymeric Films.
......................................................................................................................................... 120
Appendix Table 5.1 Apparent Permeability (P_{app}) Values of TFV in Four Hypotonic Enema
Formulations. ................................................................................................................ 144
Appendix Table 5.2 A Comparison of the Permeability of TFV and [^{14}C]-Mannitol in Three
Hypotonic Enema Formulations using the Caco-2 Monolayer Cell Model............ 145
Appendix Table 5.3 A Comparison of the Permeability of TFV in Three Hypotonic Enema
Formulations. ................................................................................................................ 145
List of Figures

Figure 2.1 Procedures of Formulation Development and Monitoring for Two Vehicle-based Combination Enema. .................................................................................................................. 29

Figure 2.2 The Initial Screening Process for the Manufacturing Procedure Development. 36

Figure 2.3 pH Stability Testing for Two Combo Formulations: a. PBS-base Combo Enema; b. Saline-base Combo Enema............................................................................................................................ 39

Figure 2.4 Osmolality Stability Testing for Two Combo Formulations: a. PBS-base Combo Enema; b. Saline-base Combo Enema.................................................................................................................. 39

Figure 2.5 Drug Content Stability: a. TFV Content in the PBS-base Combo Enema Solution; b. TFV Content in the Saline-base Combo Enema Solution...................................................... 40

Figure 2.6 Drug Content Stability: a. Q-GRFT Content in the PBS-base Combo Enema Solution; b. Q-GRFT Content in the Saline-base Combo Enema Solution................................. 40

Figure 2.7 In Vitro Caco-2 Cell Toxicity of Three Excipients in the Enema Formulations. 42

Figure 2.8 In Vitro Toxicity of Two Combination Enema Formulations using the Caco-2 Monolayer Cell Model. .................................................................................................................. 43

Figure 2.9 In Vitro Toxicity of Two Combination Enema Formulations using the Caco-2 Monolayer Cell Model. .................................................................................................................. 44

Figure 2.10 In Vitro Toxicity of Two Combination Enema Formulations using the Caco-2 Monolayer Cell Model. .................................................................................................................. 45

Figure 2.11 A Full Comparison of Multiple Enema Formulations with Different Osmolality using the Caco-2 Monolayer Cell Model................................................................. 46
Figure 2.12 P<sub>app</sub> Values of TFV in Four Hypotonic Enema Formulations using Human Colon Tissues in the Ussing Chamber

Figure 2.13 Bioactivity of gp120 Binding Efficacy Determined by ELISA.

Figure 3.1 Sugar/Sugar Polyol Selection as Cryoprotectants.

Figure 3.2 Maltitol Ratio Selection as Cryoprotectants under an accelerated condition.

Figure 3.3 Maltitol Ratio Selection as Cryoprotectants under a normal condition.

Figure 3.4 Short-term (Three Months) Stability Study.

Figure 3.5 DSC Chromatography for LMP (green line), Q-GRFT Only Lyophilized Powder (red line), Maltitol Only [lyophilized] Powder (magenta line), and Maltitol Only [unlyophilized] Powder (blue line).

Figure 3.6 XRD Patterns for Q-GRFT Only Lyophilized Powder (blue line), LMP (black line), PBS [lyophilized] (green line), and Maltitol [lyophilized] (red line).

Figure 3.7 Bioactivity of Q-GRFT LMP Determined by ELISA. N=3.

Figure 3.8 TFV Lyophilized Powder Stability.

Figure 3.9 Q-GRFT Content Detected in Individual or Combined Reconstituted Enema.

Figure 3.10 TFV Content Detected in Individual or Combined Reconstituted Enema.

Figure 4.1 The Stability Study of Q-GRFT Content after the Stress Challenges.

Figure 4.2 The Stability Study of Q-GRFT Content after the Stress Challenges on Day 60.

Figure 4.3 The Compatibility Study of Q-GRFT with Film Excipients.

Figure 4.4 Aggregation Prone Region Predictions.

Figure 4.5 A Summary of the Predicted and Selected Aggregation Prone Regions of Q-GRFT.
Figure 4.6 The Q-GRFT Homodimer Structure by Homology Modelling Shown in Two Perspectives. ................................................................. 115

Figure 4.7 Three Solvent-Cast (SC) formulations were tested and compared in the short-term stability study. .......................................................... 117

Figure 4.8 Two Hot Melt Extrusion (HME) formulations were tested and compared in a short-term stability study. .................................................. 119

Figure 4.9 Representative images of (a) Q-GRFT SC Film (F-3), and (b) Q-GRFT HME Film (F-5). ........................................................................ 120

Figure 4.10 Exploratory studies for Q-GRFT stability in the HME films (F-5). ............... 121

Appendix Figure 5.1 A Representative Figure for TEER Values Measured with Ussing Chamber. .................................................................................. 146

Appendix Figure 5.2 Epithelial Structures of the Human Colorectal Tissue using H&E Staining. ........................................................................ 147

Appendix Figure 5.3 Short-term (Ten Days) Stability Study of Q-GRFT Only (in PBS) Lyophilized Powder. ......................................................... 148

Appendix Figure 5.4 Short-term (One Month) Stability Study of Q-GRFT Only (in PBS) Lyophilized Powder. ......................................................... 149

Appendix Figure 5.5 DSC Chromatography for KCl (red), NaCl (black), Na₂HPO₄ (blue), and NaH₂PO₄ (green). ......................................................... 150

Appendix Figure 5.6 DSC Chromatography for PBS Powder (a physical combination of KCl, NaCl, Na₂HPO₄, and NaH₂PO₄ powders). ........................................... 151

Appendix Figure 5.7 XRD Patterns for the PBS Lyophilized Powder. ......................... 152
Appendix Figure 5.8 TFV Lyophilized (with Maltitol) Powder Stability. TFV was lyophilized with maltitol in two base formulations, (a) Saline-base and (b) PBS-base respectively. ......................................................................................................................................... 153

Appendix Figure 5.9 pH Determined in Individual or Combined Reconstituted Enema. 154

Appendix Figure 5.10 Osmolality is Determined in Individual or Combined Reconstituted Enema.................................................................................................................................................. 155

Appendix Figure 5.11 Long-term Stability Study for LMP...................................................... 156

Appendix Figure 5.12 SEC chromatography for the Long-term Stability Study of LMP. 157

Appendix Figure 5.13 SEC chromatograms of Q-GRFT SC film group (Formulation-1, F-1, shown in the top black line), its Placebo film group (shown in the bottom black line), and Placebo Spiked Q-GRFT group (shown in the middle red line). ...................... 158

Appendix Figure 5.14 SEC chromatograms of Q-GRFT SC film group (Formulation-2, F-2, shown in the top black line), its Placebo film group (shown in the bottom black line), and Placebo Spiked Q-GRFT group (shown in the middle red line). ...................... 159

Appendix Figure 5.15 SEC chromatograms of Q-GRFT SC film group (Formulation-3, F-3, shown in the top black line), its Placebo film group (shown in the bottom black line), and Placebo Spiked Q-GRFT group (shown in the middle red line) ......................... 160

Appendix Figure 5.16 SEC chromatograms of Q-GRFT HME film group (Formulation-5, F-5, shown in the middle black line), its Placebo film group (shown in the top blue line), and Q-GRFT reference group (shown in the bottom magenta line). ...................... 161
There have been highs and lows during my Ph.D. training. I sincerely appreciate all the people I have met in both professional and personal settings. From you, I gained strength.

I would like to thank Dr. Lisa Rohan for her kind and generous support. She gives precious advice on the courses and all the projects I participated in over the past two years. The experience she shared definitely helped me succeed in this project and beyond. Moreover, she cared for all the students and staff. The family-like working atmosphere really paves the path for scientific research.

I also want to express my gratitude to all of the lab members, Dr. Sravan Patel, Dr. Guru Valicherla, Dr. Junmei Zhang, Dr. Galit Regev, Dr. Jing Li, Dr. Sheila Grab, Dr. Lindsey Kramzer, Dr. Kunal Jhunjhunwala, Ms. Lin Wang, Mr. Philip Graebing, Ms. Christina Bagia, Mr. Prithivirajan Durairajan, Ms. Ruohui Zheng and all the other members. It is such a privilege to work with all these intelligent people. And I would like to thank the help from my committee members, Dr. Donna Huryn, Dr. Vinayak Sant, Dr. Junmei Wang, and Dr. Joshua Fuqua, as well as other faculty in the School of Pharmacy. The generous help from Dr. Wen Xie, Dr. Sam Poloyac, Dr. Maggie Folan, Ms. Lori Altenbaugh, Ms. Marian Klanica, and Ms. Dolly Hornick is also appreciated.

Last but not the least, I would like to thank my family for their unconditional support. Without their love, I certainly cannot achieve what I have. Also, I will never forget the fun and lovely moments I spent with all my friends during these years. Special thanks to Yunqi An, Xinyi Chen, Emma Liao, Chenxiao Tang, Hung-Chung Tung, Xinran Cai, and Zhongfang Zhang for
their great tolerance in our friendships. In the end, shout out to all the funniest YouTubers and cutest pets in the world for their kind company on many sleepless nights.
Dedication

This dissertation is dedicated to all the less fortunate people around the world living in fear affected by HIV.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ANN</td>
<td>Artificial Neural Network</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
</tr>
<tr>
<td>APR</td>
<td>Aggregation Prone Region</td>
</tr>
<tr>
<td>ARV</td>
<td>Anti-Retroviral</td>
</tr>
<tr>
<td>ASP</td>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CC₅₀</td>
<td>50% Cytotoxic Concentration</td>
</tr>
<tr>
<td>CMC</td>
<td>Chemistry, Manufacturing and Control</td>
</tr>
<tr>
<td>COVID-19</td>
<td>Coronavirus Disease 2019</td>
</tr>
<tr>
<td>CQA</td>
<td>Critical Quality Attribute</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug Delivery System</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DPV</td>
<td>Dapivirine</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>50% Effective Concentration</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EFdA</td>
<td>4’-ethynyl-2-fluoro-2’-deoxyadenosine; Islatravir</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FTC</td>
<td>Emtricitabine</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>GRFT/Q-GRFT</td>
<td>Griffithsin/Q-Griffithsin</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
</tr>
<tr>
<td>HEC</td>
<td>Hydroxyethyl Cellulose</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HPMC</td>
<td>Hydroxypropyl Methylcellulose</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papillomavirus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Hotspot</td>
</tr>
<tr>
<td>HST</td>
<td>Hotspot Threshold</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes Simplex Virus-2</td>
</tr>
<tr>
<td>HT</td>
<td>High Temperature</td>
</tr>
<tr>
<td>HTS</td>
<td>High-Throughput Screening</td>
</tr>
<tr>
<td>HME</td>
<td>Hot-Melt Extrusion</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Inhibitory Concentration</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese Encephalitis Virus</td>
</tr>
<tr>
<td>KBP</td>
<td>Kentucky Bioprocessing</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>KRBG</td>
<td>Krebs Ringer Bicarbonate Glucose</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption/Ionization</td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>Middle East Respiratory Syndrome-Coronavirus</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometer</td>
</tr>
<tr>
<td>MSM</td>
<td>Men Who Have Sex With Men</td>
</tr>
<tr>
<td>MTC</td>
<td>Medicine-Taking Compliance</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MVC</td>
<td>Maraviroc</td>
</tr>
<tr>
<td>N-9</td>
<td>Nonoxynol-9</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>Monosodium Phosphate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium Phosphate</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-Human Primate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside Reverse Transcriptase Inhibitor</td>
</tr>
<tr>
<td>NS/N.S.</td>
<td>No Significance</td>
</tr>
<tr>
<td>P&lt;sub&gt;app&lt;/sub&gt;</td>
<td>Apparent Permeability</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBD</td>
<td>Protein Databank</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
</tr>
<tr>
<td>PEDV</td>
<td>Porcine Epidemic Diarrhea Virus</td>
</tr>
<tr>
<td>PEG/PEO</td>
<td>Polyethylene Glycol/Polyethylene Oxide</td>
</tr>
<tr>
<td>PEP</td>
<td>Post-Exposure Prophylaxis</td>
</tr>
<tr>
<td>PEST</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PFDD</td>
<td>Patient-Focus Drug Development</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PrEP</td>
<td>Pre-Exposure Prophylaxis</td>
</tr>
<tr>
<td>PS</td>
<td>Puncture Strength</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl Alcohol</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized Controlled Trial</td>
</tr>
<tr>
<td>RT</td>
<td>Retention Time</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>Severe Acute Respiratory Syndrome-Coronavirus</td>
</tr>
<tr>
<td>SC</td>
<td>Solvent-Cast</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
</tr>
<tr>
<td>SLS</td>
<td>Sodium Lauryl Sulfate</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard Operating Procedure</td>
</tr>
<tr>
<td>SS</td>
<td>Screw Speed</td>
</tr>
</tbody>
</table>
1.0 INTRODUCTION

1.1 HIV INFECTION: A GLOBAL EPIDEMIC

With 680 thousand people dying of related illnesses worldwide in 2020 and 36.3 million cumulative deaths so far\(^1\), human immunodeficiency virus (HIV) remains a severe epidemic.

HIV can spread through bodily fluids and attack the body’s immune system after infection\(^2\). Without treatment, HIV reproduces in the host, destroying and impairing the function of immune cells\(^1\). Infected individuals gradually become immunodeficient, leading to the most severe stage of HIV infection: acquired immunodeficiency syndrome (AIDS)\(^2\). Since the height of the epidemic in the mid-1980s, tremendous efforts have been made in the field. With the advances in diagnosis, treatment, care, and prevention of HIV, the number of newly infected cases and AIDS-related deaths has dropped over the years. According to the World Health Organization (WHO)\(^1\), 37.9 million people are living with HIV/AIDS globally in 2018. However, by 2020 that number was reduced to 37.7 million\(^3\). In the United States (U.S.) alone, 3.3 million people are living with HIV in 2018\(^4\). At the end of 2019, the estimated number of people having HIV in the U.S. decreased to 1.2 million\(^5\). However, the emerging coronavirus disease 2019 (COVID-19) pandemic disrupted essential health services for HIV globally\(^7\). The disruptions in HIV prevention, testing, and treatment services slowed down the progress to end the HIV epidemic.

Therefore, to achieve the goal of at least a 90% reduction in new HIV infections by 2030\(^8\), more accessible resources should be provided, especially to the key populations with higher risks of infection.
1.1.1 Sexually Transmitted HIV Infection

Sexually transmission of HIV-1 can be initiated through vaginal, rectal, and sometimes oral sex with an infected partner. Once virus-containing body fluid (for example, semen) is deposited in the vagina or rectum, the virus binds to CD4 receptors on the host cell surface by using the envelope protein gp120\textsuperscript{9,10}. The envelope protein, altered by CD4 binding, also binds to specific chemokine co-receptors on the cell (CCR5 and CSCR4)\textsuperscript{9}. HIV primarily targets CD4\textsuperscript{+} T cells. Several other key target cells, including Langerhans cells (dendritic cells expressing the HIV CD4 receptor), and macrophages, are also present in the vaginal or rectal lumen. HIV can infect these target cells directly or bind to the cellular targets for further penetration\textsuperscript{11}. Following initial infection and local viral replication, the virus disseminates to regional lymph nodes. Within days, the infection of HIV is established\textsuperscript{12}. At about day 10, HIV becomes detectable in the blood. Without treatments, the spread of HIV continues exponentially and peaks around day 30, when the HIV antibodies become detectable. The immune systems then achieve some levels of control\textsuperscript{13}, until HIV causes progressive loss of CD4\textsuperscript{+} T cells and a host of immunological abnormalities\textsuperscript{14,15}. After years of disease progression, uncontrolled HIV loads can cause profound immunodeficiency and a characteristic infectious or oncological complication, defined as AIDS\textsuperscript{10}.

In nearly all regions of the world, certain groups of people have high HIV prevalence due to complex biological, behavioral, and social factors. The high vulnerability to HIV infection in these groups makes them key populations in an effective response to the pandemic\textsuperscript{7,10}. 
1.1.2 Key Population: Men Who Have Sex With Men (MSM)

Men who have sex with men (MSM) have been the most HIV-impacted population since the beginning of this epidemic\(^1\). Although the number of new HIV infections among MSM decreased by 9% from 2015 to 2019\(^2\), MSM is still a key population with a significant proportion of HIV incidence\(^1\). In 2019, male-to-male sexual contact accounted for 65% of all new HIV diagnoses in the U.S.\(^3\)

Among MSM, the receptive partners have the highest risk of HIV infection during unprotected anal intercourse\(^4,5\), due to anatomical and physiological characterizations of the rectum. The human rectum has an average length of 17.4 cm, a surface area of 200.9 cm\(^2\), and a thickness of 25 µm, lined with a single layer of columnar cells\(^6-8\). Compared to the much thicker vaginal epithelium (215 µm), the rectum is more susceptible to damage during intercourse, leading to an increased chance of viral entry\(^9\). The rectal environment also lacks the innate protection elicited by the low pH that is present in the vagina\(^10,11\). Studies\(^12,13\) reported that acidic, rather than neutralized, cervicovaginal mucus containing lactic acid can trap HIV. However, the normal pH of the rectum is neutral (7–8) with minimal buffering capacity\(^14\).

In addition, greater expression of CCR5, one HIV targeted receptor, was found on rectal macrophages by McElrath et al.\(^15\) Similarly, Kelley et al.\(^16\) also identified a distinct rectal environment within men who had engaged in anal intercourse that is associated with mucosal injury and repairs. Altogether, studies reported a high population of HIV-1 target cells in the rectum\(^17-19\), emphasizing the vulnerability of the rectum to HIV infection.

Besides the biological vulnerability presented in MSM, this population usually face homophobia and transphobia. These social discriminations are barriers against HIV diagnosis, prevention, and treatment\(^20-22\). The lack of information, education, and financial support also
makes it harder for some sub-populations to access healthcare services\textsuperscript{35-37}. The biological properties of the rectum, combined with the social and cultural stigma faced by MSM, make them one of the key populations that require attention\textsuperscript{3,11,31,38}.

1.1.3 Key Population: Women and Adolescent Girls

In 2020, women and girls accounted for 50% of all new HIV infections globally\textsuperscript{3}. This ratio is even higher in regions where HIV is most severe, including Sub-Saharan Africa. In Sub-Saharan Africa, six out of seven newly infected adolescents (aged 15–19) are girls\textsuperscript{3}. In addition, a higher risk of HIV infection was observed for certain overlapping populations, including transgender women\textsuperscript{6,7}, female sex workers\textsuperscript{39}, and women who inject drugs\textsuperscript{40}. Although HIV diagnoses among women have declined sharply in the U.S., there are still 7,000 women who received HIV diagnoses in 2018, accounting for one-quarter of the total diagnoses\textsuperscript{41}.

Social barriers, including sexism, racism, and HIV stigma, have a major negative impact on women’s health and well-being\textsuperscript{3,40,42}. Women and adolescent girls, who are sometimes financially and socially impaired, have limited access to HIV diagnosis, treatments, and prevention services\textsuperscript{7,40}. It is also reported that more than one-third of women (35%) around the world have encountered physical and/or sexual violence in their lives\textsuperscript{3}. Due to sexual violence, or unawareness of a partner’s status for HIV, some women may not use condoms or medicine to prevent HIV\textsuperscript{5}. In a behavioral study of heterosexual women at increased risk of HIV infection, 92% of HIV-negative women reported having vaginal sex without a condom in the previous year, and 25% reported having unprotected anal sex\textsuperscript{41}. Without prevention, women have a greater chance of contracting HIV through receptive vaginal and anal sex\textsuperscript{5,40}. 
With only 55% of countries having prevention strategies addressing adolescent girls and young women, there is an urgent need to decrease the high risk for women with high-impact HIV prevention approaches.

1.2 HIV PREVENTION: PRE-EXPOSURE PROPHYLAXIS (PrEP)

There is no cure for HIV infection. However, growing evidence has demonstrated that well-designed, targeted, and hypothesis-based prevention methods can be effective in reducing the spread of HIV. Prevention strategies, including syringe services (for people who inject drugs), condom use, Pre-Exposure Prophylaxis (PrEP), and Post-Exposure Prophylaxis (PEP), have helped slow the spread of HIV. Statistics show that the number of new HIV infections per year was reduced by 52% since the peak in 1997, down to 1.5 million people newly diagnosed with HIV globally. This recent progress was credited to the increased implementation of key prevention and treatment strategies. In addition, new prevention options, including HIV vaccines, are also under development.

1.2.1 Oral PrEP and Its Current Challenges

The most common prevention method for HIV is PrEP. The goal of PrEP is to administer anti-retroviral drugs before sex to prevent the transmission of HIV. Truvada, a fixed combination of emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF), is the daily PrEP oral pill approved by FDA in 2012. The Partner study, a randomized controlled trial (RCT), tested the efficacy of TDF and FTC/TDF. Compared to the placebo arm, the use of TDF provided a 67%
protection rate and FTC/TDF provided a 75% protection rate\textsuperscript{49}. Another RCT study conducted in France and Canada involving 400 MSM or transgender women, showed that prophylaxis had an 86% efficacy rate\textsuperscript{50}. Later in 2019, the U.S. Food and Drug Administration (FDA) approved a second PrEP medication option, Descovy\textsuperscript{51,52}. This oral pill contains FTC and tenofovir alafenamide (TAF). Because the effectiveness has not been studied in women, Descovy is only indicated for people who are HIV-negative and at-risk for sexually acquired HIV through anal sex\textsuperscript{51}.

Although both Truvada and Descovy can be prescribed to MSM at risk, certain barriers lower adherence to these oral PrEP options\textsuperscript{53}. For instance, there are concerns about long-term and short-term side effects, questions about future drug resistance, and more\textsuperscript{53}. Given that adherence is critical to maintaining therapeutic concentrations of drugs in the body, these concerns may negatively impact the effectiveness of protection\textsuperscript{53,54}.

The adherence issues are worse among women, especially in the adolescent female population. One review in 2017 analyzed 10 eligible published RCTs with 24,193 participants\textsuperscript{55}, indicating that the protective efficacy of oral PrEP increases with medicine-taking compliance (MTC). In this meta-analysis study, the subgroup analysis revealed that the MTC level of participants younger than 30 was lower than those aged 30 or older (34.9% vs. 69.6%, p<0.001); those studies that involved only women had lower MTC than those of only men or both women and men (31.3% vs. 71.7% and 31.3% vs. 71.0%, p<0.01 for both). And the differences between subgroups were associated with adherence, as the data reveals relatively lower compliance in young (<30 years old) groups of women\textsuperscript{55}. Similar effects were also observed in women in the U.S., with an extremely low (7%) administration rate of oral PrEP due to social barriers\textsuperscript{56}. 

6
Besides compliance as a clear cause for PrEP failure, differences between male and female anatomy also affect the efficacy of PrEP. It has been confirmed that after administration, TDF levels in male rectal tissue are between 10 and 100 times higher than those of female cervical/vaginal tissue\textsuperscript{57}. These results demonstrate the need for better protective options for this key population.

In addition, financial burdens, lack of policy and laws, and social stigma all lead to the low prescription rate, even in the U.S. In 2019, only 23\% of people eligible for PrEP were prescribed\textsuperscript{46}. Since no single prevention method can stop the HIV epidemic by itself, several approaches are needed for different populations to address the gaps in oral PrEP and to provide an effective reduction in HIV infection\textsuperscript{45}.

1.2.2 Topical Microbicides

To address the global HIV epidemic, the “Global health sector strategy on HIV for 2016–2021” was initiated at the Sixty-Ninth World Health Assembly\textsuperscript{1}. Under the direction “Innovation for acceleration”, expanding the HIV prevention options\textsuperscript{7} is one of the most important focus areas in the future.

Microbicides offer an important alternative for HIV prevention\textsuperscript{11,42}. Sexual transmission of HIV-1 is initiated when virus-containing body fluid (normally, semen) is deposited in the rectum or vagina, or less likely, when the virus passes from these compartments to the insertive partners. Several key target cells, including T cells, Langerhans cells (dendritic cells expressing the HIV CD4 receptor), and macrophages, are present in the vaginal or rectal lumen. HIV can infect these target cells directly or bind to the cellular targets for further penetration\textsuperscript{11}. Following initial infection and local viral replication, the virus disseminates to regional lymph nodes. Eventually,
the infection of HIV is established\textsuperscript{12}. Active pharmaceutical ingredients (APIs) in microbicides can either disrupt the virus envelope before the initial attachment of the virus to target cells (e.g. entry inhibitors) or suppress viral replication (e.g. reverse transcriptase inhibitors)\textsuperscript{11,42}.

Because of the biological differences between the rectum and vagina, microbicide development strategies have also been different for these two compartments.

\textbf{1.2.2.1 Rectal Microbicides}

Studies have demonstrated growing evidence for the need for safe and effective rectal microbicides\textsuperscript{31}. Due to biological differences between rectal and vaginal environments, including epithelium thickness, pH, and microbiota, the risk of contracting HIV for receptive partners of anal sex is 5 times higher than for vaginal sex\textsuperscript{31}. It is also pointed out that rectal microbicides as secondary barriers are beneficial during condom-using intercourse. In cases where condoms break or slip off, microbicides can still provide protection against HIV\textsuperscript{58}. Branching from the first generation of rectal microbicides, where only one API was incorporated, combination strategies have been explored in a single product\textsuperscript{58}. Since the presence of other sexually transmitted diseases (STDs) is associated with a higher risk of HIV infection\textsuperscript{11,59}, combination microbicides also serve as a multi-purpose technology to protect MSM from both STDs and HIV infection.

Several drug delivery systems have been studied as rectal microbicides, including gels, suppositories, and enemas/douches\textsuperscript{31,59-64}. Gels have been traditionally used as a topical drug delivery system. This dosage form has already been applied to vaginal delivery\textsuperscript{42,65,66}. Anti-retroviral (ARV) agents, namely TFV\textsuperscript{66} and maraviroc (MVC)\textsuperscript{67}, have been developed as vaginal gel formulations to prevent HIV. Anton et al.\textsuperscript{61} assessed the safety, acceptability, pharmacokinetic (PK), and pharmacodynamic (PD) responses to rectal administration of TFV 1\% vaginally formulated gel. Results from this study indicate that rectal dosing results in greater drug contents
in rectal tissues than oral dosing. In addition, a recent study has also reported the potential of dual compartment (rectal and vaginal) gels with the combination of TFV and FTC\textsuperscript{68}. However, the aforementioned gel formulations were not originally intended for rectal use\textsuperscript{61}.

Suppositories are semi-solid delivery systems, designed to either melt or dissolve at body temperature after insertion. As a dosage form, suppositories have been applied to many indications, including constipation, inflammatory bowel disease, and recently HIV prevention\textsuperscript{31,58}. Enemas are liquid dosage forms stored in bottles. Usually used as a treatment for constipation, enemas are also used by MSM as a cleansing agent before anal sex\textsuperscript{62,69}. Given the common practice of this behavior, researchers developed enema formulations with ARVs to provide HIV protection for this population\textsuperscript{62-64}. A clinical study\textsuperscript{70} was conducted on nine men with an enema of different osmolarities. Results indicated good acceptability of men using enemas as rectal microbicides\textsuperscript{70}.

Regardless of the dosage form, several considerations need to be included in the formulation. Firstly, the normal pH of the human rectum is around 6.6\textsuperscript{71,72}. Therefore the pH range for formulations needs to be close to neutral (6.5–8.0). Secondly, the literature suggests that hypotonic enemas are the best option compared to isotonic and hypertonic enemas\textsuperscript{63,64}. The hypotonic vehicle delivers higher drug concentrations into colon tissues, without damaging the epithelium. Other perspectives, including drug dosing, toxicity, and drug interactions with other drugs, excipients, enzymes, or transporters, should also be considered during formulation development\textsuperscript{58}.

1.2.2.2 Vaginal Microbicides

Vaginal microbicides have been developed for decades since Zena Stein first proposed a topical “virucide” that blocks HIV-1 transmission\textsuperscript{73}. From there, the first generation of non-specific microbicides, including surfactants/detergents, was designed to deliver bioactivity
Surfactants can disrupt the membranes of viruses and bacteria. Therefore, several products containing surfactants were tested clinically as vaginal microbicides, namely nonoxynol-9 (N-9) gel/sponge/film, SAVVY or C31G gel, and sodium lauryl sulfate (SLS) gel. However, these products either lacked clinical efficacy or resulted in negative clinical endpoints. Results of phase II/III clinical trials demonstrated an increase in HIV infection risk in women with frequent use (more than 3.5 vaginal applications per day) of N-9 gels, possibly due to the damage of vaginal epithelium. Despite the safety issues associated with N-9 in the vagina, N-9 films are still on the market as a vaginal microbicide for contraception, namely vaginal contraceptive films (VCF).

Due to the lack of efficacy in clinical trials for first-generation microbicides, researchers have been focusing on APIs that directly and specifically act against HIV. Among the second-generation vaginal microbicides, the TFV vaginal gel and the dapivirine (DPV) intravaginal ring are the two most clinically advanced products.

Clinical trials with TFV gels (HPTN 050 and CAPRISA 004) demonstrated good acceptability, safety, and effectiveness of TFV 1% vaginal gel. Results of the CAPRISA trial showed that tenofovir gel use was associated with an overall 39% decrease in HIV-1 acquisition. Additionally, among women with high gel adherence, the tenofovir gel reduced HIV infection by 54% when compared to placebo gel. Unfortunately, patient adherence is a significant issue for vaginal gels. In the VOICE study, 5,029 HIV-negative women were randomized to TDF, FTC/TDF, 1% TDF vaginal gel, and two placebo arms. None of the three study arms showed protection over the placebo arms, possibly due to low product adherence. The investigators later found out that the TDF levels were only detected in 30%, 29%, and 25% of those women who received TDF, FTC/TDF, and 1% TDF vaginal gel, respectively. Similar findings were reported in the FACTS-001 study, where tenofovir (TFV) 1% gel was evaluated for HIV prevention.
among women in South Africa. The study reported that the TFV gel did not prevent HIV acquisition in this population due to low patient adherence66.

The DPV intravaginal ring (DPV-VR) was recommended by WHO in 2021 as an additional prevention choice for women at risk of HIV infection85. Results of two Phase III clinical trials (The Ring Study and MTN-020-ASPIRE) assessing the safety and efficacy of the DPV-VR indicated that the product has a moderate effect on the prevention of HIV transmission overall86,87. Compared to the placebo group, the DPV-VR safely reduced HIV infection by 27–31%86. In addition, vaginal rings delivering TDF, TFV, MVC, and a combination of MVC/DPV have been completed in a series of Phase I trials87-89. Similar to other microbicides, vaginal rings do not completely eliminate the adherence challenges exhibited in clinical trials86,87,90, but the adherence issue was reduced, likely because the ring is a solid dosage form. It was reported that the DPV-VR had higher levels of adherence than several prior trials90, particularly VOICE, where vaginal gels were assessed84.

Recent research in the field has demonstrated that the vaginal polymeric film is a promising topical drug delivery system (DDS) for HIV prevention91-96. Polymeric films are thin strips with water-soluble excipients. They dissolve and release the API once applied to the vaginal mucosal surface. The development of vaginal polymeric films is widely spread from anti-fungal, antibacterial drugs97,98 to contraceptive agents. Furthermore, several ART agents, such as DPV and 4’-ethynyl-2-fluoro-2’-deoxyadenosine (EFdA), are also under development in vaginal films99,100.

Compared to other vaginal DDS, polymeric films offer accurate dosing and can be administered without applicators99. In a recent two-arm, cross-over clinical study (FAME-05), Hendrix et al.96 compared the PK and PD of single-dose TFV vaginal film and gel formulations. Results from the study demonstrated consistently higher concentrations of drug in plasma and
cervicovaginal samples in patients who received films as opposed to gel\textsuperscript{96}. Polymeric films can also provide discreet usage without any drug leakage compared to vaginal gels\textsuperscript{101}. Acceptability studies show that women prefer films over soft-gel capsules, tablets, and foams\textsuperscript{99}. Apart from their user-friendly properties, polymeric films also have low manufacturing costs and good stability during storage and transportation. Overall, the polymeric vaginal film provides women with a cheap, convenient, clean, and private option for HIV prevention. These properties are essential to achieving higher compliance. As mentioned above, the high efficacy these clinical trials showed depends heavily on patient compliance. Therefore, the polymeric vaginal film will have a promising future with better effectiveness compared to other topical DDSs for HIV prevention.

To conclude this section, researchers have demonstrated abundant evidence to address the gaps in current PrEP options by exploring other prevention methods. In particular, topical microbicides, for both the rectum and vagina, have been investigated and developed for at-risk key populations\textsuperscript{11,42,102}. A WHO progress report published in 2021 stated that, while progress was already insufficient before, the COVID-19 pandemic further deterred progress to achieve the Fast-Track commitments to end AIDS by 2030 as a public threat\textsuperscript{7}. The need for advanced microbicides was also echoed by FDA\textsuperscript{103}. More studies are needed to advance the current topical microbicide field and to provide more preventive services against HIV with better adherence and efficacy.

\textbf{1.3 Q-GRIFFITHSIN: A PROMISING CANDIDATE FOR HIV PREVENTION}

Aiming to investigate anti-HIV activities in natural product extracts, the National Cancer Institute (NCI) researchers discovered Griffithsin (GRFT) in 2004\textsuperscript{104}. GRFT, isolated from a
marine red alga, is a sugar-binding lectin. Enveloped viruses, such as HIV, have surface glycoproteins that mediate attachment and fusion with the target cell membrane\textsuperscript{105,106}. Thus, GRFT can prevent HIV entry by binding with carbohydrates on the virus surface. However, one disadvantage of GRFT is its susceptibility to oxidation\textsuperscript{107}. To reduce the degradation of GRFT caused by oxidation, a mutated protein drug, Q-Griffithsin (Q-GRFT) was developed. Q-GRFT has the same potency and safety profile as GRFT and is currently under development\textsuperscript{108}.

1.3.1 Manufacturing Process

The manufacturing processes of GRFT have been developed and improved over the years. Originally\textsuperscript{104}, GRFT was isolated from the aqueous extraction of a marine red alga, \textit{Griffithsia sp}. from the Pacific Ocean off of New Zealand. After bioassay-guided fractionations, including ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, reversed-phase chromatography, and size exclusion chromatography, a homogenous biologically active protein was yielded. SDS-PAGE and immunoblotting results confirmed a single protein band with a relative molecular mass of \textasciitilde13kDa. The protein was then sequenced by a combination of N-terminal Edman degradation, MALDI-TOF MS of intact GRFT, and a series of overlapping peptide fragments generated by chemical and enzymatic cleavages. The entire 121 amino acid sequence was established except for a single amino acid at position 31 (151.05 Da), which did not match any of the 20 known amino acids. Researchers replaced the unknown amino acid with an alanine, synthesized the corresponding DNA coding sequence, and expressed the recombinant protein in \textit{Escherichia coli} (\textit{E. coli}). The anti-HIV activities of the recombinant GRFT and the wildtype GRFT are comparable. However, the \textit{E. coli} expression system wasn’t suitable for bulk production, thus a chloroplast(plastid)-transformed
(transplastomic) plant-based expression system was developed\textsuperscript{109}. Hoelscher M. et al. demonstrated the feasibility of using transplastomic tobacco leaves as a highly efficient and cost-effective production platform for GRFT\textsuperscript{110}. The recombinant GRFT manufactured from this system displayed a similar potency and safety profile compared to wildtype GRFT.

1.3.2 The Potency and Safety Profile

GRFT is highly potent against laboratory and clinical isolates of T- and M-tropic HIV-1 and inhibits cell fusion and cell-to-cell transmission of HIV at low concentration\textsuperscript{104}. To determine the potency of GRFT, researchers used a T-tropic laboratory strain (HIV-1\textsubscript{RF}) in CEM-SS cells. Results showed concentration-dependent protection of GRFT against HIV-induced cell killing, along with a decrease of HIV-1 viral core antigen, p24. In addition, GRFT exhibited potency against the infection of other HIV strains as well as the cell-cell infusion\textsuperscript{104}. The inhibition of HIV is rapid upon contact with GRFT, which can block the virus within as little as 15 minutes of treatment\textsuperscript{104}. In addition, the viruses pretreated with GRFT lost their infectivity in CEM-SS cells, indicating that GRFT behaves as a virucide. However, GRFT became less effective when the treatment was performed after HIV inoculation or absorption onto target cells.

An \textit{in vitro} study reveals the low cytotoxicity, rapid onsite antiviral activity, and long-term stability in cervical/vaginal lavage of GRFT\textsuperscript{111}. Another \textit{in vitro} study revealed no evidence of direct cytotoxicity from GRFT to uninfected human peripheral blood mononuclear cells (PBMCs) at its active antiviral concentration\textsuperscript{112}. In addition, GRFT had no measurable effect on cell viability or the levels of T-cell activation markers\textsuperscript{113}. In an \textit{in vivo} study, where a rectal microbicide containing GRFT was evaluated in pig-tailed macaques, researchers confirmed the safety of GRFT on the rectal proteome and microbiome\textsuperscript{114}. Another study of a GRFT microbicide also
demonstrated that no induced epithelial damage or inflammatory responses were found in the vagina both *in vitro* and *in vivo*\textsuperscript{115}. Furthermore, the efficacy of GRFT remained stable throughout normal cervical/vaginal pH in macaques after 24 hours of incubation\textsuperscript{112}. Conclusively, studies demonstrate that GRFT is potent, safe, and has great potential for use in microbicides.

1.3.3 The Protein Structure

The potent anti-HIV efficacy of GRFT relates to its structure. However, there was no homology to any other proteins previously reported. As a result, the structure of GRFT was investigated by a combination of X-ray crystallography, isothermal titration calorimetry, and molecular modeling\textsuperscript{116}. These structures, solved and refined at \( \sim 1.3 \text{ Å} \), have shown GRFT to be a domain-swapped homodimer, with 3 carbohydrate-binding sites on each subunit (monomer)\textsuperscript{117}. Each subunit exhibits three repeats of an antiparallel four-strand beta-sheet. The structure of GRFT's almost perfect internal three-fold symmetry in each monomer superficially resembles a beta-prism-I motif, indicating that GRFT is a unique adaptation to the \( \beta \)-prism-I (jacalin-related) family\textsuperscript{118}.

The dimer structure of GRFT is required for its superior anti-HIV potency. In a study\textsuperscript{119}, researchers engineered a “one-armed” obligate dimer (“GRFT-linker-GRFT One Arm”) with one functional subunit and another subunit in which all three binding sites were mutated. This GRFT variant showed a drastic reduction in inhibitory function, with an 84- to 1,010-fold decrease compared to the wildtype GRFT. Furthermore, while the wildtype GRFT demonstrated an ability to alter the structure of gp120, an important glycoprotein of HIV, GRFT-linker-GRFT One Arm lost this ability. Evidence demonstrated that this domain-swapped homodimer structure of GRFT is crucial to its potent HIV inhibition.
1.3.4 The Mechanism of HIV Prevention

Enveloped viruses, such as HIV, have surface glycoproteins that mediate attachment and fusion with CD4 on the target cell membrane\textsuperscript{120,121}. ELISA results indicate that GRFT has direct interactions with these glycoproteins, namely gp120, gp160, and gp41. These interactions are indeed glycosylation-dependent for no binding was observed for GRFT with nonglycosylated sgp120. Mannose-GRFT complexes demonstrate that the three binding sites are almost identical, and each site contains one aspartic acid (Asp) residue (Asp30, Asp70, and Asp112)\textsuperscript{116}. Aspartic acids interact through carboxylation with the hydroxyl oxygens O6 and O4 of the mannose. Within the same site, oxygens O5 and O6 also form hydrogen bonds with neighboring amino acids\textsuperscript{118}. The abovementioned carbohydrate-binding sites on GRFT can bind with the carbohydrates on gp120 and therefore inhibit gp120/CD4 interactions. Thus, GRFT can prevent HIV infection as an entry inhibitor.

1.3.5 Current Applications and Future Directions

GRFT can be combined with other anti-HIV molecules, to deliver either synergistic effects or complementary effects (synergistic with tenofovir, maraviroc, and enfuvirtide\textsuperscript{122}; dapivirine\textsuperscript{123}; antibodies\textsuperscript{124}; complementary with broadly neutralizing antibodies\textsuperscript{125}). Besides, GRFT also has inhibitory effects against other enveloped viruses. To date, GRFT has already shown antiviral activities against severe acute respiratory syndrome coronavirus (SARS-CoV)\textsuperscript{118,126}, Middle East respiratory syndrome coronavirus (MERS-CoV)\textsuperscript{127}, Japanese encephalitis virus (JEV)\textsuperscript{128}, porcine epidemic diarrhea virus (PEDV)\textsuperscript{129}, and hepatitis C virus (HCV)\textsuperscript{130}. Levendosky K et al. also demonstrated that GRFT can prevent other sexually transmitted infections (STIs) such as herpes
simplex virus 2 (HSV-2) and human papillomavirus (HPV)\textsuperscript{131}. Specifically, the mechanism of action for preventing HPV, a nonenveloped virus, is different. GRFT prevents HPV by binding with $\alpha_6$ integrin, a second receptor for the virus to enter the cells. The HPV-$\alpha_6$ integrin-GRFT complex can be further internalized without leading to infection.

However, GRFT is prone to oxidation\textsuperscript{107}. To reduce the degradation of GRFT caused by oxidation, a mutated protein drug, Q-GRFT was developed. Q-GRFT has one amino acid mutation difference from GRFT. In Q-GRFT, the easily oxidized residue, methionine, is replaced with glutamine while maintaining the same high antiviral potency. Q-GRFT exists as a stable homodimer where each subunit has 121 amino acids. Each subunit is capable of binding three monosaccharides. The binding sites are almost identical and each site contains one aspartic acid residue that makes extensive contact with glycoproteins\textsuperscript{116}. Recent studies performed by Broliden et al.\textsuperscript{132} and Steinbach-Rankins et al.\textsuperscript{108} demonstrated that Q-GRFT has a similar safety and efficacy profile as GRFT in \textit{in vitro} and \textit{in vivo} models.

In summary, these results collectively support that GRFT/Q-GRFT can be safely and effectively utilized in microbicides. Because GRFT/Q-GRFT has potency against several sexually transmitted viruses (including HIV, HSV-2, and HPV), they are promising candidates to expand the current drug spectrum for PrEP. Taken together, anti-STI/HIV microbicides in a liquid state (rectal enema\textsuperscript{133,134}), semi-solid state (vaginal gel\textsuperscript{66,95}, rectal gel\textsuperscript{135}), and solid-state (vaginal polymeric films\textsuperscript{93,94}) are being developed for GRFT and its variants. Therefore, in this work, Q-GRFT was incorporated and evaluated in three drug delivery systems, including an enema solution, lyophilized powder, and polymeric films.
1.4 GAP ANALYSIS OF INCORPORATING Q-GRFT IN DOSAGE FORMS

1.4.1 The Challenges of Loading Proteins in Liquid Dosage Forms

Enemas are commonly used by MSM as a cleansing product before or after intercourse. Studies\textsuperscript{59,69,136} indicate that the receptive partners, who also have a higher risk for HIV infection, perform douching for hygiene and/or psychological reasons. There is evidence that, by combining prevention methods with this behavior, an enema can serve as a great drug delivery system for PrEP and potentially promote adherence\textsuperscript{59,136}. In addition, recent studies already heightened the feasibility and effectiveness of using an enema solution for HIV/STI prevention in MSM\textsuperscript{59,62}. Therefore, rectal enema formulations have been explored to incorporate ARVs such as microbicides against HIV infection\textsuperscript{63,64,102}.

Because commercial products usually need a shelf life of two years to be economically viable\textsuperscript{137}, formulation scientists in the biopharmaceutical industry face the challenge of creating stable liquid aqueous formulations for protein drugs\textsuperscript{137,138}. Since excipients can either have positive or negative impacts on protein stability, it is also critical for researchers to screen stable excipients with limited protein stock. In light of this, many studies have been performed to investigate the interactions between excipients and proteins\textsuperscript{138}. Moreover, recently protein-excipient interaction studies have also been studied with the help of \textit{in silico} platforms\textsuperscript{139-142}. Collectively, growing evidence in the field has provided formulation scientists with some theories to explain the stabilizing mechanisms, including preferential excluding, protein hydration, and preferential interactions\textsuperscript{143-145}. Thus, understanding protein-excipient interactions is a critical step in stabilizing proteins in liquid dosage forms.
A review published in 2018 pointed out that not all approaches to stabilizing protein drugs must be performed empirically\textsuperscript{146}. By applying rational designs, it is hoped that protein-incorporated aqueous formulations can be achieved with desired properties.

1.4.2 The Challenges of Loading Proteins in Lyophilized Powders

While enema use/douching provides potential benefits for increasing adherence\textsuperscript{69,136}, the dosage form itself can be further developed. Due to the liquid form, enemas are hard to transport and store\textsuperscript{147}. Therefore, it is important to develop a powder form of enema sachet, providing convenience and economic advantages\textsuperscript{147,148}. Enema powder, manufactured by lyophilization, can be reconstituted into an enema solution when used.

However, protein denaturation can occur in either extremely high or low temperatures. In the context of lyophilization, while normal or thermal denaturation is entropy-driven, cold denaturation is enthalpy driven\textsuperscript{149,150}. Theoretically, the calculated free energy of unfolding for proteins has a parabolic relationship with temperature. This means that a temperature of maximum stability exists, and a low temperature below the threshold can destabilize a protein\textsuperscript{151,152}. Another theory of protein instability during lyophilization relates to the excipients. The buffering agents (salts), such as disodium phosphate/monosodium phosphate, have different solubility\textsuperscript{153}. While heating or freezing a buffered protein solution, evaporation would cause different crystallization rates, finally leading to a significant pH shift\textsuperscript{154}. In addition, over-drying also has negative impacts on protein stability due to the removal of the protein “hydration shell”\textsuperscript{155,156}.

The abovementioned stresses, including low-temperature stress (cryo-stress), dehydration effects, concentration effects, and pH shifts, are involved in each step of the lyophilization
process\textsuperscript{157}. Therefore, cryoprotectant screenings and process modifications are crucial for lyophilized powder formulations with protein drugs.

1.4.3 The Challenges of Loading Proteins in Polymeric Films

Two commonly-used methods to manufacture polymeric films are solvent-casting (SC) and hot-melt extrusion (HME)\textsuperscript{158,159}. However, these methods present challenges for protein drugs as high temperature and mechanical forces are involved within the manufacturing processes.

RC-101, a synthetic analog of retrocyclin, showed \textit{in vitro} activity against HIV-1\textsuperscript{160}. In one study\textsuperscript{161}, three different PVA-base film formulations were assessed with RC-101, and short-time stability was monitored. The results showed the drug contents decreased in one month. The authors suspected the complexing or aggregation of RC-101 might explain the phenomena where the parent RC-101 HPLC peak disappeared.

Similarly, aggregation would potentially be the dominant issue for Q-GRFT. The structure of Q-GRFT is composed of three repeats of an antiparallel four-strand beta-sheet that superficially resembles a beta-prism-I motif\textsuperscript{162}. The increase of beta-sheet content is often an indication of protein aggregation and/or increased intermolecular interaction which could lead to aggregation\textsuperscript{163,164}. Thus, stabilizing proteins is one of the most important tasks in the development of Q-GRFT polymeric films.

Polymers should be explored in the screening process, as they have already been used to stabilize proteins in solution and during freeze-thawing and freeze-drying\textsuperscript{165}. For example, studies indicate that polyvinyl alcohol (PVA) and hydroxypropyl methylcellulose (HPMC) can protect rabbit muscle lactate dehydrogenase from aggregation, and hydroxyethyl cellulose (HEC) completely inhibited lyophilization-induced aggregation of aFGF\textsuperscript{166,167}. A possible protecting
mechanism by the excipients is the water replacement hypothesis. Since the loss of the protein hydro-shell would lead to protein-protein hydrophobic interaction, when the protein is exposed to a less humid condition, a water-replacing agent is needed to preserve the protein’s natural conformation. In polymeric films, excipients can form hydrogen bonds with proteins. Thus, adding stabilizing agents into the formulation can possibly prevent aggregation.

Overall, these protein-specific protecting effects need to be investigated with Q-GRFT. As a crucial step in the protein drug pre-formulation process, a comprehensive excipient compatibility study should be performed for Q-GRFT. Physiochemical characterizations of the lead products also need to be assessed as suitable vaginal delivery systems.

1.5 HYPOTHESIS AND SPECIFIC AIMS

During the development of protein drug formulations, many challenges exist that may cause protein degradation: excipient-protein interactions, pH changes, the removal of the protein hydration shell, temperature stress, and mechanical forces. To assess the potential risk of degradation, a thorough pre-formulation evaluation is required. The susceptibilities of the protein to environmental factors which may cause degradation are assessed in these studies. Outcomes from the experiments can be used to develop appropriate drug formulations as they provide critical knowledge which can impact drug product manufacture, storage, and use conditions.

Such studies were conducted for Q-GRFT. Within these studies, aggregation was identified as a major factor to consider for the development of stable Q-GRFT formulations. In order to prevent aggregation in the formulations, several strategies were explored in this dissertation work.
First, pH and osmolality need to be maintained suitable for Q-GRFT. Secondly, in cases where water is removed from the environment, excipients can provide hydrogen bonding to maintain protein structures. Thirdly, excipients in the solid dosage form can exist as hindrances to preventing protein-protein interactions. As the mechanisms behind the aggregation prevention strategies are complex, no single hypothesis can explain all findings. These will be further discussed in detail in the following chapters.

Generally, in each chapter, excipients will be screened in the pre-formulation process, and possible mechanisms will be explored. In addition, Q-GRFT’s main activity is due to its binding to viral gp120, thereby preventing HIV attachment to host cells. Thus, a gp120 binding study is crucial to assess the bioactivity of Q-GRFT in the final formulations. More importantly, there are some Critical Quality Attributes (CQAs) for each dosage form according to the delivery routes. Physiochemical properties will be evaluated to confirm the quality of final formulations as topical microbicides. Safety and pharmacokinetic profiles will also be explored with *in vitro* and *ex vivo* models if needed.

Based on the above information:

**We hypothesize that by manipulating excipients, Q-GRFT can remain stable in both liquid and solid dosage forms. We further hypothesize that the designed rectal/vaginal products can achieve physicochemical properties suitable as topical PrEP options.** This hypothesis was studied with three specific aims.

**Aim 1** explores the potential of developing stable Q-GRFT enema formulations with the combination of TFV. In Chapter 2, stability, bioactivity, safety, and permeability profiles will be established for the Q-GRFT-incorporated enema to demonstrate the capability of rectal microbicides.
**Aim 2** investigates the feasibility of manufacturing a stable Q-GRFT enema sachet (powder form) with lyophilization. In Chapter 3, cryoprotectants will be evaluated to prevent Q-GRFT aggregation. Physicochemical properties of the Q-GRFT-incorporated solid powder and the reconstituted solution will also be characterized for rectal microbicides.

**Aim 3** develops and screens polymeric vaginal film formulations to achieve stable Q-GRFT film products. In Chapter 4, excipients will be studied with experimental and computational methods. The lead film formulation will further be assessed for important attributes for vaginal microbicides.
2.0 Q-GRFT LIQUID FORMULATION DESIGN: ENEMA

Although the number of newly infected people decreased significantly over the past decades, HIV/AIDS remains a severe epidemic. With 1.5 million newly infected in 2020, and no cure available, prevention is still one of the most effective strategies to control HIV from spreading\(^{169}\).

Due to biological delicacy and cultural influences, MSM are disproportionately at risk of HIV infection\(^{170}\). Since 1981, when the syndrome was first described, MSM has had a high prevalence of HIV/AIDS despite decades of medical efforts\(^{170}\). Currently, the most commonly-used PrEP for MSM is oral tablets. There are two oral PrEP tablets approved by FDA, Truvada, and Descovy. Because of the prevention strategies, including PrEP, the new HIV infections in 2020 have declined by 31% since 2010\(^{169}\). However, several barriers that negatively impact the adherence of MSM to oral PrEP are still present\(^{53}\). For example, people have concerns about long-term and short-term side effects, and potential drug resistance\(^{53}\). Given that adherence is critical in maintaining therapeutic concentrations of drugs in the body, these concerns may negatively impact the effectiveness of protection\(^{53,54}\). In addition, a meta-analysis of sixteen observational studies and one open-label trial revealed that there is a link between MSM who take PrEP and risky sexual behaviors\(^{171}\). The increase of condomless sex among PrEP users may lead to the surge of other STIs (e.g. Chlamydia and syphilis diagnoses)\(^{171-173}\). Therefore, this chapter aims to address the urgent need for more PrEP options which can provide easier access and better adherence. Specially, we explore the potential of incorporating Q-GRFT in enema as a topical microbicide for HIV prevention.
2.1 INTRODUCTION

Douching is commonly practiced by MSM prior to engaging in rectal intercourse. Studies indicate that the receptive partners, who also have a higher susceptibility for HIV infection, douche for hygiene and/or psychological reasons. There is evidence that combining HIV prevention modalities with this practiced behavior, may potentially promote PrEP adherence. Recent studies have highlighted the feasibility and effectiveness of using an enema solution for HIV/STI prevention in MSM. In light of this, our lab developed single-entity enema solutions containing TFV or Q-GRFT (unpublished) and advanced them to clinical trials.

TFV is in the class of NRTIs. Its prodrug, TDF is used as an API in Truvada, combined with FTC. TFV has also been commonly used for HIV prevention and evaluated in various delivery systems. Q-GRFT is modified from GRFT, an entry inhibitor against HIV and some other viruses. Differing from the wild type by one amino acid, Q-GRFT is less prone to oxidation while maintaining the same high antiviral potency. Minooei et al. reported the synergistic effects of TFV and Q-GRFT observed in vitro. In this study, the co-administration of Q-GRFT and TFV resulted in reductions in the IC₅₀ of individual free APIs, tested with the TZM-bl cell assay. Therefore, this chapter set out to investigate the possibility of combining both drugs in an enema dosage form as a PrEP option. The combination enema, which contains both TFV and Q-GRFT, aims to provide synergistic efficacy against HIV infection. In this work, we developed two combination enema (combo enema) formulations with different vehicle compositions and evaluated the physicochemical characteristics, toxicity, and permeability properties using in vitro and ex vivo models. This work generated fresh insights into TFV/Q-GRFT combination enema and explored their potential as safe rectal microbicides for MSM.
2.1.1 Identification of Critical Quality Attributes (CQAs)

To develop safe and effective formulations, it is important to identify parameters that impact the quality. The consideration of the administration route, the properties of the drug substances (TFV and Q-GRFT for this work), and experience from previous development of single-entity enema solutions were taken into the CQAs. Firstly, the normal pH of the human rectum is around 6.6\textsuperscript{71,72}, therefore the range for formulation pH was set to be close to neutral (6.5–8.0). Secondly, the literature suggests that the hypotonic enema is the best option compared to isotonic and hypertonic enema\textsuperscript{63,64}. In these studies, the hypotonic vehicle delivers higher TFV concentrations into colon tissues driven by the net water movement, without causing damage to the epithelium. Thus, we designed our combo enema to be hypotonic with the osmolality of 145 mOsm/kg.

Lastly, literature and in-house products were considered to determine the drug content. A previous study compared the TFV alone enema with low (1.76 mg/ml) or high (5.28 mg/ml) TFV concentrations\textsuperscript{63}. The results showed the superiority of high TFV enema over the others. On the other hand, a Q-GRFT alone enema has been developed in our lab and is stable for 14 days (unpublished). The Q-GRFT concentration used in that study is 0.32 mg/ml. Based on these two established formulations, we designed the drug content of both drugs in our new combination formulations.

2.1.2 Development of the Manufacturing Process

This chapter also aimed to design an easy, robust, and reproducible standard operating procedure (SOP) for potential big-scale manufacturing. pH and osmolality are two important
properties of the enema. Therefore, they were monitored in the development of the manufacturing process. A step-by-step waterfall process development is implemented to precisely control the parameters within ranges for the final formulations. Two types of vehicle solutions were previously studied in the formulation development of TFV-only enema, namely PBS-base and saline-base. Thus, this chapter also set to design lead enema formulations with these two bases.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Recombinant Q-GRFT drug substance was manufactured by Kentucky Bioprocessing LLC (Owensboro, KY), and supplied by Dr. Kenneth Palmer at the University of Louisville. TFV was synthesized by WuXi AppTec (Shanghai, China). PBS 10× molecular biology grade (pH 7.4) was purchased from Mediatech Inc. (Manassa, VA). Saline buffer (sodium chloride injection, USP, sterile) was purchased from B. Braun Medical Inc. (Irvine, CA). Acetonitrile (ACN), trifluoroacetic acid (TFA), t-butylammonium bisulfate (tBAHS), potassium phosphate dibasic (Na₂HPO₄), hydrochloric acid (HCl), sodium hydroxide (NaOH), Krebs Ringer Bicarbonate Buffer containing Glucose (KRBG), Hank’s Balanced Salt Solution (HBSS) and Hematoxylin and Eosin (H&E) stain kit were obtained from Fisher Scientific (Pittsburgh, PA). A MilliQ (Millipore; Milford, MA) water filtration system operating at 18.2 MΩ cm was used for water. PBS 1x was manufactured in-house with PBS 10x stock and MilliQ-water.
2.2.2 The Manufacturing Process

2.2.2.1 The Process Development

To investigate if the vehicle strength impacts the pH and osmolality of formulations, PBS-base was used directly (1x PBS) or diluted (50% PBS) with or without the addition of TFV and Q-GRFT. To study if the vehicle composition impacts the pH and osmolality of formulations, PBS-base and saline-base were used directly (1x PBS; 1x saline) with the addition of TFV and Q-GRFT. Three formulations (50% PBS, 1x PBS, 1x saline; all with the addition of TFV and Q-GRFT) were chosen to further evaluate the effects of pH adjusting agents on osmolality. For the final screening, both vehicles (PBS-base and saline-base) were diluted into three strengths, 30%, 35%, and 40% with the addition of TFV and Q-GRFT. pH and osmolality were measured as in-line checkpoints for screening and quality assurance.

2.2.2.2 The Final Manufacturing Procedure

First, the stock buffer (10x- PBS or Saline) was diluted with Milli-Q water to a solution with lower osmolality than desired (~40 mOsm/kg lower). Then, TFV dry powder was weighed and added to the diluted PBS buffer under constant stirring (250-400 rpm). Under a pH meter, the pH was adjusted to ~7.0 with an 18% NaOH solution. TFV should be fully dissolved after pH adjustment. Finally, the Q-GRFT stock solution (in 1x PBS) was measured and added to the solution to make the final product. Both PBS- and Saline- based combination enema followed the same procedure. The final solution was assayed for pH, osmolality, and drug content (TFV and Q-GRFT) before it was aliquoted. (Describe below in Section 2.2.3)
2.2.2.3 The Stability Study Procedure

Individual enema bottles (containing 125 ml of solution) were stored under three conditions (4°C; 25°C/60%RH; 40°C/75%RH) and monitored over time (24 months for 4°C and 25°C/60%RH; 6 months for 40°C/75%RH). At each time point (1, 2, 3, 6, 9, 12, 18, and 24 months), physicochemical characteristics were evaluated and compared against the CQAs. The entire process is summarized in Figure 2.1.

Figure 2.1 Procedures of Formulation Development and Monitoring for Two Vehicle-based Combination Enema.

2.2.3 Physicochemical Characterization of Enema Solutions

2.2.3.1 Appearance

The appearance of the enema solutions was monitored by visual observation. The formulations should be clear and colorless. Any turbidity may indicate the aggregation of Q-GRFT or contaminations, warranting further investigations.
2.2.3.2 pH

The pH was determined using a pH meter (XL150 pH Benchtop Meters, Fisher Scientific). Before use, the pH meter was calibrated using standard buffers (Orion All-in-One pH Buffers Kit, Thermo Scientific) every time.

2.2.3.3 Osmolality

The osmolality of the enema solution was tested using a freeze-point osmometer (Advanced Instruments). In short, about 200 µL of sample solution was injected into the cooling chamber. Then, a supercooled condition (below the freezing temperature) was achieved by the apparatus. While the sample was in the supercooled state, a physical shock was introduced to form a partially crystallized ice-water mixture. The heat of fusion, resulting from the crystallization, raised the sample temperature to a plateau where the solid-liquid equilibrium was maintained. This temperature, representing the true freezing point of the sample, was recorded and calculated to osmolality. Before measurements, a standard osmolality solution (200 mOsm/kg, Advanced Instruments) was used to validate the apparatus. If the deviation was more than ±2 mOsm/kg, the apparatus would be calibrated before testing. For each testing group, the average osmolality values were measured in triplicate.

2.2.3.4 TFV Content Determination by Ultra Performance Liquid Chromatography

The Ultra Performance Liquid Chromatography (UPLC) method for measuring the TFV content was developed in our lab and described previously. For sample preparation, a testing solution was first filtered through a 0.22 µm PTFE filter (Target Syringe Filters, Thermo Scientific) with 1 ml syringes (BD U-100, Fisher Scientific). The samples were diluted 50 times with the mobile phase prior to UPLC analysis. After preparation, samples were quantified by
integrating the peak area with the UPLC method (Waters Acquity UPLC H-Class systems; Acquity UPLC BEH C18 columns, 130Å, 1.7µm, 2.1 x 50 mm; with VanGuard Pre-Column, 130Å, 1.7µm, 2.1 x 5mm), and the TFV content was calculated with calibration curves.

### 2.2.3.5 Q-GRFT Content Determination by High-Performance Liquid Chromatography

The High-Performance Liquid Chromatography (HPLC) method for measuring Q-GRFT content was previously developed in our lab and reported elsewhere. The samples were diluted 2 times with Milli-Q water. The Q-GRFT content was detected by an HPLC system (Waters Corporation, Milford, MA) equipped with an auto-injector (model 717), a quaternary pump (model 600), and a photodiode array detector (model 2996)) with a C5 column (Jupiter 5µm 300Å, 250 x 4.6 mm) and a C5 pre-column (Security Guard Standard Widepore).

### 2.2.4 Cell Culture and Cell Toxicity Studies

The Caco-2 cell line was kindly provided by Dr. Charlene S. Dezzutti (Magee-Womens Research Institute, Pittsburgh, PA). Passages 33 to 43 were used in the studies. The Caco-2 cells were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM) [500 ml of DMEM (Corning) containing 50 ml fetal bovine serum (FBS; Gibco Qualified One-Shot, Thermo Scientific) and 5 ml PEST (penicillin 10,000 U/ml-streptomycin, 10,000 ug/ml-glutamine 29.2 mg/ml solution (100x); Gibco, Fisher Scientific)] at 37 °C with 5% CO₂ incubation. For the determination of 50% cytotoxic concentrations (CC₅₀), Caco-2 cells were seeded on 96-well plates with 2 x 10⁴ cells/well. After overnight incubation, the samples were added to the wells and treated for 24 hours. The cell viability was detected using the CellTiter-Glo assay (Promega) with a fluorescence detector. The viability % (compared to the untreated groups) was plotted against the
concentrations of testing excipients (in log scale). CC$_{50}$ was calculated using a sigmoidal 4PL model in Prism 9 software.

### 2.2.5 Permeability and Toxicity Assessment with the Caco-2 Monolayer Cell Model

The procedure was adapted from a previously reported method to build a Caco-2 monolayer cell model and perform the permeability studies $^{177}$. Cell passages 34 to 42 were used to build the model in different batches. For preparation, a total of $3 \times 10^5$ Caco-2 cells were dispensed on the apical side of each Transwell filter. The model would be ready after 21–29 days of culture. Our model was validated with a paracellular marker, $[^{14}\text{C}]$-Mannitol. The apparent permeability ($P_{\text{app}}$) of $[^{14}\text{C}]$-Mannitol tested using this monolayer cell model was $3.3 \times 10^{-7}$ cm/s, which was at the same level as the reported value ($1.2 \times 10^{-7}$ cm/s) $^{177}$. In addition, the cell model was stained with ZO-1 Monoclonal Antibody (ZO1-1A12)-Alexa Fluor 488 (Invitrogen, Thermo Scientific) and observed using a confocal microscope (Nikon A1R confocal microscope). The cell model expressed uniform ZO-1 protein, a tight junction indicator. Z-axis imaging also confirmed that the Caco-2 cells formed a single layer on the substrate.

A toxicity study utilizing the Caco-2 model was performed prior to permeability studies to examine the safety of the complete liquid formulations. Samples were applied on the apical side of the Transwell. After 2-hour treatments, the treated samples were discarded and washed off twice with HBSS. A cell viability study was performed with an MTT/isopropanol-extraction method adapted from the literature $^{178}$. Transepithelial electrical resistance (TEER) values were also measured before and after the treatments as supplemental criteria for the toxicity study.

To study the permeability, the sample solution was gently dispensed on the apical side of the model and cultured at $37^\circ$C for 2 hours. At each time point (Time 0, 15, 30, 45, 60, 75, 90, 120
minutes), HBSS samples were collected from the basal sides and tested using UPLC (for TFV) and HPLC (for Q-GRFT). The $P_{app}$ was calculated using Equation 1.

$$
P_{app} = \frac{\left(\frac{dQ}{dt}\right)}{A \cdot C_0}
$$

Equation 1

$P_{app}$ (cm/sec): the apparent permeability coefficient; $\left(\frac{dQ}{dt}\right)$ (ng/sec): the rate of drug transportation; A (cm$^2$): the surface area of the cell layers; $C_0$ (ng/ml): The initial drug concentration on the donor side.

2.2.6 Permeability and Toxicity Assessment with the Ex Vivo Human Colon Tissue

The project used the UPMC Hillman Cancer Center and Tissue and Research Pathology/Pitt Biospecimen Core shared resource which is supported in part by award P30CA047904. Excised human colorectal tissues were obtained under the University of Pittsburgh Institutional Review Board (IRB) Protocol PRO09110431. tissue specimens were collected from patients (18–75 years old). A section of each tissue was retained for histological evaluation with an H&E staining assay. After removal of the excess stromal tissue, the epithelial tissue was placed between the donor and receptor compartments of the Ussing chamber apparatus with the epithelial side toward the donor compartment. This set-up was maintained at 37°C throughout the experiment. Both donor and receptor compartments were filled with KRBG during system calibration. Then the KRBG buffer from the donor sides was replaced with sample solutions. 50 μL was removed from the donor compartment at the beginning and the end of the experiments. At predetermined time points (Time 0, 15, 30, 45, 60, 75, 90, 120 minutes), 200 μL was removed from the receptor chamber and the same volume of fresh KRBG was added for replacement. These samples were held at 4°C until analyzed by UPLC (for TFV) or HPLC (for Q-GRFT). The $P_{app}$ values were calculated using Equation 1 as described above.
All tissues (including tissues pre-treatments and post-treatments from all groups) were processed for paraffin sectioning. The tissue sections were embedded into paraffin blocks using the Leica EG 1160 embedding station. Tissues were then sectioned at five microns (5 μm) with the Olympus CUT 4060 microtome and placed on slides for H&E staining procedures. Hematoxylin stains the nuclei of cells purple. Eosin stains the other structures of the tissue section red/pink. Microscopy was performed with a Zeiss Axioskop 40 Microscope. Micrographs were obtained with an AxioCam MRc 5 color camera and AxioVision software. All micrographs were taken with a ×10 objective.

2.2.7 Bioactivity Determination using Enzyme-linked Immunosorbent Assay

An Enzyme-linked Immunosorbent Assay (ELISA) (developed by Dr. Kenneth Palmer’s lab at the University of Louisville) was applied to evaluate the gp120 binding activity of select Q-GRFT samples. Nunc MaxiSorp 96-well plates were used for this experiment. The MaxiSorp surface is a hydrophilic/hydrophobic mix that binds to a wide range of biomolecules. In short, gp120 was bound to the wells of a 96-well plate overnight at 4°C. The HIV-1 gp120CM was purchased from Kentucky Bioprocessing (KBP; Part #C-1312). After overnight incubation, the solution of gp120 was removed, and a blocking solution (1x PBS-T [PBS with 0.05% Tween 20]) was applied for two hours at room temperature. After that, the wells were washed and incubated with various dilutions of Q-GRFT samples for one hour. Gp120 binding was detected by applying goat anti-QGRFT primary antibody (one-hour incubation) and HRP-labeled rabbit anti-goat secondary antibody (one-hour incubation), sequentially. TMB substrate was applied to the wells after washing the secondary antibody. Wells were allowed to develop (blue color) for
approximately three minutes before the application of sulfuric acid to stop the reaction (yellow color). The plates were measured at 450nm using a plate reader for the gp120 binding activity.

### 2.2.8 Statistical Analysis

All values are reported as means ± standard deviation (SD). Statistical data analyses were performed using one-way ANOVA with Tukey’s post hoc test, with p<0.05 as the minimal level of significance, p<0.01 for very significant, and p<0.001 for highly significant. All tests were performed using the GraphPad Prism software version 9.

### 2.3 RESULTS

#### 2.3.1 Determination of Manufacturing Procedure

pH and osmolality are two important properties of enema. Therefore, they were measured during the process of development. As shown in Figure 2.2, pH shifted significantly after the addition of TFV and Q-GRFT. This effect held for both vehicles. In addition, for vehicle-only groups (50% PBS and 1x PBS), osmolality changed proportionally with the dilutions. Furthermore, after adjusting the pH, osmolality increased by around 20 mOsm/kg for all three testing groups. Therefore, we could calculate the targeted osmolality for the initial vehicle solutions based on the CQAs, dilution factors, and the addition of drugs.

The initial osmolality was calculated to be approximately 105 mOsm/kg. To achieve this goal, we performed the final stage screening with three dilution levels for both vehicles, 30%,
35%, and 40%. Results were summarized in Table 2.1. For saline-base, 35% saline achieved pH and osmolality criteria. For PBS-base, both 35% and 40% dilutions met the CQAs. We finally chose two lead vehicle bases, 35% saline and 35% PBS solutions as comparisons.

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>Osmolality (mOsm/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% PBS</td>
<td>7.49</td>
<td>144.0 ± 1.4</td>
</tr>
<tr>
<td>1x PBS</td>
<td>7.39</td>
<td>285.5 ± 0.7</td>
</tr>
<tr>
<td>1x saline</td>
<td>5.18</td>
<td>287.5 ± 0.7</td>
</tr>
<tr>
<td>50% PBS + TFV+ Q-GRFT</td>
<td>3.93</td>
<td>167.0 ± 1.7</td>
</tr>
<tr>
<td>1x PBS + TFV+ Q-GRFT</td>
<td>4.38</td>
<td>304.7 ± 2.1</td>
</tr>
<tr>
<td>1x saline + TFV+ Q-GRFT</td>
<td>3.23</td>
<td>299.5 ± 0.7</td>
</tr>
</tbody>
</table>

**STAGE A**

**pH Adjustment**

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>Osmolality (mOsm/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% PBS + TFV+ Q-GRFT</td>
<td>8.01</td>
<td>271.0 ± 1.7</td>
</tr>
<tr>
<td>1x PBS + TFV+ Q-GRFT</td>
<td>7.46</td>
<td>321.3 ± 1.5</td>
</tr>
<tr>
<td>1x saline + TFV+ Q-GRFT</td>
<td>7.31</td>
<td>317.7 ± 1.2</td>
</tr>
</tbody>
</table>

**STAGE B**

**Conclusions & Calculations**

**STAGE C**

(Final Stage)

Figure 2.2 The Initial Screening Process for the Manufacturing Procedure Development.

Table 2.1 The Final Stage (Stage C) Screening Process for Both Vehicle-based Enema Formulation Development.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial</th>
<th>TFV addition &amp; pH adjustment</th>
<th>Q-GRFT addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Osmolality (mOsm/kg)</td>
<td>pH</td>
</tr>
<tr>
<td>30% PBS</td>
<td>7.57</td>
<td>85.0±0.0</td>
<td>7.13</td>
</tr>
<tr>
<td>35% PBS</td>
<td>7.54</td>
<td>97.0±1.4</td>
<td>7.35</td>
</tr>
</tbody>
</table>
2.3.2 Characterizations of the TFV/Q-GRFT Combination Enema Solutions

We designed, manufactured, and monitored two combination enema solutions with different vehicles (PBS- and Saline-base) to provide more options for HIV prevention. Parameters including pH, osmolality, and drug contents of both TFV and Q-GRFT were evaluated. As shown in Table 2.2, all the physiochemical characteristics met the CQAs. Also, no significant difference was observed between the two formulations for all the parameters.

Table 2.2 Physicochemical Characterizations of the Two Vehicle-based Combination (Combo) Enema.

<table>
<thead>
<tr>
<th>CQAs</th>
<th>PBS-base Combo Enema</th>
<th>Saline-base Combo Enema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual: Clear &amp; Colorless</td>
<td>Pass</td>
<td>Pass</td>
</tr>
<tr>
<td>pH: 6.5–8.0</td>
<td>7.12</td>
<td>7.08</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>145±22 (123–167)</td>
<td>139±0.6</td>
<td>137± 0.6</td>
</tr>
<tr>
<td>TFV content [% label claim (5.28mg/ml)]: 90%–110%</td>
<td>95.2±0.3%</td>
<td>94.4±1.7%</td>
</tr>
<tr>
<td>QGRFT content [% label claim (0.32mg/ml)]: 90%–110%</td>
<td>90.6±4.6%</td>
<td>93.4±3.5%</td>
</tr>
</tbody>
</table>
2.3.3 Stability of the Two Combination Enema Formulations

Both enema formulations were dispensed into enema bottles and stored under three different conditions for up to two years (4°C and 25°C/60%RH for 24 months; 40°C/75%RH for 6 months). CQAs were monitored at each time point (Time 0, 1 month (M), 2M, 3M, 6M, 9M, 12M, 18M, and 24M) using the methods described previously. By visual observation, the enema remained clear and colorless throughout the entire stability study. pH was also stable (Figure 2.3) for all three conditions. For osmolality, both formulations had a small increasing trend (Figure 2.4) but remained within the CQA ranges. Nonetheless, both formulations maintained hypotonic (145±22 mOsm/kg) for two years. Drug content is the most important characteristic in this stability study. TFV and Q-GRFT contents were measured by UPLC and HPLC, relatively. For each time point, drug content was compared to the label claim (TFV: 5.28 mg/ml; Q-GRFT: 0.32 mg/ml). TFV content remained within range (90%–110% of the label claim) for all time points under all three conditions (Figure 2.5). Q-GRFT content in the Saline-base formulation also fitted in the range for all time points (Figure 2.6b). Surprisingly, the PBS-base formulation at 24M had the Q-GRFT content of 89.5% and 88.1% (to the label claim) for the 25°C/60%RH and 4°C storage conditions, respectively (Figure 2.6a). Although these recovery values were slightly out of the tight range, they were not significantly different from the Q-GRFT content at Time 0 (90.6%).
Figure 2.3 pH Stability Testing for Two Combo Formulations: a. PBS-base Combo Enema; b. Saline-base Combo Enema.

Formulations were monitored under 3 conditions (4°C and 25°C/60%RH for 24 months; 40°C/75%RH for 6 months).

Figure 2.4 Osmolality Stability Testing for Two Combo Formulations: a. PBS-base Combo Enema; b. Saline-base Combo Enema.

Formulations were monitored under 3 conditions (4°C and 25°C/60%RH for 24 months; 40°C/75%RH for 6 months). For each time point, n=3.
Figure 2.5 Drug Content Stability: a. TFV Content in the PBS-base Combo Enema Solution; b. TFV Content in the Saline-base Combo Enema Solution.

TFV was tested using UPLC, and relative content (%, compared to the label claim) is presented. Formulations were monitored under 3 conditions (4°C and 25°C/60%RH for 24 months; 40°C/75%RH for 6 months). For time 0, n=6; for the rest samples, n=3.

Figure 2.6 Drug Content Stability: a. Q-GRFT Content in the PBS-base Combo Enema Solution; b. Q-GRFT Content in the Saline-base Combo Enema Solution.

Q-GRFT was tested using HPLC, and relative content (%, compared to the label claim) is presented. Formulations were monitored under 3 conditions (4°C and 25°C/60%RH for 24 months; 40°C/75%RH for 6 months). For time 0, n=6; for the rest samples, n=3.
2.3.4 *In Vitro* Cell Toxicity Studies of the Enema Solutions

We first selected the three excipients which have higher concentrations in the combo enema compared to the single-entity enema (highlighted in bold in Table 2.3, namely Na$_2$HPO$_4$, KH$_2$PO$_4$, and KCl). These selected excipients were tested for the CC$_{50}$ values. For each excipient tested, a series of dilutions was made with culture media and applied to the Caco-2 cells for 24 hours. Figure 2.7 shows the representative curves of all selected excipients. At the highest concentrations used in the combo enema formulations (indicated with the red dotted lines), Caco-2 cells remained 100% viability for all three excipients. Furthermore, the CC$_{50}$ values are significantly higher (for Na$_2$HPO$_4$, 40 times higher; for KH$_2$PO$_4$, 100 times higher; for KCl, 200 times higher) than the highest concentrations used in the formulations.

**Table 2.3 A Composition Comparison of Similar Enema Formulations That Are in Clinical Trials or on the Market.**

*Bold values indicate that the excipient content in the experiment group is higher than in the clinical group.*

<table>
<thead>
<tr>
<th>Content</th>
<th>Experiment Groups</th>
<th>Clinical Groups</th>
<th>Commercial Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ingredients (mg/ml)</td>
<td>Q-GRFT/TFV combo PBS-base (Hypotonic)</td>
<td>Q-GRFT only (Hypotonic)</td>
<td>Normosol® Fleet® (Isotonic) (hypertonic)</td>
</tr>
<tr>
<td><strong>Active Pharmaceutical Ingredients (APIs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q-GRFT</td>
<td>0.32</td>
<td>0.32</td>
<td>N/A</td>
</tr>
<tr>
<td>TFV</td>
<td>5.28</td>
<td>5.28</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Osmolytes (Salts)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.069</td>
<td>0.001</td>
<td>N/A</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.083</td>
<td>0.002</td>
<td>N/A</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$ (anhydrous)</td>
<td>0.500</td>
<td>0.010</td>
<td>N/A</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.777</td>
<td>3.117</td>
<td>8.966</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$ H$_2$O</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$ 7H$_2$O</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table and figures continue...
Figure 2.7 In Vitro Caco-2 Cell Toxicity of Three Excipients in the Enema Formulations.

The x-axis is in the log scale. a: Na₂HPO₄; b: KH₂PO₄; c: KCl. Red lines indicate the highest concentrations used in the formulations. N=8 for each group. d: CC₅₀ is calculated using the sigmoidal 4PL model in Prism9, summarized in the table.

We further wanted to evaluate the entire formulation with full compositions, modifying the Caco-2 monolayer cell model. We applied the sample liquid on the donor sides while culturing the model in the incubator. Cell viability was measured using an MTT assay with a UV plate reader. Compared to the untreated group, both of our formulation groups achieved excellent viability (118% for the PBS-base and 114% for the Saline-base), as shown in Figure 2.8. We also measured the TEER values before and after treatments (Figure 2.9). The positive control group (formaldehyde) had TEER below the threshold (165 Ω·cm²,177). This shows that formaldehyde destroyed the integrity of the cell monolayer and damaged cell viability. In contrast, the two combo enema groups maintained TEER values above the threshold, indicating the integrity of the tight junctions.
Figure 2.8 In Vitro Toxicity of Two Combination Enema Formulations using the Caco-2 Monolayer Cell Model.

Cell viability was compared to the untreated group (100%) using the MTT method. N=3.
Figure 2.9 *In Vitro* Toxicity of Two Combination Enema Formulations using the Caco-2 Monolayer Cell Model.

TEER values after the treatments. The black dot line indicates the lower threshold. For the untreated and the formaldehyde groups: N=3; for both combo enema groups: N=9.

We further evaluated the cell model after treatments using a confocal microscope. The tight junction protein (ZO-1) was stained in green and the nuclei in blue (Figure 2.10). The confocal images also supported the integrity of the cell monolayer after the 2-hour treatments.
Figure 2.10 *In Vitro* Toxicity of Two Combination Enema Formulations using the Caco-2 Monolayer Cell Model.

Confocal images were taken by a Nikon A1R confocal microscope. The tight junction protein (ZO-1) was stained in green and the nuclei in blue. Scales (20 μm) are shown in the pictures.
2.3.5 Permeability Validation of the Different Enema Formulations on the Caco-2 Monolayer Cell Model

After the toxicity study, we further evaluated the permeability profile using the same Caco-2 monolayer model for enema formulations with different osmolality. Because literature indicated the TFV can penetrate tissues via the paracellular pathway\textsuperscript{65}, we first used $[^{14}\text{C}]$-Mannitol, a paracellular marker, to test if different osmolality would result in different permeation profiles. All enema formulations were added with the same concentration of $[^{14}\text{C}]$-Mannitol and applied on the apical sides of the models for 2 hours. Figure 2.11 shows the permeation of $[^{14}\text{C}]$-Mannitol in all enema formulations used. The apparent permeability values were also calculated and summarized in Table 2.4. These results demonstrate clearly that these formulations can be divided into three groups based on their permeability profiles.

![Caco-2 Monolayer Transwell Permeability](image)

Figure 2.11 A Full Comparison of Multiple Enema Formulations with Different Osmolality using the Caco-2 Monolayer Cell Model.
$[^{14}\text{C}]-\text{Mannitol}$ was used as the paracellular marker and added to all the groups. Samples from the receptor sides were collected over two hours and measured for $[^{14}\text{C}]$. The cumulative amount of $[^{14}\text{C}]-\text{Mannitol}$ was plotted against time in the figure. N=3 for all groups.

Table 2.4 A Full Comparison of Multiple Enema Formulations with Different Osmolality using the Caco-2 Monolayer Cell Model.

$[^{14}\text{C}]-\text{Mannitol}$ was used as the paracellular marker and added to all the groups. The apparent permeability of $[^{14}\text{C}]-\text{Mannitol}$ in each formulation was calculated. (n=3)

<table>
<thead>
<tr>
<th>Group</th>
<th>Paracellular marker $[^{14}\text{C}]-\text{Mannitol}$: $P_{\text{app}}$ (cm/s)</th>
<th>Category $[^{179}]P_{\text{app}}$ (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS (negative control)</td>
<td>$2.7 \times 10^{-7} \pm 6.2 \times 10^{-8}$</td>
<td>Poorly absorbed: $&lt;1 \times 10^{-6}$</td>
</tr>
<tr>
<td>0.9% NaCl (Normasol®)</td>
<td>$1.1 \times 10^{-7} \pm 1.5 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td>PBS-base combo Enema</td>
<td>$4.9 \times 10^{-6} \pm 7.1 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td>PBS-base Placebo</td>
<td>$6.7 \times 10^{-6} \pm 1.3 \times 10^{-6}$</td>
<td>Moderately absorbed: $1–10 \times 10^{-6}$</td>
</tr>
<tr>
<td>Saline-base combo Enema</td>
<td>$4.8 \times 10^{-6} \pm 3.9 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td>Saline-base Placebo</td>
<td>$6.5 \times 10^{-6} \pm 5.7 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td>TFV only Hypo-Enema (Clinical Group)</td>
<td>$5.3 \times 10^{-6} \pm 7.7 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td>Fleet®</td>
<td>$2.5 \times 10^{-5} \pm 3.0 \times 10^{-6}$</td>
<td>Well absorbed: $&gt;10 \times 10^{-6}$</td>
</tr>
<tr>
<td>20% SDS (positive control)</td>
<td>$3.0 \times 10^{-5} \pm 1.0 \times 10^{-6}$</td>
<td></td>
</tr>
</tbody>
</table>
2.3.6 Permeability of TFV Revealed by *In Vitro* and *Ex Vivo* Models

After confirming the safety profile, we further evaluated the permeability profile using the same Caco-2 monolayer model. TFV content in the receptor sides was tested for both combination enema as well as the TFV-only Hypo enema, as a comparison. The $P_{\text{app}}$ values were calculated and summarized in Table 2.5. There is no significant difference among all three hypo-osmolar formulations, suggesting that combining with Q-GRFT did not impact the permeability of TFV.

<table>
<thead>
<tr>
<th></th>
<th>$P_{\text{app}}$ of TFV (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFV/Q-GRFT Combo Enema PBS-base (Hypotonic)</td>
<td>$1.5 \times 10^{-6} \pm 1.5 \times 10^{-7}$</td>
</tr>
<tr>
<td>TFV/Q-GRFT Combo Enema Saline-base (Hypotonic)</td>
<td>$1.8 \times 10^{-6} \pm 2.2 \times 10^{-7}$</td>
</tr>
<tr>
<td>TFV Only Enema (Clinical Group) (Hypotonic)</td>
<td>$1.8 \times 10^{-6} \pm 2.6 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

An *ex vivo* permeability model using human colorectal tissues was also performed to study the permeation of TFV. The calculated $P_{\text{app}}$ of TFV in four hypotonic enema formulations is shown in Figure 2.12 (For individual $P_{\text{app}}$, see Appendix Table 2.1). Results suggest that there is no significant difference between any two of the groups. Despite variations, which are commonly seen in human tissue studies, the mean $P_{\text{app}}$ values are all similar, indicating that the permeation of TFV is independent of formulation composition, including the addition of Q-GRFT.
The figure shows the individual value in black dots and the mean value (with SD) in bars. Data in each group contains replicate N=8–20, from 3–7 tissue donors. One-way ANOVA was performed using Prism 9. No significance was found between any two groups.

2.3.7 The Permeability of Q-GRFT Evaluated by the *Ex Vivo* Colon Tissue Model

The permeation of Q-GRFT was also studied with the *ex vivo* permeability model. A total of 10 tissues from different donors were collected and utilized in this study for the experimental and control groups. The solution from receptor sides was collected at all time points. Donor solution was also collected at the beginning and the end of the experiments. As expected, the Q-
GRFT content from the receptor sides was lower than the LLOQ of the HPLC method (1 µg/ml) (data not shown). When we compared the donor sides before and after the treatments, the Q-GRFT recovery is 98.69% and 100.72% for PBS- and Saline-base combination enema, respectively (Table 2.6).

Table 2.6 A Comparison of the Q-GRFT Permeability among Four Formulations.

Q-GRFT content on donor sides was detected with HPLC. *This data is from 5 different human colon tissues (with 3–4 replicates each).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>Q-GRFT Donor Side Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AVG</td>
</tr>
<tr>
<td>Human Colon Tissue</td>
<td>TFV/Q-GRFT Combo Enema PBS-base (Hypotonic)</td>
<td>98.69</td>
</tr>
<tr>
<td></td>
<td>TFV/Q-GRFT Combo Enema Saline-base (Hypotonic)</td>
<td>100.72</td>
</tr>
<tr>
<td>Human Cervical Tissue</td>
<td>GRFT-AlexaFluor488 (in 1×PBS, Isotonic)</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>GRFT (in 1×PBS, Isotonic)</td>
<td>101.3</td>
</tr>
</tbody>
</table>

The toxicity of the combination formulations was evaluated in this model as well. The Ussing Chamber can also monitor the TEER throughout the treatment to confirm the integrity of the epithelium. For the combination formulations, the majority of sample tissues maintained at
least 75% of the initial TEER after the 2-hour treatment (Appendix Figures 2.1), suggesting integrity of the colon epithelium. We also performed the histology study using H&E staining. No apparent morphological changes were observed (Appendix Figure 2.2), indicating that both combination formulations caused no toxicity to the human colorectal tissues.

2.3.8 Both Enema Solution Retained Bioactivity after 24 Months

Since Q-GRFT prevents viral entry by binding with the gp120 proteins on the viral surface, the binding efficacy is an important indicator of its bioactivity. Both enema solutions, stored in two conditions, were tested using ELISA. The Q-GRFT solution was used as the reference group. (Figure 2.13)

![GP120 Binding Efficacy](image)

Figure 2.13 Bioactivity of gp120 Binding Efficacy Determined by ELISA.

No significance was observed for the EC50 across groups. N=3.

The EC50 for the Q-GRFT solution was 11.70 ng/ml. Comparably, the EC50 for the PBS-base combo enema was 20.00 ng/ml (4°C) and 21.82 ng/ml (25°C/60%RH). And the EC50 for the
Saline-base combo enema was 18.39 ng/ml (4°C) and 22.28 ng/ml (25°C/60%RH). Results from all the groups fit into the target criteria (EC$_{50}$: 5–50 ng/ml), indicating the stability of Q-GRFT.

### 2.4 DISCUSSION

As indicated by the U.S. FDA, the primary goal of patient-focused drug development (PFDD) is to better incorporate the patients’ voice in drug development and evaluation$^{180}$. We chose to use an enema product as the dosage form to provide users with a behaviorally congruent product option for oral PrEP$^{53}$. These on-demand products, incorporating behavioral benefits from the targeted population (douching among MSM before sex), may potentially offer immediate protection against HIV and other STIs.

We aimed to design enema formulations that fulfill the patients’ needs by identifying the CQAs to the patients’ benefits. In reviewing literature$^{63,64,71,72}$, we considered the administration route, the properties of the drug substances (namely, TFV and Q-GRFT), and experience from previous development of single-entity enema solutions in CQAs (Figure 2.1).

The second goal of this research was to design an easy, robust, and reproducible SOP for potential large-scale manufacturing. Based on preliminary studies with TFV and Q-GRFT enema, we understood that (1) TFV, salts in buffer, and pH adjusting agents are contributors to osmolality. The addition of Q-GRFT solution does not change the osmolality significantly in the final product; (2) the pH of the solution impacts the solubility of TFV powder$^{64}$; (3) the pH of Q-GRFT stock solution is neutral (around 7.39). And pH adjustment can potentially damage the Q-GRFT stability, as instability of GRFT was observed in acidic conditions$^{107}$. Therefore, a simplified procedure for manufacturing the enema solution was developed (Figure 2.1).
Stability has always been a major issue for liquid formulations containing protein drugs. To the best of our knowledge, our study was the first to detect the long-term stability of enema formulations containing Q-GRFT. And the long-term compatibility of combined TFV and Q-GRFT has yet been explored either. The current results showed that both our formulations met the CQAs after manufacturing (Table 2.2). More importantly, physicochemical parameters, including appearance, pH, osmolality, and TFV content, remained stable under three conditions for two years. For Q-GRFT content, the study demonstrated that, despite slight fluctuations, it remained within range for up to 18 months in both formulations. Surprisingly, Q-GRFT content in the PBS-base formulation dropped slightly below 90% at the 24-month time point. We can still consider them stable, given the fact that no significant difference was found when compared to the Q-GRFT content on Time 0 (90.6%). However, caution must be applied with the small sample size. The finding does hint at the slight superiority of saline-base formulation over the PBS-base one. Overall, the results of this study demonstrated that two stable formulations were achieved. The study provided a successful example of integrating the CQAs into the formulation development process. With the presence of clear and biorelevant CQAs, product quality can be controlled by the release and during the storage.

Toxicity is another important perspective to consider in drug product development. Given the positive safety profile we obtained from clinical trials (data unpublished) of the single-entity (TFV-only or Q-GRFT-only) products, we wanted to evaluate the toxicity of the current combo enema with bridge studies. First, we performed a composition comparison for the enema formulations, as shown in Table 2.3. Because the single-entity enema solutions are both based on 1×Saline (0.9% NaCl), the compositions are all similar across these groups. Only three excipients in the PBS-base combo enema have higher concentrations (highlighted in bold in Table 2.3),
compared to the clinical enema formulations. Therefore, Na₂HPO₄, KH₂PO₄, and KCl were tested for their CC₅₀ on Caco-2 cells. In order to further evaluate the toxicity of the entire liquid formulations, we took the advantage of Caco-2 monolayer cell model. The model was established by seeding Caco-2 cells onto the polycarbonate membranes and cultured until a monolayer of cells was formed. Due to tight junctions formed by the cells, this model can mimic the colorectal epithelium. Overall, results showed that even with higher concentrations, these excipients have no toxicity in Caco-2 cells. Also, results further demonstrated that these two combination enemas do not damage the cell viability and integrity in the Caco-2 monolayer model. The formulations we designed in this study have a similar safety profile to the ones in clinical trials. Furthermore, the toxicity studies served as a foundation for the following in vitro permeability study.

Since this Caco-2 monolayer model has been widely used to predict in vivo absorption, we first utilized this model to characterize our formulations with a paracellular marker, [³¹⁴C]-Mannitol. Based on the data, our two combination enema formulations, their matching placebos, and the TFV-only Hypo(osmolar) enema (from the clinical trial) can be categorized together. This group has higher [³¹⁴C]-Mannitol permeation compared to the HBSS (negative control) and 0.9% NaCl (Normosal®) group, but lower permeation compared to the 20% SDS (positive control) and Fleet® group. These three categories also match the suggested divisions from the literature (Table 2.4). It should be noted that both formulations in the well-absorbed group have poor safety profiles, suggesting the high Pᵥᵥ values were achieved due to the damaged epithelium. The HBSS, which was used as a negative control, and the 0.9% NaCl are both iso-osmolar formulations, lacking the driving force for [³¹⁴C]-Mannitol permeation into the receptor sides. On the other hand, hypo-osmolar formulations will drive more water into the receptor sides, resulting in higher concentrations of [³¹⁴C]-Mannitol, the hydrophilic paracellular marker. Within the
moderately absorbed group, all enema formulations have similar P_app values. The data indicates that the paracellular permeability of [14C]-Mannitol mainly depends on the osmolality of the formulation, regardless of the compositions.

For TFV, permeability is an important PK parameter given it is an NRTI. In addition, the correlation between pharmacokinetics and pharmacodynamics has already been established182,183. Results generated from both in vitro and ex vivo models demonstrated good permeation of TFV. By comparing the P_app with the clinical enema (TFV-only enema), both PBS-base and saline-base combo enema achieved desired property. There is no significant difference among all three hypo-osmolar formulations, suggesting that combining with Q-GRFT did not impact the permeability of TFV.

We can also compare the P_app of TFV with the P_app of [14C]-Mannitol within each group (Appendix Table 2.2). Data suggests that TFV achieved similar permeability profiles as [14C]-Mannitol, indicating that TFV may mainly use the paracellular pathway to penetrate tissues. There is no significant difference between TFV and [14C]-Mannitol, but the P_app values of TFV are slightly lower. The results echo that TFV is not solely across the tissue via the paracellular pathway. In addition, lower P_app of TFV suggests the potential impacts of transporters. TFV is predominantly transported by MRP4, which is one of the transporters expressed in Caco-2 cells184.

Findings from the ex vivo model were also in accordance with the results generated from the in vitro studies (Appendix Table 2.3). Given that the colon epithelium is also a thin single layer, the results were consistent with that of the Caco-2 monolayer model. When we compared the permeability data across studies, P_app is generally higher in the ex vivo model than in the in vitro model. This could also be explained by the existence of MRP4, whose expression in Caco-2 cells is higher compared to in human intestine tissues184. Higher transporter expressions resulted
in lower $P_{\text{app}}$. Hence, the TFV concentration in a patient’s tissue may be higher than detected in the *in vitro* studies.

Because of the protein nature of Q-GRFT, we did not anticipate it to permeate through the tissues. Literature suggests that Q-GRFT sticks on the surface of vaginal tissue after a 6-hour treatment\textsuperscript{107}. However, it was unknown for colorectal tissues. With the vaginal epithelium being multi-layers and the colorectal epithelium being single-layer, it was important to evaluate the permeation of Q-GRFT in this study. Previous work from lab\textsuperscript{107} was compared to demonstrate the location of Q-GRFT. Two differences exist between the studies: (1) Vaginal tissues have more epithelial layers than colorectal tissues; (2) Isotonic enema delivers less drug into tissues compared to hypotonic enema. Therefore, if Q-GRFT is permeable, we would see much less recovery from the donor sides for our study (the hypotonic enema used on the colorectal tissues). Yet the recoveries are all close to 100% across studies, suggesting Q-GRFT would also remain on the surface of the colorectal epithelium, despite the high driving force from hypotonic formulations. In addition, the results had small RSDs, demonstrating low intra- and inter-patient differences.

### 2.5 Conclusion

In summary, we successfully developed two clear and hypotonic enema formulations with neutral pH. Both formulations achieved similar permeability of TFV compared to the single entity (TFV only) enema which is being studied in a clinical trial. For Q-GRFT, we suspected that it sticks to the surface of colorectal tissues where it will function as an entry inhibitor. In addition, *in vitro* and *ex vivo* assays demonstrated both formulations to be safe for HIV prevention. For the first time, we demonstrated the enema formulations remained stable for 2 years. Both products
were stable after long-term storage (in different conditions), with no significant loss for the drug contents of both small molecule and biologic APIs.

Moreover, with CQAs incorporated into the design of products, this simple, robust, and reproducible procedure is suitable for future large-scale manufacturing. In summary, these two enema formulations can successfully deliver two APIs with expected synergistic effects for HIV/STI prevention. These patient-centric products show potential for advancement to the next stages of product development.
3.0 Q-GRFT SOLID FORMULATION DESIGN: POWDER

While enema use/douching provides potential benefits of increasing adherence\textsuperscript{69,136}, the dosage form itself can be further developed. Due to its nature of liquid form, it is hard for transportation and storage\textsuperscript{147}. Thus, solid dosage forms also present economic advantages for manufacturers and distributors\textsuperscript{147,148}. On the user’s end, solid dosage forms can also offer advantages, such as convenience and privacy\textsuperscript{91,185-188}. Therefore, it is important to develop a powder form of the enema. Enema powder can be reconstituted into an enema solution by users themselves. As a powder sachet, it is easy for the users to carry around, especially during the holiday and travel when the chances of unprotected sexual behaviors/meeting new sexual partners/having sex with sex workers are higher\textsuperscript{189-191}. Moreover, protein drugs or other biologics may have limited stability in the solution. By removing water, protein drugs can achieve a longer shelf-life under ambient conditions\textsuperscript{157}.

Currently, lyophilization/freeze-drying is one of the most commonly used processes to dehydrate aqueous formulations\textsuperscript{157}. It offers a relatively cheap, safe, and continuous manufacturing process for potential big-scale productions\textsuperscript{157,192,193}. Another popular dehydration method, spray-drying, generated impurities for Q-GRFT (unpublished data), possibly due to the heat during the spraying process\textsuperscript{194}. Therefore, freeze-drying was preferred due to the lack of this unstable factor\textsuperscript{194,195}. Moreover, because of the low water content in the solid dosage form, the possibility of drug movements within the formulation is significantly decreased, leading to possible longer-term stability compared to liquid formulations\textsuperscript{157,192}.  

58
3.1 INTRODUCTION

3.1.1 Protein Instability Caused by Lyophilization

Despite all the benefits of the lyophilization aforementioned, there are still challenges remaining for the lyophilized protein formulations\textsuperscript{196}. The low temperatures, as well as the water-removing process, generate stresses for the protein drug, which may lead to protein degradation. Damaged proteins may further result in colloidal instabilities (aggregations)\textsuperscript{197,198}, conformational instabilities (structural changes)\textsuperscript{199,200}, and bioactivity loss\textsuperscript{201,202}. These stresses, which are involved in each step of the lyophilization process, include low-temperature stress (cryo-stress), dehydration effects, concentration effects, and pH shifts\textsuperscript{157}.

3.1.1.1 Cryo-stress

Literature\textsuperscript{193,203,204} has reported the instabilities of protein drugs caused by low-temperature stresses, including freeze-thaw cycles. A possible explanation is that the solvophobic interactions in proteins decreased as the temperature dropped, leading to an enthalpy-driven instability\textsuperscript{205,206}. The solvophobic thermodynamic force, coming from the interactions between the osmolytes and the protein backbone, can protect from denaturation if maintained\textsuperscript{207}. Several other studies\textsuperscript{208,209} also reported similar protein conformational changes induced by the cryo-stress.

3.1.1.2 Dehydration

In the liquid formulation, protein drugs are surrounded by water molecules, generating a hydration shell. Generally, the water content in the lyophilized end-product is less than 10\%, suggesting the elimination of the hydration shell\textsuperscript{192}. To be specific, the protein-bound water
molecules were removed during the secondary drying step. This step may disrupt the native state of the protein drug\textsuperscript{210}, or reside functionality in the active site\textsuperscript{211}. Therefore, the optimum residual moisture in lyophilized protein drugs should be carefully controlled\textsuperscript{212}. Recent studies\textsuperscript{155,156} also warrant the negative impacts of over-drying, stating the importance of hydration shell for lyophilized protein drugs.

3.1.1.3 Concentration Effects

Because of ice formation (freezing) or water elimination (drying), the solution was concentrated leading to an increase in protein concentration. Normally, the interaction slows down as the temperature decreases. However, the increased concentration can lead to an acceleration in chemical reactions\textsuperscript{213}. Moreover, a concentrated environment increases the possibility of protein-protein interactions, causing aggregations\textsuperscript{204}.

3.1.1.4 pH Shifts

The increased concentration of buffer agents may cause pH shifts\textsuperscript{153,214}. Protein drugs, which are sensitive to extreme acidic or basic conditions, may unfold or denature\textsuperscript{215,216}. One example is that the freezing of lactate dehydrogenase (LDH) solution caused the pH to drop from 7.5 to 4.5, which led to protein denaturation\textsuperscript{217}. Research by Franks (1990)\textsuperscript{218} finds that Na\textsubscript{2}HPO\textsubscript{4} crystallizes more readily than NaH\textsubscript{2}PO\textsubscript{4} during lyophilization. Because the solubility of the disodium form is considerably lower than that of the monosodium form, the molar ratio of [NaH\textsubscript{2}PO\textsubscript{4}]/[Na\textsubscript{2}HPO\textsubscript{4}] remaining in the liquid was increased, generating a significant acidic environment\textsuperscript{218}.  

60
3.1.1.5 Other Theories

There were also other theories and examples reported for the protein denaturation that happened during lyophilization. It was reported that the increased protein drug concentration may lead to liquid-liquid phase separation\textsuperscript{219}. This separation created a large excess of the interface, denaturing the protein. Other studies\textsuperscript{220,221} also reported the formation of the ice-water interface during slow freezing. Proteins can be attracted to such interfaces, changing their native conformations.

3.1.2 Cryoprotectants Prevent Protein Aggregation during Lyophilization

As one of the protein degradation mechanisms\textsuperscript{157,192,193,203}, aggregation is a common issue for protein drugs during lyophilization\textsuperscript{222-224}. Encouragingly, there is literature\textsuperscript{157,192} reported that these protein aggregations can be prevented or reversed to increase protein stability after lyophilization. Pharmaceutical proteins can be formulated with excipients that offer protection to maintain the secondary and tertiary structures\textsuperscript{145,225}. These excipients, namely stabilizers or cryoprotectants, can be categorized as follows: amino acids/proteins, polymers, surfactants, buffering agents/salts, and saccharides or sugar alcohol isomers\textsuperscript{138}.

Literature provides abundant examples of the above-mentioned excipients. For example, Amino acids can stabilize proteins by various mechanisms such as preferential hydration and direct binding as well as being buffer agents\textsuperscript{226}. Polymers have been evaluated to stabilize proteins in solution and lyophilized formulations\textsuperscript{165-167}. Surfactants are used to inhibit protein aggregation due to agitation. It was also shown that surfactants can block protein molecules from interacting with other hydrophobic surfaces\textsuperscript{227}. Buffering agents can control solution pH to maintain the higher-order structure of a protein\textsuperscript{192,214}. 
Another commonly-used family of cryoprotectants contains sugars and sugar polyols. In the liquid state, sugars are thought to stabilize proteins by preferential hydration at high protein concentrations\textsuperscript{228,229}. In the solid-state, various mechanisms are involved for sugars to protect proteins. One mechanism is the water replacement hypothesis\textsuperscript{168}. Since the loss of the protein hydro-shell would lead to protein-protein hydrophobic interaction when the protein is exposed to a less humid condition, water-replacing agents can preserve the protein’s natural conformation\textsuperscript{192,210,211}. Sugars serve as water substitutes by forming hydrogen bonds with proteins\textsuperscript{230}. On the other hand, recent studies also stated that sugars/sugar polyols form a vitrified, rigid sugar-glass matrix (vitrification hypothesis), which limits the protein degradations kinetically\textsuperscript{138,231}. Although there is experimental evidence to support both hypotheses, neither one is capable of fully explaining the protecting effects of sugars/sugar polyols\textsuperscript{231}.

The protections offered by sugars/sugar polyols are also protein-specific. One excipient may have different stabilizing/destabilizing results even to proteins from the same family\textsuperscript{225}. For instance, non-reducing disaccharides and sugar polyols, such as sucrose\textsuperscript{232,233}, trehalose\textsuperscript{234,235}, sorbitol\textsuperscript{236,237}, and mannitol\textsuperscript{238}, were reported to stabilize different proteins in various formulations\textsuperscript{232,233}. However, Singh et al.\textsuperscript{198} reported that the excess use of trehalose may lead to an increase in sugar crystallization, eventually the reduction of protein stability. Piedmonte et al.\textsuperscript{239} also reported a similar aggregation case with sorbitol crystallization. Another example that supports the complexity of cryoprotectants is lactose, a reducing sugar. Because reducing sugars can lead to protein glycation, they are normally considered incompatible in lyophilized protein formulations\textsuperscript{138}. But Ibrahim et al. challenged it by the successful development of inhaled formulations of proteins with lactose\textsuperscript{240}.
Therefore, screening and identifying excipients is one major practical challenge in protein formulation development and needs to be studied for every single protein drug entering clinical trials.

3.1.3 Critical Quality Attributes for Q-GRFT Lyophilized Powder

Previous work from Kramzer et al. (2021)\textsuperscript{107} demonstrated the good stability of GRFT during freeze-thaw cycles. After five cycles of -80°C/25°C and -20°C/25°C, the GRFT recovery was 102% and 99% respectively. These results set the foundation for successful developments of lyophilized formulations. However, GRFT was found prone to oxidation\textsuperscript{107}. A mutated protein drug, Q-GRFT was later developed to address the oxidation issue. It replaces the easily oxidized residue (methionine) with glutamine while maintaining the same high antiviral potency\textsuperscript{108}.

While oxidation was minimized as the degradation pathway, protein aggregation was identified by our group as one of the major challenges for lyophilized Q-GRFT formulations. (Appendix Figure 3.1 and Appendix Figure 3.2) When Q-GRFT was lyophilized in its original liquid formulation (Q-GRFT in 25% PBS buffer), the drug content was significantly decreased starting from Day 3 after lyophilization (stored in 25°C/60% RH, Appendix Figure 3.1). An aggregation peak (retention time (RT)=18.961 min) was found using size exclusion chromatography (SEC), eluting prior to the Q-GRFT monomer peak (RT=20.528 min). Since sugars/sugar polyols have been widely demonstrated to prevent protein aggregation\textsuperscript{139,157,237}, the major goal of this study was to screen suitable cryoprotectants and to develop a stable Q-GRFT lyophilized powder formulation.

Other physicochemical properties should also be considered as CQAs during the formulation developments. In the solid state, crystallization should be controlled as it might disrupt
the protein stability\textsuperscript{214,237,239}. In addition, water content should be monitored to minimize protein mobility, in the meantime, to avoid over-drying\textsuperscript{155,210,211}. Moreover, since the lyophilized powder has potential for further development in other solid drug delivery systems, it is preferred to demonstrate properties for large-scale manufacturing. Flowability, a critical attribute for pharmaceutical powder, should be included in the CQA\textsuperscript{241}. In the liquid state, we designed that the reconstituted Q-GRFT enema or combo enema (with TFV) still retains all the CQAs stated in Chapter 3. The CQAs for the reconstituted enema include appearance, pH, osmolality, drug contents (for both Q-GRFT and TFV), and bioactivity.

\section*{3.2 MATERIALS AND METHODS}

\subsection*{3.2.1 Materials}

Recombinant Q-GRFT drug substance was supplied by Kentucky Bioprocessing LLC (Owensboro, KY). TFV was sourced from WuXi AppTec (Shanghai, China). PBS 10\texttimes molecular biology grade (pH 7.4) was purchased from Mediatech, Inc. (Manassa, VA). Saline buffer (sodium chloride injection, USP, sterile) was purchased from B. Braun Medical Inc. (Irvine, CA). ACN, TFA, sulfuric acid, tBAHS, Na\textsubscript{2}HPO\textsubscript{4}, potassium phosphate monobasic (NaH\textsubscript{2}PO\textsubscript{4}), potassium chloride (KCl), sodium chloride (NaCl), HCl, NaOH, sucrose, trehalose, lactitol, maltitol, water content standards (Hydranal), and pH standard kits were obtained from Fisher Scientific (Pittsburgh, PA). The osmolality standards were purchased from Advanced Instruments (Norwood, MA). The protein molecular weight markers (Calbiochem) were purchased from EMD Millipore (Milford, MA). Purified water was prepared in-house utilizing a MilliQ (Millipore;
Milford, MA) filtration system at 18.2 MΩ cm. PBS 1× was manufactured in-house with PBS 10× stock and MilliQ-water.

3.2.2 Lyophilization

For samples with cryoprotectants, sugar/sugar polyol powders were weighed and added to individual scintillation vials. The Q-GRFT stock solution was then added. The sample vials were mixed until the powder dissolved. Next, the vials containing the sample solution (with or without the addition of cryoprotectants) were transferred to a -80°C freezer to freeze overnight. Until lyophilization, the samples were tilted and placed in the beaker, attached to a pre-set lyophilizer (Labconco FreeZone Freeze Dry System, Kansas City, MO). The lyophilizing process was set to maintain the environment at -50°C and 0.07 mBar. Sample vials were measured for their weights after lyophilizing for at least two days. Once the weights were stable, indicating no more water was removed, the lyophilized powder was taken out of the vials and ground with a mortar and pestle. The lyophilized powder would be packed in a tube at 4°C (unless other conditions were indicated) until further characterizations.

3.2.3 Physicochemical Characterization of the Lyophilized Powder

3.2.3.1 Appearance

The appearance of the lyophilized powder was monitored by visual observations. Color, macro particle shape, and texture were recorded.
3.2.3.2 Crystallinity Determination by Differential Scanning Calorimetry (DSC)

Lyophilized powders were characterized for their thermal properties by the Mettler Toledo DSC 3 with the STARe Excellence Software (Columbus, OH). At least 2 mg of powder was measured inside the crucibles to fully cover the bottom surface. Using an empty crucible with a punctured lid as the control, all samples were measured from 25 to 250°C at a heating rate of 10°C/min under a constant nitrogen purge of 20 ml/min. The endothermic peaks were integrated with the built-in software methods.

3.2.3.3 Crystallinity Determination by X-Ray Powder Diffraction (XRD)

Lyophilized powders were tested for crystallization by the Bruker D8 Discover SRD instrument (Billerica, MA) with third-generation Göbel Mirrors to provide maximal X-Ray flux density and an Ultra GID detector. The powders were compressed to form a thin layer on the platform. The measurements start with an angle of 3.5° and end with an angle of 95°. The scan speed is 0.40 seconds/step, and the increment is 0.04°. After scanning, the data was saved using DIFFRAC\textsuperscript{plus} BASIC Evaluation software, converted using PowDLL converter software, and graphed using Prism 9.

3.2.3.4 Flowability

The powder flow was characterized following the Angle of Repose method recorded in the International harmonization of compendial standards chapter <1174>\textsuperscript{241}. The powder was tested on the Copley BEP2 Flowability Tester (Nottingham, United Kingdom) with a Mitutoyo ABSOLUTE Digimatic Height Gage (Kanagawa, Japan). In short, the testing powder flew through a funnel with a fixed height of 74mm onto a 100mm (D, outer diameter) platform. Once the cone-like powder pile was formed, the height of the pile (H) was measured by the gauge. The angle of
repose (α) was calculated using Equation 3.1. The powder can be classified with different flow properties based on the literature\textsuperscript{242}.

\[
\tan(\alpha) = \frac{H}{0.5 \times D}
\]

Equation 3.1

3.2.3.5 Size Distribution

The powder was characterized for particle size distribution using a Humboldt HA-4325V motorized sieve shaker with sieve sizes of 1000, 850, 600, 425, 355, 150, 125, 106, 75, 45, and 20 microns (Elgin, IL). The powder was placed in the top sieve (1000 micron) and shaker at level 7 for 30 to 60 minutes. Every sieve was weighed before and after to record the powder weights in each level. Size distribution was later graphed based on the weights of powder.

3.2.3.6 Water Content

Water content was detected using a Karl-Fischer titration apparatus (Metrohm,758 KFD Titrino; Herisau, Switzerland). Powders were weighed, transferred to scintillation vials, and cramp-sealed before measuring. The apparatus was set at 120°C and calibrated with Water Content Standards (Hydranal) each time before the measurements. Water content (WC%, w/w) was calculated and reported by the software.

3.2.4 Physicochemical Characterization of the Reconstituted Enema Solution

3.2.4.1 Appearance

The appearance of the enema solutions was monitored by visual observation. The formulations should be clear and colorless. Any turbidity may indicate the aggregation of Q-GRFT or contaminations, warranting further investigations.
3.2.4.2 pH

The pH was determined by a pH meter (XL150 pH Benchtop Meters, Thermo Fisher Scientific; Waltham, MA). Before use, the pH meter was calibrated using pH standard buffers (Orion All-in-One pH Buffers Kit, Thermo Fisher Scientific; Waltham, MA) every time. The pH was recorded only after the value stayed stable.

3.2.4.3 Osmolality

The osmolality assay was performed using a freeze-point osmometer (Advanced Instruments; Norwood, MA). Every time, a standard osmolality solution (200 mOsm/kg, Advanced Instruments; Norwood, MA) was used to test the accuracy of the apparatus. If the deviation was more than ±2 mOsm/kg, a calibration procedure would be performed with a serial range of standard osmolality solutions. For each testing group, the average osmolality values were measured in triplicate.

3.2.5 Drug Content

3.2.5.1 TFV Content Determination by Ultra Performance Liquid Chromatography

The UPLC method for measuring the TFV content was developed in our lab and described previously\textsuperscript{101}. For sample preparation, a sample solution was first filtered through a 0.22 μm PTFE filter (Target Syringe Filters, Thermo Fisher Scientific) with 1 ml syringes (BD U-100, Thermo Fisher Scientific). The samples were then diluted 50 times with the mobile phase prior to UPLC analysis. After preparation, samples were quantified by integrating the peak area with the UPLC method (Waters Acquity UPLC H-Class systems; Acquity UPLC BEH C18 columns, 130Å,
1.7µm, 2.1×50 mm; with VanGuard Pre-Column, 130Å, 1.7µm, 2.1×5mm), and the TFV content was calculated with calibration curves.

3.2.5.2 Q-GRFT Content Determination by High-Performance Liquid Chromatography

The HPLC method for measuring the Q-GRFT content was developed in our lab and reported previously. The samples were diluted 2 times with Milli-Q water. The Q-GRFT content was detected by an HPLC system (Waters Corporation, Milford, MA) equipped with an auto-injector (model 717), a quaternary pump (model 600), and a photodiode array detector (model 2996) with a C5 column (Jupiter 5µm 300Å, 250×4.6 mm) and a C5 pre-column (Security Guard Standard Widepore).

3.2.5.3 Aggregation Determination by Size Exclusion Chromatography

An SEC column (TSKgel SuperSW3000 Size Exclusion HPLC column, Tosoh Bioscience) was used for the detection of aggregations. The column was connected to an HPLC system (Thermo Fisher Scientific Dionex UltiMate 3000, Waltham, MA) equipped with an auto-injector, a dual-gradient pump, and a UV detector. For sample preparations, the powders were first dissolved in MilliQ water and then directly injected into the system. The mobile phase is PBS solution at a constant rate (0.3 ml/min). A protein molecular weight marker (Calbiochem, EMD Millipore) was used as a calibration to determine the molecular weights of tested samples.

3.2.6 Bioactivity Determination by Enzyme-linked Immunosorbent Assay

An ELISA was adapted and applied to evaluate the gp120 binding activity of the Q-GRFT samples. Nunc MaxiSorp 96-well plates were used for this experiment. A detailed method was
described in Section 2.2.7. The plates were measured at 450nm using a plate reader for the gp120 binding activity.

3.2.7 Statistical Analysis

All values are reported as means ± SD. Statistical data analyses were performed using one-way ANOVA with Tukey’s post hoc test, with p<0.05 as the minimal level of significance, p<0.01 for very significant, and p<0.001 for highly significant. All statistical analyses were performed using the GraphPad Prism software version 9.

3.3 RESULTS

3.3.1 Cryoprotectant Selection Under Accelerated Condition

Because of numerous successes reported by the literature with sugars/sugar polyols, four representatives (sucrose (Suc.), trehalose (Tre.), maltitol (Mal.), and lactitol (Lac.)) were included in the study to detect their cryoprotecting effects on Q-GRFT. Q-GRFT solution was mixed with different molar ratios (3, 6, or 12) of sugar/sugar polyol to protein monomer before lyophilization. After lyophilization, the powder was stored in the scintillation vials. The Q-GRFT stability in the lyophilized powder was tested by HPLC after storing in an accelerated condition (40°C/75% RH) for 3 months. As shown in Figure 3.1, the protecting effects vary based on the type and the ratio of the sugar/sugar polyols added.
Figure 3.1 Sugar/Sugar Polyol Selection as Cryoprotectants.

Q-GRFT content was detected at three months by HPLC after storing the lyophilized powder under an accelerated condition (40 °C/75% RH). N=4. *p<0.05.

Compared to the control group, which is the Q-GRFT-only lyophilized powder group, lactitol has no protecting effects on every ratio. The maltitol and sucrose groups, have the same protecting powers, proved by significance higher Q-GRFT content on higher ratios. In addition, this protecting effect is positively correlated with the amounts of sugar/sugar polyol added. For the trehalose group, significantly higher amounts of Q-GRFT content were found in the ratio groups of ×3 and ×12 only. Despite a higher amount of Q-GRFT found in the middle ratio (×6) of the trehalose group, it is not statistically significant when compared to the control group.

3.3.2 Maltitol Ratio Selections

Given the superiority of maltitol among all four cryoprotectants shown above and in the real-time condition (25°C/60% RH, unpublished data), it was selected as the model protectant for
a more detailed ratio selection. To study if the protecting effects remain positively correlated with the amounts of maltitol, Q-GRFT solution was lyophilized with different molar ratios of maltitol to Q-GRFT monomer (1×, 3×, 9×, 18×, 27×, 36×, or 72×). The lyophilized powders were sealed in aluminum pouches, a package proposed for the final product. The pouches were stored under either an acceleration condition (40°C/75% RH) or a real-time condition (25°C/60% RH) for 3 months. For the accelerated condition, the results (shown in Figure 3.2) showed a similar trend to previous findings. There is a positive correlation between the Q-GRFT content and the ratios of maltitol used in the formulation. The recovery in this study was slightly lower compared to the previous study (for the Q-GRFT only control group), possibly due to different packages. Nonetheless, the Q-GRFT content is significantly higher in the ratio groups of 18×, 27×, and 36×.

![Q-GRFT LMP Stability T3M [40°C/ 75%RH]](image)

Figure 3.2 Maltitol Ratio Selection as Cryoprotectants under an accelerated condition.
The Maltitol ratio was defined by the molar ratio of maltitol to Q-GRFT monomer. Q-GRFT content was detected at three months by HPLC after storing the lyophilized powder under an accelerated condition (40°C/75% RH). N=4. *p<0.05; **p<0.01; ****p<0.0001.
However, the superiorit of higher maltitol ratios did not reflect in the normal condition (25°C/60% RH). As shown in Figure 3.3, significantly higher Q-GRFT contents were found in all ratio groups except the 36× and 72× groups. Under this condition, the positive trend remains up to 18×. In addition, a higher ratio of maltitol also presented potential difficulties for future productions. Q-GRFT lyophilized powder with higher maltitol contents was stickier due to the hygroscopic nature of maltitol.

**Figure 3.3 Maltitol Ratio Selection as Cryoprotectants under a normal condition.**

The Maltitol ratio was defined by the molar ratio of maltitol to Q-GRFT monomer. Q-GRFT content was detected at three months by HPLC after storing the lyophilized powder under a normal condition (25°C/60% RH). N=4. A dashed line indicates the 100% label claim.

Therefore, the ratio group of 18× maltitol to Q-GRFT (from now on, this formulation was referred to as the Q-GRFT lyophilized powder or LMP) was chosen for the following manufacturing and formulation developments.
3.3.3 Short Term Stability Study for the Q-GRFT Lyophilized Powder

Since only one single time point (3 months) was investigated in the cryoprotectant selection study, a more detailed short-term study was desired to confirm the stability of Q-GRFT in the real-time condition. The lyophilized Q-GRFT with maltitol powder (LMP) was monitored for Q-GRFT content at several time points (Day 1, 3, 5, 7, 14, 30, 60, 90) in 25°C/60% RH condition. The Q-GRFT content tested was within the range of 90% to 110% for all time points, indicating its stability for up to 3 months (Figure 3.4.A). In addition, LMP was also tested in SEC to detect any aggregation. As shown in Figure 3.4.B, there is no aggregation peak shown on SEC for LMP. In summary, maltitol at a molar ratio of 18 to Q-GRFT was chosen as the cryoprotectant in the formulation. Q-GRFT in the LMP remains stable for up to 3 months and no aggregation was detected.
Figure 3.4 Short-term (Three Months) Stability Study.

(a) Q-GRFT content was detected at different time points by HPLC after storing the lyophilized powder under a normal condition (25°C/60% RH). N=4. Dash lines indicate the 90% (lower limit), 100% (target), and 110% (upper limit) of the label claim. (b) SEC chromatography was tested on the last time point. LMP group (shown in blue) and the Q-GRFT reference group (shown in magenta) were compared. Q-GRFT elutes at the same retention time for both groups. No aggregation peaks were detected.
3.3.4 Physicochemical Characterizations of the Q-GRFT Lyophilized Powder

The LMP formulation was characterized for its physicochemical properties in both solid and reconstituted liquid states. The Q-GRFT-only lyophilized powder was also assessed as a comparison. As a pharmaceutical powder, water content (Karl-Fischer), flowability (angle of repose), and crystallinity (DSC and XRD) were measured. The powders were also reconstituted with Milli-Q water to test for osmolality, pH, and drug content, summarized in Table 3.1. Both lyophilized powders achieved water content below 10% after lyophilization, with slightly higher water in the LMP (8.47%). In addition, flowability was not impacted by the addition of maltitol in the formulation. Both powders achieved angles of repose below 30, which were categorized as excellent flowability241,242.

Table 3.1 A Comparison of Q-GRFT Only Lyophilized Powder and LMP for the Physiochemical Characterizations.

Both the Powder Form and the Reconstituted Enema Form were assessed. (N=3)

<table>
<thead>
<tr>
<th></th>
<th>Lyophilized Powder</th>
<th>Reconstituted Enema Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water Content (%)</td>
<td>Flowability (Degree°)</td>
</tr>
<tr>
<td>Q-GRFT ONLY Powder</td>
<td>6.12±1.21</td>
<td>26.94±0.52</td>
</tr>
<tr>
<td>LMP</td>
<td>8.47±0.44</td>
<td>29.89±0.88</td>
</tr>
</tbody>
</table>

In addition, lyophilized powders were reconstituted with Milli-Q water and characterized for osmolality, pH, and drug content. No difference was detected in osmolality and pH between the two groups. However, LMP has higher drug content (103.47% label claim) compared to Q-
GRFT only powder (88.03% label claim). Given that the samples were tested at Time 0, the results demonstrated the immediate protecting effects of maltitol to prevent Q-GRFT loss during lyophilization.

3.3.5 Crystallization Detections by DSC

The crystallinity of lyophilized powders (summarized in Table 3.1.) was tested by DSC and shown in Figure 3.5.

Because the Q-GRFT bulk solution comes in the PBS buffer, PBS exists throughout the entire manufacturing process. Therefore, excipients in PBS (non-lyophilized) were identified by DSC either individually (Appendix Figure 3.3) or combined (Appendix Figure 3.4). Only sodium phosphates showed significant endothermic peaks on DSC, with Na₂HPO₄ having a peak of ~93°C and NaH₂PO₄ having a peak of ~215°C (Appendix Figure 3.3). These two peaks remained significant even after being combined with NaCl and KCl, despite the predominant existence of NaCl in the formulation (80.97%, w/w to PBS). However, slight decreases were found for both phosphates (for Na₂HPO₄, a -15°C shift; for NaH₂PO₄, a -3°C shift), suggesting interactions between excipients during the heating process. In addition, the LMP formulation contains maltitol. Thus, maltitol (before and after lyophilized) powders were also identified by DSC (Figure 3.5). Before lyophilization, maltitol crystals present a signature peak on DSC ~155°C. This signature peak significantly decreased after lyophilization, as partial maltitol transformed into an amorphous state.
Figure 3.5 DSC Chromatography for LMP (green line), Q-GRFT Only Lyophilized Powder (red line), Maltitol Only [lyophilized] Powder (magenta line), and Maltitol Only [unlyophilized] Powder (blue line). Negative peaks are endothermic peaks. Relative energy (y-axis) was plotted against temperature (x-axis).

When Q-GRFT was lyophilized without any cryoprotectants, a significant peak was found around 209°C, indicating the crystallization of PBS (to be specific, NaH₂PO₄). Encouragingly, the PBS crystallization was demolished by the addition of maltitol, with no peak shown near the temperature (Figure 3.5). For the LMP group, the endothermic peak shown on DSC was maltitol (~158°C). Compared to the maltitol crystal group, this peak is significantly smaller, suggesting excessive maltitol in the LMP formulation exists as a combination of both crystallized and amorphous states.

3.3.6 Crystallinity determination by XRD

The crystallinity of lyophilized powders was also confirmed by XRD (Figure 3.6).
PBS was lyophilized and analyzed by XRD, showing some signature peaks which were identified in Appendix Figure 3.5. The maltitol-only lyophilized powder did not present any peaks by XRD, confirming its amorphous state (Figure 3.6). For the Q-GRFT only lyophilized powder, it has some signature peaks which are identical to PBS. This result indicated crystallization of PBS in the Q-GRFT only group. In the contrast, LMP did not have these peaks, suggesting the addition of maltitol prevented the crystallization of PBS.

3.3.7 LMP Retained gp120 Binding Bioactivity

The LMP group was reconstituted and tested for gp120 binding efficacy after lyophilization. The Q-GRFT solution group was used as the reference, as shown in Figure 3.7.
The Q-GRFT reference group had an EC50 of 7.02 ng/ml, while the EC50 of the LMP group was 7.57 ng/ml. In the contrast, the Q-GRFT only lyophilized powder has an EC50 of 10.40 ng/ml. Even though this value fits in the criteria range (5–50 ng/ml), it is higher than the other two groups, suggesting a decrease in gp120 binding efficacy. Results demonstrated that the addition of maltitol during lyophilization (LMP group) can retain the bioactivity of Q-GRFT in powder form.

3.3.8 Combination Powder: Short Term Stability Study for the Lyophilized TFV Powders

Given the synergetic effects of TFV and Q-GRFT, we wanted to design a combination lyophilized powder formulation. After reconstitution, the combination powder can transform into the Q-GRFT/TFV combo enema, which was described in Chapter 3. For this purpose, we developed TFV lyophilized powder with two base formulations and monitored for their stability under accelerated conditions. For both formulations, TFV content was within the 90% to 110% range of label claims (Figure 3.8). The results showed that lyophilized TFV remained stable for 3 months under the accelerated condition, suggesting a longer shelf life. In addition, TFV was also lyophilized with the addition of maltitol and monitored for short-term stability under the same
accelerated condition (Appendix Figure 3.6). Similarly, no drug loss was observed under the accelerated condition for 3 months, demonstrating good compatibility of TFV formulations with maltitol. These results set a foundation for the Q-GRFT/TFV combined powder formulations.

![TFV Content in Sachets](image)

**Figure 3.8 TFV Lyophilized Powder Stability.**

TFV was lyophilized with two base formulations (Saline-base and PBS-base). TFV content was detected at different time points by UPLC after storing the lyophilized powder under an accelerated condition (40°C/75% RH). N=3. Dash lines indicate the lower (90%) and upper (110%) limits of the label claim.

### 3.3.9 Combination Powder: the Physically Combined Powder Strategy

Since either LMP (lyophilized powder containing Q-GRFT only) or lyophilized TFV powder (both base formulations) has stable drug content individually, we wanted to study whether the combined lyophilized powder can achieve all the CQAs after reconstitution (developed and described in Chapter 3).

LMP and lyophilized TFV powder were reconstituted with Milli-Q water individually (referred to as Individual Reconstituted Enema). Individual reconstituted enema solutions were
combined (referred to as Liquid Combined Enema) to study if there are any interactions post reconstitution. Individual lyophilized powders were also combined first before reconstitution (referred to as Powder Combined Enema) to study if any interactions are involved in the solid form.

Both Q-GRFT content (Figure 3.9) and TFV content (Figure 3.10) were determined. For both drugs, all groups achieved drug content within the 90% to 100% range. Moreover, no significant difference was found among groups, indicating no potential drug-drug incompatibility at Time 0. Because pH and osmolality were also important CQAs for enema, these two attributes were also tested for the three groups. As shown in Appendix Figure 3.7, all groups achieved a pH of around 7. In addition, Appendix Figure 3.8 shows that osmolality was not impacted by the combination, either.

![Q-GRFT Content in Sachets](image)

**Figure 3.9 Q-GRFT Content Detected in Individual or Combined Reconstituted Enema.**

No significance was detected among the groups. Dash lines indicate the 90% (lower limit) and 110% (upper limit) of the label claim. N=3.
Figure 3.10 TFV Content Detected in Individual or Combined Reconstituted Enema.
No significance was detected among the groups. Dash lines indicate the 90% (lower limit) and 110% (upper limit) of the label claim. N=3.

3.4 DISCUSSION

As solid dosage forms offer many advantages over liquid dosage forms, we aimed to develop a solid drug delivery system, which can be reconstituted to enema studied in Chapter 3. The solid dosage form can ideally inherent enema’s characterizations after reconstitution, providing potential benefits of increasing adherence as well. Therefore, it was needed to develop a stable powder form of an enema sachet.
Lyophilization has been demonstrated to be one of the most commonly used processes to prepare dehydrated proteins, including protein purification and formulation developments\textsuperscript{157}. Compared to other drying methods, lyophilization is cheaper\textsuperscript{157,192,193}, and more stable for protein drugs in some cases\textsuperscript{16,17}. However, given the susceptibility of protein drugs, several stresses during the lyophilization process may impact protein stability, including low-temperature stress (cryostress), concentration effects, dehydration effects, and pH shifts\textsuperscript{157}. Although great efforts have been made in the investigation of protein stabilization during lyophilization, the mechanisms of denaturation are complex\textsuperscript{157,196,198,199,202,205,218,221,223,224}. No single theory can elucidate all reasons for protein instability during lyophilization. Excipients were studied by the formulators to stabilize protein drugs, in response to the abovementioned stresses\textsuperscript{138,208,217,232,234,240}. Literature shows that by incorporating cryoprotectants, stable protein drugs can be incorporated into the lyophilized formulations\textsuperscript{138,192,224}. Given that the protecting effects are protein-specific\textsuperscript{138}, excipients should be screened and studied for Q-GRFT.

After studying successful cases, two non-reducing disaccharides (sucrose and trehalose) and two polyols (maltitol and lactitol) were included in the initial screening\textsuperscript{139,233-235,237,239}. In addition, the molar ratio of cryoprotectants to protein drugs plays an important role in stabilization\textsuperscript{230}. Therefore, four excipients at three different molar ratios were tested for their protecting powers during lyophilization. Q-GRFT content was detected by HPLC and compared against their relative label claims at Time 0 (Figure 3.1). The four tested cryoprotectants presented three different effects: no protection (lactitol), concentration-dependent protection (maltitol and sucrose), and concentration-independent protection (trehalose). The protecting effects observed in this study can be explained by the “water replacement hypothesis”\textsuperscript{168}. Sucrose, maltitol, and trehalose, all have eight to nine hydroxyl groups, which offer hydrogen bonds with Q-GRFT. Since
the hydro-shell was maintained, the protein’s natural conformation should be preserved\textsuperscript{192,210,211}. In this theory, sugars/sugar polyols served as water substitutes to provide hydrogen bonds, in turn, to prevent aggregation\textsuperscript{230}. To our surprise, this protecting effect was not reflected in lactitol. Lactitol, which also has nine hydroxyl groups, did not stabilize Q-GRFT during lyophilization. To further explore this phenomenon, we performed computational simulation studies using molecular docking (details shown in Chapter 4). The simulated results showed that lactitol has fewer clashes than maltitol when docking with Q-GRFT. Unlike the steric hindrance provided by maltitol, the docking between lactitol and Q-GRFT is more flexible. Hence, lactitol does not impact the formation of tetramer or further aggregates. Our simulation results aligned with a recent study, which claimed the spatial differences among the sugar alcohols contribute to the lack of protecting effects of lactitol\textsuperscript{243}. Another study also hypothesized that lactitol has fewer interactions with protein because of the intramolecular hydrogen bonds\textsuperscript{244}. In the future, more studies are needed to elucidate these hypotheses.

Maltitol was selected as the model cryoprotectant for a more in-depth exploration of the protecting effects. A more detailed molar ratio panel was investigated for maltitol, echoing the concentration-dependent protecting effects in the accelerated condition (Figure 3.2). However, when samples were stored and monitored in the real-time condition, the protecting effects were independent of concentrations (Figure 3.3). The differences between the two conditions were likely due to the different temperatures. In addition to the “water replacement hypothesis”, recent studies also suggested a “vitrification hypothesis”, where sugars/sugar polyols form a vitrified, rigid sugar-glass matrix that limits the protein degradations kinetically\textsuperscript{138,231}. Maltitol, lyophilized alone, has a glass transition temperature (Tg) of 40.6–43.1\degree C\textsuperscript{237,245}. The Tg was increased with the addition of proteins or buffers, which was also expected in our study\textsuperscript{237}. DSC (Figure 3.5) and
XRD (Figure 3.6) results showed that maltitol stays in an amorphous state in LMP. When the samples were stored in the accelerated condition (40°C/75%RH), which was slightly below the Tg of LMP, annealing or “densification” might occur231. Studies found that by heating an amorphous sample below its Tg, a glass will enter the “equilibrium glassy state” asymptotically, leading to structural relaxation and enhanced protein stability246,247. As maltitol concentration increases, these effects might expand, resulting in the concentration-dependent protections. However, densification is less likely to happen in temperatures far below Tg231, which is the real-time condition (25°C/60%RH) in this study. Thus, the concentration dependence was not observed.

Nonetheless, the LMP demonstrated good stability in drug content over three months, with no aggregation detected (Figure 3.4). More importantly, the bioactivity of gp120 binding was maintained for LMP (Figure 3.7), suggesting the potential preservation of its HIV-preventing efficacy.

DSC (Figure 3.5) and XRD (Figure 3.6) results also revealed the reason why Q-GRFT was not stable without any cryoprotectants. Without cryoprotectants, maltitol to be specific, PBS buffering agents crystallized in the lyophilized powder. DSC of the Q-GRFT only lyophilized powder showed an endothermic peak (~209°C), suggesting the crystallization of NaH2PO4 (Appendix Figure 3.3). It was also supported by XRD patterns of the Q-GRFT only lyophilized powder (Figure 3.6). The Q-GRFT-only lyophilized powder group presented some signature peaks, which were the same as the PBS-only lyophilized powder214,248, shown in Appendix Figure 3.3. These results indicated the crystallization of the NaCl, KCl, and NaH2PO4. Studies have reported that there was a pronounced reduction in the pH of PBS solution during lyophilization, especially during the cooling process153,214. To be exact, the pH shifted to around 4 because of the crystallization of sodium phosphates153. In this acidic condition, the protein drug was not stable107,
which leads to a decrease in drug content in the Q-GRFT only lyophilized powder. Therefore, we speculate that Q-GRFT was unstable due to the pH shifts caused by the crystallization of PBS.

Consequently, our study offers a third possible explanation for Q-GRFT stability in LMP. Maltitol can inhibit the crystallization of PBS, preventing the Q-GRFT denaturation. As shown in Figure 3.5 and Figure 3.6, the addition of maltitol diminished the peaks of NaH₂PO₄, NaCl, and KCl, detected by DSC or XRD. Similarly, a recent study reported the reduction of sorbitol crystallization in the presence of maltitol²⁴⁹. Although further investigations are needed, our findings suggested that maltitol can suppress the crystallization of PBS, leading to the preservation of environmental pH and Q-GRFT stability. This finding is supported by the literature where researchers found that the additions of sugar/sugar polyol preserved the pH in PBS¹⁵³.

Studies for the TFV/Q-GRFT combination lyophilized powder were also included in this work. TFV lyophilized powder formulations were initially designed and developed by our lab to achieve similar physiochemical and pharmacodynamic properties as the reconstituted enema⁶³. Both of the TFV lyophilized powder formulations (PBS-base and Saline-base) monitored in this study demonstrated good stability in the accelerated condition (Figure 3.8) and maltitol compatibility (Appendix Figure 3.6). In the preliminary study, both TFV and Q-GRFT drug contents decreased in the co-lyophilized formulation (unpublished data). It was unexpected to discover the instability of both drugs during lyophilization, suspecting more complex drug-drug interactions or drug-excipient interactions occurred. In order to demolish the denaturation pathways, we wanted to incorporate TFV powder and Q-GRFT powder in the solid state, hoping to improve the compatibility in the physical mixture. After physically mixed, the drug content of both Q-GRFT (Figure 3.9) and TFV (Figure 3.10) were maintained, with the recovery above 90% for both base formulations (PBS-base and Saline-base). After the combination lyophilized powder
was reconstituted, pH (Appendix Figure 3.7) and osmolality (Appendix Figure 3.8) also fit in the CQAs. The stability study for the physically-combined powder is ongoing. Overall, the physical mixture of the TFV/Q-GRFT combination lyophilized powder showed potential to be utilized as the combo enema, similar to the products developed in Chapter 3.

The LMP also has some limitations that can be investigated in the future. The DSC graph shows that there is an endothermic peak of maltitol in LMP. Since the melting temperature of maltitol is around 149.6°C\textsuperscript{245}, results suggested that a small portion of maltitol in the lyophilized formulation crystalized. Possible protein mobility during primary or secondary drying processes can explain the partial crystallinity of maltitol, as similar effects were reported for glucose and sorbitol\textsuperscript{239}. It was reported that the crystallization of excipients, including buffer agents and cryoprotectants\textsuperscript{198}, may lead to protein drug aggregation. Therefore, the maltitol crystals need to be further monitored and studied. More studies are needed for the combination products in the next stage, including a long-term stability study for the combo powder, bioactivity studies, \textit{in vitro} and \textit{ex vivo} permeability studies, and \textit{in vivo} pharmacokinetic and pharmacodynamic studies for the reconstituted combo enema.

3.5 CONCLUSION

This work was set to explore the possibility of developing stable Q-GRFT-incorporated lyophilized powder formulations. We have screened cryoprotectants and achieved a Q-GRFT lyophilized powder (LMP) which is stable for at least three months in real-time. The LMP also demonstrated acceptable physicochemical properties, both in powder form and in reconstituted liquid form. The gp120 binding efficacy remained after lyophilization. We further characterized
the crystallinity of LMP using DSC and XRD. The results revealed a possible cryoprotecting mechanism of maltitol, which is to prevent the crystallization of PBS in the formulations. In addition, the combination powder formulations were further explored with the lyophilized TFV powder. The combination powder demonstrated potential for future development, with drug contents, pH, and osmolality all fitting in CQAs. In summary, we successfully developed a stable Q-GRFT lyophilized powder formulation and explored the hypothesis of cryoprotection. Although more in vitro, ex vivo, and in vivo studies are needed, the lyophilized powder has the potential to be used either only or combined with other synergetic drugs. The reconstituted enema products can be developed in the future to increase adherence, and in turn, to provide better HIV protection.
4.0 Q-GRFT SOLID FORMULATION DESIGN: FILMS

The polymeric film delivery system is a thin and flexible strip of polymer that incorporates the API within\textsuperscript{250,251}. The API can be either dissolved or suspended in the polymeric film\textsuperscript{250}. As a solid pharmaceutical product, APIs which are susceptible to degradation in an aqueous environment may have a longer shelf-life in films\textsuperscript{91,251}. Compared to liquid dosage forms (e.g. Enema, Chapter 2), polymeric films are easily transported and stored\textsuperscript{252}. Compared to lyophilized powder (Chapter 3), polymeric films offer precise dosing and convenient application\textsuperscript{250}. Overall, these economic, pharmaceutical, and clinical benefits make polymeric films an emerging dosage platform for drug delivery in recent years\textsuperscript{91,160,185,253}.

Various delivery routes have been explored for polymeric films, including oral strips, buccal films, ophthalmic films, and vaginal films\textsuperscript{91,250,251,254,255}. In the context of this chapter, I will focus on the polymeric vaginal films, and designing products from a woman’s perspective\textsuperscript{256,257}.

As a drug delivery site, the vagina presents several advantages, such as a large surface area, abundant blood supply, no first-pass metabolism, and relatively high permeability to several drugs\textsuperscript{91,185}. This biological advantage allows some APIs to be quickly absorbed through the vagina into the blood and then distributed systemically\textsuperscript{185}. Thus, it has potential for both local and systemic drug delivery through the vagina\textsuperscript{91,158,185}. As a result, polymeric films have been studied to incorporate a range of compounds, including contraceptives, microbicides, or anti-HIV drugs over the years\textsuperscript{92,99,101,103,161,252,258-261}. 
4.1 INTRODUCTION

4.1.1 Advantages of Polymeric Vaginal Films

To date, literature reported several products taking the advantage of vaginal delivery to improve women’s health, including vaginal rings\textsuperscript{188,262}, tablets\textsuperscript{185}, hydrogels\textsuperscript{263,264}, and polymeric films\textsuperscript{160,187}. Among these vaginal DDS, the polymeric film is one of the most attractive and viable dosage forms for women\textsuperscript{91,186}. Compared to vaginal gels and foams, polymeric vaginal films are preferred due to their patient-friendly application and better residence time\textsuperscript{101,158,160,251}. Patients complain a lot less about the leakages of vaginal films than vaginal gels or creams\textsuperscript{91,158}. In addition, literature reported that vaginal film formulations have fewer negative impacts on the innate antiviral immunity in the vagina\textsuperscript{265}. The films can be individually wrapped in flat, sealed packages\textsuperscript{251}. The portability and discretion of films provide an advantage over traditional vaginal dosage forms\textsuperscript{158,185}. Hence, the polymeric vaginal film has good acceptability among women as demonstrated by several published studies\textsuperscript{93,186,252}. However, some aspects should be considered for the development of the polymeric vaginal film, including cultural and religious differences, personal hygiene issues, and insertion difficulties\textsuperscript{266}.

4.1.2 FDA Approved Polymeric Vaginal Films

By far, the VCF is the only FDA-approved vaginal film used for contraception. VCF is a two-inch square soft soluble film that is manually inserted into the vagina at least 15 minutes before intercourse. VCF contains N-9, an FDA-approved organic compound that can be used as a
spermicide\textsuperscript{267}. N-9 can immobilize sperm and is widely used in three vaginal contraceptive forms: suppositories, gels, and films\textsuperscript{261,267,268}.

However, as a spermicide, the N-9 VCF has a relatively higher failure rate, compared to other birth control methods. A clinical study indicated that five vaginal contraceptives containing N-9 as spermicide have typical-use failure rates ranging from 10\% to 20\%\textsuperscript{269}. The N-9 VCF has also been associated with an increased risk of STIs and damaged mucosal surfaces\textsuperscript{261,268}.

### 4.1.3 Film Manufacturing Methods

The most commonly used method to manufacture polymeric films is the solvent-casting method\textsuperscript{158}. The traditional method first dissolved or dispersed all excipients and APIs in the solvents. And then the solution/suspension was poured into a mold and cast into films under heating (or room temperature)\textsuperscript{161,254,260}. The traditional SC method was later modified to a modernized semi-automatic manufacturing process utilizing a thin-film applicator (Elcometer). Several polymeric vaginal films have been developed and manufactured using the SC method, incorporating small molecule drugs\textsuperscript{92,270-273}, peptides\textsuperscript{161}, antibodies\textsuperscript{261}, and nanoparticles\textsuperscript{123,274,275}.

Recently polymeric films have also been manufactured using another manufacturing process, namely Hot Melt Extrusion\textsuperscript{159,276-278}. HME is an efficient large-scale manufacturing process well-used in the food and pharmaceutical industry\textsuperscript{276,279}. Since it does not involve an aqueous solvent throughout the process, it is the preferred method for APIs that are hydrophobic or hydrolysis-sensitive\textsuperscript{276}. Regev et al.\textsuperscript{276} recently reported the possibility to manipulate physicochemical properties for HME films by changing HME process parameters. After modifications of barrel/zone temperature, screw speed, and feed rate, HME films can achieve parameters well within the CQAs for vaginal films\textsuperscript{276}.
4.1.4 Excipients in the Polymeric Vaginal Film Formulations

Other than APIs, film formulations usually include polymers, plasticizers, fillers, colorants, flavoring agents, and other agents\textsuperscript{99}. Excipients should be non-toxic, non-irritant, and inexpensive to manufacture. From the processing perspective, polymers should also possess good wetting (for SC), spreadability, peelability, and moderate mechanical properties\textsuperscript{251}. Among all the excipients, the type of polymer and its molecular weight dramatically influence the properties of the film\textsuperscript{91,280}. Polyacrylates, polyethylene glycol (PEG), polyvinyl alcohol, and cellulose derivatives are the polymers well-studied and commonly used in vaginal films\textsuperscript{280,281}. Plasticizers are added to decrease the intermolecular forces along polymer chains\textsuperscript{282}. By improving chain mobility, plasticizers provide film flexibility and pliability\textsuperscript{99}. Colorants and flavoring agents can be included for better patient compliance. Other excipients, such as antioxidants, can be added as needed\textsuperscript{159}. The excipients in the film formulation should be carefully selected, as they may impact not only the physiochemical properties of the film\textsuperscript{100,283} but also the toxicity\textsuperscript{267} and compatibility with the innate antiviral immunity\textsuperscript{24,265}.

4.1.5 Critical Quality Attributes for the Polymeric Vaginal Film

Technological characteristics of developed films on various esthetic, chemical, physical, mechanical, and performance parameters should be tested\textsuperscript{100,283}. One review paper\textsuperscript{91} summarized all the characteristics of vaginal films. Among these, appearance, color, and transparency are the main visual characteristics to be evaluated for films\textsuperscript{93}. In addition, a behavioral clinical study\textsuperscript{284} asked participants to compare a variety of vaginal films using a set of \textit{in mano} perceptibility survey items. Women most frequently preferred vaginal films to be thin, smooth, and translucent.
Weight, thickness, and surface morphology are physical parameters that characterize vaginal films\textsuperscript{92,276}. The size of films also mattered to some women, but the preferred size differed individually\textsuperscript{93}. As for mechanical properties, puncture strength, elongation percentage, stiffness (Young’s modulus), and fold endurance are important for films\textsuperscript{251}. The water content of films needs to be controlled, as water impacts the mechanical properties of the film\textsuperscript{211,282}. In addition, low water content affects film disintegration while high water content might contribute to microbial growth over time\textsuperscript{100,285}. More importantly, drug content, content uniformity, \textit{in vitro} drug release and film pharmacokinetics can be dependent on these film chemical and physical properties\textsuperscript{285}.

In summary, physicochemical properties, including appearance, thickness, weight, puncture strength, water content, disintegration time and drug content should be tested for controlling the product quality. CQAs should be defined prior to product development.

### 4.1.6 Challenges for Protein Drugs in the Polymeric Vaginal Film

Protein drugs exhibit additional challenges in the abovementioned manufacturing processes of vaginal films. One study\textsuperscript{161} has reported the attempt of incorporating RC-101, a synthetic analog of retrocyclin, into the polymeric vaginal film. In this study, three different PVA-base formulations were assessed with short time RC-101 stabilities. The results showed the drug contents started to decrease from Day 15 in the accelerated condition (40°C/75%RH). The authors suspected the complexing or aggregation of RC-101 might explain the phenomena where the parent RC-101 HPLC peak decreased. In addition, a recent study\textsuperscript{261} reported another attempt to incorporate an antibody in the film. It was stated that the antibody remained stable in a PVA-base formulation for 3 weeks at room temperature (25°C), but long-term stability was not explored\textsuperscript{261}. 
Other studies involving antibodies in vaginal films (namely, MB66) did not report stability data\textsuperscript{286,287}.

While these cases all utilized the SC method, attempts of using HME to incorporate proteins in solid dosage forms have also been made\textsuperscript{288}. Cossé et al.\textsuperscript{289} reported that the bovine serum albumin (BSA) content stays stable for eight weeks in a 30°C/75%RH condition. However, the secondary structure changed after 6 weeks, indicating instability\textsuperscript{289}. A similar finding was reported in another study\textsuperscript{290}, where aggregation was observed as a major problem for BSA instability after HME.

Similarly, aggregation would possibly be a dominant issue for stabilizing Q-GRFT in films. Aggregation of Q-GRFT was studied and stated in the previous chapter. The Q-GRFT susceptibility of aggregation was due to its unique structure. In one Q-GRFT monomer, there are three repeats of an antiparallel four-strand beta-sheet that superficially resembles a beta-prism-I motif\textsuperscript{162}. Studies showed that the increase of beta-sheet content is often an indication of protein aggregation and/or increased intermolecular interaction which could lead to aggregation\textsuperscript{163,164}. Thus, as a crucial step in the protein drug pre-formulation process\textsuperscript{144}, an excipient-drug compatibility study should be performed for Q-GRFT. Q-GRFT content and extent of protein aggregation should be closely monitored during formulation development.

The goal of this project was to demonstrate the capacity of film platforms to incorporate Q-GRFT for vaginal delivery. To achieve this goal, I aimed to screen excipients and formulations for stable Q-GRFT polymeric films. The films were also characterized for various physicochemical properties and assessed for potential future development.
4.2 MATERIALS AND METHODS

4.2.1 Materials

Recombinant Q-GRFT drug substance was supplied by Kentucky Bioprocessing LLC (Owensboro, KY). ACN, TFA, HCl, and NaOH were obtained from Fisher Scientific (Pittsburgh, PA). PEG 400, vitamin E acetate, triacetin, and glycerin were purchased from Spectrum (Gardena, CA). HPMC (METHOCEL® E5), PEG 4000, PEG 8000, and poly (ethylene oxide) (PEO N10; PEO N80) were obtained from Dow Chemical Company (Midland, MI). PVA 40-88, HEC, and carboxymethyl cellulose (CMC) were purchased from Millipore Sigma (Temecula, CA). A MilliQ (Millipore; Milford, MA) water filtration system operated at 18.2 MΩ cm was used for water. PBS 1× was prepared in-house with PBS 10× stock and MilliQ-water.

4.2.2 The Q-GRFT Lyophilized Powder Stress-Testing under the Processing Condition

Before the Q-GRFT polymeric films were manufactured, the Q-GRFT lyophilized powder (LMP, Chapter 3) was first challenged under stressed conditions similar to the film manufacturing condition to test the protein stability. Since the high temperature (65°C) is involved in both SC and HME processing and mechanical forces (from the screws) are involved in the HME processing, three different conditions were tested for LMP (Table 4.1).

Table 4.1 Stress Testing Conditions (Mimicking HME Processing) for LMP.

<table>
<thead>
<tr>
<th>Condition</th>
<th>High Temperature (65°C)</th>
<th>Screw Speed (100 rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Condition 2</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Samples were treated for one hour, which was longer than the normal film manufacturing time, representing the worst scenario. The LMP was then packed in individual aluminum packages and stored at 25°C/60% RH. LMP samples were taken at different time points (Time 0, Day 5, 10, 30, and 60) for Q-GRFT content detection using HPLC. At the last time point, aggregations were also measured using SEC.

4.2.3 Compatibility Study

As a critical step in the pre-formulation development, a comparability study of Q-GRFT with the excipients was warranted. Based on the previous lab experience and literature, 12 commonly used excipients were selected for the study. These excipients can be divided into five different categories, including (1) film-forming agents, (2) disintegration agents, (3) plasticizers, (4) dispersing aids, and (5) lubricants, as summarized in Table 4.2.

Table 4.2 A List of Excipients Used in the Compatibility Study.

<table>
<thead>
<tr>
<th>Excipient Name</th>
<th>Category</th>
<th>Intended Manufacturing Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPMC</td>
<td>Film Forming Agent</td>
<td>SC</td>
</tr>
<tr>
<td>HEC</td>
<td>Film Forming Agent</td>
<td>SC</td>
</tr>
<tr>
<td>CMC</td>
<td>Film Forming Agent; Disintegration Agent</td>
<td>SC</td>
</tr>
<tr>
<td>PVA 40-88</td>
<td>Film Forming Agent</td>
<td>SC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>PEO N10</strong></td>
<td>Film Forming Agent</td>
<td>HME</td>
</tr>
<tr>
<td><strong>PEO N80</strong></td>
<td>Film Forming Agent</td>
<td>HME</td>
</tr>
<tr>
<td><strong>PEG 4000</strong></td>
<td>Disintegration Agent</td>
<td>HME</td>
</tr>
<tr>
<td><strong>PEG 8000</strong></td>
<td>Disintegration Agent</td>
<td>HME</td>
</tr>
<tr>
<td><strong>Propylene glycol</strong></td>
<td>Plasticizer; Dispersing Aid</td>
<td>SC</td>
</tr>
<tr>
<td><strong>Glycerin</strong></td>
<td>Plasticizer; Dispersing Aid</td>
<td>SC</td>
</tr>
<tr>
<td><strong>PEG 400</strong></td>
<td>Lubricant; Plasticizer</td>
<td>HME</td>
</tr>
<tr>
<td><strong>Triacetin</strong></td>
<td>Lubricant; Plasticizer</td>
<td>HME</td>
</tr>
</tbody>
</table>

To mimic the manufacturing processes, excipients were first mixed with Q-GRFT at the same ratios as used in the formulations. Then, for excipients used in the SC films, mixtures were challenged on the thin film applicator at 65°C. For excipients from the HME film formulations, mixtures were also treated under twin screws at a speed of 100 rpm, in addition to high temperature (65°C). Samples were treated for 30 minutes for both processing methods and later packed in individual aluminum packages. Q-GRFT content was determined by HPLC after storing samples at 25°C/60% RH for 10 days.

### 4.2.4 The Q-GRFT SC Film Formulations and the Manufacturing Procedure

Three SC formulations (Table 4.3, Table 4.4, and Table 4.5) were manufactured and compared in a short-term stability study.
### Table 4.3 Formulation-1 (F-1) for the Q-GRFT SC Polymeric Films.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Role</th>
<th>Ratio (w/w %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEC (Natrosol 250L)</td>
<td>Film Forming</td>
<td>5.99</td>
</tr>
<tr>
<td>HPMC (METHOCELTM E5)</td>
<td>Film Forming</td>
<td>5.99</td>
</tr>
<tr>
<td>CMC</td>
<td>Disintegration Agent</td>
<td>1.99</td>
</tr>
<tr>
<td>Glycerin</td>
<td>Plasticizer/Dispersing Aid</td>
<td>1.99</td>
</tr>
<tr>
<td>Q-GRFT</td>
<td>API</td>
<td>0.22</td>
</tr>
<tr>
<td>Milli-Q Water</td>
<td>Solvent</td>
<td>83.82</td>
</tr>
</tbody>
</table>

### Table 4.4 Formulation-2 (F-2) for the Q-GRFT SC Polymeric Films.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Role</th>
<th>Ratio (w/w %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA 40-88</td>
<td>Film Forming</td>
<td>6.97</td>
</tr>
<tr>
<td>HPMC (METHOCELTM E5)</td>
<td>Film Forming</td>
<td>1.74</td>
</tr>
<tr>
<td>PEG 8000</td>
<td>Disintegration Agent</td>
<td>2.32</td>
</tr>
<tr>
<td>Glycerin</td>
<td>Plasticizer/Dispersing Aid</td>
<td>0.72</td>
</tr>
<tr>
<td>Propylene Glycol</td>
<td>Plasticizer/Dispersing Aid</td>
<td>0.72</td>
</tr>
<tr>
<td>Q-GRFT</td>
<td>API</td>
<td>0.89</td>
</tr>
<tr>
<td>Milli-Q Water</td>
<td>Solvent</td>
<td>86.64</td>
</tr>
</tbody>
</table>
Table 4.5 Formulation-3 (F-3) for the Q-GRFT SC Polymeric Films.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Role</th>
<th>Ratio (w/w %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA 40-88</td>
<td>Film Forming</td>
<td>3.57</td>
</tr>
<tr>
<td>HPMC ( METHOCELTM K4M )</td>
<td>Film Forming</td>
<td>1.49</td>
</tr>
<tr>
<td>PEG 8000</td>
<td>Disintegration Agent</td>
<td>2.38</td>
</tr>
<tr>
<td>Glycerin</td>
<td>Plasticizer/Dispersing Aid</td>
<td>0.74</td>
</tr>
<tr>
<td>Propylene Glycol</td>
<td>Plasticizer/Dispersing Aid</td>
<td>0.74</td>
</tr>
<tr>
<td>Q-GRFT</td>
<td>API</td>
<td>0.89</td>
</tr>
<tr>
<td>Milli-Q Water</td>
<td>Solvent</td>
<td>90.19</td>
</tr>
</tbody>
</table>

All three formulations utilized a similar SC procedure reported previously\textsuperscript{270}. (1) To make film solution: In short, for F-1, HEC, HPMC, and CMC were added to the Milli-Q water in sequence and stirred at 200 rpm with an overhead mixer. After the film solution/suspension was uniform, glycerin and Q-GRFT were added and mixed until homogenous. For F-2 and F-3, briefly, PVA was first mixed in Milli-Q water and dissolved after a water bath (90°C for 10 minutes). And then HPMC and PEG 8000 were added sequentially until homogenous with an overhead mixer. The film solution was stirred at the lowest speed possible (100 or 120 rpm) overnight to diminish air entrapment. Plasticizers (glycerin and propylene glycol mixture) and Q-GRFT were added at last and stirred until uniform. (2) The film solution was cast on a polyester substrate by an automatic thin-film applicator (Elcometer 4340, Manchester, UK) using an 11-inch doctor blade. The thickness was controlled to 110 µm. The casted film solution was allowed to dry for 15
minutes at 65°C before the sheet was removed from the substrate. (3) Once film sheets were peeled, individual films were cut using a die press into 1 inch by 1 inch (1”×1”) unit doses. Individual films were then packed into separate aluminum packages for storage or further characterizations.

A weight-to-weight ratio (individual excipient to Q-GRFT) comparison of all excipients used in these three formulations is summarized in Table 4.6.

Table 4.6 A Comparison of Excipient Amounts in Three Q-GRFT SC Polymeric Film Formulations.

Ratios shown in the table are excipient weight to Q-GRFT weight ratio.

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Formulation-1 (F-1)</th>
<th>Formulation-2 (F-2)</th>
<th>Formulation-3 (F-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPMC</td>
<td>27:1</td>
<td>12:1</td>
<td>5:1</td>
</tr>
<tr>
<td>PVA</td>
<td>N/A</td>
<td>46:1</td>
<td>12:1</td>
</tr>
<tr>
<td>HEC</td>
<td>27:1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CMC</td>
<td>9:1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PEG 8000</td>
<td>N/A</td>
<td>16:1</td>
<td>8:1</td>
</tr>
<tr>
<td>Plasticizers</td>
<td>9:1</td>
<td>5:1</td>
<td>2.5:1</td>
</tr>
</tbody>
</table>

4.2.5 The Q-GRFT HME Film Formulations and the Manufacturing Procedure

Q-GRFT-incorporated polymeric films can also be manufactured using the HME method. The formulations are shown in Table 4.7 and Table 4.8.
Table 4.7 Formulation-4 (F-4) for the Q-GRFT HME Polymeric Films.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Role</th>
<th>Ratio (w/w %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO N10</td>
<td>Film Forming</td>
<td>51.19</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>Disintegration Agent</td>
<td>34.13</td>
</tr>
<tr>
<td>PEG 400</td>
<td>Lubricant/ Plasticizer</td>
<td>8.53</td>
</tr>
<tr>
<td>Vitamin E Acetate</td>
<td>Anti-oxidant</td>
<td>3.41</td>
</tr>
<tr>
<td>Q-GRFT LMP</td>
<td>API</td>
<td>2.75</td>
</tr>
</tbody>
</table>

Table 4.8 Formulation-5 (F-5) for the Q-GRFT HME Polymeric Films.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Role</th>
<th>Ratio (w/w %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO N10</td>
<td>Film Forming</td>
<td>51.19</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>Disintegration Agent</td>
<td>34.13</td>
</tr>
<tr>
<td>Triacetin</td>
<td>Lubricant/ Plasticizer</td>
<td>8.53</td>
</tr>
<tr>
<td>Vitamin E Acetate</td>
<td>Anti-oxidant</td>
<td>3.41</td>
</tr>
<tr>
<td>Q-GRFT LMP</td>
<td>API</td>
<td>2.75</td>
</tr>
</tbody>
</table>

All films were manufactured by a twin-screw HAAKE MiniLab 3 Micro Compounder (Thermo Fisher Scientific, Waltham, MA). A fast-dissolving film formulation was used as the base formulation. Briefly, the extruder was set to 100 rpm and the temperature was set to 65°C. Either
PEG 400 or triacetin (lubricant/plasticizer) was mixed with vitamin E acetate (anti-oxidant) and added to the appliance first. Then, PEG 4000 was mixed with one-third (1/3) of the PEO N10 and added to the appliance for 10 minutes or until homogenous. Lastly, Q-GRFT LMP and the remaining PEO N10 were mixed and added to the appliance at 40 rpm. The mixture was then extruded. The extruded products were then pressed to individual filaments by a thin film presser. The filaments were finally cut into 0.5”×1” units and packed in pouches for further characterization.

4.2.6 Drug Content Determination by High-Performance Liquid Chromatography

The HPLC method for measuring the Q-GRFT content was developed in our lab and reported elsewhere107. The stability study samples were dissolved and further diluted as needed with Milli-Q water. The Q-GRFT content was detected by an HPLC system (Waters Corporation, Milford, MA) equipped with an auto-injector (model 717), a quaternary pump (model 600), and a photodiode array detector (model 2996) with a C5 column (Jupiter 5µm 300Å, 250mm×4.6mm) and a C5 pre-column (Security Guard Standard Widepore).

4.2.7 Aggregation Determination by Size Exclusion Chromatography

An SEC column (TSKgel SuperSW3000 Size Exclusion HPLC column, Tosoh Bioscience) was used for the detection of aggregations. The samples were first dissolved in Milli-Q water and then directly injected into the system. The mobile phase is PBS solution at a constant rate (0.3 ml/min). A protein molecular weight marker (Calbiochem, EMD Millipore) was used for calibration.
4.2.8 Physicochemical Characterizations of Polymeric Films

4.2.8.1 Appearance

The appearance of the polymeric films was monitored by visual observation. The formulations should be white or off-white. The surface and edges of the films were preferably to be smooth. Any deviation should be recorded.

4.2.8.2 Weight

Individual film weight was measured using an analytical balance (Mettler Toledo XS105, Columbus, OH). An average value was calculated and recorded for each batch.

4.2.8.3 Thickness

Individual film thickness was measured using a thickness gauge (Mitutoyo Corporation, Kanagawa, Japan). Measurements were taken at five points (four corners and the middle point). An average thickness was calculated and recorded.

4.2.8.4 Water Content

Water content was measured using a Karl-Fisher apparatus (Metrohm, 758KFD Titrino, Herisau, Switzerland) following the titration method specified by the manufacturer. Films were weighed and sealed in a glass bottle. The oven temperature was set up at 120°C for extracting the residual water in the film. Data were acquired and analyzed by Tiamo 2.2 software.
4.2.8.5 Puncture Strength

Puncture strength (PS) was characterized with a texture analyzer (TA.XT.Plus, Hamilton, MA). In short, the film was fixed in the TA-108S5 fixture with five 15 mm openings, and the maximum force required to puncture the film using a rounded end ball probe (8A, 1/8”) was recorded by the Exponent software.

4.2.9 Bioactivity Determination by ELISA

An ELISA method was applied to evaluate the gp120 binding activity of selected Q-GRFT samples. Nunc MaxiSorp 96-well plates were used for this experiment. The MaxiSorp surface is a hydrophilic/hydrophobic mix that binds to a wide range of biomolecules. In short, gp120 was bound to the wells of a 96-well plate overnight at 4°C. The HIV-1 gp120CM was obtained from Kentucky Bioprocessing (KBP; Part #C-1312). After overnight incubation, the solution of gp120 was removed, and a blocking solution (1× PBS-T [PBS with 0.05% Tween 20]) was applied for two hours at room temperature. After that, the wells were washed and incubated with various dilutions of Q-GRFT samples for one hour. Gp120 binding was detected by sequentially applying goat anti-QGRFT primary antibody (one-hour incubation) and HRP-labeled rabbit anti-goat secondary antibody (one-hour incubation). TMB substrate was applied to the wells after washing the secondary antibody. Wells were allowed to develop (blue color) for approximately three minutes before the application of sulfuric acid to stop the reaction (yellow color). The plates were measured at 450nm using a plate reader for the gp120 binding activity.
4.2.10 Computational Study: Homology Modeling of Q-GRFT

The X-ray structure of GRFT was used in this work to build a homology model of Q-GRFT. The crystal structure of unliganded GRFT\textsuperscript{118} (Protein Data Bank [PDB] entry: 2GTY; resolution, 1.30Å; method, X-ray diffraction) was downloaded from the protein data bank and used as the template. The Prime package in Maestro 11.2 was utilized to build the homology model. In detail, from the GRFT monomer template, the methionine on position 78 (M78) was changed to glutamine (Q78). Both GRFT monomers were modified to achieve the Q-GRFT homodimer structure. The Q-GRFT was then prepared by the ProteinPrep in the Maestro software for the following studies.

4.2.11 Computational Study: Selections of the Aggregation Prone Regions

The selection of the aggregation-prone regions (APRs) was performed using three databases, AggreScan\textsuperscript{291,292}, CamSol\textsuperscript{293,294}, and NetCSSP\textsuperscript{295}. The Q-GRFT homology model was used in these online servers to predict the APRs. These predictions are based on an aggregation-propensity scale for natural amino acids derived from \textit{in vivo} experiments and on the assumption that short and specific sequence stretches modulate protein aggregation.

Specifically, AggreScan calculates aggregation-propensity values per amino acid (aaAV, or a3v) derived previously from experimental data. The “hot spot” threshold (HST) has been defined as the average of the a3v of the 20 amino acids weighted by their frequencies in the SwissProt database. A region in the polypeptide sequence is considered an aggregation “hot spot” (HS) if there are 5 or more sequentially continuous residues with an a4v (a3V average) larger than the HST. Similarly, CamSol calculates the intrinsic residue solubility of each amino acid within
the protein sequence based on the experimental data. And the amino acids with the solubility values below -1.0 were identified as the aggregation-promoting regions. NetCSSP utilized dual artificial neural networks (ANNs) to predict secondary structure changes with high accuracy (83%). The regions with a propensity score >6 were highlighted as the predicted regions for structural changes.

4.2.12 Computational Study: Molecular Docking between Q-GRFT and Selected Excipients

Molecular docking studies were performed using the embedded docking package in Maestro 11.2. Based on the APRs identified and the tertiary structure of Q-GRFT, six docking grids were generated. Based on the name and location of the center amino acids, the six grids are 24R (arginine), 51T (threonine), 54S (serine), 97N (asparagine), 99K (lysine), and 121Y (tyrosine). Grids were explored with the size of both 20Å and 30Å. For molecular docking, the hydrogen atoms of the protein were allowed to move. The number of additional starting conformations per molecule was set to 20. The maximum number of poses per ligand was set to 200. The docking scores were calculated by the default package in the Maestro software. And the scores from all six grids (size: 30Å) were recorded and compared for all excipients tested.

4.2.13 Statistical Analysis

All values are reported as means ± standard deviation. Statistical data analyses were performed using one-way ANOVA with Tukey’s post hoc test, with p<0.05 as the minimal level of significance, p<0.01 for very significant, and p<0.001 for highly significant. All tests were performed using the GraphPad Prism software version 9.
4.3 RESULTS

4.3.1 Stress Testings for the Q-GRFT Lyophilized Powder

As summarized in Table 4.1, three conditions were utilized to challenge the Q-GRFT lyophilized powder, mimicking the film manufacturing processes. Q-GRFT content was measured in the post-challenged samples at every time point. The drug content was reported as the Q-GRFT weight/LMP weight ratio, as shown in Figure 4.1.

![Figure 4.1 The Stability Study of Q-GRFT Content after the Stress Challenges.](image)

Q-GRFT content was measured by HPLC and reported as the Q-GRFT weight/LMP weight ratio (%).

Condition 1: High Temperature (HT)+, Screw Speed (SS)+; Condition 2: HT+, SS--; Condition 3: HT-, SS-. A two-way analysis was performed. For Time 0, N=6; for other time points, N=4. N.S.: no significance.
At Time 0, no difference was observed in the Q-GRFT content across three groups, suggesting no immediate loss during the heating or the mixing process. After storage over time, no significant difference was found, indicating good stability of LMP. Q-GRFT content stayed at a similar level after two months. In addition, no aggregation was detected for all three conditions compared to the reference group (Figure 4.2), demonstrating the protective effects of maltitol remained during either high-temperature shock or mechanical forces.

Figure 4.2 The Stability Study of Q-GRFT Content after the Stress Challenges on Day 60.
SEC chromatography was compared for all three condition groups and the Q-GRFT reference. Condition 1: High Temperature (HT)+, Screw Speed (SS)+; Condition 2: HT+, SS−; Condition 3: HT−, SS−. Samples were tested in duplicate, and one representative sample was shown for each group. No aggregation was detected.
4.3.2 Compatibility Study of Q-GRFT with CommonExcipients in Film Formulations

Q-GRFT was mixed with individual excipients and treated under the manufacturing process. After storage at 25°C/60% RH for 10 days, Q-GRFT content was measured using HPLC, as shown in Figure 4.3.

![Excipient Compatibility Study](image)

**Figure 4.3** The Compatibility Study of Q-GRFT with Film Excipients.

Q-GRFT solution was used as the reference. Other groups were stored under 25°C/60% RH and tested for Q-GRFT content on Day 10 by HPLC. A two-way ANOVA analysis was performed. N=4. Ns, no significance; ***, p<0.001.

Compared to the reference group (Q-GRFT solution), Q-GRFT content remained at the same level for the Q-GRFT LMP group, validating the stability of LMP. However, when the Q-GRFT content on Day 10 was compared to Time 0, significant decreases were found in the groups of LMP with either HPMC, HEC, CMC, PVA, or PEG 400. Encouragingly, no decrease was found
for PEG 4000, PEG 8000, PEO N10, PEO N80, glycerin, triacetin, and propylene glycol, suggesting good compatibilities for these excipients.

4.3.3 Homology Modeling for Q-GRFT and Aggregation Prone Region Identifications

A computational study was performed to further investigate the protein-excipient interactions. As preparation, the Q-GRFT structure needed to build with homology modeling. Compared to the GRFT monomer, only one amino acid replacement was needed to obtain the structure of Q-GRFT, suggesting a high accuracy for the modeling. The methionine at the position of 78 was changed to glutamine (in both monomers) to achieve the homodimer homology model of Q-GRFT. This model was later used in the three online servers (AggreScan, CamSol, and NetCSSP) to screen for the aggregation-prone regions (APRs). The predictions generated from AggreScan and CamSol were shown in Figure 4.4.a. and Figure 4.4.b. respectively. Results from NetCSSP can be assessed through the link as interactive webpages ([http://compbio.sookmyung.ac.kr/~cssp/data/108280.html](http://compbio.sookmyung.ac.kr/~cssp/data/108280.html)).

Protein-protein interactions were also investigated using the Z-dock online server ([https://zdock.umassmed.edu](https://zdock.umassmed.edu)). Q-GRFT homology structure was used as both the proteins in the system to perform the protein-protein docking. This docking mimicked the nucleation of aggregates. And the binding regions were identified as APRs. Results from multiple Z-dock simulations showed that Q-GRFT would bind with another Q-GRFT in similar regions. These regions have high similarities despite whether monomers or homodimers were used in the study. Additionally, these regions are also covered in the APRs that were identified above.
Figure 4.4 Aggregation Prone Region Predictions.

Figures were generated by (a) AggreScan and (b) CamSol using the Q-GRFT homology model. (a) The average aggregation-propensity values per amino acid (Ave. aaAV, or a4v) was calculated by AggreScan (y-axis) and plotted against the protein sequence (x-axis). The regions where the a4v values were higher than the pre-determined threshold were identified as the Hot Spot Area (HSA), shown in the figure as peaks. (b) The predicted solubility for amino acids was calculated by CamSol (y-axis) and plotted against the protein sequence (x-axis). The regions where the predicted solubility values were below -1.0 were identified as the Aggregation Promoting Regions, highlighted in red.
Based on the findings from the three APR predicting tools, regions that could potentially cause aggregations were summarized in Figure 4.5. The summarized results from the predictions showed common regions were successfully recognized by these methods, despite their different predicting mechanisms. Combining the findings and the tertiary structure of Q-GRFT, the final selection of the APRs was shown in Figure 4.5. The three-dimensional (3D) Q-GRFT structures were shown in Figure 4.6 from two perspectives, with the selected APRs highlighted in colors (green, orange, blue, purple, and magenta).
Figure 4.5 A Summary of the Predicted and Selected Aggregation Prone Regions of Q-GRFT.

The position of individual amino acids was shown at the top. Q-GRFT monomer sequences were shown in black. APRs identified by AggreScan, CamSol, or NetCSSP were summarized and highlighted in red respectively. The final selection of APRs was highlighted in green, orange, blue, purple, and magenta as different regions.
Figure 4.6 The Q-GRFT Homodimer Structure by Homology Modelling Shown in Two Perspectives.

The Grey (with multiple colors) chain is Chain A, and the Yellow chain is Chain B. Chain A and B are identical. Five APRs are identified with different colors (green, orange, blue, purple, and magenta).
4.3.4 Molecular Docking Study for Protein-Excipient Interactions

The homology model of Q-GRFT was used for evaluating protein-excipient interactions. Based on the APRs identified and the tertiary structure, six grids were generated for the molecular docking. Based on the name and location of the center amino acids, the six grids are 24R (arginine), 51T (threonine), 54S (serine), 97N (asparaginate), 99K (lysine), and 121Y (tyrosine). The selected excipients were performed in all six grids to explore their binding affinity of them with Q-GRFT. Docking scores calculated by the Maestro 11.2 and the lowest scores (representing the strongest binding affinities) were summarized in Table 4.9.

Table 4.9 A Summary Table for the Docking Scores.

The six molecular docking grids were generated to cover the aggregation-prone regions. Individual grids were named after the center amino acid respectively.

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Aggregation Prone Region (APR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer</td>
<td>24R</td>
</tr>
<tr>
<td>HEC</td>
<td>-6.069</td>
</tr>
<tr>
<td>CMC</td>
<td>-3.353</td>
</tr>
<tr>
<td>Plasticizer</td>
<td>PEG400</td>
</tr>
<tr>
<td>Glycerin</td>
<td>-1.799</td>
</tr>
</tbody>
</table>

Despite different docking grids, HEC and HPMC had the lowest docking scores of all the excipients. Both HEC and HPMC achieve scores below -4.0 for all grids, indicating strong binding
affinities between HEC/HPMC and Q-GRFT. Among the four polymers performed in this study, PVA had the lowest interactions with Q-GRFT, represented by the highest scores in 5 out of 6 grids tested. In general, polymers had stronger binding affinities than plasticizers, shown by lower average docking scores. Surprisingly, PEG 400 had an average docking score below -4.0. Except for one grid (24R), PEG 400 showed a strong binding affinity with Q-GRFT in all the other five regions. The strong interactions between excipients (HEC, HPMC, CMC, and PEG 400) and Q-GRFT might be an indicator of the incompatibility.

4.3.5 Formulation Selection for the Q-GRFT SC Films

Based on the aforementioned experimental and computational studies, we hypothesized the Q-GRFT stability would be different in various formulation compositions. Therefore, in this proof-of-concept study, three formulations (Table 4.3, Table 4.4, and Table 4.5) were applied to incorporate Q-GRFT. After storage at 25°C/60% RH, films were sampled and tested for Q-GRFT content on Day 90, as shown in Figure 4.7.

Figure 4.7 Three Solvent-Cast (SC) formulations were tested and compared in the short-term stability study. Q-GRFT contents in film (a) Formulation-1 (F-1), (b) Formulation-2 (F-2), and (c) Formulation-3 (F-3) on Day 90 were compared with Time 0. T-tests were applied for all three comparisons. For Time 0, N=6; for Day 90, N=3. NS, No Significance; *, p<0.05; ****, p<0.0001.
The drug content in F-1 (Figure 4.7.a) and F-2 (Figure 4.7.b) both significantly decreased after 90 days, suggesting strong impacts of the formulation. Q-GRFT stability in F-1 was the worst among the three formulations, with only 55% recovery compared to Time 0. Encouragingly, Q-GRFT remained stable in F-3 for up to 3 months, with a 99% recovery (Figure 4.7.c). SEC was also utilized to detect any aggregation in the films. Results showed aggregations in F-1 and F-2 but none in F-3 (Appendix Figure 4.3). The SEC chromatograms supported the HPLC data, suggesting the superiority of F-3 among the three SC formulations, based on the lack of potential interactions between Q-GRFT and the excipients.

4.3.6 Formulation Selection for the Q-GRFT HME Films

Previously we have developed one HME formulation (F-4, Table 4.7). Even though most of the excipients in this formulation had good compatibility with Q-GRFT, one excipient (PEG 400) showed negative impacts on Q-GRFT stability (Section 4.3.2). Thus, we modified the formulation by substituting PEG 400 with triacetin (Table 4.8), to seek better protection from the formulation. Drug contents were measured by HPLC on Day 90, after storing at 25°C/60% RH (Figure 4.8).
Figure 4.8 Two Hot Melt Extrusion (HME) formulations were tested and compared in a short-term stability study.

Q-GRFT contents in film (a) Formulation-4 (F-4), and (b) Formulation-5 (F-5) on Day 90 were compared with Time 0. T-tests were applied for both comparisons. For Time 0, N=6; for Day 90, N=3. NS, No Significance; ****, p<0.0001.

Q-GRFT content decreased significantly in F-4 on Day 90, with only 55% recovery compared to Time 0. In the contrast, the modified formulation (F-5) demonstrated Q-GRFT stability with 102% recovery on Day 90. There was no significant difference between the drug contents of the two-time points. In addition, SEC results confirmed that there was no aggregation in F-5 (Appendix Figure 4.4).

4.3.7 Physicochemical Characterizations of Q-GRFT Polymeric Films

One formulation was selected from either the SC or the HME manufacturing method. Physiochemical properties, including appearance, weight, thickness, water content, and puncture strength were measured and compared (Table 4.10 and Figure 4.9).
Table 4.10 Physicochemical Characterizations of Q-GRFT-incorporated Polymeric Films.

Data reported as average value ± standard deviations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Manufacturing Methods</th>
<th>Appearance</th>
<th>Weight (mg)</th>
<th>Thickness (mm)</th>
<th>Water Content (%)</th>
<th>Puncture Strength (kg/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-3</td>
<td>SC</td>
<td>Opaque, White, Bumpy, Soft</td>
<td>17.57 ± 4.06</td>
<td>0.06 ± 0.01</td>
<td>6.05 ± 1.79</td>
<td>2.86 ± 0.67</td>
</tr>
<tr>
<td>F-5</td>
<td>HME</td>
<td>Translucent, Off-white, Smooth, Soft</td>
<td>32.58 ± 4.23</td>
<td>0.09 ± 0.02</td>
<td>0.64 ± 0.17</td>
<td>1.82 ± 0.66</td>
</tr>
</tbody>
</table>

Figure 4.9 Representative images of (a) Q-GRFT SC Film (F-3), and (b) Q-GRFT HME Film (F-5).

Images were taken on a black-and-white marble rock surface to test the transparency of films.

On average, HME films are heavier and thicker than SC films. However, SC films have higher water contents and higher puncture strength than HME films. Both films are soft and flexible, demonstrating good mechanical properties. But the SC films (Figure 4.9.a) have a bumpy and slightly rough surface due to air-trapping in the formulation solution. Except for a few spots, HME films are translucent. Overall, both films have CQAs that are comparable with other vaginal films.
4.3.8 Stability Studies for the Selected Q-GRFT HME Films

The selected Q-GRFT HME films (F-5) were stored under two conditions to explore the drug stability, shown in Figure 4.10.

![Graphs showing drug content and label claim for Q-GRFT HME films under different conditions.](image)

**Figure 4.10 Exploratory studies for Q-GRFT stability in the HME films (F-5).**

Films were stored in (a) the accelerated condition [40°C/75%RH] for 3 months, and (b) the real-life condition [25°C/60%RH] for 24 months. Drug contents were determined by HPLC. Both Q-GRFT weight/Film weight ratios (left y-axis) and %Label Claim (right y-axis) were reported. For Time 0, N=6; For other time points, N=3.

In the 40°C/75% RH condition, films retained only 72.38% of drug content after 3 months compared to Time 0. Surprisingly, films stored in the 25°C/60% RH still achieve 82.86% after 24 months.

4.4 DISCUSSION

The vaginal polymeric film is one of the preferred dosage forms by women\(^{91,186}\), compared to other traditional vaginal delivery systems. It offers discreet and on-demand use, ease of self-administration, low cost, and non-leakiness\(^{158,185,251}\). However, protein drugs, which are
susceptible to high temperature or mechanical forces, are challenging to be incorporated into polymeric vaginal films. Previous chapters and literature demonstrated the instability of Q-GRFT can be prevented or reversed with selected excipients in the formulations. Therefore, the primary aim of this chapter was to screen excipients and formulations to achieve stable Q-GRFT polymeric vaginal film products. In addition, we performed the physiochemical characterizations of the lead formulations and compared them with the internal CQAs.

We first tested out the stability of LMP (Chapter 3) under two manufacturing stresses, namely high temperature (65°C) and mechanical forces. Results (Figure 4.1) demonstrated good stability of LMP after stress challenges. No significant drug loss nor aggregations (Figure 4.2) were found for both stressed groups. The data validates the good protecting effects of maltitol observed in the previous chapter. Also, the findings are supported by recent studies, where the authors stated protecting effects of trehalose after HME. Trehalose and maltitol were both reported to increase protein drug stability. Therefore, it is not surprising to observe the protective effects of Q-GRFT, especially this phenomenon was also demonstrated in Chapter 3.

A recent review introduced the concept of physical aging of an amorphous sample by thermal treatment, also called annealing or “densification”. By heating an amorphous sample below its Tg (glass transition temperature), the sample will enter the “equilibrium glassy state” asymptotically, leading to structural relaxation and enhanced protein stability. Maltitol in LMP was proven to be in the amorphous state. Given that the Tg of maltitol would increase to 56°C or higher with the addition of protein drugs, a short-time challenge at 65°C would possibly densify the LMP and cause Q-GRFT relaxation. More studies in the future are needed to validate this hypothesis.
We further combined Q-GRFT (LMP) with some commonly-used excipients and performed an excipient screening study. These mixture groups were stressed under high temperatures (65°C, for SC and HME) and mechanical forces (HME) to mimic the real manufacturing challenges. As shown in Figure 4.3, HPMC, HEC, CMC, PVA, and PEG 400 showed significant drug loss after 10 days, indicating strong incompatibilities. We hypothesized two possible explanations, including charges and hydroxyl groups of these excipients.

Among these excipients, CMC was reported to be negatively charged in an aqueous solution\textsuperscript{296}. HPMC solution also has a negative charge with a zeta potential range of -2.14 to -3.40 mV\textsuperscript{297}. PVA is slightly negatively charged, as a result of incomplete hydrolysis. In theory, PVA is classified as a nonionic polymer\textsuperscript{298,299}. However, the PVA used in this study (PVA 40-88) was only 88\% hydrolyzed\textsuperscript{300}. Due to the existence of the acetate groups, there is a migration of partial negative charge from the -CH\textsubscript{2}- groups (located at α positions relative to the acetate groups) to the carbonyl oxygen of the acetate group\textsuperscript{299}. Therefore, PVA macromolecules have negative charges.

Given that Q-GRFT was tested to be positively charged (≈+40 mV) in the buffer (unpublished data), there might be excipient-protein interactions in the formulation solution. It was reported that the electrostatic interactions of excipient-protein may negatively impact protein stability\textsuperscript{143}. Studies\textsuperscript{301-303} intended to follow the Hofmeister series\textsuperscript{138} stated that ion binding can modulate protein aggregation due to temperature and agitation stresses by neutralizing protein charges. Therefore, negatively charged excipients, such as CMC, HPMC, and PVA may cause protein aggregation by impacting Q-GRFT’s surface charges. In the contrast, PEO, which did not impact Q-GRFT stability, was reported to be positively charged\textsuperscript{304}. However, the electrostatic interactions cannot explain all scenarios, as certain polyanions were reported to thermally stabilize proteins\textsuperscript{143}. Moreover, a recent study by Zalar et al.\textsuperscript{305} argued that the binding of excipients might
be a poor indicator for protein aggregation kinetics. Thus, this hypothesis needs to be further validated.

Another hypothesis was that the hydroxyl groups on the excipient (HEC, HPMC, CMC, and PVA) side chains may interact with Q-GRFT. Some studies stated that polymers can stabilize protein drugs by providing hydrogen bonds, while others warrant that this effect is protein-specific. In the liquid state, the hydro-shell is important to preserve a protein’s natural conformation. Since the hydroxyl groups can form hydrogen bonds with the protein, polymers can serve as water substitutes to prevent aggregation. However, the film-forming process transits from the liquid state to the (semi-) solid state. As polymers crosslink to a physical network, new intermolecular associations of linear or branched polymers may have formed. Thus, the abovementioned hydrogen bonds (with Q-GRFT) were impacted, leading to possible conformational changes and aggregations.

Molecular docking has been extensively used in drug discovery both for the design of new drugs to specific binding sites, or to repurpose a small molecule by finding other binding sites. Most recently, molecular docking has also been applied to protein-excipient screening by comparing the hotspots from both protein-protein interactions and protein-excipient interactions. By implementing molecular docking, we could have a better understanding of the interactions between proteins and excipients. Correlating the virtual predictions with the experimental data, the computer-aid approach can support the screening results. Since the crystal structure of Q-GRFT was not reported, homology modeling was performed. For GRFT, structural information can be obtained in a public dataset, the protein data bank (https://www.rcsb.org/). The crystal structure of unliganded GRFT (PDB entry: 2GTY) with great resolution (1.3Å) was downloaded. This homology model was predicted to have a high accuracy as Q-GRFT was only
one amino acid different from the template (GRFT). In one monomer, methionine at the position of 78 was replaced with glutamine to achieve the Q-GRFT structure.

The molecular docking study was then performed to investigate the protein-excipient interactions. The docking scores calculated represented the predicted binding affinity between excipient and protein, where lower scores meant stronger interactions. Judging from the average scores from all six docking grids in the study, HEC showed the strongest binding affinity with Q-GRFT, followed by HPMC and CMC. Although the average docking score of PVA was not lower than -4.0 (an empirical threshold of strong binding affinity), it did show middle to strong levels of interactions in some locations (54S, 97N, 99K, and 121Y). As for the plasticizers, though most of them (3 out of 4) showed a weak binding affinity with Q-GRFT, PEG 400 had an average docking score of -4.021, with stronger interactions shown in 54S and 99K. Although future studies are needed, the docking study showed a correlation between the docking scores and the incompatibility ranks (Figure 4.3).

Nonetheless, we successfully screened excipients that are compatible with Q-GRFT in this study. These excipients were incorporated into proof-of-concept formulation studies. Our lab has already developed an SC polymeric vaginal film platform. In a study, the PVA-base formulation was used for vaginal delivery of DPV, a potent anti-retroviral molecule. This quick-dissolving film provides rapid drug release, lacks toxicity to the innate microflora, maintains stability, and retains the bioactivity of APIs. In addition, Lai et al. recently reported a successful case where they incorporated an IgG antibody in a PVA-base vaginal film formulation. The films were stored at room temperature (and -70°C) and remained stable for 3 weeks. Therefore, we manufactured the Q-GRFT SC films in three formulations and monitored them for drug content (Figure 4.7). F-1, which contains HEC, CMC, and HPMC (Table 4.3), demonstrated
the worst Q-GRFT stability, with aggregation detected in the film as well (Appendix Figure 4.1). F-2 (Table 4.4) and F-3 (Table 4.5) have similar compositions, but different excipient-protein ratios (Table 4.6). With higher PVA content in the formulation, F-2 showed less Q-GRFT content on Day90. Aggregations were also detected in the F-2 films (Appendix Figure 4.2). Despite small amounts of PVA existing in F-3, Q-GRFT content was not decreased for 90 days. There was no aggregation either, suggesting good stability of Q-GRFT in F-3 (Appendix Figure 4.3).

Similar results were also obtained for the HME films. The only difference between F-4 (Table 4.7) and F-5 (Table 4.8) was the plasticizer (PEG 400 v.s. triacetin). As PEG 400 showed negative impacts on Q-GRFT (Figure 4.3), we observed a consequential result in Figure 4.8. The Q-GRFT content was significantly decreased in the F-4 films, while remaining at the same level in the F-5 films, echoing the excipient study. Moreover, SEC results supported the stability of Q-GRFT in F-5, with no aggregation peak detected (Appendix Figure 4.4).

We chose the two formulations with stable Q-GRFT content, F-3 in the SC group and F-5 in the HME group, respectively, as our lead formulations. Overall, the Q-GRFT films were found to be thin, soft, and flexible, which are critical physical and tactile attributes shown above to be preferred by women\textsuperscript{91,93}. Despite different polymers, formulations, and methods used, these physiochemical characterizations are also comparable to other polymeric vaginal films\textsuperscript{123,161,276,310}, including one protein film product. The HME films were found to be heavier, and thicker than the SC films. HME films also had lower water content due to the lack of aqueous solution throughout the process. A similar observation was reported by Regev et al.\textsuperscript{276} The study also compared HME films and SC films with dapivirine as the API and found the same trends for the physicochemical properties as the corresponding films with Q-GRFT. In this study, the puncture strength of the HME films was found to be similar to that of SC films, suggesting similar mechanical properties.
However, between the two films, the SC films had rougher and bumpier surfaces because of the air entrapment during casting. The tactile difference can impact user acceptability. As a result, HME films were chosen to perform an exploratory long-term stability study. The Q-GRFT content in F-5 films was decreased to 80% of label claim after 3 months in the accelerated condition, and after 24 months in the unstressed condition. Given that the stability of protein-incorporated vaginal films was reported to be only weeks, our findings demonstrated the possibility of longer shelf life for protein films. In the future, a full stability study should be performed for more detailed physiochemical characterizations of the films.

4.5 CONCLUSION

In summary, we have described the screening of excipients and formulations for the development of Q-GRFT-incorporated polymeric vaginal films. The protein-excipient interactions were explored using in silico methods including molecular docking. We have also successfully identified excipients that are incompatible with Q-GRFT and validated the results by two proof-of-concept formulation studies. The selected SC and HME films successfully incorporated Q-GRFT and were found to have acceptable visual and physicochemical characteristics. Furthermore, the Q-GRFT HME film was found to be stable for at least 3 months.

Overall, this study explored the potential of Q-GRFT in another solid dosage form, polymeric films. This chapter also set the groundwork for future research to further modify and advance this platform to incorporate protein drugs.
5.0 DISCUSSION

HIV/AIDS continues as a global pandemic, with 1.5 million people becoming newly infected with HIV globally in 2020. The vast majority of these new HIV infections are through sexual transmission. Public health agencies, including WHO and CDC, identified key populations (men who have sex with men, and women) who are continuously at risk of HIV infections.

MSM has been the most HIV-impacted population since the beginning of this epidemic. Due to biological, social, and cultural stigma and discrimination, MSM has a significant proportion of HIV incidence, accounting for around 65% of all new HIV diagnoses in the U.S. Although oral PrEP options are available for MSM at risk, there are still concerns regarding side effects and drug resistance which negatively impact adherence. To address the adherence issue, current research has been combining prevention methods with normally practiced behavioral routines. To this end work toward the development of antiviral drug containing enema products is being conducted as a topical PrEP product.

Women are undergoing the disproportional burden of HIV infection, which is attributed to physiological vulnerability and unequal socioeconomic status. Millions of new HIV infections in women every year urge the development of effective female-controlled prevention strategies to decelerate the spread of HIV. The vaginal polymeric film is one of the potential female-controlled topical microbicides. This product has demonstrated high acceptability, favorable safety, and pharmacokinetic profiles in clinical trials.

The work presented within this dissertation contributes to the development of dosage form options for rectal and vaginal application as topical pre-exposure prophylactics for HIV infection.
Specifically, it focuses on developing novel formulations for the delivery of Q-GRFT which can be utilized in these highly susceptible populations to prevent HIV infection.

In this work, we found that: (1) Q-GRFT can remain stable with TFV, an NRTI that has synergetic effects against HIV infection; (2) protein-excipient interactions impact the stability of Q-GRFT; (3) formulation modifications can overcome the challenges during manufacturing processes. This work sought to achieve three primary goals: (1) explore the potential of combing Q-GRFT and TFV in both the liquid form (Chapter 2) and the solid dosage form (Chapter 3); (2) investigate the protein-excipient interactions and their impact on Q-GRFT stability (Chapter 3 and Chapter 4); (3) develop stable Q-GRFT-incorporated formulations that meet CQAs as topical PrEP options (Chapter 2, Chapter 3, and Chapter 4). The products generated from this work can be used as topical PrEP options that expand the current HIV prevention strategies for the key population at risk.

This chapter presents a summary of major findings, and the implication of this work, and provides discussions on some of the limitations within the work presented. Additionally, the significance and innovation of this work are described and future directions for further development of the products generated are discussed.

5.1 MAJOR FINDINGS

5.1.1 Addressing the Adherence Issue with Patient-Centered Product Designs

There is a growing body of evidence that highlights the importance of patient adherence in HIV prevention product effectiveness. Therefore, when topical PrEP options were
under development, it is important to incorporate strategies that may reduce the adherence issue. Two considerations explored in this work are discussed below.

### 5.1.1.1 Utilizing Pre-Existing Behaviors

Enema products are widely used as a cleansing procedure by MSM engaging in receptive anal intercourse before and/or after the intercourse. Behavioral studies have demonstrated the benefits of combing HIV prevention modalities with such enema products. In light of this, researchers have developed several enema formulations with ARVs to serve as rectal topical PrEP options. In particular, TFV, a common NRTI, was developed as an enema and characterized *in vitro* and *in vivo*. Additionally, Q-GRFT enema has also been developed in our lab and evaluated in a clinical trial. Therefore, in Chapter 2, we explored the possibility of combining these two APIs which have synergistic effects against HIV in the enema solution.

Results from the *in vitro* Caco-2 cell model and *ex vivo* human colorectal tissue model demonstrated no epithelial damages observed. Data collectively suggest low to no potential toxicity for the combination enema used in rectal environments. More importantly, the combination of TFV and Q-GRFT in enema did not impact their respective permeability profiles. Our results showed that this high-permeable characteristic of TFV remained in the hypotonic combination enema, while Q-GRFT was not permeated.

On one hand, TFV was previously shown to penetrate more into tissues in a hypotonic enema than in an isotonic enema. As an NRTI, TFV needs to be present in the cell to provide its protective effects. With comparable permeability of the TFV-only enema, which is being elevated in clinical trials, our combination enema was expected to have the same tissue concentrations of TFV. On the other hand, Q-GRFT was preferably not permeable due to its binding mechanism. The previous research demonstrated that GRFT adhered to the superficial cells of the human
ectocervical epithelium. Similarly, we assessed the combination enema using the \textit{ex vivo} human colorectal tissues with the same experimental set-ups. Results showed no significant decrease of Q-GRFT content on the donor side, indicating no drug permeated through the mounted colorectal tissues. The data suggested that Q-GRFT, as a lectin, may also adhere to the apical side of the rectal epithelium, supporting the literature findings.

5.1.1.2 Advancing to Solid Dosage Forms

While enema use/douching provides potential benefits of increasing adherence\textsuperscript{69,136}, it is hard for transportation and storage as liquid dosages\textsuperscript{147}. Thus, solid dosage forms were further explored in Chapter 3 and Chapter 4 to offer economic advantages\textsuperscript{147,148}, convenience, and privacy\textsuperscript{91,185-188}.

In Chapter 3, a lyophilized powder formulation incorporating Q-GRFT was developed. Maltitol at a molar ratio of 18:1 to Q-GRFT was applied as the cryoprotectant during the lyophilization process. The Q-GRFT (with maltitol) lyophilized powder (LMP) was found to be stable under the real-time condition (25°C/60% RH) for at least three months. More importantly, the bioactivity of Q-GRFT was maintained in the powder formulation supported by the gp120 binding study. Although more studies are needed, a preliminary long-term elucidated that LMP retained Q-GRFT content for 12 months with no significant drug loss. After 24 months, the drug content decreased to 88.76% (of label claim) with small aggregation peaks detected. The TFV/Q-GRFT combination powder formulations were also assessed in Chapter 3, where two formulations of lyophilized TFV powder (PBS-base and Saline-base) were combined respectively with LMP. The physical mixture of the combination powder exhibited physiochemical properties that fit with the ranges, including pH, osmolality, and drug contents of both TFV and Q-GRFT. The results suggested the good compatibility of two lyophilized powders in the solid form.
Chapter 4 investigated the potential of incorporating Q-GRFT in the polymeric film dosage form. Among the vaginal DDS, the polymeric film is one of the most attractive and viable dosage forms for women\textsuperscript{91,186}. The vaginal films offer advantages including patient-friendly applications, better residence time, less messiness, convenience, and discretion\textsuperscript{101,158,160,251}. Due to the superiority of vaginal films, they have been developed to incorporate small molecule drugs\textsuperscript{92,270-273}, peptides\textsuperscript{161}, antibodies\textsuperscript{261}, and nanoparticles\textsuperscript{123,274,275}. In Chapter 4, Q-GRFT was successfully incorporated into the polymeric films by two commonly-used manufacturing methods, the solvent-cast method, and the hot-melt extrusion method. Two lead formulations (one from each manufacturing method) were chosen based on the short-time stability study. Results from which showed no decrease in Q-GRFT content under the real-time condition for three months, suggesting the maintenance of stable Q-GRFT. These two lead formulations were further assessed for their physiochemical properties. Both polymeric films, despite different formulations and manufacturing processes, were soft and flexible. The HME films were heavier and thicker because the thickness was not controlled as precisely as the SC films. In addition, because of the nonaqueous processing, HME films had much less water content in the final product. HME films also showed lower puncture strength, possibly because of lower plasticizer content in the final products. Literature\textsuperscript{315,316} reported that stronger mechanical properties were reflected in polymeric films with higher plasticizer contents, supporting our findings. The same trends for the physiochemical properties were also observed by Regev et al.\textsuperscript{276}, in which study HME films and SC films, both containing dapivirine, were compared. Nonetheless, these characteristics were found comparable to other polymeric films previously developed in our lab. The film products developed in this work aimed to achieve properties preferred by women, potentially leading to higher acceptability and adherence.
5.1.2 Reducing the Aggregation Issue for Q-GRFT-Incorporated Formulations

Protein drugs have been demonstrating instability in both liquid and solid formulations historically\textsuperscript{157,196,198,199,202,205,218,221,223,224}. Over the years, formulation scientists investigated the protein-excipient interactions and provided several hypotheses and cases to protect protein drugs from degradation. Q-GRFT, as a protein drug, may also face various stresses caused by the manufacturing processes. These stresses, including low temperature (cryo-stress), high temperature, concentration effects, pH shifts, dehydration, and mechanical stress, were studied and discussed in this dissertation work.

Preliminary data identified aggregation as one of the major degradation pathways for Q-GRFT. Several studies set to prevent protein aggregation have reported successful cases by utilizing excipients. Although the protecting effects are protein-specific\textsuperscript{138}, the excipients can potentially prevent Q-GRFT from aggregation, which can be further explained by different theories.

Lyophilization is one of the most commonly used processes to prepare dehydrated proteins\textsuperscript{157}. Although it has been demonstrated as a cheap and easy manufacturing procedure\textsuperscript{157,192,193}, stresses from the lyophilization process may impact protein stability\textsuperscript{157}. Great efforts have been made in the investigation of protein stabilization using excipients\textsuperscript{138,208,217,232,234,240}. Particularly, literature shows that by incorporating cryoprotectants, stable protein drugs can be incorporated into the lyophilized formulations\textsuperscript{138,192,224}. Therefore, Chapter 3 screened cryoprotectants and explored their protecting effects for Q-GRFT. Four cryoprotectants, including two non-reducing disaccharides (sucrose and trehalose) and two sugar polyols (maltitol and lactitol), were included due to their success from literature\textsuperscript{139,233-235,237,239}. Among the four tested cryoprotectants, maltitol and sucrose presented concentration-dependent
protection of Q-GRFT. Furthermore, a more detailed molar ratio panel was investigated for maltitol, demonstrating the same concentration-dependent protecting profile in the accelerated condition. Results from the study supported existing findings, outlining the critical role of cryoprotectant content in stabilization\textsuperscript{230}. The final lyophilized Q-GRFT (with maltitol) powder (LMP) was further evaluated for the drug content. In the short-term stability study, no significant drug loss was found by HPLC, and no aggregation was observed by SEC.

The manufacturing procedure of polymeric films requires high temperature (65°C) and mechanical force, which may also induce aggregation of Q-GRFT. Therefore, the protecting effects of excipients were also evaluated in Chapter 4. Findings from the excipient screening study revealed some excipients have better protecting effects over the others. The various effects for protecting Q-GRFT from aggregation were further supported by the formulation study. In the proof-of-concept developments of formulations, formulations demonstrated different protecting effects aligning with their respective compositions. Excipients that presented instability with Q-GRFT carried through the same impact in the formulation, with a significant decrease in drug content and aggregation peaks observed on the chromatographs. On the brighter side, formulations with no to low content of such excipients stabilized Q-GRFT and prevent aggregations.

Literature has offered several possible explanations for the protecting effects of excipients. The “water replacement hypothesis” is widely-used to explain the protecting effects of sugar and sugar polyols\textsuperscript{168}. In the LMP, maltitol has nine hydroxyl groups, which offer hydrogen bonds with Q-GRFT. Since the hydro-shell was maintained, the protein’s natural conformation can be preserved\textsuperscript{192,210,211}. In this theory, sugars/sugar polyols served as water substitutes to provide hydrogen bonds, in turn, to prevent aggregation\textsuperscript{230}. To our surprise, this protecting effect was not reflected in lactitol. Lactitol, which also has nine hydroxyl groups, did not stabilize Q-GRFT.
during lyophilization. Computational approaches were also explored to address this phenomenon. As more studies are needed to elucidate these effects, the lack of protecting effects of lactitol can be explained by the spatial differences among the sugar alcohols or intramolecular hydrogen bonds.

However, the same hydrogen-bonding effects may have negative impacts on the stability of Q-GRFT in polymeric films. In Chapter 4, the hydroxyl groups on the excipient (HEC, HPMC, CMC, and PVA) side chains may form hydrogen bonds with Q-GRFT. During the film-forming process, the environment transited from the liquid state to the (semi-) solid state. As polymers crosslink to a physical network, new intermolecular associations of linear or branched polymers formed. Thus, the abovementioned hydrogen bonds with Q-GRFT were impacted, leading to possible conformational changes and aggregations. Although further studies are needed, this hypothesis can explain the incompatibility of Q-GRFT with HEC, HPMC, CMC, and PVA.

In addition to the “water replacement hypothesis”, the “vitrification hypothesis” was also mentioned in the literature, where sugars/sugar polyols form a vitrified, rigid sugar-glass matrix that limits the protein degradations kinetically. In Chapter 3, DSC and XRD results showed that maltitol presents in an amorphous state in LMP. Maltitol, when lyophilized alone, has a glass transition temperature (Tg) of 40.6–43.1°C. The Tg was increased with the addition of proteins or buffers, which was also expected in our study. Therefore, when LMP was challenged under high temperature (65°C) in Chapter 4, annealing or “densification” might occur. Studies found that under such conditions, a glass may enter the “equilibrium glassy state” asymptotically, leading to structural relaxation and enhanced protein stability.

Our study offers an additional explanation for Q-GRFT stability in LMP. Finding from Chapter 3 proved the inhibition of PBS crystallization with the presence of maltitol. Research
finds that the unbalanced rate in the crystallization of PBS can generate a significant acidic environment during lyophilization\textsuperscript{218}. It can cause the pH to drop from 7.5 to 4.5, leading to protein denaturation\textsuperscript{217}. When maltitol was added to the formulation, the reduction of PBS crystallization may lead to the preservation of environmental pH and Q-GRFT stability. Results from the dissertation work supported the existing reports where the additions of mannitol or trehalose decreased the magnitude changes of pH in PBS\textsuperscript{153}.

The last hypothesis we discussed in the dissertation work is the electronic charges carried by the excipients. Given that Q-GRFT was tested to be positively charged (\(\sim+40\) mV) in the buffer (unpublished data), there might be excipient-protein interactions in the formulation solution. It has been reported that the electrostatic interactions of excipient-protein may negatively impact protein stability\textsuperscript{143}. Specifically, when a hydrated protein is exposed to a water-poor environment (such as films), it tends to transfer protons to ionized carboxyl groups and thus abolishes as many charges as possible in the protein\textsuperscript{317}. The decreased charge density may facilitate protein-protein hydrophobic interaction, causing protein aggregation. The excipients which showed incompatibility with Q-GRFT were mostly negative-charged, including CMC\textsuperscript{296}, HPMC\textsuperscript{297}, and PVA\textsuperscript{298,299}. When negatively-charged excipients were combined with positively-charged protein drugs, the ion binding can modulate protein aggregation by neutralizing protein charges\textsuperscript{138}. Similar effects were reported by several studies\textsuperscript{301-303}.

Overall, we successfully prevent the aggregation of Q-GRFT by modifying the formulations. In addition, several possible explanations were explored and discussed above. Although great efforts have been made in the investigation of protein stabilization, the mechanisms of denaturation are complex\textsuperscript{157,196,198,199,202,205,218,221,223,224}. As no single theory can elucidate all
reasons for protein instability, we want to set the foundation for future fundamental studies of stabilizing Q-GRFT in this dissertation work.

5.1.3 Developing Stable Q-GRFT-Incorporated Topical PrEP options for HIV Prevention

Previous work in our lab has demonstrated the potential of developing a Q-GRFT enema solution. However, the stability of the Q-GRFT enema was only monitored for weeks. As biological products normally require months to years of stability to have economic values, we explored the potential of stabilizing Q-GRFT in the enema for a longer time. In addition, the combination of TFV and Q-GRFT was never reported in the liquid form to our knowledge. Therefore, we explored the stability of the TFV/Q-GRFT combination enema. Results demonstrated no drug loss for both APIs for up to 24 months. More importantly, the binding efficacy of Q-GRFT was also maintained for all formulations tested in the gp120 binding study, suggesting good compatibility of the combination. Other CQAs were also monitored and found to be stable within the range, including appearance, pH, and osmolality. No cell toxicity and epithelial damage were observed for both designed formulations, proven by in vitro and ex vivo models. More importantly, results from these models also showed a similar permeable profile of TFV in the combination enema with the single enema, which has already been identified as the desired characteristic previously. Overall, the combination enema developed in Chapter 2 demonstrated the potential for further development and assessment with in vivo models and future clinical trials.

The lyophilized powder form of the Q-GRFT and Q-GRFT/TFV combination was developed in Chapter 3. As a pharmaceutical powder dosage form, the lyophilized powder should demonstrate good appearance, flowability, and drug stability. The LMP manufactured was white to off-white. After grinding with a mortar and pestle, LMP showed excellent flowability. More
importantly, when the powder was reconstituted, the enema solution should retain the same CQAs
designed in Chapter 2. To our expectations, the Q-GRFT only reconstituted solution had osmolality around 0, due to the lack of buffered agents. By combing with the TFV powder, which was lyophilized in either PBS-base or Saline-base formulations, the combination enema was hypotonic. The osmolality of the reconstituted combination enema fit within the range (145±22 mOsm/kg). The appearance of all reconstituted solutions was clear and transparent. The pH of all groups was also neutral. Overall, the final LMP demonstrated good stability in drug content over three months, with no aggregation detected. Moreover, the bioactivity of gp120 binding was maintained for LMP, suggesting the potential preservation of its HIV-preventing efficacy. The stability of drug content was also shown in the combination powder, with both Q-GRFT and TFV content above 90% of the label claims. Although more in vitro and in vivo studies are needed, the products developed in Chapter 3 are promising rectal PrEP options that have the potential for future development.

We further explored the potential of incorporating Q-GRFT in the polymeric films in Chapter 4. As a vaginal delivery system, polymeric films were preferred to be soft, smooth, and flexible. Additional desirable physicochemical properties also include strong mechanical properties, relatively low moisture content, and stable drug content. After a series of excipient and formulation screenings, one lead SC formulation and one lead HME formulation were chosen to compare for their CQAs. Although the physiochemical characterizations for both lead formulations were comparable to other polymeric films previously reported, the HME films demonstrated superiority based on the smooth surface. Although the bubbles from the air-trapping during solvent-casting can be reduced in big-scale manufacturing, HME is still preferred due to the advantages including compact sizes, small carbon footprint, and streamlined production.
Therefore, the HME films were further monitored in a preliminary long-term stability study for Q-GRFT content. Results from the study showed that 82% of the label claim for the Q-GRFT content was recovered in the HME films after 24 months, suggesting the potential for long-term stabilization of protein drugs in the polymeric films.

5.2 LIMITATIONS

Although pre-set CQAs were met for the three rectal or vaginal PrEP options, the designed products from this dissertation work need further evaluations before advancing into the next stages.

First of all, the safety evaluation of the topical PrEP options was only addressed in in vitro and ex vivo models for the combination enema. Although the individual enema solutions are currently evaluated in clinical trials, the potential toxicity resulting from the combination was unknown. In the dissertation work, we lack evidence for the immune response caused by the topical microbicides. In addition, as a part of innate immunity, the compatibility of our products with common microbiota in a healthy human should be assessed to complete the safety evaluations.

Secondly, the gp120 binding assay is not a marker of in vitro bioactivity. The ELISA only detects the binding affinity of our protein drug (Q-GRFT) to the target molecule (gp120). Since no virus is involved in this biochemistry assay, the prevention bioactivity of Q-GRFT was not detected for the products. Therefore, investigating Q-GRFT bioactivity in an in vitro cell-based model, like the TZM-bl cell culture, or in an ex vivo tissue explant challenge model would provide efficacy data to support the gp120 binding ELISA experiments.

Thirdly, only aggregation was monitored and discussed within the context of this dissertation work. Even though Q-GRFT was mutated to reduce the oxidation of the protein drug,
this degradation pathway along with deamination remains a potential challenge. Due to the limited assays available, oxidized or deaminized degradants were not detectable. To rule out the possibility or to better stabilize Q-GRFT in the formulations, bioassays that can distinguish these denatured products need to be performed. In addition, accelerated conditions (with temperatures of 40°C or 65°C) were utilized as well as the real-time condition (with a temperature of 25°C). There has been an ongoing debate whether the shelf life of biologics can be determined the same way as small molecule APIs. The protein drugs don’t necessarily follow the Arrhenius equation in higher temperatures as different mechanisms of degradation may take place. With such concerns, the degradation pathways at higher temperatures should be investigated for Q-GRFT.

Fourthly, in this dissertation project, the excipient screening is still empirical based on previous experience within groups or literature examples\textsuperscript{138}. Excipient selections using empirical strategy do not usually consider the 3D structure of the protein drug\textsuperscript{139}. Moreover, the current strategy depends heavily on the formulation of scientists’ experience and labor. Due to the variety of classes of excipients and numerous combinations, it will be impossible to systemically evaluate all the formulations based on empirical screening.

Additionally, the excipient-protein interactions were not fully studied. Literature has reported studies for the excipient-protein interactions using nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FTIR), Raman spectroscopy, or other methods. However, with limited resources, these experimental investigations were not performed. Even though several hypotheses were discussed based on the literature, more evidence is needed to support these theories.

Lastly, the long-term stability study for the LMP and the polymeric films was only preliminary. And only drug contents were monitored in the study. As physiochemical
characterizations were critical contributors to the quality of the products, a full panel of assessments should be performed for the rectal LMP and vaginal polymeric films. The current long-term stability study only monitored the 12- or 24-month timepoints. To determine or predict the shelf-life for marketable products, more time points should be included in the future.

5.3 CONTRIBUTIONS

This project was the first to address the issue of Q-GRFT aggregation in solid dosage forms. The understanding of causes was the first study related to this issue. Based on the results generated from this work, several possible explanations were proposed. The data also illustrated the importance of formulation modification, both stabilizing agents and choices of excipients. To the best of our knowledge, the formulations developed from this project were the first to report in the field, including the Q-GRFT/TFV combination enema, Q-GRFT only, and combination (with TFV) powder, and Q-GRFT polymeric vaginal films. This dissertation work will shed light on future novel developments of other protein formulations affected by aggregation. The findings, after further investigations, will support the current hypotheses on excipient-protein interactions and their effects to prevent protein degradation.

5.4 FUTURE DIRECTIONS

The dissertation work has set the stage for several future directions, which can contribute to (1) advancing the rectal and vaginal products to the next stages. (2) a better understanding of
the excipient-protein interactions for Q-GRFT. (3) exploring the potential of utilizing *in silico* platforms for excipient screening and formulation developments.

The non-human primate (NHP) model is a well-established animal model used previously for vaginal and rectal PrEP options. Many studies evaluated topical PrEP options with this *in vivo* model due to its high similarity of biological environments with humans. Literature has reported a pigtailed macaque model with anatomical and physiological similarities to humans. This model allows a more reliable translation of results to humans than *in vitro* studies. Therefore, it is desired to assess the toxicity, pharmacokinetic and pharmacodynamic profiles of our designed topical PrEP options *in vivo* as the next step.

A more detailed long-term drug substance stability should be performed to validate the data generated in this dissertation work. Storage at 25°C/60%RH for 24 months and storage at 40°C/75% RH for six months (or longer) should be evaluated. Assessments during this time frame should include evaluations of chemical and physical stability, gp120 binding, bioactivity (TZM-bl cells viral challenging studies), and safety (microbiome compatibility studies and *ex vivo* tissue toxicity studies).

It was also discussed prior that more experimental explorations on the protein-excipient interactions could be conducted in this dissertation project. Methods, including NMR, FTIR, and Raman spectroscopy, should be performed in the future if we have access to more Q-GRFT. Besides, high-throughput screening, a fast-grown excipient screening process\textsuperscript{145,225}, can also be explored to aid the conventional process.

Recent progress in the field has been made to take the advantage of computational methods. Conventional screening or high-throughput screening has limitations such as the lack of full mechanistic information\textsuperscript{138}. Thus, computational approaches have been reported to provide
alternative strategies to overcome these limitations. Studies\cite{318-320} have stated the possibility of using simulation models to predict lyophilized products. Researchers also reported using computational methods to study the interactions between protein and excipients which are commonly used in the formulations. Hence, we wanted to combine the \textit{in-silico} platform to explain the mechanisms behind the aggregation reduction by excipients.

Molecular docking has been extensively used in drug discovery both for the design of new drugs to specific binding sites\cite{307}, or to repurpose a small molecule by finding other binding sites\cite{308}. Most recently, molecular docking has also been applied to protein-excipient screening by comparing the hotspots from both protein-protein interactions and protein-excipient interactions\cite{139}. Therefore, we explored a molecular docking approach to investigate the excipient-protein interactions in this project. However, the work presented only set the foundation for future studies to better understand the binding sites and the bonds between excipients and Q-GRFT. Other computational approaches, including molecular dynamics simulations, can be implemented in the future. With more studies to correlate the virtual predictions with the experimental data, it is hopeful to have computer-aid approaches for the excipient screening and formulation development.
Appendix A Appendix Materials for Chapter 2

Appendix Table 5.1 Apparent Permeability ($P_{app}$) Values of TFV in Four Hypotonic Enema Formulations.

Enema was tested with human colon tissues in the Ussing chamber.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>TFV Only Enema Phosphate-base (Hypotonic)</th>
<th>TFV Only Enema NaCl/NaOH-base (Hypotonic)</th>
<th>TFV/Q-GRFT Combo Enema PBS-base (Hypotonic)</th>
<th>TFV/Q-GRFT Combo Enema Saline-base (Hypotonic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon Tissue #</td>
<td>T#3128</td>
<td>T#3128</td>
<td>T#3128</td>
<td>T#3128</td>
</tr>
<tr>
<td>Individual $P_{app}$ (cm/s)</td>
<td>$2.2 \times 10^{-6}$</td>
<td>$1.9 \times 10^{-7}$</td>
<td>$1.0 \times 10^{-6}$</td>
<td>$2.0 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$2.0 \times 10^{-4}$</td>
<td>$3.3 \times 10^{-7}$</td>
<td>$3.3 \times 10^{-10}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2.8 \times 10^{-6}$</td>
<td>$1.1 \times 10^{-6}$</td>
<td>$2.4 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$4.4 \times 10^{-4}$</td>
<td>$1.2 \times 10^{-9}$</td>
<td>$8.3 \times 10^{-10}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$6.1 \times 10^{-7}$</td>
<td>$5.3 \times 10^{-7}$</td>
<td>$8.0 \times 10^{-8}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2.8 \times 10^{-6}$</td>
<td>$4.0 \times 10^{-4}$</td>
<td>$2.6 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$1.0 \times 10^{-6}$</td>
<td>$1.5 \times 10^{-10}$</td>
<td>$9.2 \times 10^{-8}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$1.2 \times 10^{-6}$</td>
<td>$1.7 \times 10^{-10}$</td>
<td>$1.3 \times 10^{-10}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$5.8 \times 10^{-7}$</td>
<td>$2.1 \times 10^{-10}$</td>
<td>$2.7 \times 10^{-10}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$5.4 \times 10^{-7}$</td>
<td>$6.7 \times 10^{-7}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$1.0 \times 10^{-6}$</td>
<td>$3.8 \times 10^{-7}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2.5 \times 10^{-7}$</td>
<td>$2.6 \times 10^{-7}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$3.5 \times 10^{-7}$</td>
<td>$1.5 \times 10^{-7}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2.5 \times 10^{-8}$</td>
<td>$1.9 \times 10^{-7}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average $P_{app}$ (cm/s)</td>
<td>$1.3 \times 10^{-6} \pm 9.4 \times 10^{-7}$</td>
<td>$2.1 \times 10^{-6} \pm 1.8 \times 10^{-6}$</td>
<td>$2.1 \times 10^{-6} \pm 1.4 \times 10^{-6}$</td>
<td>$2.5 \times 10^{-6} \pm 2.2 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
Appendix Table 5.2 A Comparison of the Permeability of TFV and $^{14}$C-Mannitol in Three Hypotonic Enema Formulations using the Caco-2 Monolayer Cell Model.

$^{14}$C-Mannitol was used as the paracellular marker and added to all the groups (n=3–6).

<table>
<thead>
<tr>
<th></th>
<th>$P_{app}$ of TFV (cm/s)</th>
<th>$P_{app}$ of $^{14}$C-Mannitol (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFV/Q-GRFT Combo Enema PBS-base (Hypotonic)</td>
<td>$1.5 \times 10^{-6} \pm 1.5 \times 10^{-7}$</td>
<td>$4.9 \times 10^{-6} \pm 7.1 \times 10^{-7}$</td>
</tr>
<tr>
<td>TFV/Q-GRFT Combo Enema Saline-base (Hypotonic)</td>
<td>$1.8 \times 10^{-6} \pm 2.2 \times 10^{-7}$</td>
<td>$4.8 \times 10^{-6} \pm 3.9 \times 10^{-7}$</td>
</tr>
<tr>
<td>TFV Only Enema (Clinical Group) (Hypotonic)</td>
<td>$1.8 \times 10^{-6} \pm 2.6 \times 10^{-7}$</td>
<td>$5.3 \times 10^{-6} \pm 7.7 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Appendix Table 5.3 A Comparison of the Permeability of TFV in Three Hypotonic Enema Formulations.

Studies used either the Caco-2 monolayer cell model (n=3–6) or the human colon tissue model (n=8–20).

<table>
<thead>
<tr>
<th>$P_{app}$ of TFV (cm/s)</th>
<th>Caco-2 Monolayer Model</th>
<th>Human Colon Tissue Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFV/Q-GRFT Combo Enema PBS-base (Hypotonic)</td>
<td>$1.5 \times 10^{-6} \pm 1.5 \times 10^{-7}$</td>
<td>$2.1 \times 10^{-6} \pm 1.4 \times 10^{-6}$</td>
</tr>
<tr>
<td>TFV/Q-GRFT Combo Enema Saline-base (Hypotonic)</td>
<td>$1.8 \times 10^{-6} \pm 2.2 \times 10^{-7}$</td>
<td>$2.5 \times 10^{-6} \pm 2.2 \times 10^{-6}$</td>
</tr>
<tr>
<td>TFV Only Enema (Clinical Group) (Hypotonic)</td>
<td>$1.8 \times 10^{-6} \pm 2.6 \times 10^{-7}$</td>
<td>$2.1 \times 10^{-6} \pm 1.8 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
Appendix Figure 5.1 A Representative Figure for TEER Values Measured with Ussing Chamber.

Six pieces of the colorectal tissue were obtained from the same donor patients. TEER values were monitored throughout the experiments (2 hours) and presented as % of the Time 0 TEER value. Both PBS- and Saline-base combo formulations were performed in triplicates, numbered 1,2, and 3 respectively, and shown with different colors.
Appendix Figure 5.2 Epithelial Structures of the Human Colorectal Tissue using H&E Staining.

a. Pre-Treatment Tissue (before the 2-hour treatment); b. Post-treatment with PBS-base combo enema; c. Post-treatment with Saline-base combo enema.
Appendix B Appendix Materials for Chapter 3

Appendix Figure 5.3 Short-term (Ten Days) Stability Study of Q-GRFT Only (in PBS) Lyophilized Powder.

Samples were stored in the real-time condition (25°C/60%RH). Q-GRFT content was detected at different time points by HPLC after storing the lyophilized powder under a normal condition (25 °C/60% RH). N=3. Significance (p<0.05) was found for Day 3, 4, 7, 8, and 10, comparing the drug content to Day 0.
Appendix Figure 5.4 Short-term (One Month) Stability Study of Q-GRFT Only (in PBS) Lyophilized Powder.

Samples were stored in the real-time condition (25°C/60% RH). SEC chromatographs of the Q-GRFT-only lyophilized powder group (bottom) and the Q-GRFT reference group (top) on Day 0 (left) and Day 30 (right). Q-GRFT monomer peaks elute at the same retention time for all four chromatographs. Aggregation was detected for the Q-GRFT-only lyophilized powder group on Day 30 (pointed).
Appendix Figure 5.5 DSC Chromatography for KCl (red), NaCl (black), Na$_2$HPO$_4$ (blue), and NaH$_2$PO$_4$ (green).

Negative peaks are endothermic peaks. Relative energy (y-axis) was plotted against temperature (x-axis). Integration of the peak was shown in the figure.
Appendix Figure 5.6 DSC Chromatography for PBS Powder (a physical combination of KCl, NaCl, Na$_2$HPO$_4$, and NaH$_2$PO$_4$ powders).

Negative peaks are endothermic peaks. Relative energy (y-axis) was plotted against temperature (x-axis). Integration of the peak (Na$_2$HPO$_4$ [blue], NaH$_2$PO$_4$ [green]) was shown in the figure.
Appendix Figure 5.7 XRD Patterns for the PBS Lyophilized Powder.

The figure was plotted using Prism 9. Signature peaks were identified based on the data from the literature\textsuperscript{214,248}. Intensity (y-axis) was plotted against 2θ (x-axis).
Appendix Figure 5.8 TFV Lyophilized (with Maltitol) Powder Stability. TFV was lyophilized with maltitol in two base formulations, (a) Saline-base and (b) PBS-base respectively.

TFV content was detected at different time points by UPLC after storing the lyophilized powder under an accelerated condition (40°C/75% RH). N=3.

Dash lines indicate the lower (90%) and upper (110%) limits of the label claim.
Appendix Figure 5.9 pH Determined in Individual or Combined Reconstituted Enema.

The target pH range is 6.5–8. N=3.
Appendix Figure 5.10 Osmolality is Determined in Individual or Combined Reconstituted Enema.

Dash lines show the 85% (lower limit) and 115% (upper limit) of the target osmolality. Target osmolality for combo enema is 145 mOsm/kg. LMP has an osmolality close to 0 due to the lack of buffering agents. N=3.
Appendix Figure 5.11 Long-term Stability Study for LMP.

Q-GRFT Content was detected at different time points by HPLC after storing the lyophilized powder under a real-time condition (25°C/60% RH). For Time 0 and 12 months, N=4. For the Time 24 months, N=6. Dash lines indicate the 90% (lower limit), 100% (target), and 110% (upper limit) of the label claim. NS=No significance.
Appendix Figure 5.12 SEC chromatography for the Long-term Stability Study of LMP.

Samples were stored under a real-time condition (25°C/60% RH) and tested at the last time point (24 months). LMP group (shown in black) and the Q-GRFT reference group (shown in blue) were compared. Q-GRFT elutes at the same retention time for both groups. An aggregation peak was detected as indicated in the figure.
Appendix C Appendix Materials for Chapter 4

Appendix Figure 5.13 SEC chromatograms of Q-GRFT SC film group (Formulation-1, F-1, shown in the top black line), its Placebo film group (shown in the bottom black line), and Placebo Spiked Q-GRFT group (shown in the middle red line).

Film excipient peaks, Q-GRFT peaks, and aggregate peaks were indicated on the graph.
Appendix Figure 5.14 SEC chromatograms of Q-GRFT SC film group (Formulation-2, F-2, shown in the top black line), its Placebo film group (shown in the bottom black line), and Placebo Spiked Q-GRFT group (shown in the middle red line).

Film excipient peaks, Q-GRFT peaks, and aggregate peaks were indicated on the graph.
Appendix Figure 5.15 SEC chromatograms of Q-GRFT SC film group (Formulation-3, F-3, shown in the top black line), its Placebo film group (shown in the bottom black line), and Placebo Spiked Q-GRFT group (shown in the middle red line).

Film excipient peaks and Q-GRFT peaks were indicated on the graph. No aggregates were detected.
Appendix Figure 5.16 SEC chromatograms of Q-GRFT HME film group (Formulation-5, F-5, shown in the middle black line), its Placebo film group (shown in the top blue line), and Q-GRFT reference group (shown in the bottom magenta line).

Film excipient peaks and Q-GRFT peaks were indicated on the graph. Q-GRFT monomer peaks elute at the same RT for both drug-containing groups.

No aggregates were detected.
2. CDC. About HIV.
5. CDC. Basic Statistics.


40. WOMEN, U. Facts and figures: HIV and AIDS.


51. FDA. FDA approves second drug to prevent HIV infection as part of ongoing efforts to end the HIV epidemic. Vol. 2022 (2019).


delivery among women and their male partners in KwaZulu-Natal, South Africa. *The
European journal of contraception & reproductive health care : the official journal of the
European Society of Contraception* 24, 390-398 (2019).

96. Robinson, J.A., et al. Comparison of the Pharmacokinetics and Pharmacodynamics of
Single-Dose Tenofovir Vaginal Film and Gel Formulation (FAME 05). *Journal of

delivery: design, optimization, and physicodynamic characterization. *Aaps Pharmscitech*
10, 951 (2009).


vaginal delivery film containing EFdA, a novel anti-HIV nucleoside reverse transcriptase

100. Akil, A., et al. Development and characterization of a vaginal film containing dapivirine,
a non-nucleoside reverse transcriptase inhibitor (NNRTI), for prevention of HIV-1 sexual

combinations of antiretrovirals (ARVs) for HIV prevention. *Pharmaceutical research* 32,

102. Mcgowan, I. & Dezzutti, C. Rectal Microbicide Development. in *Current Topics in
Microbiology and Immunology* 117-136 (Springer Berlin Heidelberg, 2013).

103. Office of Communications, D.o.D.I., Center for Drug Evaluation and Research, Food and
Drug Administration. Guidance for Industry: Vaginal Microbicides: Development for the

104. Mori, T., Delos, S.E., Brecher, M. & Schornberg, K. Structures and mechanisms of
viral membrane fusion proteins: multiple variations on a common theme. *Critical reviews
in biochemistry and molecular biology* 43, 189-219 (2008).

perspectives in medicine* 2, a006866 (2012).


Study on Synergistic Interactions Between Free and Encapsulated Q-Griffithsin and

108. Fuqua, J.L., Hamorsky, K., Khalsa, G., Matoba, N. & Palmer, K.E. Bulk production of the

109. Hoelscher, M., et al. High-level expression of the HIV entry inhibitor griffithsin from the
plastid genome and retention of biological activity in dried tobacco leaves. *Plant molecular

110. Emau, P., et al. Griffithsin, a potent HIV entry inhibitor, is an excellent candidate for anti-
HIV microbicide. *Journal of medical primatology* 36, 244-253 (2007).


311. WHO. People Living with HIV/AIDS.


314. Fuqua, J.L. Q-GRFT Enema Development Supporting a Multi-Administration Clinical Study.


