

SMART HYDROGEL FOR ENZYME RESPONSIVE VAGINAL DELIVERY OF
ANTI-HIV PEPTIDE THERAPEUTICS

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by
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2021

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Taj Kumari Yeruva, Doctor of Philosophy

University of Missouri-Kansas City, 2021

ABSTRACT

In response to an urgent need for advanced formulations for the delivery of anti-retrovirals, a stimuli sensitive hydrogel formulation that intravaginally delivers HIV-1 entry inhibitor upon being exposed to a specific protease was developed. The hydrogel formulation consists of PEG-azide and PEG-DBCO covalently linked to the entry inhibitor peptide, Enfuvirtide via substrate linker. The substrate linker is designed to undergo proteolysis by prostate specific antigen (PSA) present in seminal fluid and release innate Enfuvirtide.

Of the tested PSA substrate linkers (HSSKLQYY, GISSFYSSK, AYLMYY and AYLMGRR), HSSKLQ was found to be an ideal candidate for PEG-based hydrogel with k_{cat}/K_M of $2.2 \text{ M}^{-1} \text{ s}^{-1}$. The PEG-based hydrogel displayed a pseudoplastic, thixotropic behavior with overall viscosity varying between 1516 Pa. s to 2.2 Pa. s, within the biologically relevant shear rates of $0.01\text{-}100 \text{ s}^{-1}$. It also exhibited viscoelastic properties appropriate for uniform spreading and being retained in vagina.

PEG-based hydrogels were loaded with N3-HSSKLQ-Enfuvirtide (HF42) that is customarily synthesized Enfuvirtide prodrug with its N-terminus connected to HSSKLQ linker. The stimuli sensitive PEG-based hydrogel formulations released $31.3 \pm 8.7\%$ of Enfuvirtide upon being exposed to PSA at pH 7.4 and $45.5 \pm 6.5 \%$ of Enfuvirtide at pH 8.6 over 24 hr., both of which are significantly greater than its IC_{50} . The PEG-based hydrogel was non-cytotoxic to vaginal epithelial cells (VK2/E6E7) and murine macrophages (RAW 264.7) and did not significantly induce production of nitric oxide, an inflammatory mediator.

The PEG-based hydrogel is found to have suitable physicochemical properties for an intravaginal formulation of the PSA substrate linked anti-retrovirals and is safe towards vaginal epithelium. It is capable of delivering Enfuvirtide with effective concentrations and has the potential to be used in clinical setting for effective prevention of HIV-1.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies, have examined the dissertation titled "Smart Hydrogel for Enzyme Responsive Vaginal Delivery of Anti-HIV Peptide Therapeutics" presented by Taj Kumari Yeruva, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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CONTENTS

ABSTRACT	iii
LIST OF ILLUSTRATIONS	ix
LIST OF TABLES.....	xv
ACKNOWLEDGEMENTS.....	xvi
Chapter	
1. INTRODUCTION	1
1.1. HIV- Causative Agent of AIDS	1
1.2. Global Burden of HIV	7
1.3. HIV Infection in Women.....	11
1.4. HIV Transmission	13
1.5. HIV Treatment.....	18
1.6. HIV Prevention.....	25
2. BIOMEDICAL STRATEGIES TO PREVENT VAGINAL TRANSMISSION OF HIV IN WOMEN.....	33
2.1. Introduction	33
2.2. Vaginal Acquisition of HIV	34
2.3. Prevention Strategies for Vaginal Transmission of HIV-1	41
2.4. Microbicide Formulation and Delivery	51

3. CURRENT STATE OF STIMULI SENSITIVE MICROBICIDES.....	69
3.1. Introduction	69
3.2. Temperature Responsive Microbicides	71
3.3. pH Responsive Microbicides.....	74
3.4. gp120 Responsive Microbicides.....	78
3.5. Enzyme Responsive Microbicides	81
4. DEVELOPMENT AND EVALUATION OF SMART HYDROGEL FOR HIV-1 PREVENTION	84
4.1. Rationale.....	84
4.2. Design of PEG-Based Smart Hydrogel	86
4.3. Development of PEG-Based Smart Hydrogel.....	88
4.4. Physicochemical Characterization of PEG-based Smart Hydrogel	126
4.5. Assessment of Efficacy and Safety of Hydrogel.....	130
4.6. Conclusions.....	137
5. SUMMARY, SCOPE, AND RECOMMENDATIONS.....	138
5.1. Summary	138
5.2. Scope	139
5.3. Recommendations.....	141
APPENDIX	142
REFERENCES.....	150

VITA182

LIST OF ILLUSTRATIONS

Figure 1. Depiction of HIV structure. a) schematic diagram of HIV indicating location of structural proteins, b) electron micrograph of HIV virion. (reprinted with permission from ref. ⁷ and ⁴).....	4
Figure 2. Schematic diagram of HIV-1 genome with viral genes drawn based on relative orientation in the entire RNA genome. (reprinted from ref. ¹¹)	4
Figure 3. HIV infection and AIDS. a) Changes in HIV RNA levels with progression of diseases during prototypic HIV infection. b) Typical CD4 ⁺ T cell number from the initial point of acquisition until development of AIDs. (Reprinted with permission from ref. ¹²)	6
Figure 4. People living with HIV in 2020 in different regions across the world. Data obtained from UNAIDS.	8
Figure 5. Death rates from HIV/AIDS measured as the number of deaths per 100,000 individuals across various age categories. (Graph adopted from ref. ¹⁵).....	9
Figure 6. Global prevalence, new cases, and deaths from HIV/AIDS over the years from 1990 to 2017. (Graph adopted from ref. ¹⁵).....	10
Figure 7. Higher number of HIV infection in females compared to males since 1990. (Graph adopted from ref. ¹⁵).....	12

Figure 8. Different classes of antiretrovirals were developed to target different stages in HIV life cycle for inhibition of viral replication. (Reprinted from “HIV Sites for Therapeutic Intervention” by BioRender.com) 19

Figure 9. Millions of lives saved since the first approval of ART and introduction of combination ART in 1990s ¹⁵..... 25

Figure 10. Implementation of PMTCT reduced HIV transmission from mother to child and averted new HIV infections in children ¹⁵. 30

Figure 11. Histological Structure of the Female Reproductive Tract. A. Simple columnar epithelium of the endocervix. B. Stratified squamous epithelium of the ectocervix and vagina. Fluorescent deconvolution images of rhesus macaques C. endocervical epithelium. D. ectocervical epithelium. E. vaginal epithelium. (Adopted from ref. ⁵⁸) 35

Figure 12. Potential mechanisms for HIV-1 transmission across mucosal epithelium. (Reprinted with permission from ref. ²⁵)..... 37

Figure 13. Time frame, sites and major events in vaginal transmission and window of opportunities for prevention. (Reprinted with permission from ref. ⁶³)..... 40

Figure 14. Topical strategies to prevent vaginal HIV transmission. (reprinted with permission from ref. ⁶⁶)..... 42

Figure 15. Depiction of Intravaginal ring. a. Vaginal ring is flexible, and image showing ring position after insertion. b. Women holding the dapivirine vaginal ring developed by international partnership for microbicides (IPM).	59
Figure 16. Sol-to-Gel transition of nanoparticle embedded thermosensitive gel following vaginal application.	72
Figure 17. Design of PEG-based hydrogel and release of anti-retroviral peptide upon exposure to prostate specific antigen (PSA) as an enzymatic stimulus.	87
Figure 18. A. HPLC chromatogram of purified HSKLQYY and B. corresponding ESI-MS spectrum showing MH ⁺	91
Figure 19. Hydrolysis of HSKLQYY by PSA to products HSKLQ and YY.	92
Figure 20. A. Chromatogram for product (YY) peak of HSKLQYY hydrolysis over 4 hr., B. ESI-MS spectrum of YY showing MH ⁺	95
Figure 21. C. PSA progress curve with varying concentrations of HSKLQ, D. Michaelis-Menten saturation curve.	96
Figure 22. A. HPLC chromatogram of purified GISSFYSSK and B. corresponding ESI-MS spectrum showing MH ⁺	98
Figure 23. Hydrolysis of GISSFYSSK by PSA to products GISSFY and SSK.	99
Figure 24. A. Chromatogram for product (GISSFY) peak of GISSFYSSK hydrolysis over 4 hr., B. ESI-MS spectrum of GISSFY showing MH ⁺	101

Figure 25. A. PSA progress curve with varying concentrations of GISSFYSSK, B. Michaelis-Menten saturation curve.....	102
Figure 26. A. HPLC chromatogram of purified AYLMYY and B. corresponding ESI-MS spectrum showing MH ⁺	104
Figure 27. A. HPLC chromatogram of purified AYLMYY and B. corresponding ESI-MS spectrum showing MH ⁺	106
Figure 28. Chromatogram of AYLMGRR depicting its stability upon exposure to PSA.	108
Figure 29. Chromatogram of Enfuvirtide depicting its stability upon exposure to PSA.	110
Figure 30. Synthetic scheme of 4 arm PEG-DBCO from 4 arm PEG-amine	112
Figure 31. Synthetic scheme of 4 arm PEG-azide from 4 arm PEG-OH.....	113
Figure 32. A. NMR spectrum of purified PEG DBCO and B. FTIR spectrum of amide peaks (c=o stretching at 1662 cm ⁻¹ and N-H bending at 1558 cm ⁻¹) as PEG DBCO is formed from PEG-NH ₂	115
Figure 33. A. NMR spectrum of purified PEG azide, and B. FTIR spectrum showing azide peak at 2098 cm ⁻¹ as PEG azide is formed from PEG OH.....	116
Figure 34. Hydrolysis of CF43 by PSA to its product Enfuvirtide.....	117
Figure 35. Chromatogram shows decrease in substrate (CF43) and increase in product (Enfuvirtide) after 4 hours of proteolysis by PSA.	118

Figure 36. Synthesis of Azide-CF43 from CF43 and Azido-PEG3-Maleimide using thiol-maleimide click reaction.....	120
Figure 37. Reconstructed mass of Azide-CF43.	120
Figure 38. Hydrolysis of Azide-CF43 by PSA to its product Enfuvirtide.....	121
Figure 39. Chromatogram shows decrease in substrate (Azide-CF43) and increase in product (Enfuvirtide) over 24 hours of proteolysis by PSA.	121
Figure 40. Proteolysis of HF42 by PSA in sodium carbonate buffer over 24 hr. A. Chromatograms show decrease in substrate (HF42) and increase in product (Enfuvirtide). B. Enfuvirtide was released from the formulation in a linear fashion. C. Reconstructed mass of substrate (HF42) D. Reconstructed mass of Enfuvirtide.	123
Figure 41. FTIR spectra of PEG-DBCO, HF42 and the conjugated product. The azide peak was not detected as a result of triazole formation between HF42 and PEG DBCO.	125
Figure 42. SEM images of hydrogel showed highly porous structure with interconnecting micro and macropores.	127
Figure 43. Rheological properties of the PEG hydrogel. A. Shear modulus, loss modulus and $\tan \delta$ as a function of frequency at 15% strain, B. Creep at a stress of 10 Pa for 320 sec. followed by recovery upon unloading, C. Flow rheogram shows pseudoplastic flow with zero shear rate and infinite shear viscosities, D. Hysteresis in upwards/downward flow sweep of hydrogel.....	129

Figure 44. The amount of enfuvirtide released from hydrogel at pH 7.4 (A) and pH 8.6 (B). The control represents the drug loaded hydrogel that is not treated with PSA, and the Test represents drug loaded hydrogel treated with PSA. Data shows mean \pm SD (n=3). T-test on the data shows a significant difference between the means of the groups ($p < 0.001$)..... 131

Figure 45. in vitro cytotoxicity studies showing A) cell viability and B) nitric oxide (NO) production of VK2/E6E7 and RAW 264.7 cells..... 135

Figure 46. Design of long-term delivery system for PSA responsive delivery of anti-retrovirals..... 140

LIST OF TABLES

Table 1. Estimated per-act probability of acquiring HIV by exposure route.	14
Table 2. Probability of HIV-1 transmission.	17
Table 3. FDA approved HIV medicines.....	23
Table 4. HPLC method for monitoring HSSKLQYY hydrolysis by PSA.....	93
Table 5. HPLC method for monitoring GISSFYSSK hydrolysis by PSA.....	100
Table 6. HPLC method for monitoring AYLMGRR hydrolysis by PSA.....	107
Table 7. The rate constants of substrate linkers hydrolyzed by PSA.	109
Table 8. HPLC method for monitoring enfuvirtide hydrolysis by PSA	110

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DEDICATION

This dissertation is dedicated to my mother Saida Kumari Lakshmi Rajyam Yeruva
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CHAPTER 1

INTRODUCTION

1.1. HIV – Causative Agent Of AIDS

On June 5, 1981 – CDC discussed *Pneumocystis pneumonia*, a rare lung infection among group of young active gay men in Los Angeles, California in *Morbidity and mortality weekly report (MMWR)*¹. Such unusual opportunistic infections among healthy homosexual men were noticed by public health workers and physicians in the years prior. However, CDC recorded the phenomenon for the first time and was termed as Acquired Immune Deficiency Syndrome (AIDS) on July 27th, 1982².

Reports of AIDS quickly emerged across the globe indicating rapid spread of the disease and the world was suddenly faced with devastating new epidemic. Social stigma and policy makers inactivity compounded the issue. Medical and scientific community reacted promptly and worked with limited resources to identify the cause of this deadly epidemic. Based on the evidence obtained from epidemiologic data, cause of AIDS was speculated to be an infectious agent³. Reports of AIDS in hemophiliacs gave a clue about the nature of the transmissible agent. In 1983, Luc Montagnier at Institute Pasteur, Paris, France has isolated a retrovirus, now described as Human Immunodeficiency Virus (HIV-1) for the first time from a lymph node biopsy sample of a 33-year-old homosexual patient who showed symptoms that

precede AIDS ⁴. Later several research groups isolated the virus from AIDS patients and confirmed the cause of AIDS as HIV ⁵. Another virus was isolated in 1986 from west African AIDS patients and was called as HIV-2 ⁴. Majority of HIV infections globally are associated with more dominant and pathogenic strain i.e., HIV-1.

Origin of HIV

Reasons for sudden emergence of HIV and its unique pathogenicity were intensely studied since the discovery of HIV. Isolation of HIV-2 gave the first clue about the origin of HIV as it was closely related to simian immunodeficiency virus (SIV) ⁶. More than 40 different non-human primate species carry species specific SIV. HIV originated from several zoonotic transmissions of SIVs from non-human primates such as chimpanzees and sooty mangabeys to humans in West and Central Africa. Interestingly, SIVs are not pathogenic in their natural hosts. The source of cross-species transmission is still under debate. However, the most likely source of transmission was reported to be exposure of humans to animal blood and mucosal secretions because of hunting and eating bushmeat. Several HIV lineages: HIV-1 groups M to P and HIV-2 groups A-H were generated from independent zoonotic transmissions of SIV to HIV. Each group contributes to the epidemic in different proportions ⁷. HIV-1 group M is responsible for global pandemic, whereas group O causes tens of thousands of infections. Group N and P are known to cause only a handful of infections ⁸.

Structure and genome of HIV

HIV belongs to the *Lentivirus* genus of the *Retroviridae* family. Overall size of HIV ranges from 119 to 207 nm and is spherical in shape as shown in Figure 1b⁹. Like other retroviruses, each HIV virion contains two identical single strands of RNA. This genetic information along with viral enzymes, protease, reverse transcriptase, and integrase are packaged inside a cone shaped capsid that is distinct to HIV. Capsid consists of nucleocapsid protein p7/p9 and major capsid protein p24. Matrix protein p17 surrounds the viral capsid and is located under the virion envelope. The viral envelope is composed of lipid bilayer membrane and tetrameric envelope protein complex which consists of outer envelope protein (SU) gp120 and transmembrane protein (TM) gp41 as depicted in Figure 1a¹⁰.

Reverse transcription of viral RNA to DNA generates proviral DNA which contains approximately 9800 nucleotide base pairs. Ends of provirus are flanked by long terminal repeat promoter regions with genes encoding structural, regulatory, and accessory proteins as shown in Figure 2. First open reading frame *gag* encodes for

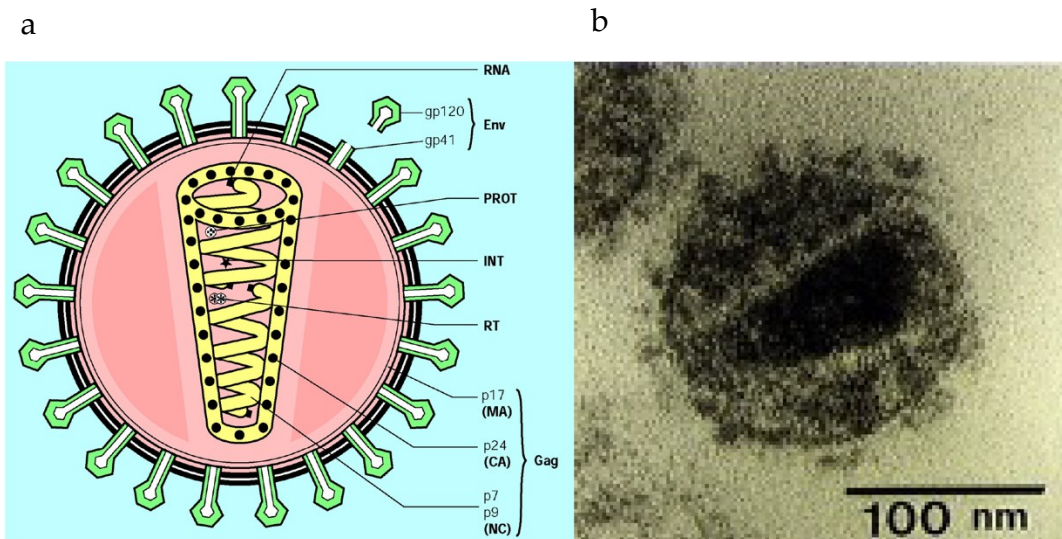


Figure 1. Depiction of HIV structure. a) schematic diagram of HIV indicating location of structural proteins, b) electron micrograph of HIV virion. (reprinted with permission from ref. ⁷ and ⁴)

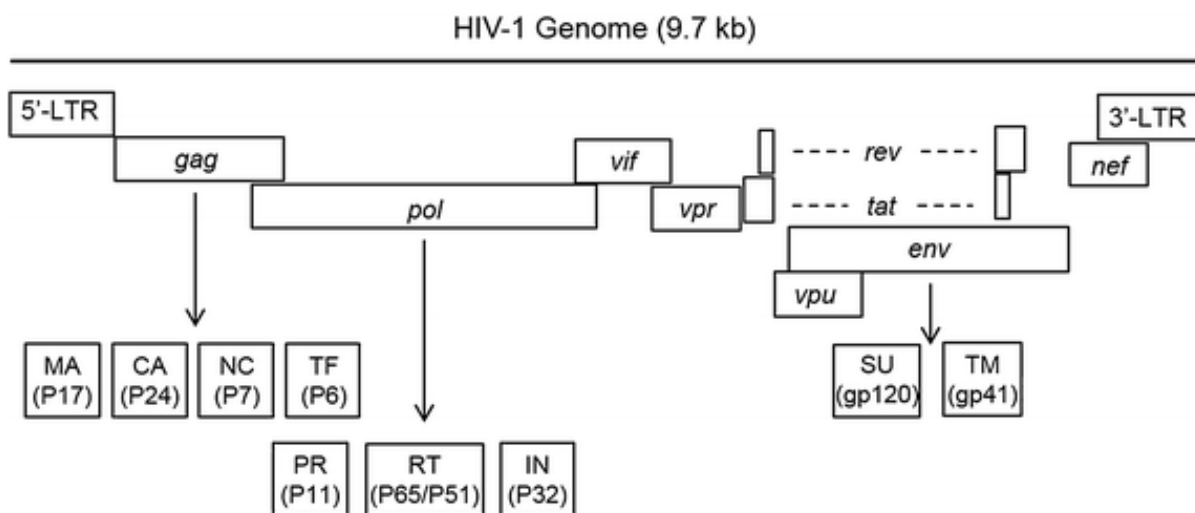
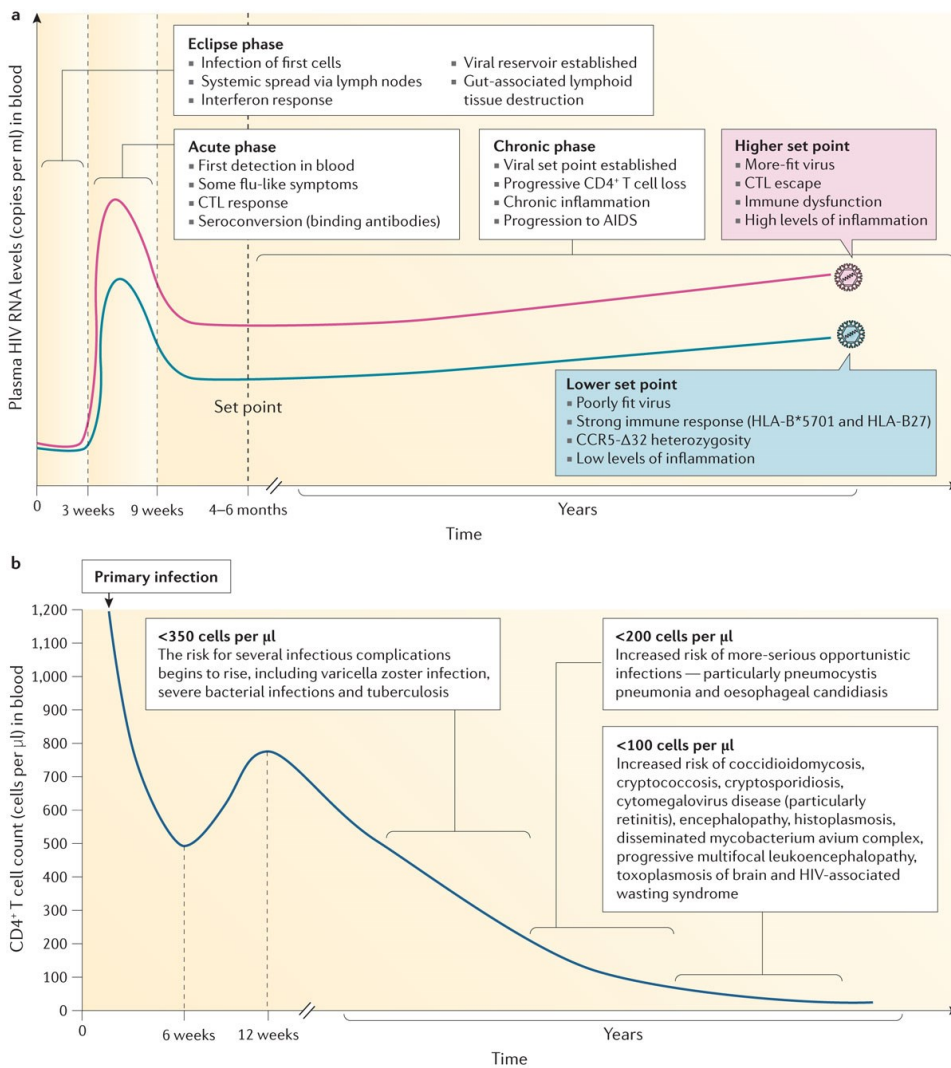


Figure 2. Schematic diagram of HIV-1 genome with viral genes drawn based on relative orientation in the entire RNA genome. (reprinted from ref. ¹¹)

the matrix protein p17, major capsid protein p24 and nucleic capsid protein p7/p9. Matrix protein plays a role in the formation and transport of pre-integration DNA complex into the host cell nucleus. The enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) are encoded by second open reading frame *pol*. *Env* gene encodes for the envelope proteins gp120 and gp41¹¹. The envelope protein complex of gp120 and gp41 are integral for fusion of virion with host cell membrane. In addition, HIV genome consists of *vif*, *vpr*, *vpu*, *rev*, *tat* and *nef* genes encoding regulatory and accessory proteins. Accessory proteins play a role in immune evasions and vital for viral spread *in vivo*.

HIV infection and disease progression

HIV primarily infects CD4⁺ T lymphocytes. Host receptor CD4 and co-receptors CCR5 and CXCR4 are essential for virus-host interactions and subsequent infection of individual cells. After initial transmission event, HIV takes hold in the mucosal tissues and spreads to the draining lymph nodes within days. The virus can be detected in blood at about 10th day and replicates exponentially for few weeks and often peaks at day 30. This period of exponential increase of viral RNA and drastic decrease in CD4⁺ T cells is called acute phase. Individuals are most infectious during acute phase because of high viral titer. CD4⁺ T cells are briefly recovered, and viral



Nature Reviews | Disease Primers

Figure 3. HIV infection and AIDS. a) Changes in HIV RNA levels with progression of diseases during prototypic HIV infection. b) Typical CD4⁺ T cell number from the initial point of acquisition until development of AIDS. (Reprinted with permission from ref. ¹²)

load is decreased following acute phase as the immune system gains a degree of control, but then viral load is increased, and a set point is established with relatively stable replication level of HIV for years. This stage of stable HIV viral load is associated with chronic phase and causes progressive loss of CD4⁺ T cells ¹³.

As the CD4⁺ T cells play an important role in the body's immune system and defense against invading pathogens, loss of 1-2 billion CD4 T cells daily causes immunological abnormalities. After several years of initial transmission, individual develops characteristic infections and oncological complications due to profound immunodeficiency. This is the end stage of HIV infection where CD4⁺ T cells per microliter of blood falls below 200 is termed as acquired immunodeficiency syndrome (AIDS). Figure 3 shows the progression of HIV to AIDS graphically. Typical person progresses to the stage of AIDS over a period of 10 years with some progressing rapidly and a some slowly ¹³.

1.2. Global Burden Of HIV

79 million people have contracted HIV since the first cases of AIDS were reported 40 years ago. Since then, 36 million people have died due to AIDS related illness ¹⁴. Initial years of the epidemic were full of suffering with little hope. Individuals, families, and communities were devastated by the epidemic. The epidemic has disrupted economic growth, community life and left millions of children

orphaned. Majority of global infection are in developing nations and communities with poor health infrastructure and social inequalities.

As shown in **Figure 4**, African continent was most hit by the pandemic. Sub-Saharan Africa has the highest infection level with lowest economic stability and access to care. AIDS death rate is highest among the age group of 15 - 49 years which are most economically productive years in human life and effects family's financial situation and country's economy negatively as shown in Figure 5. In addition, HIV increases the burden of other infections such as Tuberculosis.

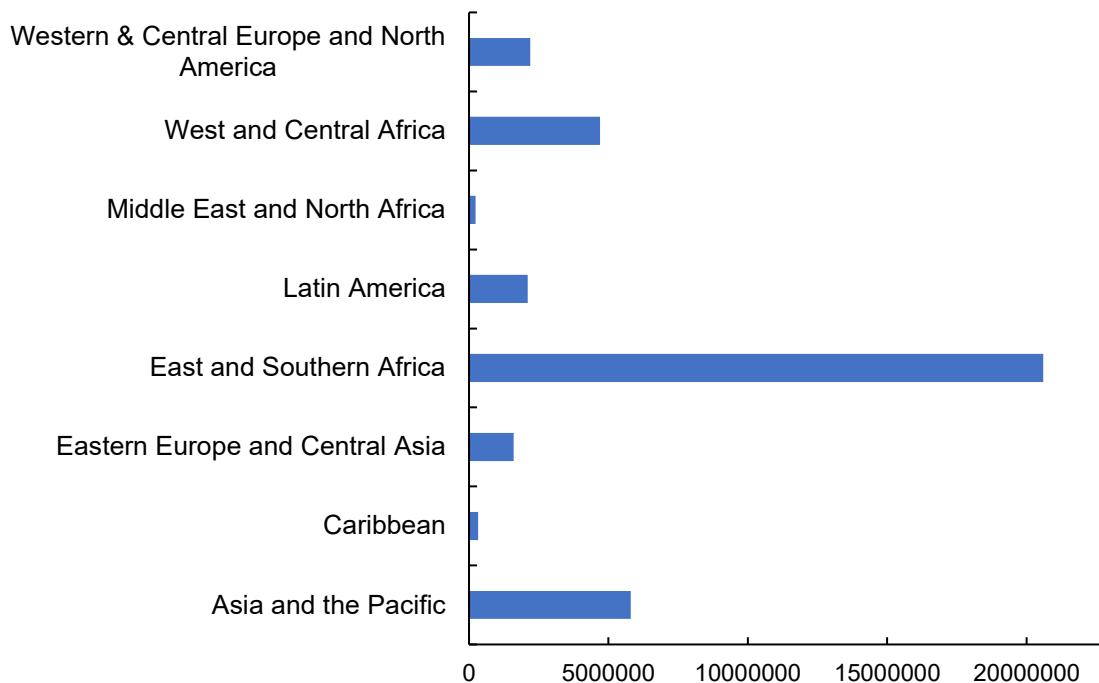


Figure 4. People living with HIV in 2020 in different regions across the world. Data obtained from UNAIDS.

HIV/AIDS death rates by age, World, 1990 to 2017

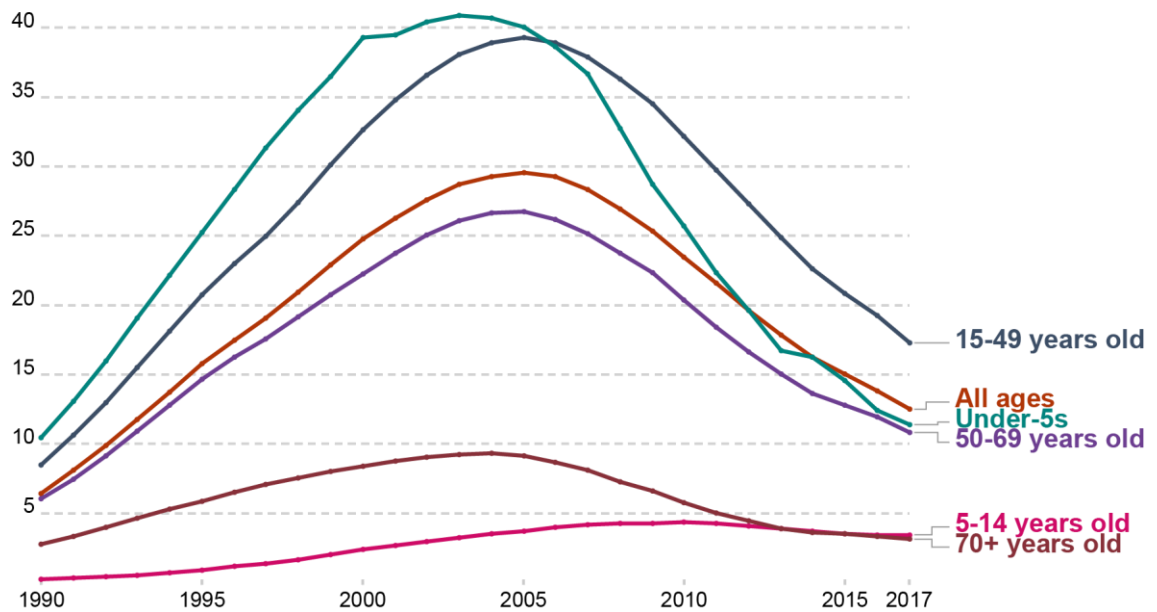


Figure 5. Death rates from HIV/AIDS measured as the number of deaths per 100,000 individuals across various age categories. (Graph adopted from ref. ¹⁵)

New HIV infections were reduced by 47% since its peak in 1998 and AIDS related deaths declined by 64 % since the peak in 2004. However, we are very far from eradication ¹⁴. In 2020 alone, 680,000 people died of AIDS related illness and 1.5 million people got infected with HIV ¹⁴. HIV is one of the main causes of mortality worldwide. Furthermore, COVID 19 had complicated the HIV management and people living with HIV experienced severe outcomes and comorbidities compared with people who do not have HIV. Sub-Saharan Africa which is home to 67% of total HIV infections did not have access to COVID 19 vaccines which increases the HIV burden as risk of dying from COVID 19 is double in people with HIV than general

population ¹⁴. In addition, HIV testing decreased by 41% during first lockdown in 2020 compared with 2019 as per data collected from African and Asian countries ¹⁴. Due to the impact of COVID 19, AIDS can become resurgent pandemic. We have gained knowledge from 4 decades of research and experience from dealing with the AIDS pandemic. We should implement the tools necessary to prevent HIV infections and stop it from being a never-ending pandemic.

Prevalence, new cases and deaths from HIV/AIDS, World, 1990 to 2017

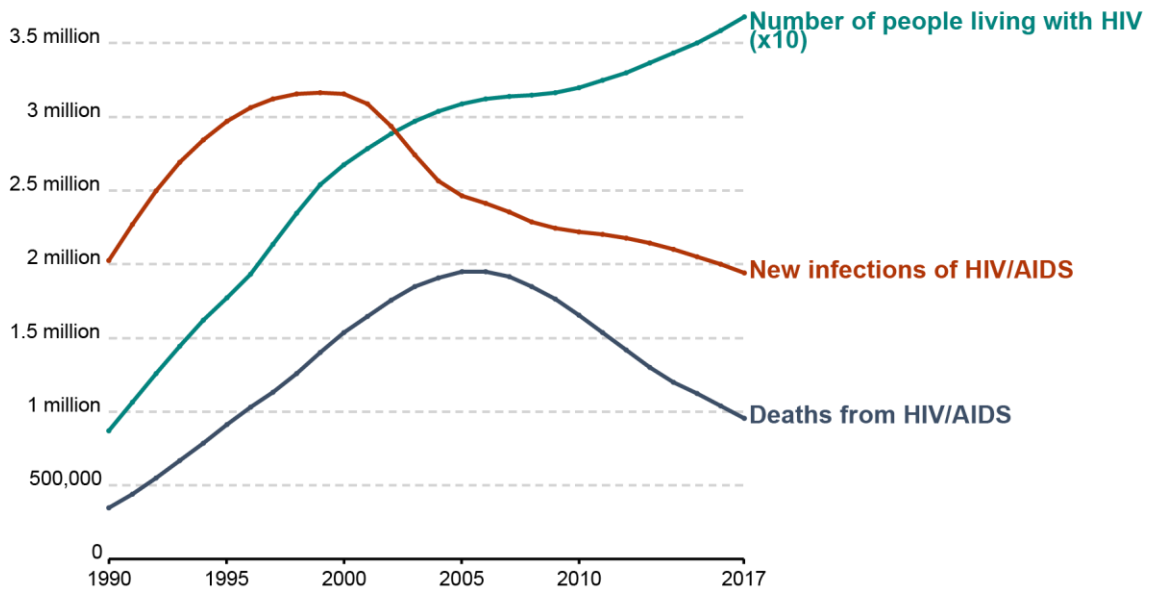


Figure 6. Global prevalence, new cases, and deaths from HIV/AIDS over the years from 1990 to 2017. (Graph adopted from ref. ¹⁵)

1.3. HIV Infection in Women

HIV is prevalent among women at unacceptably high incident rates as per epidemiological evidence. Women and girls account for 53 % of global HIV population. In 2020, 50 % of all new infections are among women and girls ¹⁴. Statistics show consistently higher number of infections among adolescent (10-19) and young (15-24) females compared to males of same age group. HIV infections among young women are 60 % higher than men of same age. Young women and adolescent girls are highly vulnerable to HIV infection and 7000 young women get infected with HIV every week ¹⁶. Figure 7 shows the higher number of infections among females compared to males consistently over the decades. In Sub-Saharan Africa, six in seven new infections among adolescents are girls and 63 % of all new infections correspond to women and girls in 2020. Transgender women are 34 times riskier at acquiring HIV ¹⁴. In addition, HIV infected women contribute to HIV infections in children by vertical transmission which contributes to global disease burden. More than 90% infections in children are acquired from their mother. HIV infections in women also leads to other complications such as adverse pregnancy outcomes and invasive cervical cancer which is not the case for men. Women living with HIV are five times more likely to develop cervical cancer ¹⁷.

Adults (15+) living with HIV - by sex

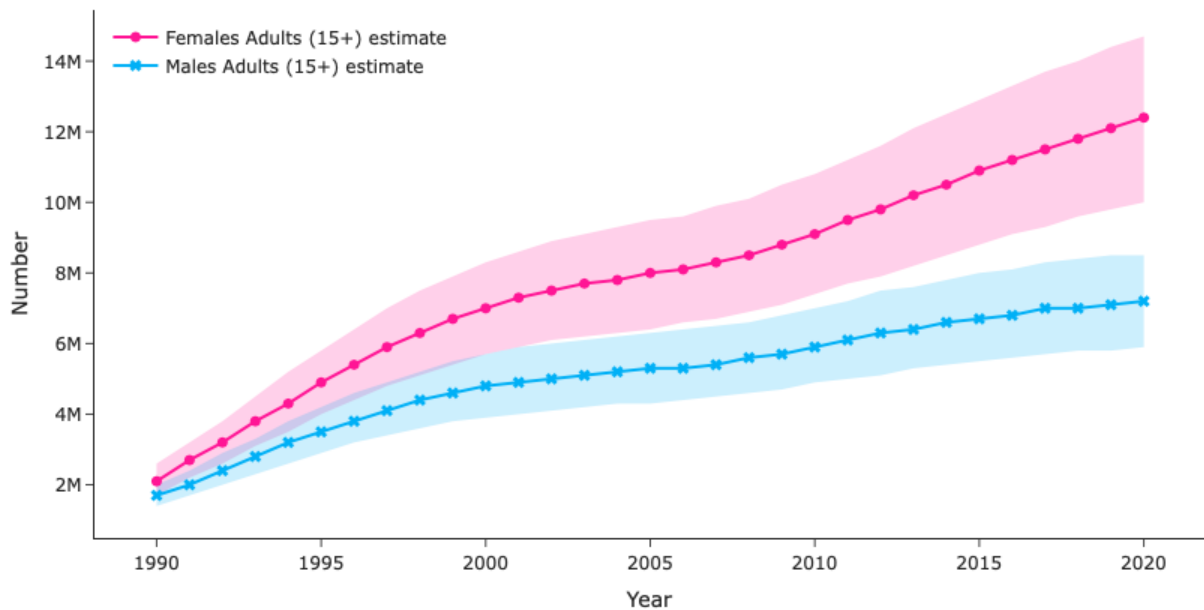


Figure 7. Higher number of HIV infection in females compared to males since 1990. (Graph adopted from ref. ¹⁵)

This disparity of HIV infection among males and females can be attributed to several factors including biological, social, cultural, and structural ¹⁸. Women are biologically more susceptible to acquiring HIV than men as they have greater mucosal surface area exposed to infectious fluids for longer periods during sexual intercourse ¹⁸. Younger women are particularly at high risk as cervical ectopy facilitates high exposure to pathogens ¹⁸. Dominant patriarchal culture and gender inequality in developing countries especially in Sub-Saharan Africa create disparities in their health status. In some regions, women are not allowed to make sexual decisions and would face serious consequences if they were to refuse sexual advances or suggest condom

use. Gender based violence is a global epidemic and is a risk factor for acquiring HIV. One in three women worldwide experienced sexual violence at least once in their lifetime ¹⁴. As per epidemiological estimates, 243 million women and girls globally experienced violence perpetrated by intimate partner in last 12 months ¹⁹. Also, women who have experienced violence are 1.5 time more likely to get infected with HIV than women who have not experienced violence ¹⁶. Rape which is a form of sexual violence is one of the least reported crimes in South Africa. It is estimated that one million rapes occur every year in some regions ²⁰. Poverty and poor economic position are another driving forces for HIV infections in women. Many women opt for transactional sex for their livelihood and young women are forced into sexual activities with older men to earn survival ^{18 20}. Female sex workers are 30 times more likely of getting infected with HIV compared with general population. Biomedical, behavioral, and structural interventions must be investigated in the context of HIV incidence and prevention in women and girls.

1.4. HIV Transmission

Understanding routes of HIV transmission is important in assessing the risk and spread of infection among a population and to design appropriate prevention strategies. Like other viruses, HIV cannot survive outside the cell. HIV must be transmitted from person to person, and it passes through exchange of infected body

fluids such as blood and blood products, seminal fluid, vaginal secretions, and breast milk. Risk of acquiring HIV through different routes was discussed in Table 1.

Table 1. Estimated per-act probability of acquiring HIV by exposure route.

Exposure route	Risk per 10,000 exposures to an infected source
<i>Parenteral exposure</i>	
Blood transfusion	9250
Needle-sharing injection drug use	63
Percutaneous needle stick	23
<i>Sexual exposure</i>	
Receptive anal intercourse	138
Insertive anal intercourse	11
Receptive penile-vaginal intercourse	8
Insertive penile-vaginal intercourse	4
Receptive oral sex	Low
Insertive oral sex	Low
<i>Vertical transmission</i>	
Mother-to-child transmission	2260

Table reproduced with permission from ref. ²¹

Transmission through contaminated blood

Transmission of HIV through transfer of infected blood to uninfected person was clearly established. This has been major mode of transmission among injection drug users who share needles. Blood transfusion has the highest estimated per act

transmission risk. Transmission of HIV through blood transfusions and organ transplantations was once a concern in some countries has been almost eliminated through routine screening for HIV antibodies ²². HIV transmission was only implicated in transplantation of highly vascular tissues such as skin and large bone whereas transmission from transplantation of avascular tissues was not reported ²³. These observations suggest the risk of transmission through infected blood. Rigorous screening of donors and allografts for HIV antibodies along with p24 antigen will eliminate the infections due to organ transplantations and blood transfusions.

Mother to child transmission

Transmission of HIV from HIV infected mother to child before or during childbirth or post-natal transmission via breast feeding is called mother to child transmission (MTCT). MTCT transmission rate was estimated to be 2260 per 10,000 exposures ²¹. MTCT can occur “a) in utero by direct hematogenous transplacental spread or ascending infection of the amniotic membranes and fluids, b) during delivery by mucocutaneous contact of the infant with maternal blood, amniotic fluid, and cervicovaginal secretions during passage through the birth canal; ascending infection from the cervix; or maternal-fetal transfusion from uterine contractions at labor and delivery, c) during breastfeeding by breastmilk” ²⁴. Risk of MTCT of HIV was influenced by viral, host and obstetric factors. Viral factors include maternal viral load in plasma, peripheral blood, genitourinary tract, breast milk and viral

characteristics such as phenotype, antiretroviral resistance, and capacity for immune escape. Host factors include stage of HIV disease, maternal and breast milk immune factors. Obstetric factors include mode of delivery, timing of delivery, invasive monitoring, obstetric procedures, and duration of ruptured amniotic membranes ²⁴.

Sexual transmission

Sexual contact is an effective means of HIV transmission as the tissues of anus, rectum and vagina are mucosal surfaces that can contain infected body fluids and can be easily injured allowing the viral invasion. Even though AIDS was first reported in homosexual men in United states and male to male transmission dominates in industrialized countries, heterosexual transmission is the predominant mode of transmission worldwide and is responsible for nearly 80% of HIV-1 infections despite the lower transmission probability per coital act which is estimated to be 0.0001 – 0.0040 depending on route of transmission which is indicated in Table 2 ²⁵. As the anal tissues are more prone to damage during sexual activity, risk of acquiring HIV through anal intercourse is five times higher than vaginal intercourse. Passing of HIV from a woman to a man during intercourse is less likely as the skin of the penis is not easily damaged. However, a woman is eight times more likely to get infected from a man through vaginal intercourse ²⁶. Penile-vaginal transmission of HIV-1 was reported at a frequency as high as 1 in 10 exposures depending on confounding risk cofactors such as genital ulcer disease, male circumcision, HIV disease stage, and

exposure route ^{22 26 27}. Though rare, female to female transmission of HIV is possible due to potential exposure of mucosal membranes to menstrual blood and vaginal secretions. It is less likely to transmit HIV during oral intercourse compared to anal or vaginal as oral tissues are less likely to get injured than vaginal or anal tissues during sexual activity ²⁶. However, risk of HIV acquisition through oral route increases if the person has oral infections or injuries ²⁶.

Table 2. Probability of HIV-1 transmission.

Route	Probability per coital act
Male to male transmission	1/10 - 1/1600
Male to female transmission	1/200 - 1/2000
Female to male transmission	1/200 - 1/10000

Table reproduced with permission from ref. ²⁵

Heterosexual transmission has been associated with several risk factors including high viral load, decreased CD4⁺ T cells in infected partner, presence of other sexually transmitted infections, and lack of circumcision. Epidemiological evidence strongly suggests the direct association between transmission and viral load in genital fluids. Presence of ulcerative and non-ulcerative sexually transmitted STDs promotes HIV infectiousness by disrupting normal mucosal barrier and recruiting immune cells

to the genital tract allowing access to HIV. Understanding mechanisms of HIV transmission through genital tract and associated risk factors is vital in developing effective prevention strategies as majority of infections are contributed by Heterosexual transmission.

1.5. HIV Treatment

Treatment of HIV patients with antiretroviral drugs has been the most significant advancement in the management of HIV-1. Anti-retroviral drugs (ARVs) stop HIV from multiplying and suppress viral load to undetectable levels. As a result, body retains ability to generate CD4⁺ T cells, maintains strong immunity, and wards off opportunistic pathogens. ARVs act by inhibiting different stages of viral replication process. Therefore, understanding HIV life cycle is needed to understand the mechanism of action of ARV drugs.

HIV life cycle and Antiretrovirals

HIV-1 life cycle is a complex multistep process that can be divided into several steps. It depends on sequential interactions between viral and host cell factors and offers multiple possibilities for therapeutic intervention as shown in Figure 8 ²⁸.

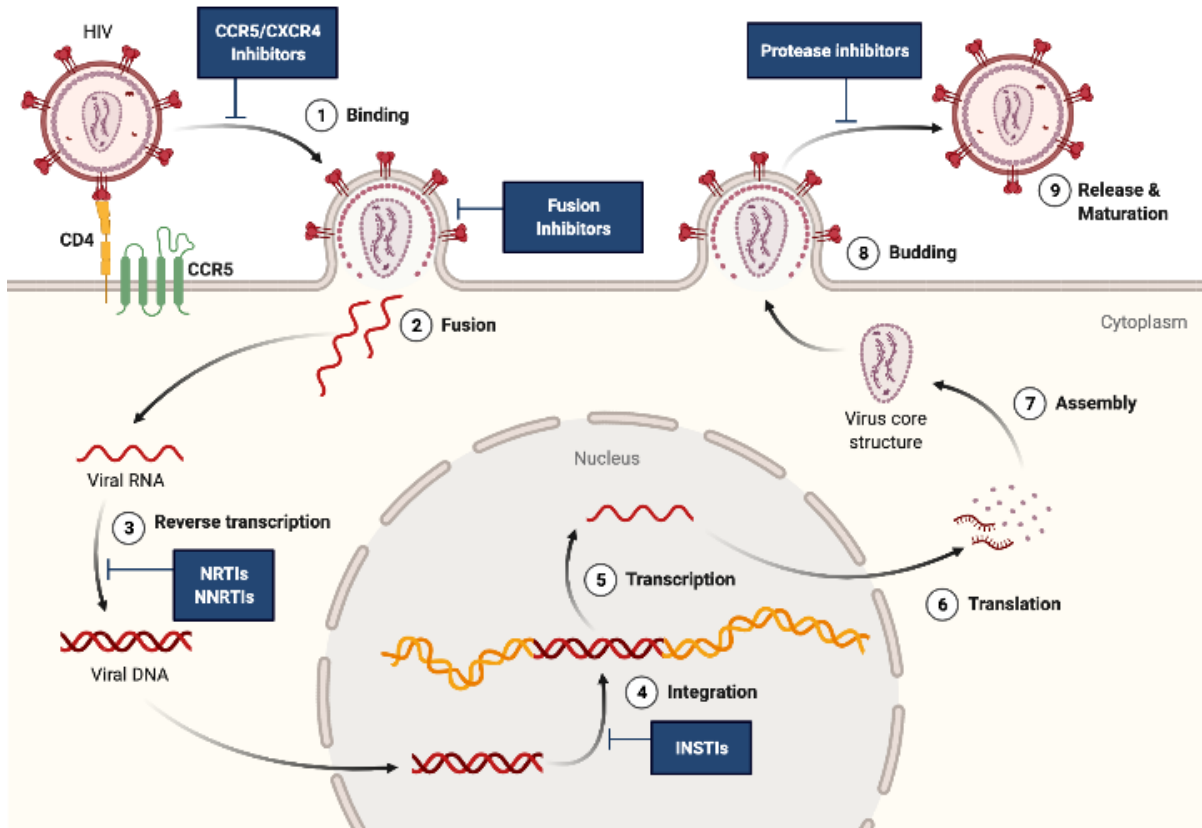


Figure 8. Different classes of antiretrovirals were developed to target different stages in HIV life cycle for inhibition of viral replication. (Reprinted from “HIV Sites for Therapeutic Intervention” by BioRender.com)

1) Viral entry: HIV has a very short time to find and infect a target cell as cell free virions have half-life of about 20-30 minutes in infected individual²⁹. CD4 is the primary receptor and chemokine receptors CCR5 and CXCR4 are the main co-receptors for HIV entry. Infection process is initiated by interaction of viral glycoprotein gp120 with CD4 receptor on the surface of T-lymphocytes and macrophages inducing conformational changes in envelope trimer allowing interaction of gp120 with either CXCR4 or CCR5 co-receptors. Additional

confirmation changes are induced because of co-receptor interaction allowing gp41 transmembrane protein to insert its hydrophobic fusion peptide into the cell membrane. This is followed by helical bundle formation pulling cellular and viral membranes together allowing viral fusion and release of viral contents into the host cell ²⁹. Antiretroviral that target these initial stages of replication process are called entry inhibitors which include coreceptor antagonists also called CXCR4/CCR5 inhibitors and fusion inhibitors.

2) Reverse transcription: Viral fusion into the host cell is followed by reverse transcription of single stranded viral RNA into double stranded DNA which is catalyzed by reverse transcriptase enzyme ²⁹. Nucleoside reverse-transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are two classes of drugs that inhibit this stage of viral replication. NRTIs act by inhibiting growing viral DNA chain where as NNRTIs act blocking reverse transcriptase enzyme.

3) Integration: Reverse transcribed double stranded DNA is transported across the nuclear membrane and inserted into the host genome by an enzyme called Integrase. The proviral DNA is replicated along with the host DNA and the host cell is infected for the rest of its life span. Infected host cell can undergo active viral production or remain latent ²⁹. Compounds that inhibit the integration of viral DNA into host genome are called Integrase inhibitors.

4) Transcription and translation: The Integrated DNA are transcribed to viral RNA and translated to long-chain polypeptide ²⁹. It is then cleaved into smaller individual structural and enzymatic proteins. Viral proteins and RNA are accumulated at the plasma membrane to assemble viral particles.

5) Budding and maturation: Progeny virions are release from the infected cell which is called budding. Release HIV particles are immature and noninfectious ³⁰. Viral protease turns these noninfectious particles to infectious and mature particles by cleaving Gag and Gag-pol precursors to generate electron-dense conical inner core ²⁹. Drugs that block this last step of HIV replication cycle are called protease inhibitors.

Antiretroviral therapy

The treatment of HIV infection using anti-retroviral drugs is called Anti-retroviral therapy. In the early years of the epidemic, the average life expectancy of a patient diagnosed with AIDS was approximately one year. First antiretroviral drug Zidovudine (3'-azido-3'-deoxythymidine (AZT)) was approved by FDA in 1987 ³¹. However, monotherapy was ineffective in long-term and lead to drug resistance. The development of combination antiretroviral therapy (cART) in the 1990s was one of the impressive achievements of medical science as patients who are treated with cART early in the course of HIV infection can have near normal life span ³¹. cART is composed of three drug combinations which consists of two NRTIs plus a third active drug from a different class. Contemporary cART is very effective at suppressing

viremia and lifelong, chronic therapy without interruption is the standard of care ³¹. Figure 9 shows the number of deaths due to treatment of HIV patients with ART. Incomplete adherence to the therapy results in development of drug resistance. Significant progress has been made towards regimen simplification through co-formulation of drugs to reduce pill burden and increase patient adherence ³². To address the limitation of everyday ART, long acting injectables were being investigated. In 2021, FDA approved CABENUVA, an intramuscular monthly injection containing cabotegravir and rilpivirine. Table 3 discusses the FDA approved drugs including cART since the start of the epidemic ³³. WHO recommends the initiation of ART for all people living with HIV regardless of CD4 cell count following confirmed diagnosis of the infection.

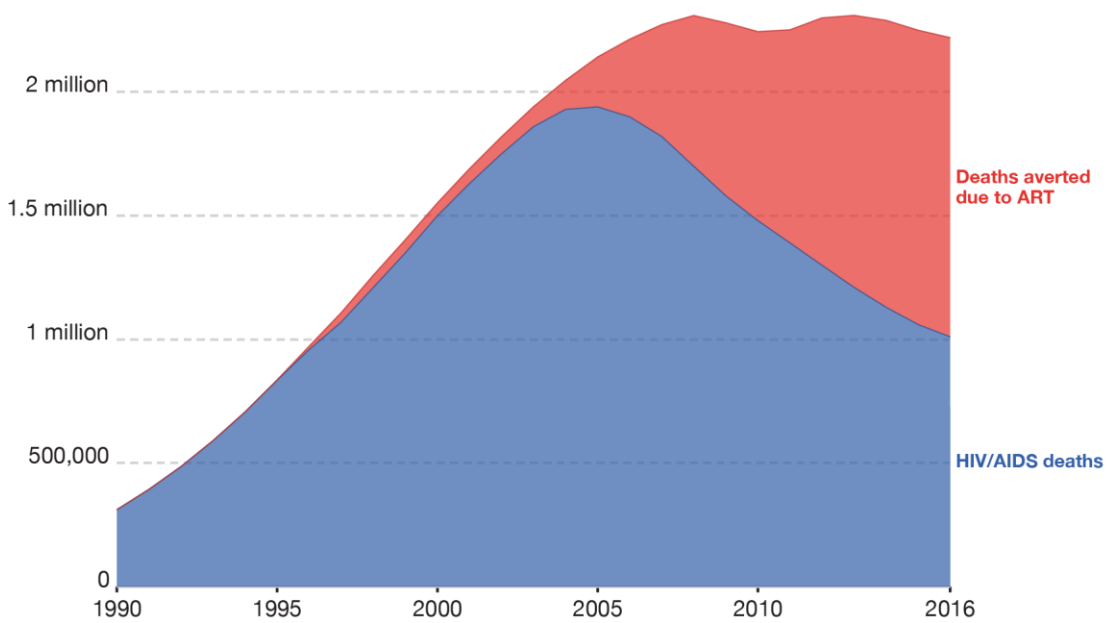
Table 3. FDA approved HIV medicines.

Drug Class	Generic name	Brand name	FDA approval year
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	abacavir (abacavir sulfate, ABS)	Ziagen	1998
	emtricitabine (FTC)	Emtriva	2003
	lamivudine (3TC)	Epivir	1995
	tenofovir disproxil fumarate (tenofovir DF, TDF)	Viread	2001
	Zidovudine (azidothymidine, AZT, ZDV)	Retrovir	1987
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	doravirine (DOR)	Pifeltro	2018
	efavirenz (EFV)	Sustiva	1998
	etravirine (ETR)	Intelence	2008
	nevirapine (extended-release nevirapine, NVP)	Viramune XR (extended release)	1996
	rilpivirine (rilpivirine hydrochloride, RPV)	Edurant	2011
Protease Inhibitors (PIs)	atazanavir (atazanavir sulfate, ATV)	Reyataz	2003
	darunavir (darunavir ethanolate, DRV)	Prezista	2006
	fosamprenavir (fosamprenavir calcium, FOS-APV, FPV)	Lexiva	2003
	ritonavir (RTV)	Norvir	1996
	saquinavir (saquinavir mesylate, SQV)	Invirase	1995
	tipranavir (TPV)	Aptivus	2005
Fusion Inhibitors	enfuvirtide (T-20)	Fuzeon	2003
CCR5 Antagonists	maraviroc (MVC)	Selzentry	2007
Table continued...			

Drug Class	Generic name	Brand name	FDA approval year
Integrase Strand Transfer Inhibitor (INSTIs)	cabotegravir (cabotegravir sodium, CAB)	Vocabria	2021
	dolutegravir (dolutegravir sodium, DTG)	Tivicay	2013
	raltegravir (raltegravir potassium, RAL)	Isentress Isentress HD	2007 2017
Attachment Inhibitors	fostemsavir (fostemsavir tromethamine, FTR)	Rukobia	2020
Post-Attachment Inhibitors	ibalizumab-uiyk (Hu5A8, IBA, Ibalizumab, TMB-355, TNX-355)	Trogarzo	2018
Pharmacokinetic Enhancers	cobicistat (COBI, c)	Tybost	2014

Table was replicated from ref. ³³

HIV/AIDS deaths and deaths averted due to antiretroviral therapy (ART), World, 1990 to 2016



Source: UNAIDS

OurWorldInData.org/hiv-aids • CC BY

Figure 9. Millions of lives saved since the first approval of ART and introduction of combination ART in 1990s ¹⁵.

1.6. HIV Prevention

Decline in new HIV infections has been very slow which increases the need for efficient prevention strategies. HIV prevention is complex and cannot be achieved by single strategy. Combination of strategies at individual and societal levels are required including structural and biomedical interventions. The primary goal of structural strategies is to change the individual risk behaviors to HIV infection.

Behavioral strategies

Behavioral strategies include attempts to stop sharing needles during substance use and avoiding risky sexual behavior. Individual behavioral changes such as limiting number of sexual partners, abstinence from sex, use of male and female condoms, never sharing needles, use of clean needles and syringes are facilitated around the globe by rendering condoms accessible, changing cultural practices, and promoting public policies that reduce stigma associated with HIV³⁴. Another key and unexpected element of behavioral strategies includes HIV testing. Frequent HIV testing and knowing HIV status early on has shown low HIV incidence in some communities.

Several instances of success were seen in lowering HIV incidence through behavioral strategies. For example, community-based program in Accra, Ghana, trained prostitutes to educate and distribute condoms among their peers. Reported condom use has increased dramatically 6 months after the intervention. Similar output was obtained from community-based program conducted among full-time prostitutes in Cross river state, Nigeria. Countries including Kenya, Haiti, Swaziland, Malawi, Zimbabwe, Namibia, Tanzania, and Brazil have reported decrease in HIV transmission associated with changes in sexual behaviors.

Lessons were learnt from intervention programs across the globe. Changes in HIV transmission rate requires radical behavioral changes from substantially large

numbers of people. Choice should be provided in terms of risk reduction strategies rather than imposing or prioritizing one over the other. Involvement from people who are affected by HIV in designing campaigns and disseminating messages is crucial for motivating behavioral change. Social norms and other structural and environmental factors along with individual behaviors should be focused for successful interventional programs ³⁵. Clear and repeated messages should be given using multiple available mass media channels for creating awareness and initiating behavior change.

Success in HIV prevention cannot be reduced to one or two elements. Despite the lessons learnt and the awareness of their impact, designing and implementing strategies that reverse or even halt HIV transmission rate was not successful. Factors such as external pressure, fear, denial, and economic status influences people from changing their behaviors and makes it harder to see change by mere implementation of behavioral strategies.

Vaccines

Successful vaccine is essential for ending HIV pandemic that takes lives of nearly one million people every year. Some of the most feared diseases such as polio, smallpox and yellow fever were only eradicated by use of vaccines. Scientists have been working on both preventive and therapeutic HIV vaccine. 40 years of scientific

work and billions of dollars spent on HIV vaccine research failed to develop successful vaccine for clinical use ³⁶.

Challenges in developing immunity against HIV were the reasons for such a failure. Lack of natural immunity against HIV made it harder to develop effective vaccine as traditional approaches such as attenuated virus may not induce immune response and are too dangerous to use in humans. In addition, continuous mutation of HIV as it replicates thousands of times in each infected cell produces several different variants making it impossible to develop a vaccine that can trigger immune system against all the strains. Highly glycosylated envelope of HIV adds to the existing complexity by evading detection by immune system. Another obstacle in developing effective vaccine was lack of animal model for HIV.

Efforts are put forth in many directions for development of vaccine with advances in recombinant technologies and understanding of molecular biology of HIV. Initial studies were based on B-cell targeted and T-targeted approaches. The cytotoxic T lymphocyte (CTL) approach was designed to lower viral set point and delay disease progression ³⁷. Several clinical trials were conducted which gave disappointing results such as VaxGen's AIDSVAXgp120 and MRKAd5 HIV-1 Gag/Pol/Nef vaccines. RV144 clinical trial conducted in Thailand using recombination of AIDSVAX B/E gp120 and ALVAC canarypox was a landmark study as it provided a glimmer of hope for HIV vaccine research with first signal of efficacy by showing

31% reduction in HIV infection³⁸. Results from HVTN 702 (Uhambo) trial that was built on the results of RV 144 gave disappointing results. The trial was stopped in 2021 as there was no difference in the rate of HIV infection between people who received the vaccine and people who did not³⁹. Studies on broadly neutralizing antibodies (bNAbs) reported that 20% of HIV infected individuals develop bNAbs after few years of infection. However, these bNAbs do not control disease progression due to continuously emerging escape viruses⁴⁰. Recent studies on passive transfer of bNAbs have shown promising results in nonhuman primates. Induction of bNAbs by active immunization is pursued as another vaccine strategy based on the success of passive transfer studies³⁷.

Though progress has been achieved in understanding vaccine development for HIV, results from Uhambo trial signify the amount of time it might take for developing moderately effective vaccine and underlines the importance of other prevention strategies.

Prevention of mother to child (PMTCT)

ART can be used to reduce viral load in the mother to lower the infectiousness and as a prophylaxis for the newborn. HIV transmission rate can be reduced to less than 5% if ART is given to pregnant women and continued through breastfeeding period¹⁵. Along with ART for pregnant women and infants, PMTCT has three other elements which include universal maternal testing and prenatal counseling, elective

cesarean delivery in cases of viral load exceeding 1000 RNA copies/ml, and breast feeding ⁴¹. Approximately 1.4 million new infections in children are prevented due to implementation of PMTCT services as can be seen in Figure 10 ⁴¹.

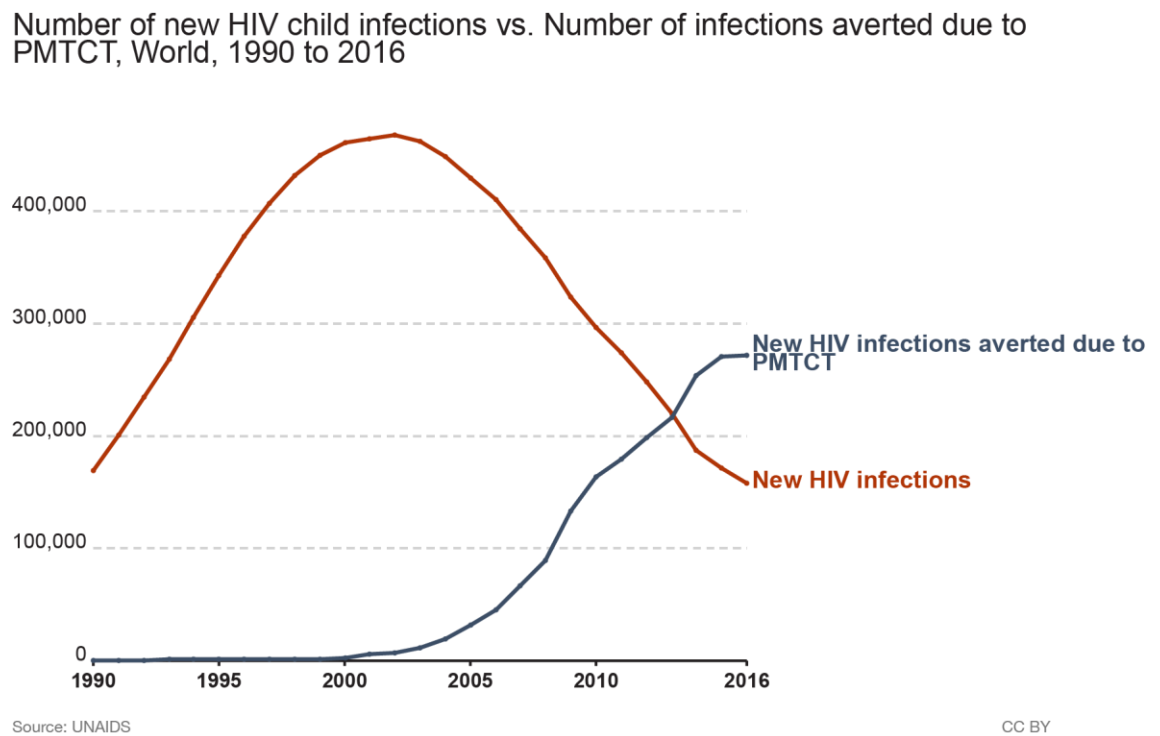


Figure 10. Implementation of PMTCT reduced HIV transmission from mother to child and averted new HIV infections in children ¹⁵.

Post-exposure prophylaxis (PEP)

Post-exposure prophylaxis is the short-term use of ART to lower the risk of HIV infection following an exposure. It is a 28-day regimen of oral ART and must be

given within 72 hours of possible exposure ⁴². It takes about 72 hours for HIV to be detected in regional lymph nodes and up to 5 to 10 days to be detected in blood offering window of opportunity for therapeutic intervention to prevent viral replication or dissemination ⁴³. WHO recommends the use of three drugs for more effective PEP regimen. TDF + FTC with a third drug such as DTG or ATV/r or DRV/r is the backbone HIV PEP regimen recommended by WHO ⁴⁴. PEP is very effective if the person adheres to the regimen. Adherence to PEP is often poor and several risks such as side effects due to ART and drug resistance are associated with PEP.

Pre-exposure prophylaxis (PrEP)

Pre-exposure prophylaxis PrEP is the use of ARV drugs to reduce the acquisition of HIV infection by HIV-negative individuals. It is a daily pill of ART and prescribed for people who are at higher risk of acquiring HIV. CDC recommends daily oral PrEP containing tenofovir in combination with Emtricitabine and was reported to significantly reduce HIV acquisition in high-risk heterosexuals, men who have sex with men and injection drug users who share needles ⁴⁵. Efficacy of daily oral PrEP is directly correlated with adherence ^{46 47 48}.

Patient adherence and cost effectiveness are biggest challenges for the use of PrEP by women, young individuals, transgender women, racial and ethnic minorities ^{49 50}. It promotes risky sexual behaviors and might increase risk of HIV infection in case of interruptions in adherence ⁵¹. Since PrEP requires daily intake of ART, body is

exposed to continuous doses of drug, and causes side effects. Generally prescribed PrEP drugs Tenofovir disoproxil fumarate (TDF), Emtricitabine (FTC) or combination of TDF/FTC were reported to show side effects such as nausea, decreases bone mineral density, hyperglycemia, and renal failure etc^{52 53}. Though these are short term side effects, no long-term studies were performed to determine their toxicity over time. These shortcomings of lack of patient adherence, cost effectiveness, short term side effects and possible long-term adverse effects does not make oral PrEP the best choice for prevention. New PrEP agents that are being investigated and developed include novel oral agents, long acting injectables, subdermal implants, multipurpose technologies, broadly neutralizing antibodies, vaginal rings, and topical products⁵⁴.

Several women and girls around the world have limited or no decision-making power about their sexual and reproductive lives and their own health care. Gender discrimination in the context of violence, poverty and insecurity blocks access to HIV prevention and treatment services, heightens their risks of acquiring HIV. Therefore, there is a need to respond to women's needs and provide a simple, convenient, and suitable prevention options such as vaginal rings and topical products for vaginal application.

CHAPTER 2

BIOMEDICAL STRATEGIES TO PREVENT VAGINAL TRANSMISSION OF HIV IN WOMEN

2.1. Introduction

Brunt of the epidemic has been faced by women with disproportionate number of infections especially in Sub-Saharan Africa with 63 % of new infections in 2020. Despite representing 10% of the population in Sub-Saharan Africa, adolescent girls, and young women account for 25% of HIV infections ⁵⁵. As discussed in previous chapter, gender inequality underpinned by harmful gender norms restrict women's access to HIV and sexual health services. Even the use of female condoms requires negotiation with male partner which is not an option for majority of women worldwide. Pre-exposure prophylaxis, only available biomedical intervention for HIV prevention requires continuous access to ART which is a challenge for women in resource poor regions that were hardest hit by the pandemic. Women initiated and women-controlled HIV transmission barriers were given little attention for a long time. Scientists should prioritize the development of preventive methods women could use without the fear of their partner finding out. Infections through vaginal route contribute to approximately 30-40% of new HIV infections in women despite lower transmission probability per exposure event ⁵⁶. Therefore, understanding the

key factors involved in vaginal transmission of HIV is valuable to design efficient prevention strategies.

2.2. Vaginal Acquisition of HIV

Vaginal transmission is mediated by exposure to semen containing infectious HIV-1 or infected cells. Both sources of cell free and cell-associated virus should be targeted by intervention strategies although relative transmissibility is uncertain ⁵⁷.

Vaginal physiology and natural barriers against HIV

Innate anatomical barriers of the vagina in a healthy individual provides robust barricades against HIV transmission and prevents vaginal transmission of HIV. Cervico-vaginal epithelia is the primary barrier of the female reproductive tract (FRT) and consists of stratified squamous epithelium of the ectocervix and vagina and the simple columnar epithelium of the endocervix as shown in Figure 11. Squamous epithelium is a multi-layered structure of approximately 100-200 microns thick ⁵⁸. It lines the most exposed regions of the female genital mucosa and has limited permeability to particles having diameter greater than 30 nm ²⁵. It constitutes a significant physical barrier against HIV as the diameter of HIV virion is approximately 100 nm. Amorphous lipoidal material and intercellular desmosomes are the crucial components of epithelial barrier integrity. Corneal epithelial cells become flattened and keratinized as they progress from basal layer to apical layer.

Outermost layer of the epithelial barrier consists of a superficial dead epithelial cell layer which is impervious to the virus ⁵⁸.

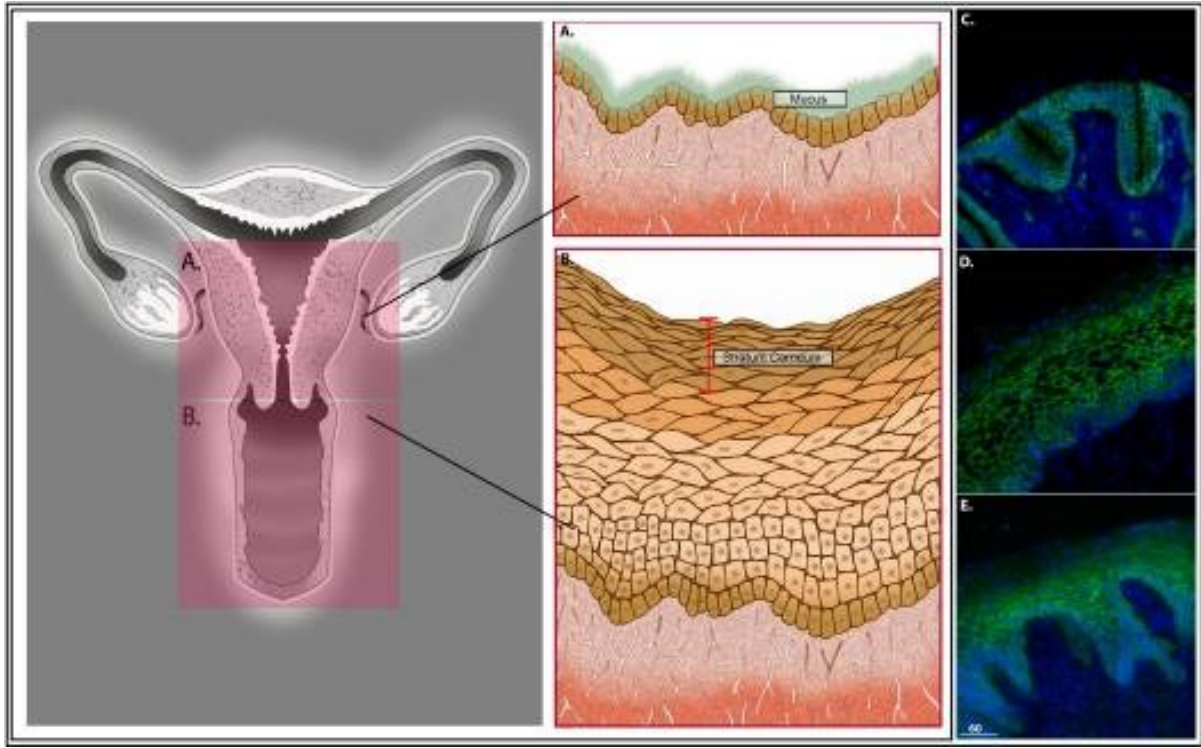


Figure 11. Histological Structure of the Female Reproductive Tract. A. Simple columnar epithelium of the endocervix. B. Stratified squamous epithelium of the ectocervix and vagina. Fluorescent deconvolution images of rhesus macaques C. endocervical epithelium. D. ectocervical epithelium. E. vaginal epithelium. (Reprinted from ref. ⁵⁸)

In contrast to the squamous epithelium of the lower FRT, upper FRT consists of columnar epithelium which is composed of single layer of cuboidal epithelial cells of approximately 10 to 30 microns thick with high concentration of HIV target cells closer to the lumen of the upper FRT ²⁵. Area of the columnar epithelium of the FRT is approximately 15 times smaller than the stratified squamous epithelium despite

being considerably thinner which leads to less exposure to the HIV containing seminal fluid. Furthermore, mucus produced by the intra-epithelial goblet cells of the endocervical epithelium protect from invading pathogens by impeding their mobility as a physical barrier. Additionally, cervical mucus also contains antiviral proteins such as secretory leukocyte protease inhibitor and other enzymes which play a role in viral defense ⁵⁸. Region of transformation from simple columnar epithelium of the endocervix to squamous epithelium of the ectocervix is called transformation zone. This region can have CD4⁺ T cells which may increase the susceptibility to HIV entry ⁵⁹.

Vaginal transmission

Several possible mechanisms of vaginal transmission of HIV-1 have been proposed at different tissue sites of viral entry bases on studies conducted *in vitro* on different epithelial types as shown in Figure 12. Free HIV virions or virions released from infected seminal cells interact with epithelial cells and traverse the epithelium through several pathways including transcytosis, endocytosis, and subsequent exocytosis or merely through transmigration ²⁵. Stratified squamous epithelial cells of the ectocervix are not susceptible to HIV-1 infection and do not transcytoses viral particles. However, the stratified epithelial layer is populated with Langerhans cells (LCs) and T cells which are targets of HIV-1 facilitating infection *in trans* ⁵⁶.

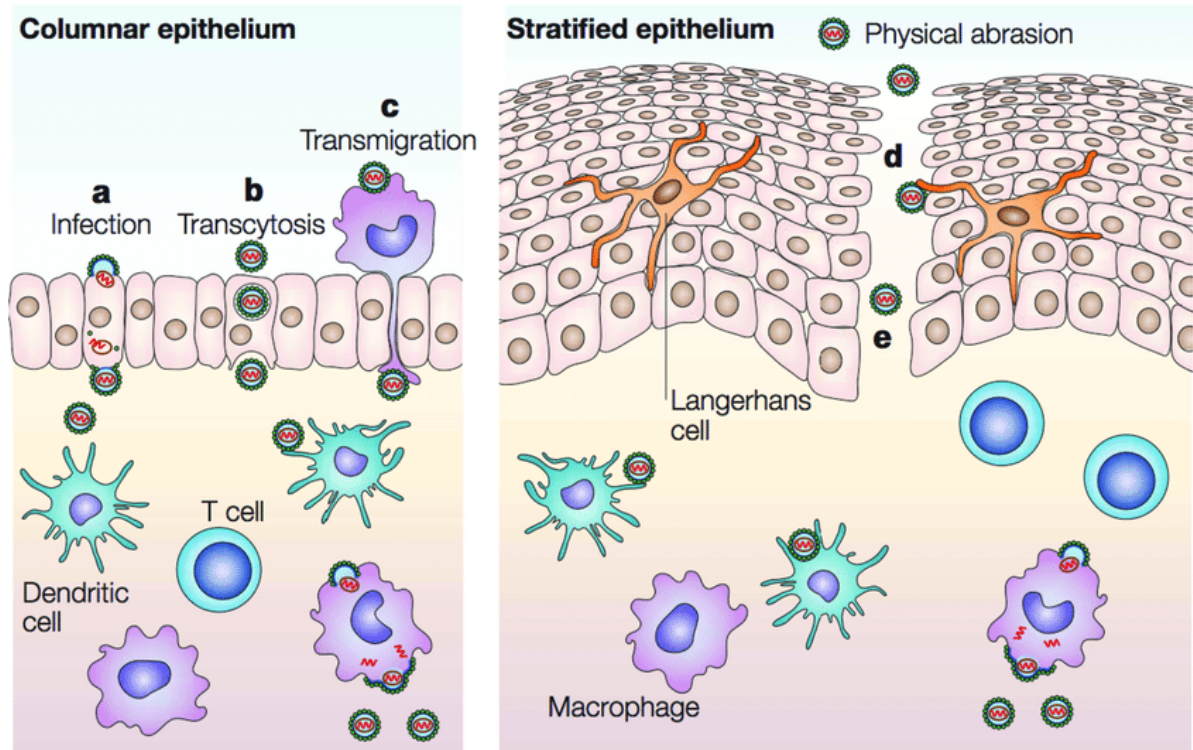


Figure 12. Potential mechanisms for HIV-1 transmission across mucosal epithelium. (Reprinted with permission from ref. ²⁵)

As discussed earlier, vaginal physiology provides a major hurdle for HIV-1 transmission. *In vivo* transmission of HIV-1 requires at least some degree of breakdown in epithelial integrity which happens frequently. 60% of women after a consensual intercourse are examined to have epithelial micro abrasions ²⁵. Conditions that disrupt epithelial barrier such as ulcerations, trauma and inflammation can increase susceptibility to HIV-1 infection. HIV-1 takes advantage of these abrasions and traverses through these gaps and gains direct access to macrophages, dendritic cells, and Langerhans cells ⁶⁰. The earliest focal collection of infected cells is CD4 T

cells comprising 90% of productively infected cells at the portal of entry. CD4 T-cell availability in the vagina, ecto-and endocervix is higher compared to other HIV-1 target cells such as macrophages and dendritic cells making it the majorly infected cell type ⁶¹⁶².

Stages of vaginal transmission and opportunities for prevention

Studies were conducted on non-human primate (NHP) model of HIV-1 transmission to women to understand mucosal events critical for establishing systemic infection of HIV. Conclusions derived from the studies on rhesus macaques vaginally inoculated with high dose of viral particles twice daily provides a window to view the mucosal transmission and first two weeks of infection ⁶³. As shown in Figure 13, within hours following mucosal exposure to high doses of SIV, virus crosses the mucosal epithelium and establishes small founder population of infected cells. During the first week of infection, founder population undergoes local expansion to generate sufficient virus and infected cells to disseminate and establish systemic infection. Replication explodes in the second week of infection in the lymphatic tissues which consist of susceptible target cells in high concentrations compared to the portal of entry. Virus levels in the blood peak by the end of second week and eventually decline and become stable near the end of four weeks. Similar to the disease progression in human, infected lymphatics comprise viral reservoirs

becoming the site of CD4 T-cell depletion leading to disease progression in SIV-infected rhesus macaques ⁶⁴.

Based on the observations made on mucosal transmission in high dose vaginal challenge NHP model, prevention strategies should be targeted at the earliest stage of infection to take advantage of viral vulnerabilities, prevention opportunities and to further avoid uncontrollable systemic infection after first week of viral exposure. Establishment of small founder population is the first viral vulnerability and provides an opportunity for prevention as it requires sustained basic reproductive rate, $R_0 > 1$. Early intervention methods that reduce the reproductive rate to less than 1 aborts the infection ⁶³. Another opportunity in the early stage of infection is to prevent local expansion. This consequently leads to lower production of virus and infected cells and further abortion of viral dissemination and systemic infection.

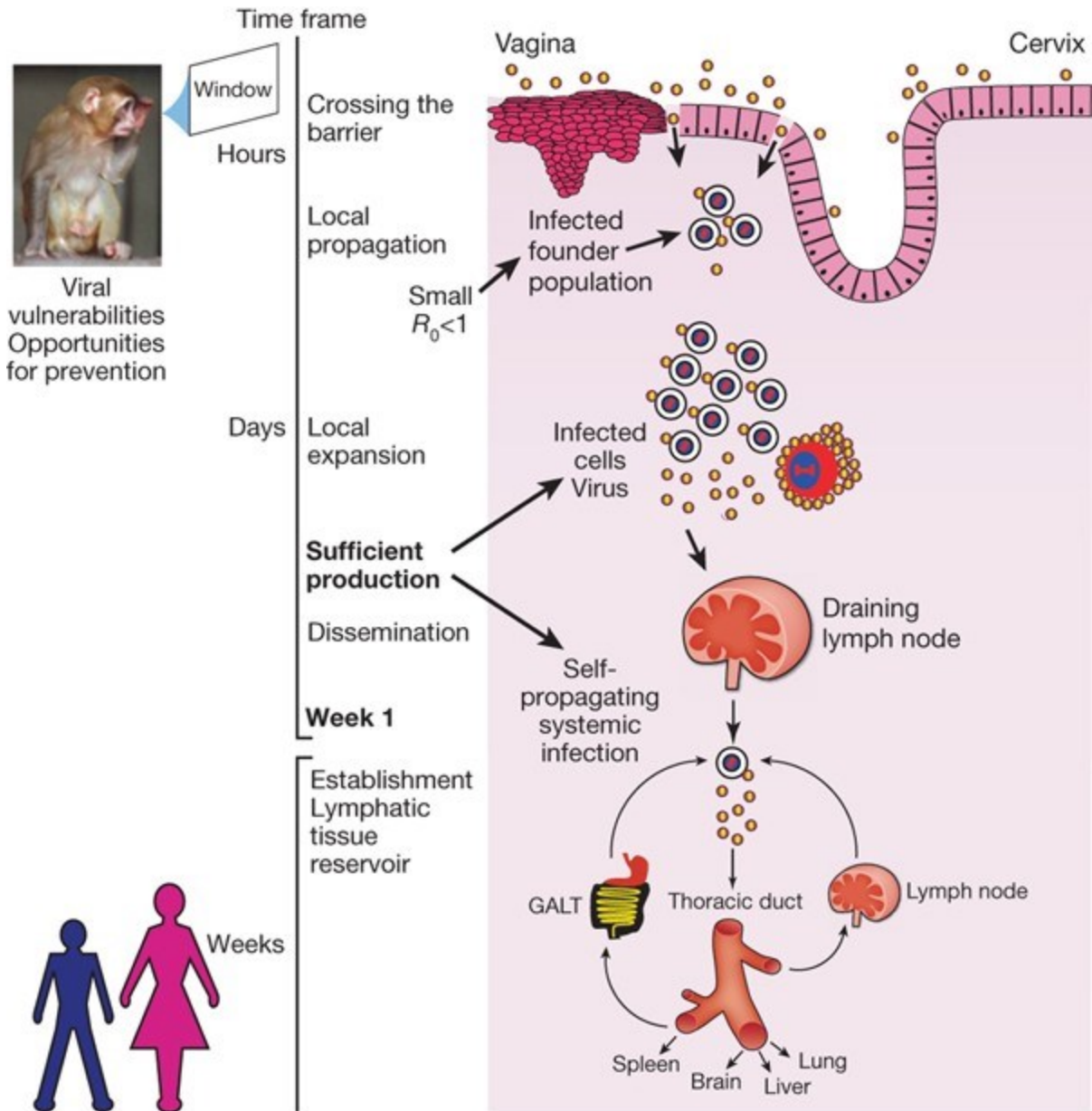


Figure 13. Time frame, sites and major events in vaginal transmission and window of opportunities for prevention. (Reprinted with permission from ref. ⁶³)

2.3. Prevention Strategies for Vaginal Transmission of HIV-1

Microbicides offer a potential intervention strategy to inhibit earliest steps of HIV transmission at the vaginal or rectal mucosa. Microbicides are topical products such as gels, creams, films, rings, capsules, and tablets designed to apply inside the vagina or rectum to protect against HIV-1. Vaginal microbicides offer a discreet prevention method that can be used and controlled by women without the cooperation from their sexual partner. Unlike condoms, microbicides offer protection for women who also want to conceive children. As microbicides are intended for topical application at the site of viral entry, higher drug concentration can be delivered without systemic exposure lowering long term toxicity in healthy but at-risk individuals ⁶⁵. Figure 14 summarizes the microbicial prevention strategies for vaginal transmission of HIV.

Nonspecific microbicides

Early development of microbicides was intended to offer protection against most sexually transmitted infections. The idea was to generate microbicides using simple nondrug compounds and provide over the counter (OTC) for broader availability even in resource poor countries. Therefore, compounds that inactivate cell free or cell associated HIV in semen such as detergents, surfactants and buffering agents are the main compounds in this category ⁶⁵.

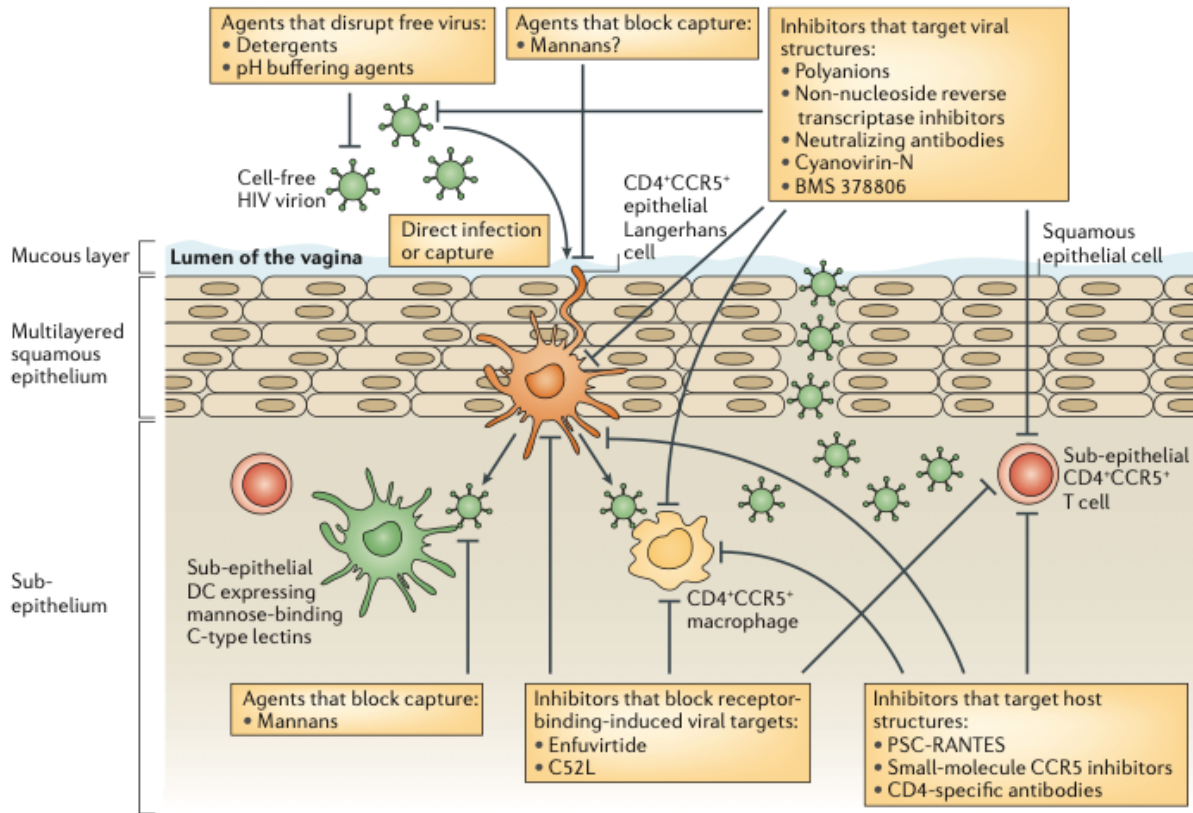


Figure 14. Topical strategies to prevent vaginal HIV transmission. (reprinted with permission from ref. ⁶⁶)

Surfactants

Surfactants disrupt the viral membrane non-specifically and are the earliest compounds that have been clinically evaluated. Nonoxynol-9 (N9), an anionic surfactant was the first vaginal microbicide to be studied ⁶⁷. N9 is an inexpensive and effective spermicide which works by disaggregating the lipid membranes of the spermatozoa ⁶⁸. It has similar effect on sexually transmitted pathogens such as HIV and genital herpes virus. The first large scale clinical trial using commercially available contraceptive sponge impregnated with 1000 mg of nonoxynol-9 gave

disappointing results as it did not protect against HIV and caused genital ulcers in some women ⁶⁹. Several subsequent clinical trials with different formulations such as N9 film and N9 gel showed insufficient protection against HIV. They even showed increase in incidence of HIV which is linked vaginal epithelial breach and genital tract lesions ⁷⁰. Development of N9 based microbicides was stopped due to these disappointing results and WHO no longer recommends N9 for HIV prevention ⁷¹.

Research continued with other surfactants such as C31G (SAVVY). It consists of an equimolar mixture of cetyl betaine and myristamine oxide and disrupts viral membrane with its alkyl moiety ⁷⁰. Clinical studies conducted using different concentrations of surfactants showed no efficacy for HIV prevention. One of the clinical studies reported discontinuation of product due to associated burning sensation. Sodium dodecyl sulfate (SDS) also called sodium laurilsulfate (SLS) are negatively charged surfactants that denature viral and cell membrane proteins ⁷⁰. In addition, SLS affects glycoproteins of HIV envelope and compromises its adsorption to target cells ⁷². SLS has been formulated as an “invisible condom” as it has thermosensitive property i.e., it is a liquid at room temperature and can cover vaginal wall and transforms into gel at body temperature ⁶⁸. In vitro studies have shown inhibition of HIV and herpes simplex virus (HSV) by SLS. Safety of SLS has been reported in in vivo studies and two-phase II clinical trials. However, phase III trial did not exhibit efficacy^{73 74}.

Buffering agents

In normal conditions, vaginal pH is acidic (3.5 – 4.5) due to the production of lactic acid and H₂O₂ by commensal lactobacillus. Acidic pH of the cervicovaginal fluid acts as a natural defense against HIV through viral inactivation. In contrast, seminal fluid which is basic (pH 7.1 – 8.0) raises vaginal pH and diminishes natural defense mechanisms⁷⁰. Buffering agents maintain natural vaginal pH directly through acidifying agents or indirectly by enhancing lactobacillus production⁶⁸.

Carbopol 974P (BufferGel) which buffers twice its volume of seminal fluid to a pH less than 5 has been shown in vitro as a spermicidal and virucidal. Two phase I clinical trials of BufferGel reported it as safe. It is now being tested in phase IIb trial for both safety and effectiveness. ACIDFORM gel which is already approved as a sexual lubricant gel is now being tested as a microbicide due to its acid-buffering and bio adhesive properties. Phase I trial showed that ACIDFORM was well tolerated and safe. An efficacy trial is ongoing to test its ability to prevent sexually transmitted infections⁶⁸. Recently, probiotic strategy was getting attention as recolonization with lactobacillus correlated with decreased HIV proliferation. Widely available substance and common household items such as lemon juice, vinegar and carbonated drinks have also been suggested to acidify vaginal environment. However, studies indicated that they could irritate and damage vaginal lining and may even increase risk of

acquiring HIV. Thus, potential microbicides are thoroughly investigated for safety and efficacy before being promoted for their use ⁷⁵.

Anionic polymers

Anionic polymers do not disrupt the HIV membrane like surfactants but work by interacting with HIV viral envelope and interfering with viral fusion. It is proposed that they act as sticky molecules by binding to positively charged viral envelope through their negative charge and block viral entry into target cells ⁶⁹. Polyanions make CXCR4 viruses particularly vulnerable due to their highly positively charged V3 regions. This is not the case for CCR5 viruses and polyanions are less effective against CCR5 viruses. Several polyanionic compounds such as naphthalene sulfonate (PRO 2000, Indevus Pharmaceuticals), carrageenan (Carraguard, Population Council), cellulose sulfate (Ushercell, Polydex Pharmaceuticals) and cellulose acetate phthalate (CAP) are being investigated as potential microbicide candidates. Anionic polymers are poorly absorbed due to their high molecular mass reducing risk of systemic toxicity ⁶⁹.

PRO 2000 has better therapeutic index than nonoxynol-9 and retain antiviral activity in presence of semen. Phase I trials reported safety and acceptability of PRO 2000 at low doses and adverse events at high doses. However, phase III trials again reported disappointing results like surfactants. Carraguard which is a sulfated polysaccharide blocks HIV from infecting cervical epithelial cells. It also blocks

migration of infected macrophages across vaginal epithelia to regional lymph nodes⁷⁶. Several phase I and phase II trials of carrageenan-based formulations showed safety in both HIV positive and HIV negative individuals. However, phase III trials showed lack of efficacy which is contributed to poor adherence and is no longer being developed⁷⁷. Cellulose sulfate has broad activity against STI pathogens and inhibits both CXCR4 and CCR5-tropic viruses. It was found to be safe in phase I clinical trials. Two phase III efficacy trials reported showed higher HIV seroincidence in the cellulose sulfate arm compared to placebo arm⁷⁸. CAP which is a carboxylate polymer shows in vitro virucidal activity against HIV-1 and HSV. CAP is developed as a micronized gel as well as film and preclinical evaluation of CAP reported minimal induction of inflammatory change. CAP was also shown effective in low dose infection of macaques by SHIV and is now pursued in human clinical trials. Dendrimers are newest category of anionic polymers. They possess the ability to bind to multiple cells at multiple locations because of their size and surface groups. Several of these compounds are in different stages of preclinical and clinical evaluation^{79 68}⁷⁰.

Specific microbicides

The early setback of non-specific microbicides led to development of specific microbicides that target different stages of viral entry and replication cycle. Specific microbicides are fabricated using anti-retroviral drugs that target adsorption and

fusion of virus with target cells, uncoating, reverse transcription of viral RNA, and integration of proviral DNA into host genome which are potential targets for intervention in mucosal transmission of HIV-1.

Microbicides targeting viral entry

Microbicides targeting viral entry prevents virus particles entering target cells and further blocks transmission of infected macrophages and dendritic cells to lymphoid cells and is the most promising prevention strategy. Viral entry inhibitors can be targeted at host cell membrane receptors or at viral envelope proteins. HIV viral envelope is highly glycosylated and plays an important role in viral defense mechanism. Carbohydrate binding agents (CBA) are used to target mannose rich glycans on the viral surface. CBAs neutralize broad variety of HIV clades including HIV-2. Examples of CBAs include lectins such as Cyanovirin-N (CV-N), 11 kDa protein isolated from cyanobacterium *Nostoc ellipsosporum*. Topically applied CV-N has shown efficacy in macaque models of vaginal SHIV challenge. Attempts are ongoing to bioengineer commensal lactobacillus to produce small lectins that bind to viral envelope to provide live microbicides ⁷⁰. Before widely testing in clinical trials, potential side effects of CBAs should be monitored as many human cell types contains glycoprotein on its surface. In addition, use of CBAs such as CV-N can lead to emergence of escape mutants that lack N-linked glycan structure ⁸⁰. Other microbicidal agents targeting viral envelope include monoclonal antibodies (mAbs)

such as 2G12 and b12 to membrane proteins ⁸¹. PRO-542, an engineered CD4 immunoglobulin fusion protein and recently discovered small molecule inhibitor BMS-806 are potential candidate that binds to gp120 and blocks interaction with host CD4 receptor and co-receptors CXCR4 and CCR5 ⁶⁶.

Conformational changes following gp120 binding with CD4 unmasks conserved regions of enveloped-protein complex including gp41 which proves as a useful target for microbicide intervention. gp41 binding agents such as enfuvirtide, a synthetic 36 amino-acid peptide blocks gp41 from folding into six-helix bundle and subsequent fusion with host cell. gp41 specific antibody 2F5 has shown efficacy in vaginal SHIV challenged macaque model ^{82 83}.

Host factors essential for viral life cycle provide better therapeutic target as they cannot be influenced by viral evolution that leads to rapid generation of escape mutants. Entry inhibitors that target host encoded proteins without compromising host-cell function are being investigated as potential microbicides. CD4 receptor which is the main target of HIV can be blocked by monoclonal antibodies such as TNX355. Cyclotriazadisulfonamide (CADA), a novel class of molecules that reversibly down regulate CD4 receptor temporarily blocks the virus from entering the cells. C-type lectin receptors (CLR) on macrophages and dendritic cells such as DC-SIGN bind to mannose residues on HIV envelope. CLR directed mAbs and mannose rich glycans such as mannan may competitively block the virus from interacting with

dendritic cells and macrophages which play a prominent role in transmission of virus to draining lymph nodes.

Inhibiting CXCR4 and CCR5, co-receptors for HIV entry is another potential strategy for targeting host factors essential for viral entry. CCR5 tropic strains are most predominant in early stages of infection and can be targeted by mAbs such as PRO-140, modified chemokines such as NNY-RANTES, PSC-RANTES and small molecules such as SCH-C, SCH-D and UK-427857⁷⁰. PSC-RANTES has shown in-vitro efficacy against all HIV clades and complete protection from SHIV SF162 in macaques with no evidence of toxicity. CMPD167, another CCR5 receptor antagonist is cyclopentene based compound and exhibited protection from vaginal SHIV in macaques⁶⁸. Maraviroc, FDA approved CCR5 antagonist has also shown prevention of SHIV infection in macaques when formulated as a microbicide⁶⁵. The bicyclam AMD 3100 and AMD-070 are highly specific small molecule CXCR4 inhibitors that are currently under investigation⁸⁴. Combination of CCR5 and CXCR4 antagonists should be considered for microbicidal development although CXCR4 tropic virus less important in sexual transmission. This lowers the evolutionary pressure on HIV to use non-CCR5 mediated entry into host cells⁸⁵.

Microbicides targeting viral replication

Microbicides that target different stages post entry is necessary to inhibit proviral incorporation and further multiplication of viruses that already entered host

cells. Many studies have been done using reverse transcriptase inhibitors as potential microbicides. Tenofovir, an NRTI was the first choice for development of ARV based microbicide due to its high barrier for resistance and established safety and efficacy profile. CAPRISA 004, a Phase IIb, double-blind, randomized, placebo-controlled study, assessed the effectiveness and the safety of a vaginal gel containing 1% tenofovir for the prevention of HIV infection in South African women. Results showed 39% reduction in HIV infection rate with 54% reduction in high adherence population ⁸⁶. FACTS 001 clinical trial which was expanded to higher study population based on CAPRISA 004 did not confirm the effectiveness of Tenofovir gel highlighting the adherence challenges ⁸⁷. Tenofovir has long tissue half-life keeping it active for prolonged periods. Several other approved NRTIs along with tenofovir are currently under clinical development as microbicides. Most advanced NNRTIs in clinical trials are dapivirine (TMC-120), UC-781, MIV-150 and MC1220. Dapivirine is most advanced of all these compounds and developed as a long-term prevention option by taking advantage of its lipophilicity ⁶⁶.

Inhibition of viral integration remains an important strategy for microbicide development. Raltegravir and dolutegravir are only FDA approved integrase inhibitors and are under preclinical development as microbicide. Protease inhibitors have the highest barrier to resistance and are interesting candidates for microbicides. Although unclear, protease inhibitors may block dissemination of virus from initial

focus until localized infection is eliminated. They are being studied *in vitro* and *in vivo* for microbicidal activity and will be developed as microbicides for clinical use if they show promising results ⁶⁵.

2.4. Microbicide Formulation and Delivery

Development of appropriate formulation and delivery tools in addition to identifying best targets for topical agents is central for successful vaginal prevention strategies ⁶⁶. Ideal microbicide formulations are easy to use, well tolerated, adequately distributed, and retained in the vagina for several hours with sufficient tissue penetration if necessary ⁶⁹. Long term delivery tools even allow less frequent administration increasing patient adherence. The choice of formulation is determined by chemical characteristic of the microbicide compound, required performance of the final product, consumer preference and economic considerations ⁸⁸. Microbicides can be formulated as solutions, suspensions, semisolid dosage forms such as hydrogels, ointments, creams, or solid dosage forms such as suppositories, tablets, capsules, films, and intravaginal rings. They are administered before coitus (semisolid formulations), daily (tablets, films, suppositories), or less frequently (intravaginal rings). Microbicide formulations provide higher bioavailability in vaginal epithelium compared to oral dosing, while drastically reducing systemic exposure lowering long term toxicity. The most important requirement for microbicidal dosage forms is their

inertness towards the vaginal epithelium. Dosage forms that are actively pursued in preclinical and clinical development of microbicides were discussed in this section ⁸⁹.

Semi-solid dosage forms

Semi-solid formulation for vaginal delivery includes gels, ointments, and creams. All types of semisolid dosage forms can be messy, leaky, and have short residence time in the vaginal lumen after application ⁹⁰. They require frequent administration to attain the required therapeutic effect which is inconvenient and leads to low adherence of therapy. However, they have high acceptability, low production cost and ease of application ⁸⁹. Semisolid dosage forms are more likely to be preferred over solid dosage forms and may vary depending on socioeconomic and cultural factors. Thus, it is important to provide prevention tool in various forms and sizes to suit specific needs of the women across the globe.

Ointments and creams

Ointments consist of a single-phase base in which solids or liquids are dispersed. In contrast, creams consist of two liquid phases dispersed in each other. Terameprocol is a transcription inhibitor with anti-HIV and HPV activity. It was formulated as a 2% w/w ointment and tested in phase I clinical trial of daily vaginal application. Results showed well tolerance of the ointment among women without serious adverse events ⁹¹. Vaginal creams of metronidazole and clindamycin are found to be safe and effective ⁹². Currently, several vaginal creams are in market such as

clotrimazole and terconazole vaginal creams for treatment of fungal infections. Although, vaginal ointments and creams are well tolerated and currently in market for various disease conditions, studies conducted on women's preference for ideal characteristic of a microbicide showed preference for clear aqueous base products such as gels over white ointment or cream ⁹³.

Gels

Gels are most frequently used and extensively studied dosage forms for microbicides. Gels can be defined as a semi rigid system containing two components i.e., dispersed phase and dispersing medium. Dispersed phase consists of a three-dimensional network of small inorganic particles or large organic molecules restricting the movement of dispersing medium. Dispersing mediums such as water, aqueous buffers and hydroalcoholic solutions are common in pharmaceutical applications. Gels formed by physical interaction between molecules of dispersed phase are reversible, whereas gels formed by covalent crosslinking are irreversible. Gels most used in vaginal drug delivery are hydrogels which are obtained with polymer gelling agents dispersed in aqueous medium. Gels have an advantage of lubrication during sexual intercourse, good spreadability and familiarity of the dosage form among users [37, 41, 42, 43].

All first-generation microbicides were developed in the form of gels by taking inspiration from OTC sexual lubrication products which were gels. Vaginal gels are

manufactured by mixing gelling polymers along with excipients such as co-solvents, buffers, humectants, and preservatives in water. Hydroxy ethyl cellulose (HEC) and Carbopol are most used polymers due to their cost-effectiveness and safety. Most of the clinical trials use 2.7% w/w HEC gel as the placebo control ⁹⁴. ACIDFORM, acid-buffering vaginal gel formulated to maintain acidic vaginal pH along with long residence time and to form protective layer in vaginal lumen was compared against universal placebo gel i.e., HEC gel showing safety with some participants reporting irritation and burning. Other nonspecific microbicides such as BufferGEL, carraguard, sodium cellulose sulphate and PRO 2000 were formulated as gels and evaluated for safety and efficacy. Results indicated that the dosage form is safe and well tolerated among both partners although lack of efficacy and adverse events were reported which were due to the active ingredient rather than the dosage form ⁹³.

Tenofovir was the first anti-retroviral containing microbicide formulated as gel. The formulation contains 1% tenofovir with excipients such as citric acid, glycerin, edetate disodium, methylparaben and propylparaben in a HEC gel base at a pH of 4.5. Gel was evaluated for safety and efficacy in phase III clinical study with 12 h before sex and 12 h after sex regimen (BAT-24 regimen). Although gel was safe, it did not prevent women from acquisition of HIV which was attributed to lack of adherence ⁹⁷. Another phase I study was conducted in South Africa using dapivirine gel (0.001%, 0.005%, or 0.02%) for once daily dosing regimen. Results showed the wide distribution

of dapivirine throughout lower genital tract with low systemic absorption and reported to be safe and well tolerated ⁹⁸. Conclusively, microbicide gels are safe and well tolerated along with need for multiple applications, leakage and stickiness reported as negative experiences. Gels should be formulated by optimizing physicochemical parameters such as volume, rheological properties, pH, and osmolality to ensure dosage requirements are met, uniform distribution of the gel to the whole vaginal epithelium is achieved and to avoid mucosal irritation and damage of vaginal epithelium. Drawbacks of semi-solid dosage forms can be eliminated by solid dosage forms which are discussed in this section ⁸⁹.

Solid dosage forms

Solid dosage forms for microbicide delivery include tablets, suppositories, films, discs, and intravaginal rings (IVRs). Solid dosage forms stabilize drugs that would else be degraded in semi-solid dosage forms due to presence of aqueous solvents. Thus, solid dosage forms have higher shelf life reducing final production cost and are easily portable. However, they have increased risk of vaginal irritation and need for high vaginal hydration for uniform distribution of the product ⁸⁹.

Tablets and suppositories

Tablets are made by blending and compressing active pharmaceutical ingredients (APIs) and excipients which are usually in powder form into solid shapes. Although, tablets are primarily used for oral delivery of anti-retrovirals, vaginal

tablets are also explored to deliver drugs that have stability concerns with gels and to provide another prevention choice for women. Typical shapes for vaginal tablets include double convex or ovule to aid in insertion. Tablets are designed to disperse or dissolve when it comes in contact with vaginal fluid. Ovule shaped vaginal tablet containing NNRTI dapivirine and gp120 inhibitor DS003 was developed by the International Partnership for Microbicides (IPM) using direct blend methodology with standard tableting excipients and techniques. Placebo version of this tablet was placed in product acceptability study along with softgel capsule and a film which showed greater acceptability for film and softgel and negative reaction to the tablet formulation from end users. However, Praneem polyherbal vaginal tablet developed by Panacea Biotech received acceptability among women. Disparity among acceptability results can be due to difference in formulations as Praneem polyherbal consist of purified extracts from *A. indica* tree leaves and saponins from *S. mukerosi* [41, 36].

Suppositories are generally rod shaped, conical or wedge shaped and are designed to melt inside the vaginal lumen ⁸⁹. They are widely used for vaginal delivery of probiotics and are currently in market such as Peach body ⁹⁹. Naturally occurring probiotics or genetically engineered commensal bacteria can have great potential for prevention of HIV through production of antiviral agents [47,48]. Suppositories and tablets are under explored in microbicide field and have the

potential to offer discreet, portable, and inexpensive prevention tool provided formulation is optimized for safety and user acceptability.

Films and Discs

Films are thin strips of water-soluble polymers incorporated with active ingredients. They offer a promising platform for microbicide delivery and dissolves to release active ingredient when applied into the vagina. Moreover, microbicide dosage form acceptability studies among women reported acceptance of films and preference over foam tablets and softgel capsules. The reasons for acceptability could be due to non-leakiness of the films as opposed to gels and unimpeded sexual activities. Broad range of drugs such as hydrophilic, hydrophobic, biomolecules and bacteria can be delivered using vaginal films. Films are formulated using water soluble polymers such as polyvinyl alcohol, pullulan and cellulose based polymers, plasticizers such as polyethylene glycol, glycerol, and phthalate derivatives to provide flexibility, fillers, disintegration agents, stabilizers and colors if required.

C-Film, a contraceptive vaginal film containing nonoxynol-9 was the first vaginal film introduced. Currently, several films are being commercially marketed such as vaginal contraceptive film (VCF), lubricating film and vaginal scented film⁹⁴. Several non-specific and specific microbicides were evaluated for HIV prevention using film formulations such as sodium dodecyl sulfate, cellulose acetate phthalate, dapivirine, zidovudine and RC-101. Recently, a HPMC based nanoparticle

incorporated film for combination delivery of tenofovir and efavirenz was developed. Results showed enhanced vaginal retention of drug with low systemic exposure and safe histological profile ¹⁰².

A recent study reported formulation of vaginal discs as a microbicide. Discs are formulated by freeze-drying gels to solve leakage and messiness associated with most frequently used dosage form i.e., gels. Discs formed by free-drying of gels based on sodium dodecyl sulphate and 2-hydroxypropyl- β -cyclodextrin were shown to have adequate mechanical strength and mucoadhesiveness for release of tenofovir and dapivirine. These freeze-dried gels are convenient to administer and quickly rehydrate in vaginal fluids to allow rapid release of active substance. Discs offer a comfortable alternative to gels for on-demand use by women ¹⁰³.

Intravaginal rings (IVRs)

Intravaginal rings are elastomeric rings that are flexible, and torus shaped intended to be self-inserted and removed. Figure 15 shows the example of a vaginal ring in position after insertion and dapivirine ring approved by the FDA for HIV prevention. IVRs are designed to provide reservoir of drug for controlled and sustained release of ARVs in the vaginal lumen. Matrix based formulation where the drug is dispersed uniformly in the polymeric matrix is the most basic design. Drug release is directly proportional to the amount of drug loading and surface area of the ring in the matrix-based design. Other designs that are currently under investigation

are reservoir or core design where drug loaded polymer core is encapsulated in blank polymer sheath and novel designs such as pod insert design and multi-segmented rings. IVRs are categorized based on the polymer used for their fabrication such as silicone elastomer rings and thermoplastic rings. Silicone elastomers are manufactured crosslinking functional siloxanes, whereas thermoplastic rings are manufactured using thermoplastic polymers such as poly (ethylene vinyl acetate) and segmented polyurethane either by injection molding or hot melt extrusion ^{89,104,105}.

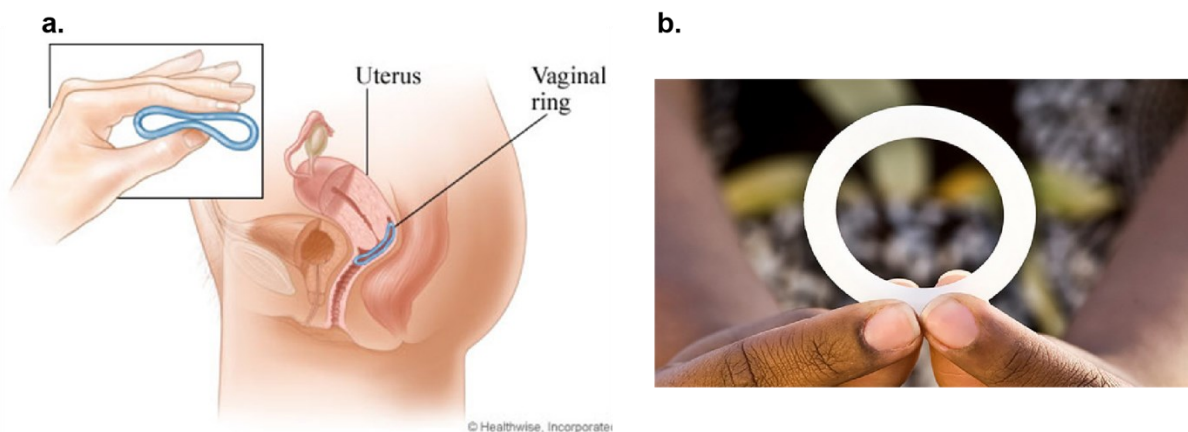


Figure 15. Depiction of Intravaginal ring. a. Vaginal ring is flexible, and image showing ring position after insertion. b. Women holding the dapivirine vaginal ring developed by international partnership for microbicides (IPM).

Several IVRs are under development such as dapivirine silicone rings in combination with marviroc and a hormonal contraceptive, MIV-150 thermoplastic ring and tenofovir thermoplastic ring in combination with levonorgestrel. Monthly Dapivirine vaginal ring was prepared as a long-acting prevention method was the

only ring clinically approved for HIV prevention. Ring is made of silicone elastomer and loaded with 25mg of Dapivirine and slowly release the drug into the vaginal lumen. Two phase III clinical trials named The Ring Study and ASPIRE was conducted to evaluate safety and efficacy of the Dapivirine vaginal ring. Results showed 30% reduction in HIV incidence compared to placebo and well tolerance of the ring^{106,107}. Based on these results, open label extension studies HOPE, and DREAM were conducted to evaluate safety, adherence, and efficacy. These extension studies resulted in similar safety profile and efficacy as Phase III clinical trials with better adherence. Positive results suggest that vaginal ring is an advancement of female-initiated HIV prevention tool. However, it is still not an ideal method of prevention. Safety results from all these studies showed no significant differences between Dapivirine arm and placebo arm. More than 50% of participants reported serious adverse events in all four studies¹⁰⁸. These events may occur due to the presence of ring itself. Silicone ring must be investigated in more detail and the occurrence of these adverse events must be taken into consideration. Along with safety concerns, the ring is not viable economically. There are also concerns among small percent of women about knowledge of the ring presence to their sexual partner. Although acceptability studies showed preference of ring over other dosage forms among male partners, lower ring adherence was reported among participants experiencing social

harm by male partner. Scientists must work simultaneously on cheaper and better alternatives for long term prevention ¹⁰⁹.

Applications of nanotechnology in the development of microbicides

Advent of nanotechnology has brought innovations in the field of anti-viral therapeutics by not only solving drug solubility, physicochemical instability and bioavailability issues but also acting as antiviral agents themselves. Nanomedicine provides several advantages for microbicide development. Nanosizing drug molecules is an important strategy to improve drug solubility by dramatically increasing the surface area of solid drugs available for solvation. Although this strategy has been used in oral and parenteral antiviral therapies, application for microbicides needs to be researched. Use of nanocarriers protects drug payloads during manufacturing, storage, and from physiological factors such as low vaginal pH and enzymes. Other potential features of nanocarriers include ability to modulate drug release, enhancing interactions between active moieties with virus or host cell targets, targeting drug delivery to HIV-susceptible cells when compounds with intracellular mechanism of action are used, reducing drug toxicity by modulating drug availability to vaginal epithelial cell damage, increasing drug activity, modulating mucoadhesive behavior and providing effective *in vivo* mucosal drug distribution and tissue penetration ¹¹⁰.

Microbicidal nanosystems can be classified as systems with inherent anti-HIV activity and carriers for antiretroviral compounds. Presence of surface moieties that interact with HIV and inhibit viral entry into target cells come under the category of inherent anti-HIV molecules. Dendrimers, gold, and silver nanoparticles have been explored for such activity. Several nanosystems were studied and increasingly being developed as active carriers of anti-retroviral drugs owing to the advantages discussed above. It should be stressed that most of the nanocarriers have to be incorporated in vehicles such as gels, films, and vaginal rings as they lack required formulation characteristics to be used as an independent microbicide formulation^{89,110}.

Dendrimers

Dendrimers are nano sized, three dimensional, highly branched symmetric molecules with well-defined monodisperse structure consisting of core or central focal point from which all bonds emerge radially, an inner shell which has several repeating units and an outer shell with multiple peripheral functional groups¹¹¹. Physicochemical properties of dendrimers can be controlled by modulating the core structure, the branching units, and the surface of functional groups⁸⁹. Dendrimers with different anionic end groups were designed to inhibit HIV fusion with target cells based on the evidence that linear polyanions inhibit viral fusion through binding to viral glycoproteins.

Fourth generation naphthalene disulfonate dendrimer SPL7013 which was formulated as a gel named VivaGel® by Starpharma was the most studied and efficient dendrimer-based microbicide towards HSV and HIV-1. SPL7013 was shown to inhibit both cell free and cell associated HIV infection *in vitro* and presented low cytotoxicity even when formulated as gel. VivaGel® was found nontoxic and effective in human ectocervical explants which was further supported by experimentation in pig-tailed macaque model. VivaGel® containing 3% SPL7013 was tested in phase 1 and phase 2 clinical trials whose results support the safety and efficacy of the gel. However, further clinical testing revealed mild genital irritation and inflammation. Overall clinical data supports the use of VivaGel® and efforts are currently underway for its clinical use.

Another novel microbicidal dendrimers currently under investigation are carbosilane dendrimers characterized by the presence of silica in their composition. Carbosilane dendrimers with anionic surface groups such as sulfate, sulfonate and naphthylsulfonate were being developed as microbicides. Polyanionic carbosilane dendrimers were shown considerably effective against HIV-1 and HIV-2 and safe for vaginal administration without affecting commensal microbiota and sperm motility. G2-S16, a second-generation dendrimer with 16 sulfonate surface groups was the most promising candidate among different carbosilane dendrimers proposed. Hydroxyethylcellulose-based gel containing 3% G2-S16 was evaluated for efficacy in

BLT mouse model which showed prevention of HIV vaginal transmission by 84% with no apparent vaginal inflammation or irritation. Polyanionic carbosilane dendrimers in combination with ARVs such as maraviroc has shown synergistic effect of HIV prevention. Targeted dendrimers such as poly (propylene imine) dendrimer terminated with targeting moieties as tuftsin and mannose loaded with ARV drugs demonstrated higher efficacy against inhibition of HIV with decreased cytotoxicity compared to non-targeted dendrimers ^{89,90,110}. Conclusively, dendrimers offer a promising platform for vaginal microbicide development.

Polymer based nanoformulations

Polymer based nanoformulations include polymeric nanoparticles and nanofibers. Nanoparticles (NPs) can be defined as particles of sizes under 1000 nm. Biodegradable and biocompatible polymers are used in formulating nano sized dosage forms as they are nontoxic and can be reabsorbed by the body. Poly (lactic-co-glycolic acid) (PLGA) has been extensively used for vaginal delivery of microbicides. PLGA NPs loaded with PSC-RANTES has shown increased drug uptake compared to free drug. PEGylated PLGA NPs loaded with fluorescent NPs demonstrated higher vaginal retention when compared with no surface modification and avidin modification. PLGA NPs were also used to study the delivery of acyclovir, tenofovir, raltegravir, efavirenz and lopinavir. Dapivirine poses formulation challenges due to low aqueous solubility despite being a potent microbicide. Poly (ethylene oxide)

(PEO) modified poly caprolactone (PCL) based nanoparticles emerged as optimal system for delivery of dapivirine by increasing topical bioavailability and subsequent efficacy. Chitosan NPs were recently studied as a microbicide which has added advantages of low immunogenicity, improved targeting due to cationic surface and instability in acidic environment which leads to fast drug release when taken up by endosomes. Nanoparticles can be used to solve variety of issues such as low solubility of drugs, low permeation and retention in vaginal mucosa and should be taken advantage of while developing microbicides ^{95,110}.

Nanofibers are string like structures that have cross-sectional diameter within nanometer range, whereas length extends up to centimeters. Electrospinning has been widely used to fabricate nanofibers among several methods available such as thermally induced phase separation, molecular self-assembly and centrifugal spinning. Nanofibers are increasingly investigated for drug delivery due to their unique properties such as high surface area to volume ratio, well-defined porosity, and ability to incorporate multiple compounds with different physicochemical properties. PCL and PLGA blended electrospun fibers were investigated for tunable release of anti-HIV drugs such as maraviroc, tenofovir and raltegravir. Changing polymer ratio led to sustained release ranging from one day to one month. Core-sheath design achieved by coaxial electrospinning was employed in few studies to remove burst release observed by uniaxial spinning ^{110,112,113}. Recently, a study was

reported using nanofibers fabricated with ethyl cellulose shell and polyvinylpyrrolidone core which showed tunable maraviroc release ¹¹⁴.

Lipid based nanoformulations

Lipid based formulations such as liposomes and solid lipid nanoparticles (SLN) are biodegradable, biocompatible, nontoxic and nonimmunogenic as they are composed of natural components such as cholesterol and phospholipids. A variety of lipids have been studied for delivery of antiviral compounds. Lecithins, fatty acids, triglycerides, and glyceryl palmitostearate are commonly used lipids for nanoformulations. Liposomes are majorly studied for delivery of HIV vaccines or siRNA for gene therapy applications. However, few studied their application in prevention of vaginal transmission of HIV. Evaluation of a liposomal formulation loaded with MC-1220, a hydrophobic NNRTI showed that gel loaded with liposomes showed protection from SHIV in macaque vaginal challenge, whereas simple hydrogel with dispersed drug did not show protection. Testing of these two formulations in New Zealand white rabbits showed faster and better absorption of drug compared to simple gel. Another study evaluated liposomal system composed of 1% octylglycerol (OG). Gel formulation with OG liposomes showed greater efficacy compared to conventional gel formulation and maintained efficacy for at least 2 months. Another study reported significant anti-HIV activity of cardiolipin incorporated liposomes and their retention in vaginal cavity for 24h following

administration without causing vaginal irritation. SLNs have improved stability, high drug loading capacity, controlled, and targeted drug release profile. SLNs were not widely explored for microbicidal development. One study reported the functionalized SLNs loaded with tenofovir for HIV prevention and was shown to be well tolerated and non-toxic to vaginal epithelial cells ¹¹⁵. Overall, lipid-based formulations are safe and offer advantages for vaginal delivery of drugs, especially for sparingly soluble drugs ^{77,89,105}.

Inorganic nanoformulations

Inorganic nanoformulations can be defined as systems derived from metals such as gold, semiconductors, oxides such as iron oxide, carbon such as carbon dots and carbon nanotubes ¹¹⁶. They are being investigated for therapeutic and diagnostic purposes in biomedicine. Metal nanoparticles have been shown to inactivate viruses due to their intrinsic nature and interaction with viral proteins can be further enhanced through surface functionalization. Conjugation of gold nanoparticles with a derivative of HIV fusion inhibitor has shown high anti-HIV activity despite having no activity for the nanoparticles alone. Some other examples of increased HIV inhibitory activity through surface functionalization include gold nanoparticles decorated with monosaccharide, aptamer functionalized gold nanoparticles, polyvinyl pyrrolidone coated silver nanoparticles and 20-deoxyribonucleosidetriphosphate functionalized silica nanoparticles. This list is not

exhaustive and several other studied are evaluating the application of inorganic nanoparticles for microbicide development ^{89,113,117}.

CHAPTER 3

CURRENT STATE OF STIMULI SENSITIVE MICROBICIDES

3.1. Introduction

Stimuli responsive drug delivery systems respond to physicochemical and pathological stimuli by dramatically changing their properties such as charge reversal, ligand disintegration and size reduction allowing release of drugs on demand ¹¹⁸. Thus, engineering delivery systems to specific stimuli allows precise spatiotemporal control on drug delivery which minimizes off target effects, reduces inherent toxicity of drugs due to prolonged tissue exposure and improves drug efficacy. Both external and internal stimuli can be used as triggers for these systems depending on the desired application. External stimulus is artificially applied from outside such as light, electric field, magnetic field, and ultrasound, whereas internal stimulus is endogenous to the pathological tissues such as redox condition, temperature, pH, and enzyme. These systems can be employed for all routes of delivery such as parenteral, peroral, ocular, nasal, transdermal, rectal, and vaginal drug delivery.

Stimuli responsive systems have been majorly investigated for cancer and inflammatory therapies. Several delivery systems were developed using characteristics of tumor microenvironment as triggers for release of drugs to effectively reach solid tumors without systemic toxicity. For example, hypoxia

responsive delivery systems demonstrated effectiveness in cancer chemotherapy. Study using hypoxia responsive liposomes for delivery of siRNA reported selective cellular uptake under hypoxic and low-pH conditions of tumor to treat glioma. Another study using cathepsin B sensitive doxorubicin – peptide conjugates showed enhanced anticancer activity compared to doxorubicin alone in an in vivo tumor xenograft model ¹¹⁹. Reactive oxygen species (ROS) responsive delivery systems developed using boronic ester materials and polyoxalate materials has shown efficacy in treating acute oxidative injury and gained importance as ROS is overproduced during inflammatory conditions of several diseases ¹²⁰. Photo-activated depot developed by a group of researchers for minimally invasive insulin delivery is an example of using external stimuli such as light to trigger drug release. Release of insulin from the depot injected under the skin was controlled by external light source making the system minimally invasive to deal with drawbacks associated with insulin pumps ¹²¹.

Similar principles apply to development of safe and effective microbicides. Endogenous stimuli including temperature, pH, pathogen surface moieties, and enzymes have been explored for the development of stimuli responsive microbicides so far. This chapter exclusively discusses the stimuli sensitive systems developed for prevention of vaginal transmission of HIV.

3.2. Temperature Responsive Microbicides

Temperature sensitive drug delivery systems developed for cancer therapy undergo sharp change in the properties of the systems in response to variation in surrounding temperature triggering drug release. As these delivery systems such as liposomes or nanocarriers travel throughout the body, rapid and specific release of drug is achieved in tumor tissue which has elevated temperature of 40-42 °C compared to body temperature of 37 °C. In case of microbicides, temperature is not used as a trigger for drug release. Indeed, it is used as a trigger for phase transition of delivery systems for ease of administration in vaginal lumen. Thermosensitive gels are majorly investigated as microbicides which are liquids at room temperature and transform to highly viscous gels at body temperature following vaginal administration. Sol-gel transition temperature of the polymers is critical in designing such gels.

Ploxamers such as Pluronic F-127 and poly(*N*-isopropylacrylamide) are most used thermosensitive polymers whose gelation temperature depends on the polymer weight percent used and are capable of showing sol-gel transition near body temperature. Study conducted on barrier properties of pluronic (F-127)-hydroxypropylmethylcellulose (HPMC) hydrogel reported that it decreased mobility of HIV-1 mimicking nanoparticles compared to macaque cervicovaginal mucus and traditional hydroxyethylcellulose hydrogel. In addition, loading of F127-HPMC

hydrogel with mini-CD4 M48U1 did not alter its therapeutic activity with 93% of peptide release in controlled fashion suggesting its potential for delivery of anti-retrovirals and creating efficient barrier for HIV-1 diffusion ^{122,123}.

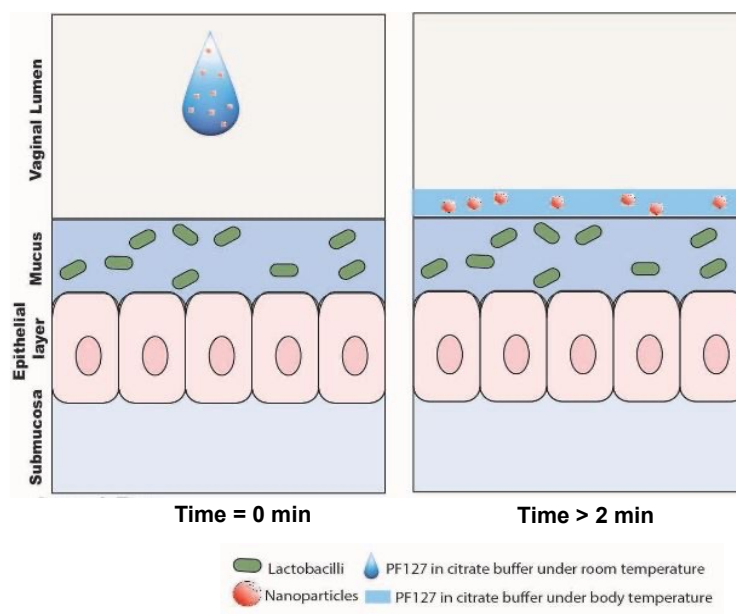


Figure 16. Sol-to-Gel transition of nanoparticle embedded thermosensitive gel following vaginal application.

Several studies reported nanoparticle embedded thermosensitive vaginal gels for safe and effective delivery of antiretrovirals. A study was conducted using PLGA nanoparticles (NPs) loaded with efavirenz (EFV), FDA approved NNRTI and raltegravir (RAL), FDA approved integrase inhibitor, which were embedded in

thermosensitive vaginal gel made of Pluronic F127 (20% w/v) and Pluronic F68 (1% w/v). Figure 16 shows the phase transition of such gels from liquids to semi solids at body temperature following vaginal administration. Results showed that the nanoparticles did not aggregate when incorporated in gel showing inhibition of HIV-1_{NL4-3} in TZM-bl indicator cells. RAL+EFV-NPs gel was also reported to be safe against HeLa cells with rapid transfer of NPs from gel and further uptake by HeLa cells ¹²⁴. Another study using rilpivirine encapsulated PLGA nanoparticles embedded in a thermosensitive gel showed significant protection against vaginal high dose HIV-1 challenge in BLT humanized mice 1.5 hours post administration. These results indicate that topically applied rilpivirine containing NPs embedded thermosensitive gel offers coitus dependent protection from HIV infection ¹²⁵. Investigation of cellulose acetate phthalate (CAP) - efavirenz combination nanoparticles embedded in thermosensitive gel has shown safety against HeLa cells with significantly higher prophylaxis against HIV-1 infection to TZM-bl cells for both short term (1 day) and long term (3 days). This indicates the potential of CAP-EFV-NP-Gel as long-term microbicide ¹²⁶.

Advantages of thermosensitive vaginal gels include ease of administration, uniform distribution of the gel with improved contact with vaginal folds and crevices. It also increases retention time in the vaginal lumen due to its higher viscosity after gelation compared to traditional gels allowing prolonged release of antiretrovirals. In

addition, nanoparticle embedded thermosensitive gel allows delivery of both hydrophilic and hydrophobic drugs. However, thermosensitive microbicide systems release drugs in continuous fashion unlike thermosensitive cancer therapies. They do not provide any advantages pertaining to stimuli sensitive systems such as on demand drug delivery and may lead to unwanted side effects to vaginal mucosa due to continuous exposure to the drug.

3.3. pH Responsive Microbicides

pH as a stimulus for vaginal microbicide delivery has been extensively studied and several studies on pH responsive microbicides were reported. Vaginal environment is colonized by lactobacillus in a healthy individual. Lactobacillus produces lactic acid and hydrogen peroxide that maintains acidic environment in vaginal lumen with pH ranging from 3.5 to 4.5 aiding in defense against pathogens¹²⁷. Several values were reported for seminal fluid pH which range from 7.19 to 8.47. Seminal fluid has very high buffering capacity compared to any other fluid in the body. Seminal fluid neutralizes vaginal environment and maintains pH near neutral when it deposits into the vaginal lumen during intercourse which is critical for survival and entry of sperm into the cervical mucus^{128,129}. Vaginal pH changed from 4.3 to 7.2 with in 8 seconds of arrival of semen in vaginal lumen as measured by radiotelemetry¹³⁰. This immediate change in pH of the vaginal environment was taken advantage for designing pH-responsive microbicides.

Carboxylic acid of several polymers such as Eudragit, polyacrylic acid, and cellulose acetate phthalate was used as the pH triggering moiety as it remains protonated at low pH of vagina and deprotonated at high pH of seminal fluid. One of the earliest reported pH responsive delivery systems was a hydrogel which is also temperature sensitive. Random terpolymer of N-isopropyl acrylamide, butyl methacrylate, and acrylic acid was used to develop the hydrogel with temperature induced gelation along with seminal pH triggered drug release. Polymer remained as solution below body temperature which will aid in vaginal application and uniform distribution in the lumen. It gelled at body temperature and remained as semi solid under vaginal pH and transitioned to solution at seminal pH triggering burst release of the entrapped model drugs for both small molecules and macro molecules. In vitro cytotoxicity studies of terpolymer showed similar profile as commercially used Carbopol indicating its potential for in vivo use ¹³¹. This system has shown low level of drug release in vaginal simulant fluid and in the absence of seminal simulant fluid indicating drug release in the absence of stimulus.

Studies were reported using Eudragit nanoparticles for pH-sensitive release of anti-retrovirals. Loaded drugs were protected from degradation under rigorous acidic conditions of vagina and quick release of drugs was observed under physiological pH of approximately 7.4. Nanoparticles were shown to be safe for vaginal epithelial cells and its membrane integrity ^{132,133}. Similar system was reported using a blend of PLGA

and Eudragit S-100 polymers for the pH sensitive release of tenofovir. In vitro testing of these nanoparticles has shown higher drug release in seminal fluid simulant and cytocompatibility to vaginal epithelial cells and *Lactobacillus crispatus* ¹³⁴. Although, these nanoparticles exhibited pH responsive drug release, they are taken up by the epithelial cells upon application and drug will be released in neutral pH of the cytosol. This pattern of nanoparticle uptake and drug release does not impede continuous release of drug and related side effects. In addition, this system will not protect the drug depot from exhausting.

Cellulose acetate phthalate (CAP) fibers and nanoparticles were reported as potential pH-sensitive microbicides. CAP is commercially used for enteric coating of tablets because of its ability to be stable in acidic environment and rapid dissolution in basic environment. In addition to the pH responsive behavior, CAP exhibits anti-HIV activity. CAP fiber webs are prepared by simple process of electrospinning. They were also loaded with fluorescent molecule rhodamine as a model drug to study pH sensitive release of entrapped moieties. Results showed abrupt release of rhodamine at pH 7.4 which contrasts with the negligible release at pH 4.2. Further *in vitro* studies showed compatibility of fibers with vaginal epithelial cells and HIV inhibitory activity ¹³⁵. More recently reported CAP based system was dolutegravir loaded CAP nanoparticles embedded in thermosensitive gel which has shown pH sensitive drug release and biocompatibility ¹³⁶. However, this system is limited like other

nanoparticle-based systems due to immediate uptake of nanoparticles by the vaginal epithelial cells and subsequent release of drugs in physiological pH of cytosol.

Reversibly pH-responsive polyurethane membranes are the only reported stimuli sensitive system with potential for use as a long-term delivery system. These membranes showed sharp on and off drug release at pH 7 and pH 4.5 respectively. They can be incorporated in reservoir type intravaginal rings for long-term use. These membranes also showed safety towards vaginal epithelial cells ¹³⁷. Few other novel systems of pH-responsive microbicides were reported such as freeze dried bigels with accelerated release of tenofovir in seminal simulant fluid ¹³⁸. Griffithsin loaded electrospun fibers with selective release in simulated seminal fluid was also reported as potential microbicide ¹³⁹. Reversible crosslinked hydrogel based on phenylboronate and salicylhydroxamate that impeded HIV transport through pH depended modulation of viscoelasticity is different from drug releasing systems but can be used as a prevention tool for male to female sexual transmission of HIV ¹⁴⁰. Although, pH responsive systems seem promising, the drug release can occur in conditions of elevated vaginal pH such as bacterial vaginosis, menopause, and vaginitis. In addition, the pH responsive systems developed so far have entrapped the drug in polymeric matrix allowing a minimal amount of drug leaching even in the absence of seminal fluid.

3.4. gp120 Responsive Microbicides

HIV-1 envelope glycoprotein (Env) has been extensively investigated as it is accessible to antibodies and the only targeting moiety available for designing vaccines. Env is a trimer composed of heterodimers of gp120 and gp41. HIV gp120 is a surface protein which plays a vital role in host cell entry of the virus by binding to CD4 receptor and CCR5 or CXCR4 co-receptors on target cells. HIV gp41 is a transmembrane protein and assists in fusion of the virus with host cell. HIV gp160 is the precursor for these glycoproteins that was extensively modified with high mannose glycans and gets proteolytically cleaved in the trans-Golgi. This extensive glycosylation contributes to more than half of the mass of Env and influence viral biology such as proper folding of Env, formation of glucan shield to evade detection by immune system, viral entry, and infectivity. Studies on HIV gp120 revealed that it's glycan portion predominantly consists of oligomannoses of five to nine mannose residues with 20-35 N-glycosylation sites. Contrast to the gp120, gp41 consists of more complex carbohydrates and fewer oligomannoses and fewer N-glycosylation sites ¹⁴¹.

Various natural and synthetic lectins have been studied as HIV entry inhibitors owing to high mannose content of gp120. These lectins specifically bind to gp120 oligomannoses preventing its interaction with host cell receptors. Examples of natural lectins include cyanobacterial lectin cyanovirin-N (CV-N), plant lectin concavalin A (con A) extracted from jack bean, and red algae lectin Griffithsin (GRFT) isolated from

Griffithsia. Synthetic lectins include carbohydrate binding agents such as benzoboroxole and phenyl boronic acid-based lectins. Formulation of lectin based anti-HIV therapy is challenging due to several factors such as solubility, stability, difficulty in manufacturing, toxicity, and resistance. Lectin direct targeting is a potential strategy for developing anti-HIV nano formulations. Immune cells are often targeted in this way. For example, HIV-1 DNA encapsulated mannan coated cationic liposomes targeting mannose receptors have triggered immunological responses by activating cytotoxic T cells and IgA. Lectin indirect targeting is another strategy for anti-HIV formulations based on lectins where lectins are included in the formulation to capture viral particles by interacting with surface glycoproteins. A study was reported on HIV-1 capture using lectin-immobilized polystyrene nanospheres [11]. Polystyrene nanospheres immobilized with Con A achieved significant reduction in viral infectivity when incubated with viral suspension compared to Con A-free nanospheres indicating its potential as HIV prevention tool ¹⁴².

Binding of Con A to oligomannoses of HIV gp120 was taken advantage by a group of researchers to engineer gp120 responsive microbicide to address challenges such as undesired drug release associated with traditional microbicides. They first studied binding interaction of Con A-glycogen and Con A-mannan as their binding constants are critical parameters in the core-shell design employed for gp120 responsive delivery system ¹⁴³. Mannose responsive particles (MRP) were prepared

by layer-by-layer coating of tenofovir entrapped calcium carbonate particles with Con A which is a mannose specific lectin and glycogen which is a polysaccharide cross-linker. These particles were termed as core containing (C^+ MRP) and the particles with $CaCO_3$ core dissolved are termed as core dissolved MRPs (C^- MRP). C^+ MRPs were well tolerated and non-cytotoxic even at higher concentrations compared to C^- MRPs when tested on vaginal epithelial cells and raw macrophages for viability and proinflammatory cytokine release. C^+ MRPs tested for gp120 triggered drug release showed maximal tenofovir release within 4 to 8 hours in the presence of mannose-rich HIV-1 recombinant gp120 and the amount of drug release was higher compared to untreated conditions i.e., in the presence of mannose free seminal and vaginal fluid simulants. HIV-1 gp120 triggered release was achieved by erosion of Con A/Glycogen layers as Con A competitively binds with mannose moieties in gp120. Erosion of MRP layers exposes $CaCO_3$ core particles that contribute to core dissolution by chelating calcium ions with citric acid present in seminal fluid enhancing drug release ¹⁴⁴. This system was further tested in C57BL/6 mice model. Results showed that the formulation was safe to vaginal epithelium up to 24 hours ¹⁴⁵.

Even though enhanced drug release was observed in response to gp120MRPs, basal drug release of about 6% was seen in the absence of gp120 i.e., in vaginal simulant fluid alone. This basal drug level itself means that the system is not entirely gp120 responsive i.e., unintended drug is released in the absence of stimulus

exhausting drug depot and may cause side effects to vaginal epithelium. In addition, vaginal canal is lined with adherent and secreted mucins which are highly glycosylated ¹⁴⁶. These glycosylation patterns could trigger the drug release from mannose responsive particles and makes the delivery system non-specific. MRPs are nanoparticles which need to be further embedded in a gel or film for vaginal administration. This adds a layer of barrier impeding interaction with gp120 of HIV and the *in vitro* studies on gp120 responsive release does not predict *in situ* sensitivity to HIV virions. Thus, better delivery systems in appropriate dosage forms need to be designed for specific stimuli sensitive drug delivery.

3.5. Enzyme Responsive Microbicides

Enzyme responsive delivery systems have shown growing interest in recent years due to the high specificity of enzymes to its substrates. This allows site specific release of drugs as in tumor microenvironment. Enzyme triggered microbicides are designed to release drugs in response to enzymes present in seminal fluid. Seminal fluid is the carrier of HIV in male-female transmission of infection. Semen is composed of several components such as citric acid, fructose, zinc, and enzymes such as acid phosphatase, prostate specific antigen (PSA), hyaluronidase and fibrinolysin to keep sperm cells viable and to aid in their motility ¹⁴⁷. Enzyme responsive microbicides allow more specificity unlike mannose and pH – responsive delivery systems due to absence of these enzymes in vaginal fluid. PSA triggered and

hyaluronidase triggered microbicides are reported so far as high concentrations of these enzymes are present in seminal fluid.

PSA degradable microparticles were prepared by crosslinking hydroxypropylmethacrylamide, bis-methacrylamide with peptide substrate for PSA. Prepared microparticles were loaded with poly(styrene-4-sulfonate) (pSS), an entry inhibitor of HIV. Microparticles were observed to be degraded when treated with human seminal plasma (HSP). Drug release studies showed conditional release of pSS from microparticles in the presence of HSP. Released pSS was shown to inactivate 60% of viral activity within 30 minutes of exposure and up to 90% upon prolonged exposure. However, the vaginal retention of microparticles was not studied and may be taken up by epithelial cells like nanoparticles. In that instance, delivery system will not stay in the vaginal lumen for it to get triggered by the seminal fluid. In addition, pSS is an anionic polymer that binds to HIV envelope for its inactivation. Recent clinical trials have shown lack of efficacy of anionic polymers as microbicide candidates. The drug was also physically entrapped which would lead to leaching in the absence of stimulus. PSA triggered microbicide is a promising idea for microbicide prevention and needs to be improved for vaginal retention, absolute drug release in the presence of stimulus and efficacy ¹⁴⁸.

Hyaluronidase sensitive delivery systems were made using hyaluronic acid, biodegradable, naturally occurring polysaccharide. Hyaluronic acid has been widely

used in recent years for biomedical applications owing to its non-immunogenicity and excellent physico-chemical properties. Hyaluronic acid nanoparticles loaded with tenofovir were shown to release higher amount of tenofovir in the presence of hyaluronidase. They were also non cytotoxic to vaginal epithelial cells and *Lactobacillus crispatus*, dominant species of healthy vaginal microbiome ¹⁴⁹. As nanofibers have more drug loading capacity, higher vaginal retention and ease of application compared to nanoparticles, hyaluronic acid nanofibers were prepared. Similar to nanoparticles, hyaluronic acid nanofibers showed triggered release of tenofovir in the presence of hyaluronidase. They were non cytotoxic and safe towards vaginal epithelium of C57BL/6 mice ¹⁵⁰. Main disadvantages of hyaluronidase-based systems include the significant amount of drug release in the absence of hyaluronidase. In addition, amount of hyaluronidase used in the experiments depict the amount of enzyme in *in vivo* conditions. However, this amount varies based on health condition of the male and number of sperms present in the ejaculate. Therefore, drug release can be lower than expected if low amount of the enzyme is present in seminal fluid. As optimal pH of the hyaluronidase is acidic, drug release can be further affected by the pH of the fluid during exposure of delivery system to the enzyme. Hyaluronidase sensitive systems need to be further improved to minimize drug release in the absence of hyaluronidase and tested in various pH conditions and enzyme quantities.

CHAPTER 4

DEVELOPMENT AND EVALUATION OF SMART HYDROGEL FOR HIV-1

PREVENTION

4.1. Rationale

HIV has been debilitating epidemic since first cases were reported four decades ago. Millions of lives were lost to HIV, and it is still one of the main causes of mortality worldwide. COVID 19 had further complicated management of HIV and the lives of HIV patients causing severe comorbidities. It is important to note that women and girls account for 53% of global HIV population. 7000 young women get infected with HIV every week. In Sub-Saharan Africa, 63 % of all new infections correspond to women and girls in 2020. Moreover, HIV infected women contribute to HIV infections in children through pregnancy, childbirth, and breastfeeding with global average mother to child transmission rate of 11.4%. HIV infections in women is a serious problem as more than 90% infections in children are acquired from their mother.

Several factors such as biological, social, cultural, and structural are the reasons for higher infection rate among females compared to males. Heterosexual transmission through vaginal route is the predominant mode of HIV transmission among women. Anti-retroviral therapy i.e., daily use of oral anti-retroviral (ARV) drugs is currently the standard of care for HIV patients to prolong their life span. It was reported that daily use of oral ARVs leads to viral resistance and causes several

side effects ^{151,152}. Prevention methods that are clinically applicable include condoms, pre-exposure prophylaxis and post-exposure prophylaxis. However, these are not accessible to women in resource poor settings due to barriers at individual, community, and societal levels. Several women and girls around the world have limited or no decision-making power about their sexual and reproductive lives and their own health care. Gender discrimination in the context of violence, poverty and insecurity blocks access to HIV prevention and treatment services and heightens their risks of acquiring HIV. Therefore, there is a need to respond to women's needs and provide a simple, convenient, and suitable prevention options to lower the spread of infection to next generation.

Microbicides i.e., topical products such as gels, films and rings that are designed to apply inside vagina or rectum are developed as a discreet prevention method that can be controlled by women. Presently, microbicides are controlled release formulations that continuously release anti-retroviral drugs to inhibit earliest steps of HIV transmission at the vaginal mucosa. However, such continuous exposure to drugs may disrupt vaginal epithelium enhancing susceptibility to HIV infection. Other concerns from women on the use of such devices include fear of ring coming out and discomfort during menstruation complexify its application ¹⁵³.

As discussed in chapter 3, stimuli responsive drug delivery systems offer on demand delivery which reduces toxicity associated with prolonged tissue exposure

to drugs. They improve drug efficacy by providing supra therapeutic levels of drugs in the event of infection. Enzyme responsive microbicides provide specificity as opposed to pH and mannose responsive microbicides as some enzymes are exclusively present in seminal fluid but absent in vaginal fluid. Therefore, the goal of the study is to design safe and effective enzyme responsive delivery system for vaginal application to prevent HIV infection in women.

4.2. Design of PEG-Based Smart Hydrogel

Prostate specific antigen (PSA) is a serine protease present in seminal fluid. It aids in liquefaction of semen and is present in high concentrations that range between 0.39 to 5 mg/ml¹⁵⁴⁻¹⁵⁶. In this study, an innovative microbicial complex was designed by attaching a PSA labile moiety (PLM) to peptide-based FDA approved entry inhibitors, such as enfuvirtide and ibalizumab. Stimuli sensitive hydrogel based on PEG conjugated microbicial complex was developed that delivers drug in response to PSA in semen, primary body fluid involved with heterosexual HIV-1 transmission as shown in Figure 18.

It was hypothesized that hydrogel based on PEG-DBCO, and PEG-azide conjugated with PLM linked microbicide is an efficient stimulus- triggered microbicial delivery system for protecting women and homosexual men against HIV-1 infection. Enfuvirtide, an FDA approved HIV-1 entry inhibitor, was selected for preparation of a prototype PLM linked anti-retroviral. PEG-based hydrogels

contain chemically cross-linked network which has highly porous structure and limited swelling property. They can be conveniently applied to the vaginal lumen owing to their flexibility and avoiding potential leakage in the presence of seminal fluid. Subsequently, we have developed and evaluated PEG-based hydrogel that delivers an entry inhibitor peptide, Enfuvirtide, upon exposure to an enzymatic stimulus (i.e., PSA). The physicochemical properties of PEG-based hydrogel were further optimized for the intravaginal delivery of Enfuvirtide. This system efficiently prevents from being infected with HIV-1 and simultaneously reduces risk of toxicity associated with continuous exposure of microbicides.

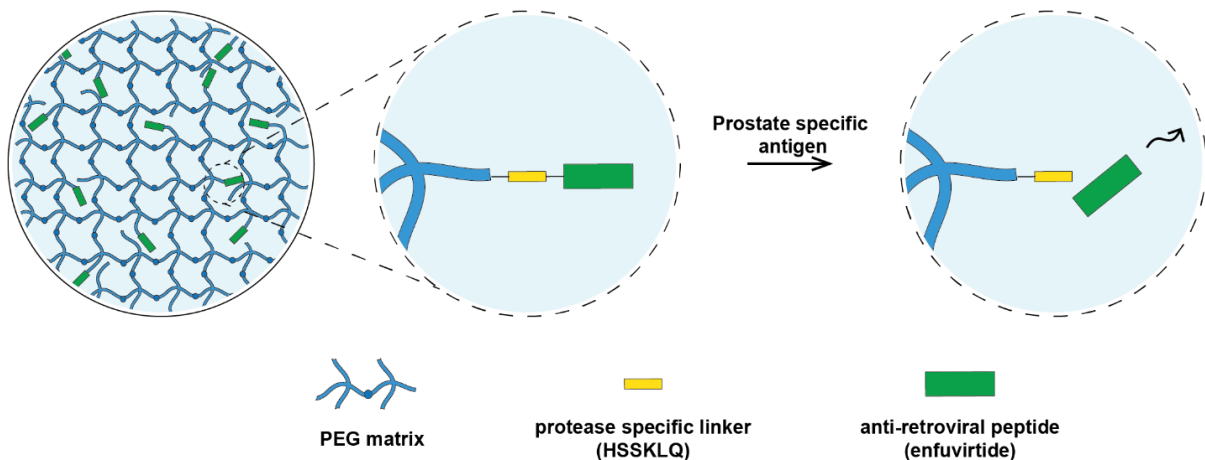


Figure 17. Design of PEG-based hydrogel and release of anti-retroviral peptide upon exposure to prostate specific antigen (PSA) as an enzymatic stimulus.

4.3. Development of PEG-Based Smart Hydrogel

4.3.1. Materials

2, 6 dichlorobenzoyl chloride (DCB), Tris base, sodium azide were purchased from Sigma Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA), diisopropylethylamine (DIEA), diethyl ether, dimethylformamide (DMF), acetonitrile HPLC grade, water HPLC grade, dichloromethane (DCM), triethylamine, Fmoc amino acids were purchased from Oakwood chemicals (Estill, SC). Sodium chloride (NaCl), hydrochloric acid (HCl) was purchased from Fischer Scientific (Waltham, MA). Wang resin (4-benzoyloxybenzyl alcohol resin), piperidine were purchased from Chem-Impex International Ltd (Wood Dale, IL. O-(7-Azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU) was purchased from Accela ChemBio Inc (San Diego, CA). 4 arm PEG-OH 10 kDa was purchased from Jenkem Technology USA (Plano, TX). 4 arm PEG-NH₂ 10kDa was purchased from Biochempeg (Watertown, MA) and DBCO-NHS was purchased from Conju-Probe (San Diego, CA). Prostate specific antigen (PSA) was purchased from Fitzgerald Industries International (Acton, MA). T-20 (Enfuvirtide, catalog number 12732) was obtained through the AIDS Reagent Program, Division of AIDS, NIAID. N₃-HSSKLQ-Enfuvirtide (HF42) peptide was custom synthesized from LifeTein LLC (Somerset, NJ). Vaginal epithelial cell line VK2E6/E7 (ATCC CRL-2616™) and Murine

macrophage cell line RAW 264.7 (ATCC TIB-71™) were purchased from ATCC (Manassas, VA).

4.3.2. Screening of PSA substrates for ideal PSA labile moiety (PLM)

Several studies have already discussed PSA substrate requirements.^{157,158} In this study, three sequences of peptides were studied from the literature to be linked to the drug. The best of the three substrates will be constructed with the hydrogel for optimal delivery and retaining biological activity of the drug after proteolysis. The sequences were designed based on the established substrates for PSA including HSKLQ-AMC, GISSFYSSK and AYLM^{148,157-159}. Of these three sequences, AYLM is designed by us under the hypothesis that it will be superior based on the previous observations on the inclusion of methionine residue before the cleavage site¹⁶⁰.

It should be noted that the kinetics of hydrolysis was determined using fluorogenic substrates in previous studies. In this study, HPLC method was developed for determination of the hydrolysis rates of the substrates. For this reason, YY was incorporated after the cleavage site as a chromophore for HSKLQ and AYLM to monitor the product chromatographically.

Study of HSSKLQYY as PLM

Synthesis and purification of HSSKLQYY

HSSKLQYY was synthesized using Fmoc solid phase peptide synthesis (SPPS) procedures. 0.5 g of wang resin was suspended in 11.6 mL of anhydrous DMF to make final concentration of 60 mM 4-benzyloxybenzyl alcohol. Fmoc amino acid (300 mM) and pyridine (495 mM) were added to the suspension of DMF. DCB (300 mM) was added to this mixture that was shaken for 18 hr.¹⁶¹. After loading of C-terminal amino acid corresponding to each specific substrate on the resin, peptide structure was built using the standard method involved with the HATU activation process¹⁶²⁻¹⁶⁴. At the end of the synthetic process, the HSSKLQYY was cleaved from the resin using TFA/water (95/5) for 1 hr. After evaporating TFA with nitrogen gas, peptide precipitate was redissolved in 3 ml of TFA. 100 ml of ice-cold diethyl ether was added to the vial to precipitate out the peptides. Obtained precipitate was purified using HPLC (Shimadzu LC-2010A, Lenexa, KS).

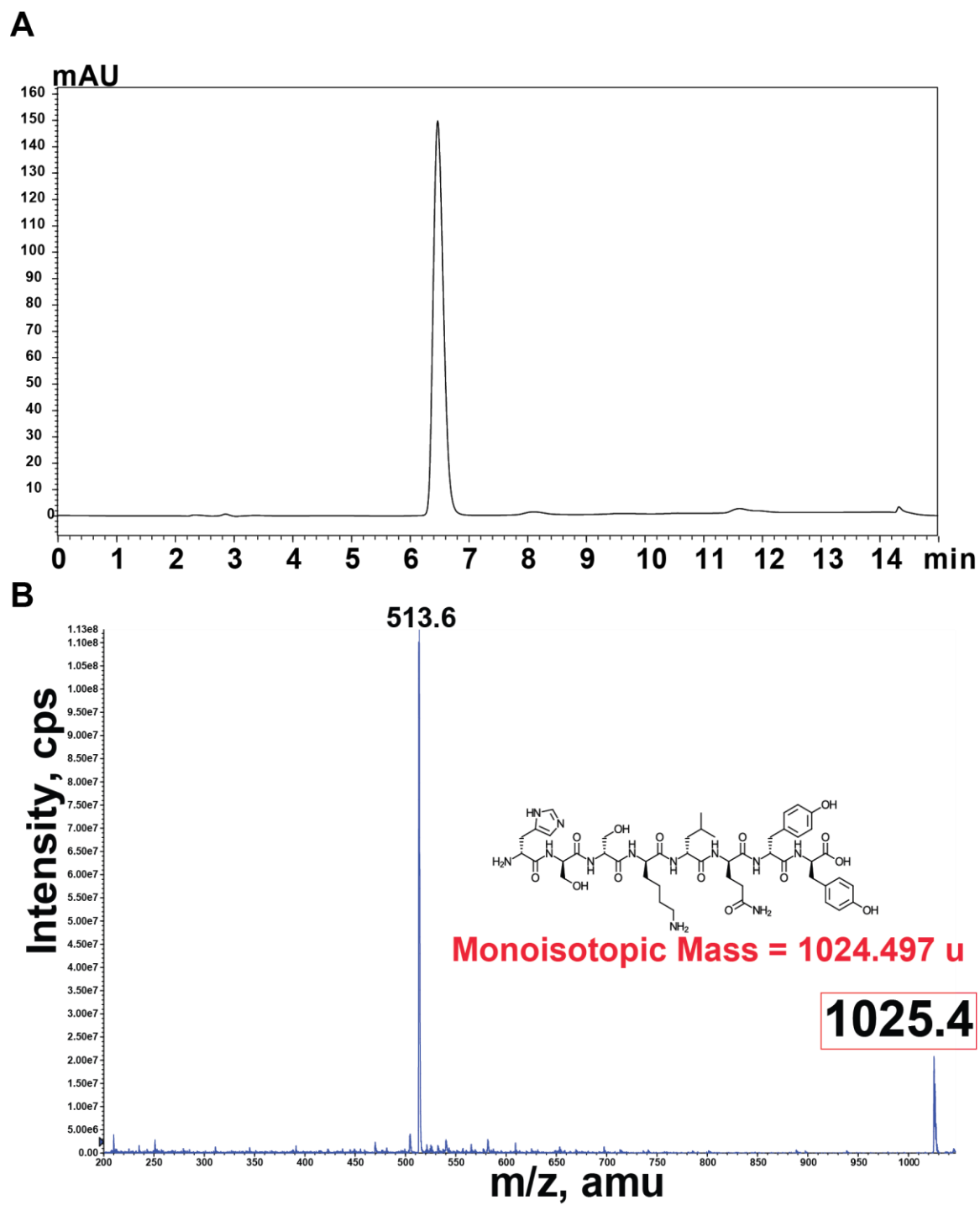


Figure 18. A. HPLC chromatogram of purified HSKLQYY and B. corresponding ESI-MS spectrum showing MH^+ .

Pure peptide was lyophilized and dissolved in 200 μL of Tris-buffer (50 mM Tris, 150 mM NaCl, 0.01% Tween 80, pH 7.8), and used as the stock solution. HSSKLQYY stock solution was quantified using UV spectroscopy using its extinction coefficient ($\epsilon_{280} = 2560 \text{ M}^{-1} \text{ cm}^{-1}$). As shown in Figure 19, purified HSSKLQYY displayed single peak in HPLC chromatograms. When the sample of HSSKLQYY peak was infused into the mass spectrometer at a flow rate of 10 $\mu\text{l}/\text{min}$, it displayed a m/z of 1025.4 corresponding to MH^+ ion (i.e., its monoisotopic mass M is 1024.4 Da).

Study of HSSKLQYY hydrolysis by PSA

Figure 20 shows the chemical structures of the products HSSKLQ and YY expected from enzymatic cleavage of HSSKLQYY by PSA.

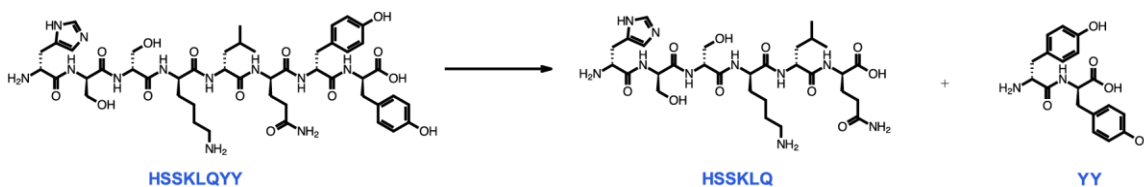


Figure 19. Hydrolysis of HSSKLQYY by PSA to products HSSKLQ and YY.

The hydrolysis rate of HSSKLQYY by PSA was examined in the Tris-buffer solution (50 mM Tris, 150 mM NaCl, 0.01% Tween 80, pH 7.8)¹⁵⁹. The hydrolysis reaction of HSSKLQ was conducted with varying concentrations loaded in a HPLC vial. The conversion of the PSA substrate linker HSSKLQYY to its product YY in the

presence of PSA was monitored by HPLC. PSA at the final concentration of 0.87 μM was added to the solution of the substrate linkers (200 μL), and the mixture was gently and homogenously mixed. HPLC analysis was performed using Nucleosil 150 X 3.2 mm 5 μm C18 column (Supelco Analytical, Sigma Aldrich (St. Louis, MO)) on samples every 15 min for 4 hr. HPLC method in Table 4 was observed to resolve substrate and product peaks. A refers to 99.9% water with 0.1% TFA and B refers to 99.9% acetonitrile with 0.1% TFA.

Table 4. HPLC method for monitoring HSSKLQYY hydrolysis by PSA

Time (min)	Mobile phase
0	15% B
10	100% B
10.1	15% B
15	15% B

The products YY was verified by infusion of the isolated material YY peak collected from HPLC in mass spectrometer (SCIEX 3200 QTRAP, Framingham, MA). The amounts of the substrate linker HSSKLQYY and the product YY at any specific time was determined based on the area under the curve (AUC) that is compared with the standard curve for each species. The hydrolysis rates were calculated from the linear slope for the formation of the product over time. The hydrolysis rates of the substrate linkers with varying concentrations were fitted to Michaelis-Menten

equation using Sigma Plot 14.5 to calculate the rate constant (K_M) and turnover number (k_{cat}).

As shown in Figure 21 and Figure 22, the substrate peak of HSKLQYY appeared at the retention time (RT) of 6.4 min, and the product peak did at 4.64 min. The product amount continuously increased over a period of 4 hr. The product peak has the mass spectrum of 345.1 Da, corresponding to MH^+ ion, confirming the cleavage of YY from HSKLQYY, as the monoisotopic mass of YY is 344.1 Da. The rate constants of HSKLQYY based on Michealis-Menten equation were K_M of 1003 μM and k_{cat} of 0.0022 s^{-1} .

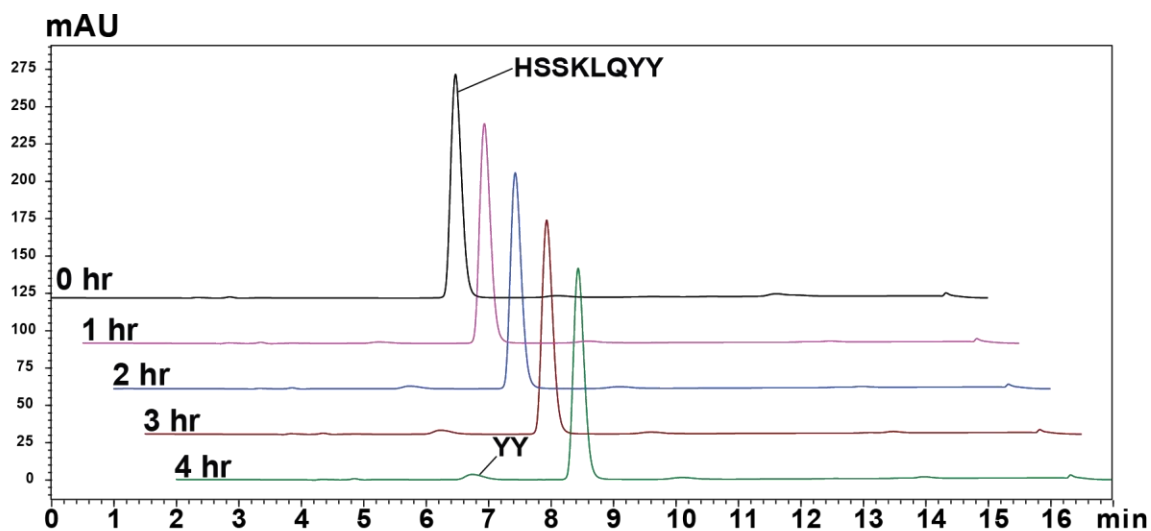
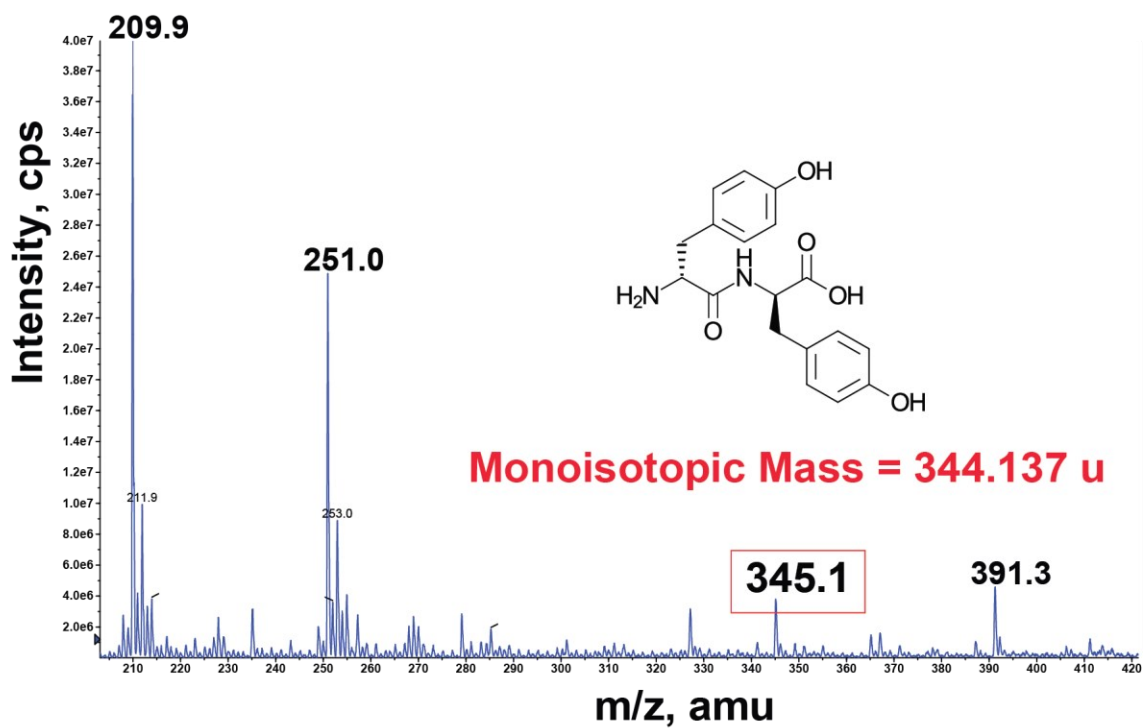
A**B**

Figure 20. A. Chromatogram for product (YY) peak of HSSKLQYY hydrolysis over 4 hr., B. ESI-MS spectrum of YY showing MH^+ .

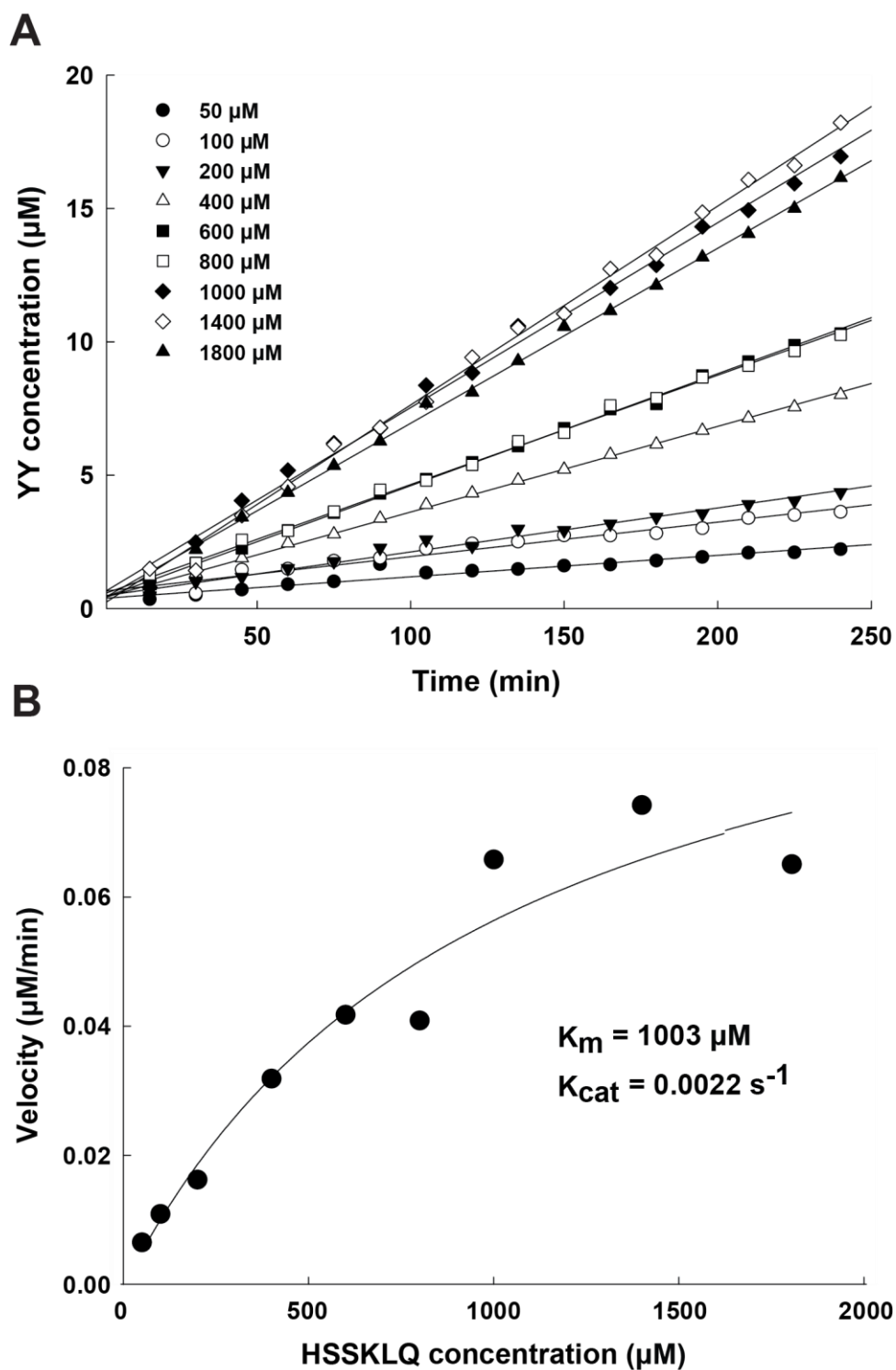


Figure 21. C. PSA progress curve with varying concentrations of HSSKLQ, D. Michaelis-Menten saturation curve.

Study of GISSFYSSK as PLM

Synthesis and purification of GISSFYSSK

GISSFYSSK was synthesized and purified using the same procedure described for HSSKLQYY. Pure peptide was lyophilized and dissolved in 200 μL of Tris-buffer (50 mM Tris, 150 mM NaCl, 0.01% Tween 80, pH 7.8), and used as the stock solution. GISSFYSSK stock solution was quantified using UV spectroscopy using its extinction coefficient ($\epsilon_{280} = 1280 \text{ M}^{-1} \text{ cm}^{-1}$). As shown in Figure 23, purified GISSFYSSK displayed single peak in its HPLC chromatogram. When a sample of GISSFYSSK peak was infused into the mass spectrometer at a flow rate of 10 $\mu\text{l}/\text{min}$, it displayed a m/z of 975.4 corresponding to MH^+ ion (i.e., its monoisotopic mass M is 974.4 Da).

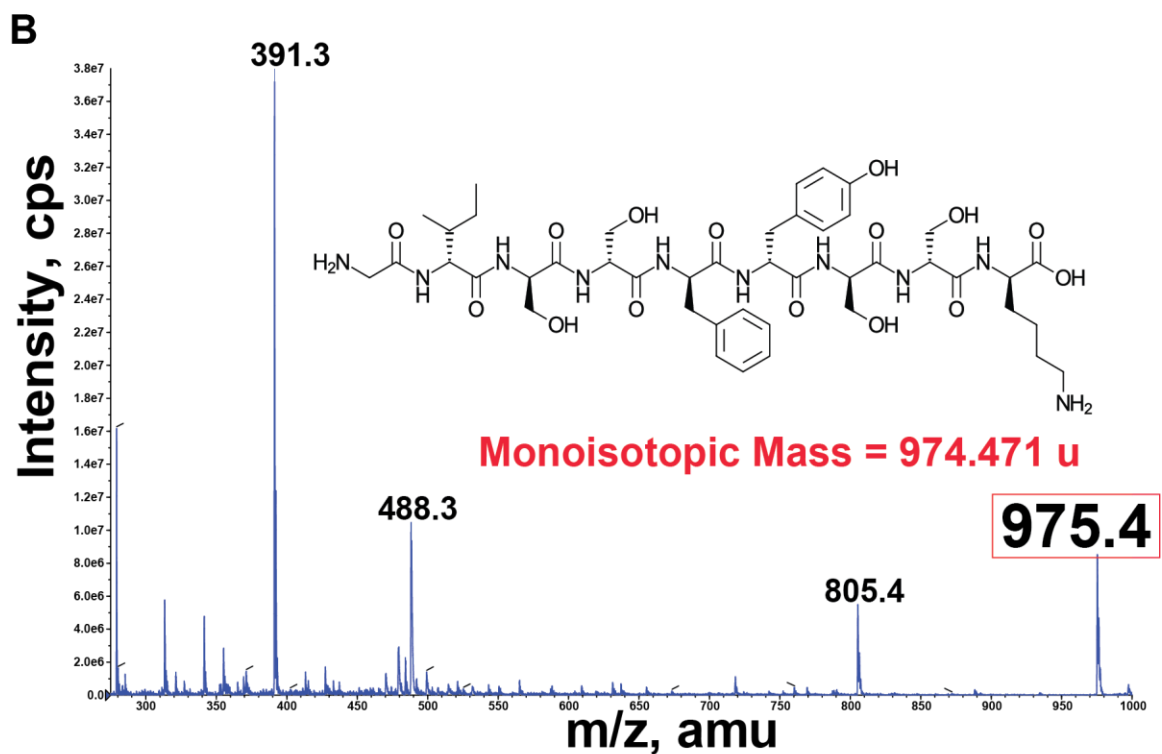
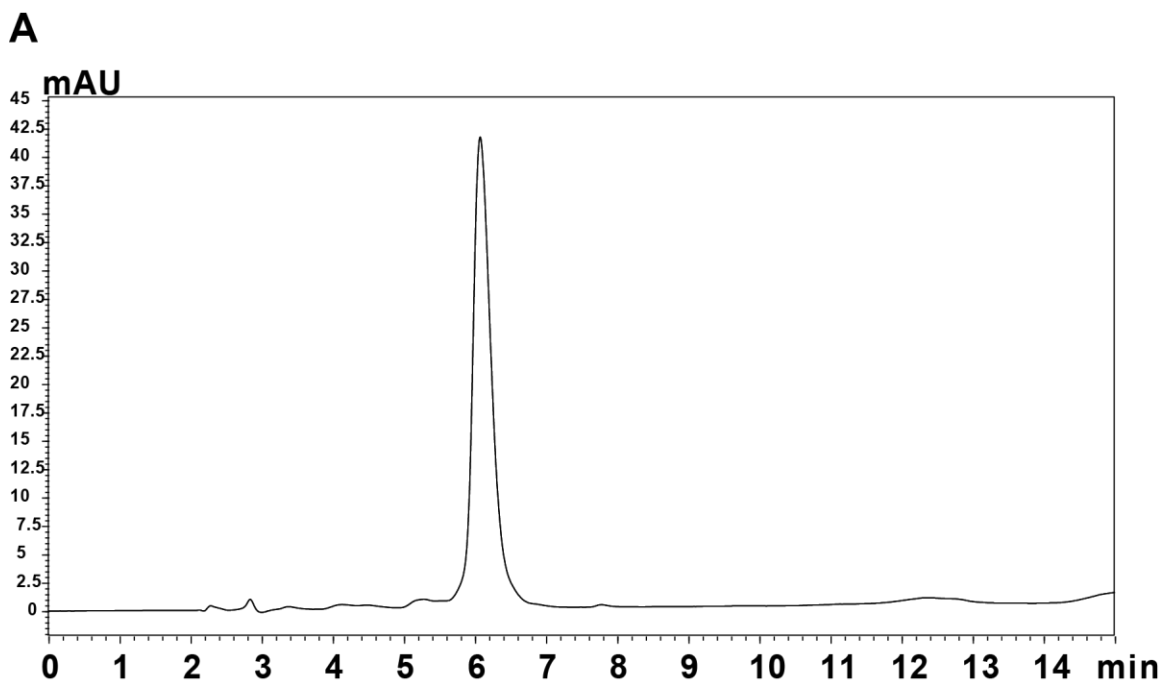


Figure 22. A. HPLC chromatogram of purified GISSFYSSK and B. corresponding ESI-MS spectrum showing MH^+

Study of GISSFYSSK hydrolysis by PSA

Figure 24 shows the chemical structures of the products GISSFY and SSK expected from enzymatic cleavage of GISSFYSSK by PSA.

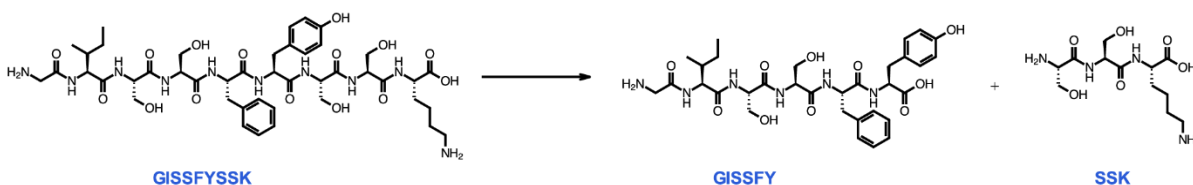


Figure 23. Hydrolysis of GISSFYSSK by PSA to products GISSFY and SSK.

The hydrolysis rate of GISSFYSSK by PSA was examined in the Tris-buffer solution (50 mM Tris, 150 mM NaCl, 0.01% Tween 80, pH 7.8). The hydrolysis reaction of GISSFYSSK was conducted with varying concentrations loaded in a HPLC vial. The conversion of the PSA substrate linker GISSFYSSK to its product GISSFY in the presence of PSA was monitored by HPLC. PSA at the final concentration of 0.87 μ M was added to the solution of the substrate linkers (200 μ L), and the mixture was gently and homogenously mixed. HPLC analysis was performed using Nucleosil 150 X 3.2 mm 5 μ m C18 column (Supelco Analytical, Sigma Aldrich (St. Louis, MO)) on samples every 15 min for 4 hr. HPLC method in Table 5 was observed to resolve substrate and product peaks. A refers to 99.9% water with 0.1% TFA and B refers to 99.9% acetonitrile with 0.1% TFA.

Table 5. HPLC method for monitoring GISSFYSSK hydrolysis by PSA

Time (min)	Mobile phase
0	20% B
10	50% B
12	100% B
12.1	20% B
15	20% B

The product of GISSFYSSK was verified by infusion of the isolated material GISSFY peak collected from HPLC in mass spectrometer (SCIEX 3200 QTRAP, Framingham, MA). The amounts of the substrate linker GISSFYSSK and the product GISSFY at any specific time was determined based on the area under the curve (AUC) that is compared with the standard curve for each species. The hydrolysis rates were calculated from the linear slope for the formation of the product over time. The hydrolysis rates of the substrate linkers with varying concentrations were fitted to Michaelis-Menten equation using Sigma Plot 14.5 to calculate the rate constant (K_M) and turnover number (k_{cat}).

As shown in Figure 25 and Figure 26, the substrate (GISSFYSSK) and product (GISSFY) eluted at 4.4 and 7.4 min, respectively. There was a difference in retention time before and after the hydrolysis process. The amount of the product continuously increased over a period of 4 hr. The product peak has the mass spectrum of 345.1, corresponding to MH^+ ion, confirming the cleavage of SSK from GISSFYSSK, as the

monoisotopic mass of GISSFY is 672.3 Da. The rate constants of GISSFYSSK based on Michealis-Menten equation were K_M of 480 μM and k_{cat} of 0.016 s^{-1} .

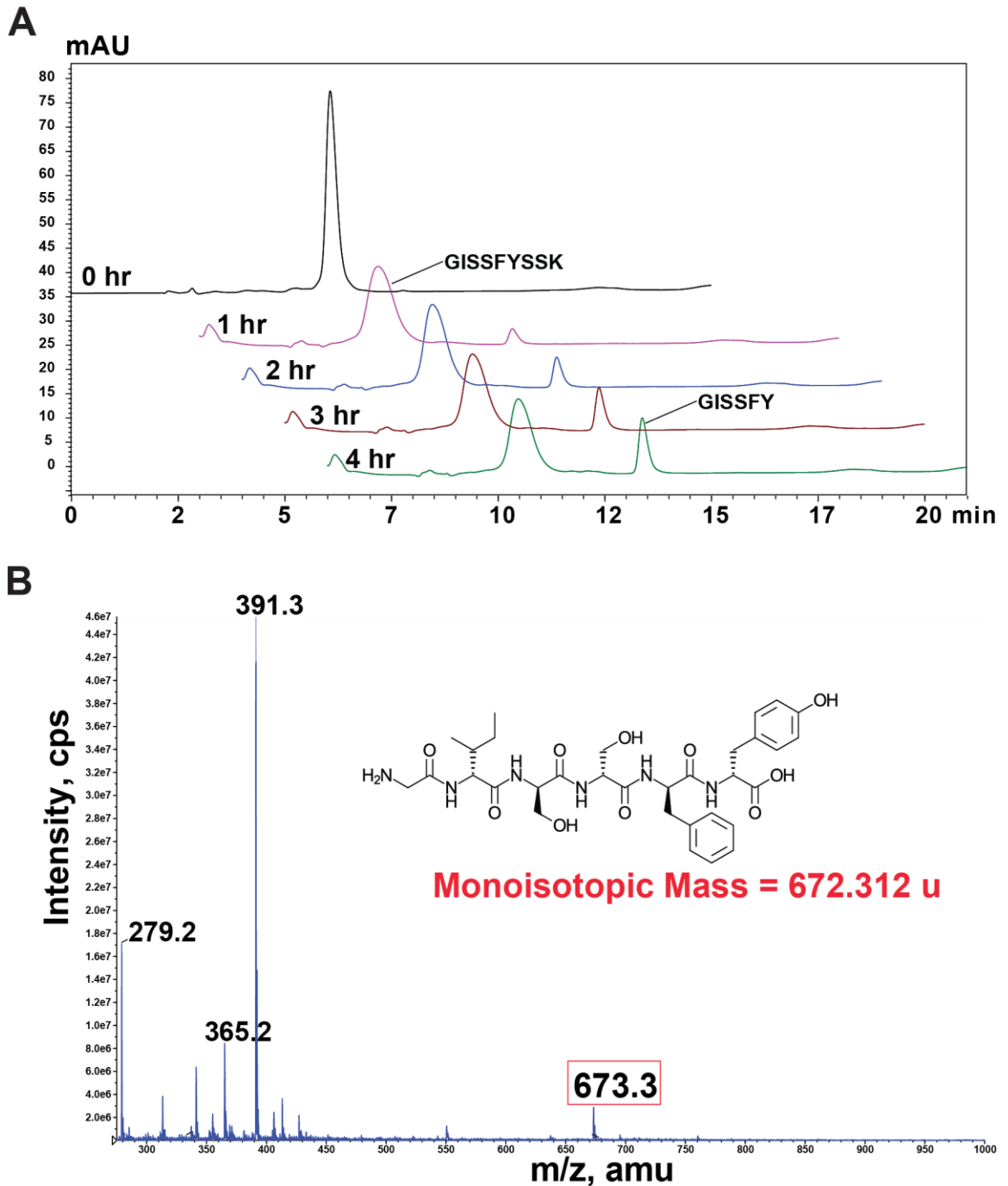


Figure 24. A. Chromatogram for product (GISSFY) peak of GISSFYSSK hydrolysis over 4 hr., B. ESI-MS spectrum of GISSFY showing MH^+ .

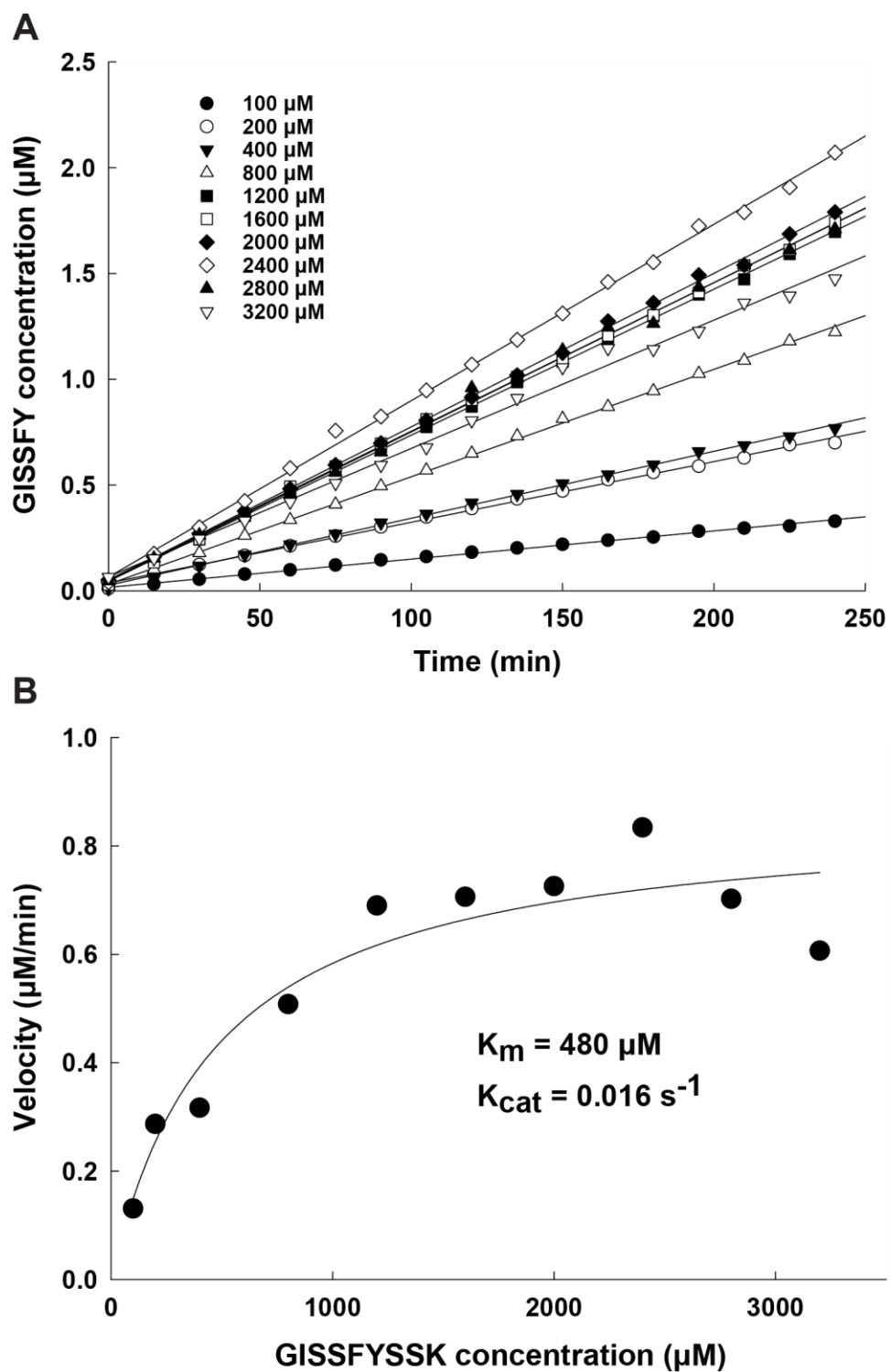


Figure 25. A. PSA progress curve with varying concentrations of GISSFYSSK, B. Michaelis-Menten saturation curve.

Study of AYLMYY as PLM

Synthesis and purification of AYLMYY

AYLMYY was synthesized using the same procedure described for HSKLQYY. At the end of the synthetic process, the peptides were cleaved from the resin using the cocktail of TFA: phenol: water: triisopropylsilane (88:5:5:2)¹⁶¹. After evaporating TFA with nitrogen gas, precipitate was dissolved in DMSO and purified by HPLC. Pure peptide was lyophilized and dissolved in 1000 μL of Tris-buffer (50 mM Tris, 150 mM NaCl, 0.01% Tween 80, pH 7.8), and used as the stock solution. AYLMYY stock solution was quantified using UV spectroscopy using its extinction coefficient ($\epsilon_{280} = 3840 \text{ M}^{-1} \text{ cm}^{-1}$). As shown in Figure 26, purified AYLMYY displayed single peak in its HPLC chromatogram. When a sample of AYLMYY peak was infused into the mass spectrometer at a flow rate of 10 $\mu\text{L}/\text{min}$, it displayed a m/z of 824.1 corresponding to MH^+ ion (i.e., its monoisotopic mass M is 822.3 Da).

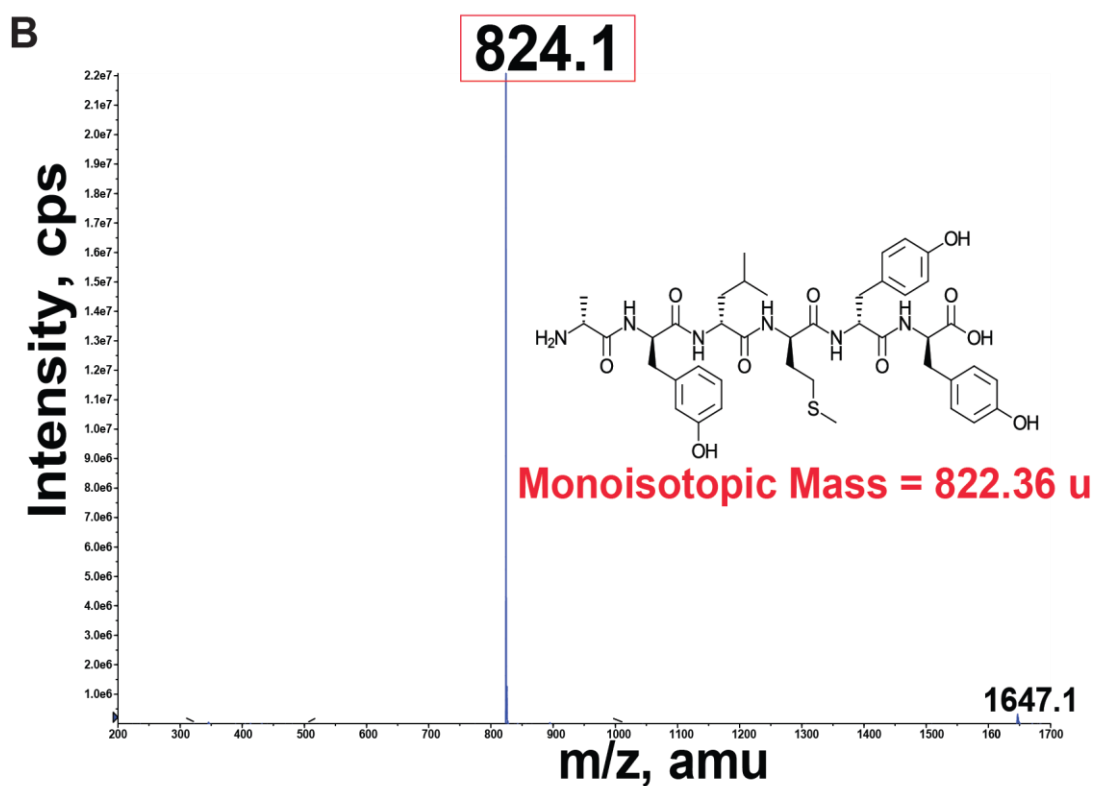
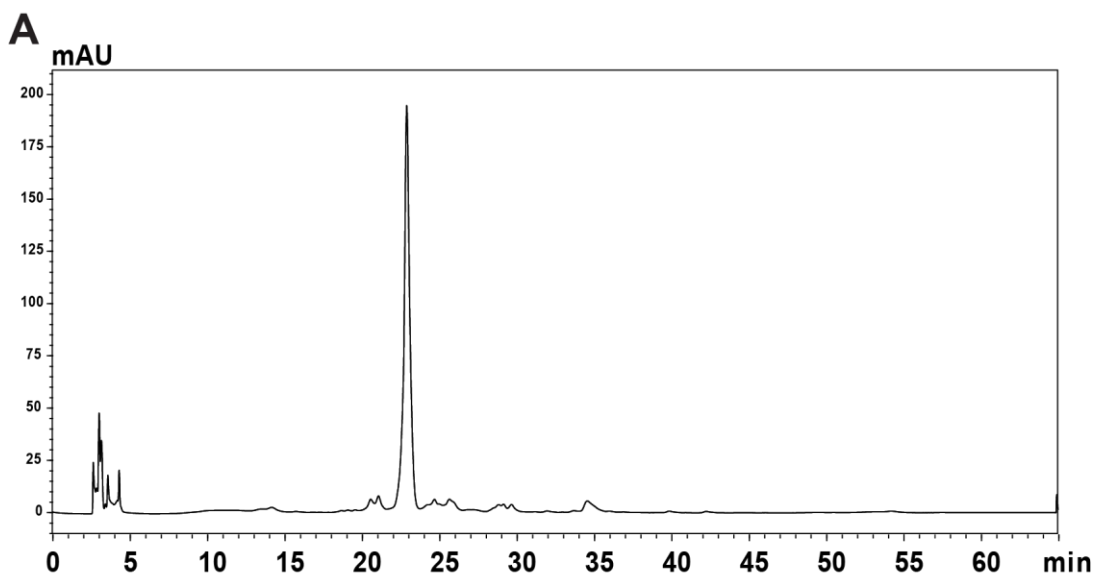


Figure 26. A. HPLC chromatogram of purified AYLMYY and B. corresponding ESI-MS spectrum showing MH^+

Study of AYLMYY hydrolysis by PSA

The substrate, AYLMY, is found to be scarcely soluble in the hydrolysis buffer, even in the presence of 10% DMSO. It was observed that AYLMY saturates in the tris buffer at 0.1 mM concentration. As typical concentration range of substrate for hydrolysis assay by PSA is 0.1 mM to 2 mM, another substrate, AYLMGRR, was designed to obtain the AYLM substrate with the enhanced the solubility.

Study of AYLMGRR as PLM

Synthesis and purification of AYLMGRR

AYLMGRR was synthesized and purified using the same procedure described for AYLMY. Pure peptide was lyophilized and dissolved in 200 μ L of Tris-buffer (50 mM Tris, 150 mM NaCl, 0.01% Tween 80, pH 7.8), and used as the stock solution. AYLMGRR stock solution was quantified using UV spectroscopy using its extinction coefficient ($\epsilon_{280} = 1280 \text{ M}^{-1} \text{ cm}^{-1}$). As shown in Figure 27, purified AYLMGRR displayed single peak in its HPLC chromatogram. When a sample of AYLMGRR peak was infused into the mass spectrometer at a flow rate of 10 μ L/min, it displayed a m/z of 866.6 corresponding to MH^+ ion (i.e., its monoisotopic mass M is 865.4 Da).

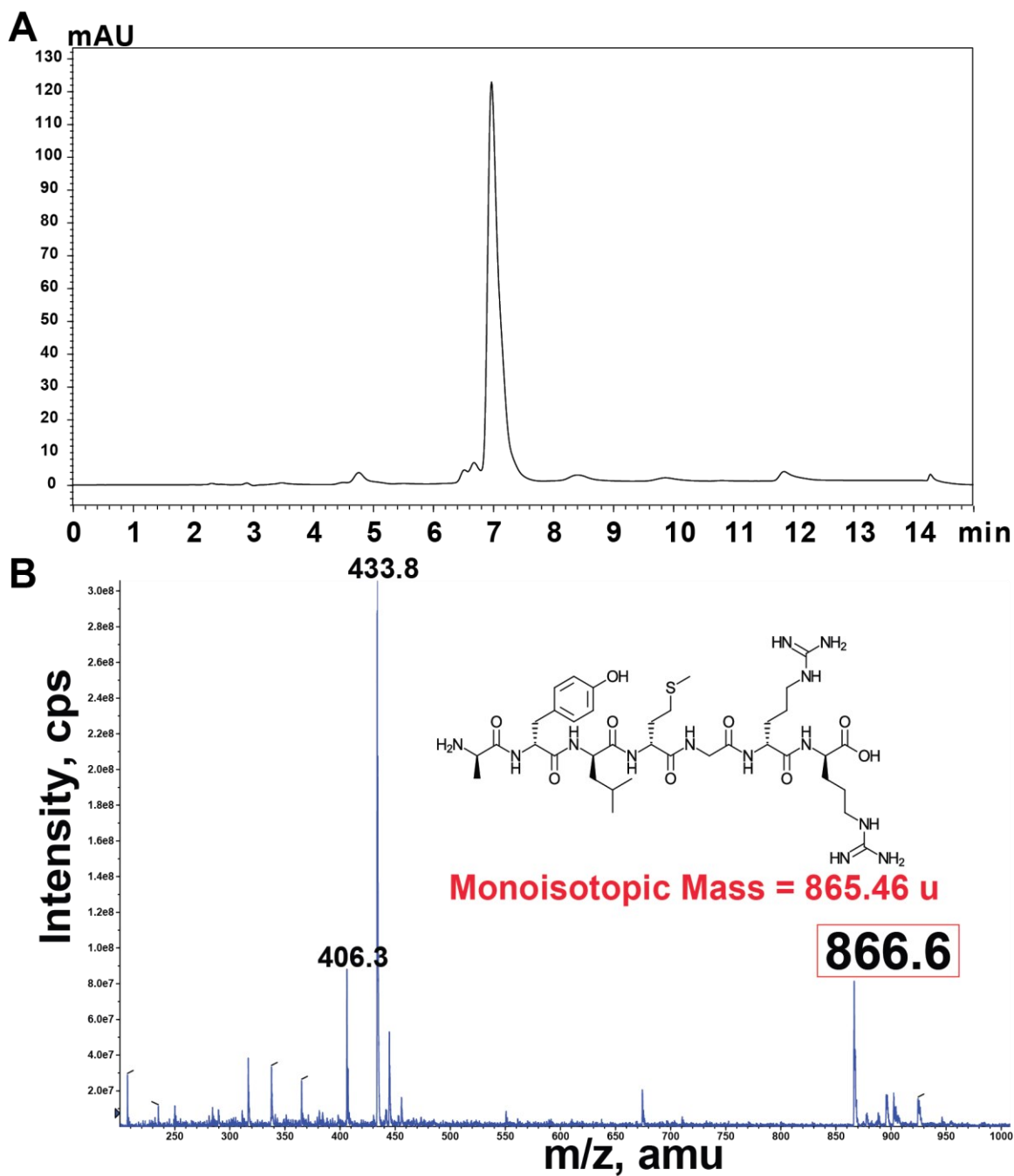


Figure 27. A. HPLC chromatogram of purified AYLMYY and B. corresponding ESI-MS spectrum showing MH^+

Study of AYLMGRR hydrolysis by PSA

The hydrolysis rate of AYLMGRR by PSA was examined in the Tris-buffer solution (50 mM Tris, 150 mM NaCl, 0.01% Tween 80, pH 7.8). The hydrolysis reaction of AYLMGRR was conducted with varying concentrations loaded in a HPLC vial. The breakdown of AYLMGRR in the presence of PSA was monitored by HPLC. PSA at the final concentration of 0.87 μ M was added to the solution of the substrate linkers (200 μ L), and the mixture was gently and homogenously mixed. HPLC analysis was performed using Nucleosil 150 X 3.2 mm 5 μ m C18 column (Supelco Analytical, Sigma Aldrich (St. Louis, MO)) on samples every 15 min for 4 hr. Below HPLC method was used to monitor substrate and product peaks. A refers to 99.9% water with 0.1% TFA and B refers to 99.9% acetonitrile with 0.1% TFA.

Table 6. HPLC method for monitoring AYLMGRR hydrolysis by PSA

Time (min)	Mobile phase
0	10% B
10	100% B
10.1	10% B
15	10% B

AYLMGRR was stable upon exposure to PSA and no product peak was observed as shown in Figure 28.

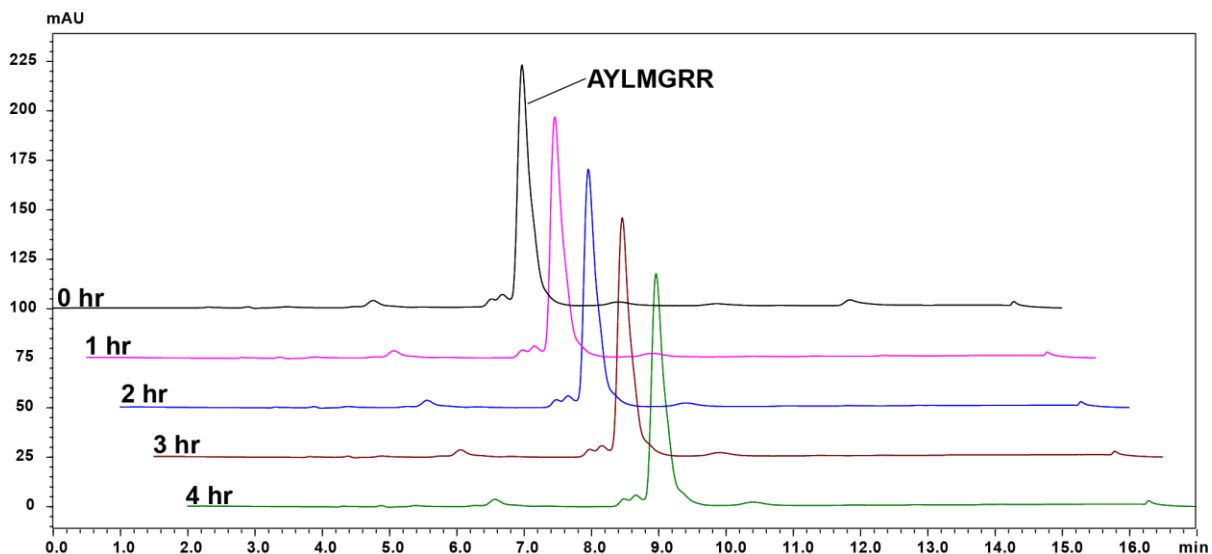


Figure 28. Chromatogram of AYLMGRR depicting its stability upon exposure to PSA.

Reason for choosing HSSKLQ as PLM for the study

The catalytic rate constants calculated based on the Michealis-Menten equation for all the substrates (HSSKLQYY, GISSGYSSK, AYLMYY and AYLMGRR) are summarized in Table 7. Hydrolysis rates may vary depending on target peptide drugs in any related studies,¹⁵⁹ as the cleavage rate of coumarinyl fluorogenic substrate HSSKLQ-AMC was affected by substitution of AMC with YY amino acids. GISSFY-SSK has higher k_{cat}/K_M than HSSKLQ-YY, but HSSKLQ has its own advantage in the design of hydrogel, as the drug cleaved from HSSKLQ-Drug was free of any residual groups from the linker and offered greater biological activity as opposed to GISSFY-SSK-Drug. AYLM-YY was found to be not suitable due to its insolubility and AYLM-GRR was not hydrolyzed in the presence of PSA even after 24 hr. HSSKLQ has

demonstrated its effectiveness in triggering YY release in response to a proteolytic stimulus, PSA, and further studies were conducted with HSKLQ.

Table 7. The rate constants of substrate linkers hydrolyzed by PSA.

Substrate	K_M [μM]	k_{cat}/K_M [$\text{M}^{-1}\text{s}^{-1}$]
HSKLQ-YY	1003	2.19
GISSFY-SSK	480	33.3
AYLM-YY	low solubility	
AYLM-GRR	no hydrolysis	

4.3.3. Assessment of Enfuvirtide as anti-retroviral peptide for the study

Assessment of Enfuvirtide stability during proteolysis mediated by PSA

As Enfuvirtide is peptidic in nature and loses its pharmacological activity upon degradation, stability of Enfuvirtide during the proteolytic process mediated by PSA was evaluated. For the stability assessment of Enfuvirtide in the presence of PSA, Enfuvirtide (1 mM, 200 μL) was mixed with PSA (0.87 μM) in a HPLC vial. The mixture was sampled every 15 min for 4 hr. and analyzed using HPLC. HPLC method in Table 8 was used to monitor substrate and product peaks. A refers to 99.9% water with 0.1% TFA and B refers to 99.9% acetonitrile with 0.1% TFA.

Table 8. HPLC method for monitoring enfuvirtide hydrolysis by PSA

Time (min)	Mobile phase
0	0% B
10	100% B
10.1	0% B
15	0% B

As shown in Figure 29, Enfuvirtide peak was observed at RT of 10.5 min. HPLC Chromatograms monitored for 4 hours did not display any extra peak depicting no breakdown of Enfuvirtide (1 mM) upon exposure to PSA enzyme, guaranteeing its stability during the proteolytic process.

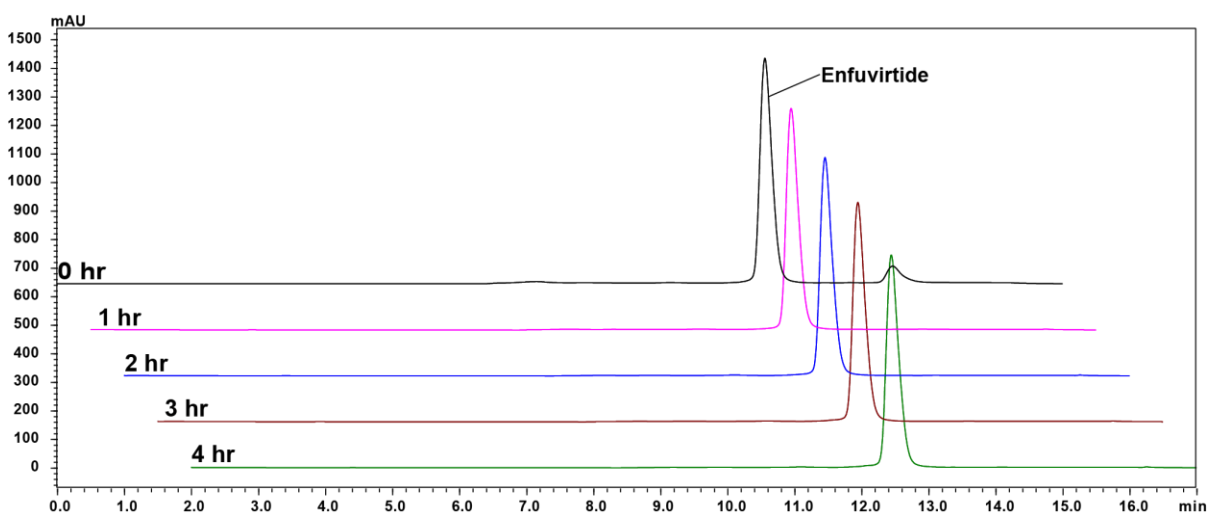


Figure 29. Chromatogram of Enfuvirtide depicting its stability upon exposure to PSA.

The rationale behind the use of Enfuvirtide over other classes of antiretrovirals, such as NRTI, NNRTI and protease inhibitors, is to target prevention at the initial

stages of viral infection over the later stages of replication. Moreover, Enfuvirtide has been effective in individuals who developed resistance for other classes of anti-retrovirals ¹⁶⁵⁻¹⁶⁷. Additional advantages with enfuvirtide include its nature of being peptidic. As it is feasible to conjugate enfuvirtide with the hydrogel through the PSA hydrolysable peptide linker, chemical conjugation of the peptide overcomes the issues of leaching and diffusion at the site of application. Furthermore, enfuvirtide is found to be stable towards proteolytic action of PSA.

4.3.4. Development of PSA responsive hydrogel

PEG polymer was used for hydrogel backbone as its biocompatibility is well studied for many decades. 4 arm PEG DBCO and 4 arm PEG-azide were chosen to form crosslinking matrix of the hydrogel as DBCO, and azide functional groups undergo spontaneous click reaction. In addition, they allow covalent attachment of the PLM linked enfuvirtide.

Synthesis and analysis of PEG-DBCO

PEG-DBCO was synthesized as described previously and synthetic scheme was shown in Figure 30 ¹⁶⁸. Briefly, 400 mg of 10 kD PEG amine (0.01 mmol) was dissolved in 1.5 mL of DCM. 40 μ L of triethylamine was added and stirred at room temperature for 15 min. 100 mg of DBCO-NHS (0.06 mmol) was added to the reaction mixture and the stirring was continued for 24 hr. The reaction mixture was then precipitated in cold diethyl ether. The precipitate was isolated using vacuum filter

and left dried over the filter. The dried precipitate was dialyzed against deionized water using 3.5 kD MWCO membrane for 48 hr. The solution of polymer was freeze dried to obtain a light brownish dry PEG-DBCO. Formation of PEG-DBCO was verified using NMR spectrum (Varian Inova 400 MHz) obtained in D₂O and FTIR (Nicolet iS10 FTIR Spectrometer).

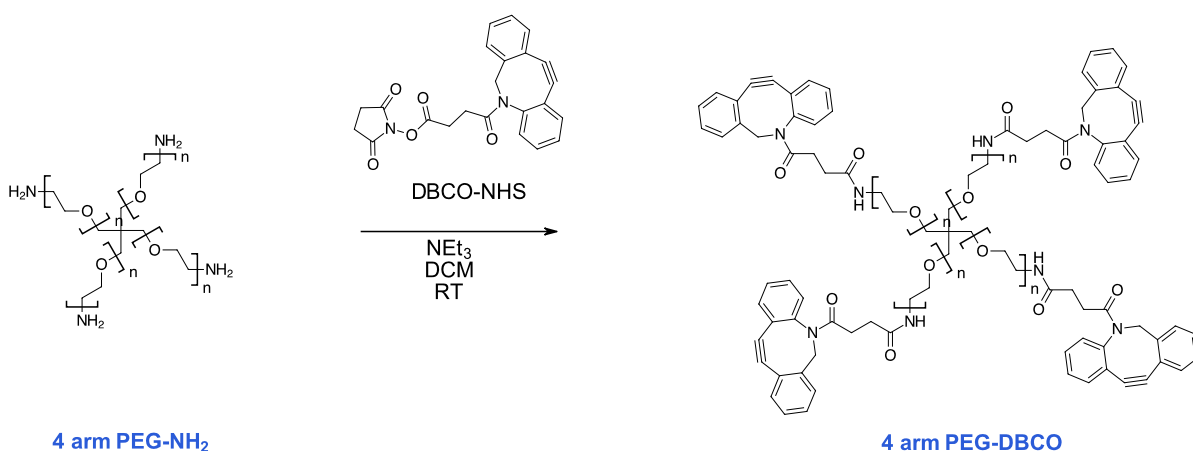


Figure 30. Synthetic scheme of 4 arm PEG-DBCO from 4 arm PEG-amine

As shown in Figure 32, PEG-DBCO formation was verified using NMR and FTIR. The signals of aromatic protons of DBCO moiety in deuterated water were detected by NMR. Additionally, FTIR confirms the presence of an amide bond with signals at 1662 cm⁻¹ and 1558 cm⁻¹.

Synthesis and analysis of PEG-azide

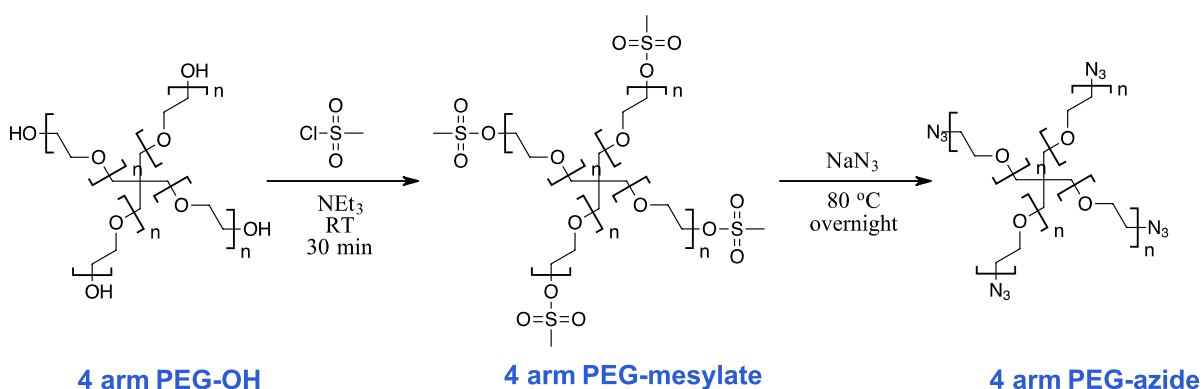


Figure 31. Synthetic scheme of 4 arm PEG-azide from 4 arm PEG-OH

PEG-azide was synthesized using the method previously reported and synthetic scheme was shown in Figure 31¹⁶⁸. Briefly, 2 g of 10 kD PEG-OH (0.1 mmol) was dissolved in 16 ml DCM. Triethylamine (0.5 mmol, 140 μ l) was added to the solution. In addition, methyl sulfonyl chloride (0.5 mmol, 78 μ l) was added dropwise to the mixture at 0 °C on ice and stirred for 30 min at room temperature. The reaction mixture was left for precipitation in cold diethyl ether. The precipitate containing PEG-mesylate was filtered using vacuum and dried on the filter. PEG-mesylate was dissolved in DMF, and subsequently added with 32.5 mg of sodium azide to react with methyl sulfonyl chloride in equimolar ratio. The mixture was kept at 80 °C overnight. It was cooled to room temperature and precipitated in cold diethyl ether. The precipitate was filtered using vacuum and dried on the filter. The dried

precipitate was dissolved in deionized water and dialyzed against deionized water using a 3.5kD MWCO membrane for 48 hr. The solution containing PEG-azide was freeze dried. Synthesized PEG azide was verified using NMR and FTIR.

The formation of PEG-azide from PEG-amine and sodium azide was also confirmed using NMR and FTIR, as shown in Figure 33. The results of NMR analysis performed in CDCl_3 showed the loss of amine protons. Subsequently, the presence of azide is verified by the signal at 2098 cm^{-1} in infrared spectroscopy.

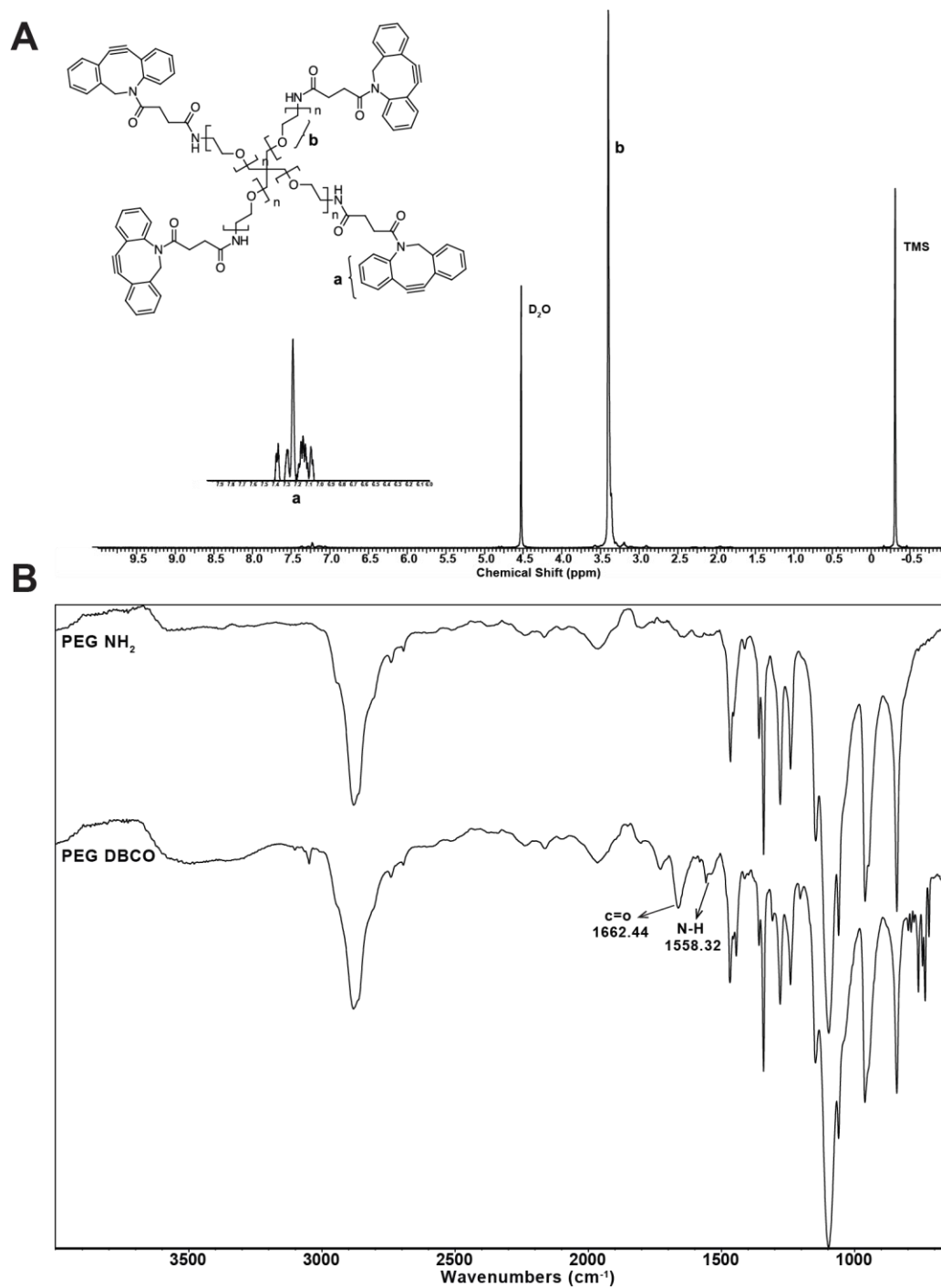


Figure 32. A. NMR spectrum of purified PEG DBCO and B. FTIR spectrum of amide peaks ($\text{C}=\text{O}$ stretching at 1662 cm^{-1} and N-H bending at 1558 cm^{-1}) as PEG DBCO is formed from PEG-NH₂.

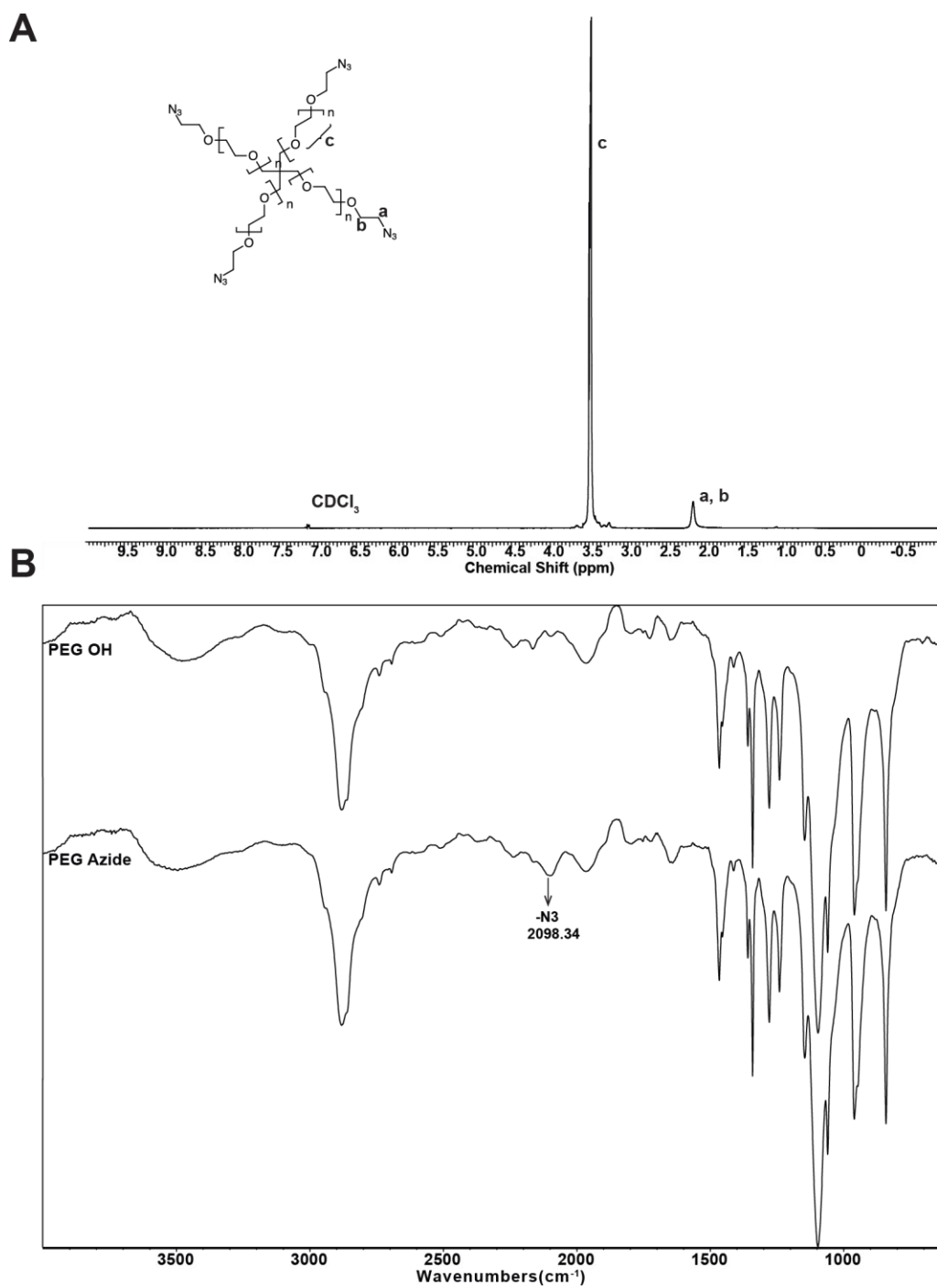


Figure 33. A. NMR spectrum of purified PEG azide, and B. FTIR spectrum showing azide peak at 2098 cm^{-1} as PEG azide is formed from PEG OH.

Optimizing chemistry for conjugation of PEG with PLM linked Enfuvirtide

Attempt 1 using DBCO-thiol click chemistry

PLM linked enfuvirtide with cysteine end group on the N terminus of the peptide was ordered from the company. It is referred as CF43 after its N terminus, C terminus and total number of amino acids. Cysteine end group containing peptide was designed to use thiol functional group to covalently link to the DBCO functional group of the PEG polymer. As shown in Figure 34, CF43 was expected to hydrolyze by PSA to form enfuvirtide.

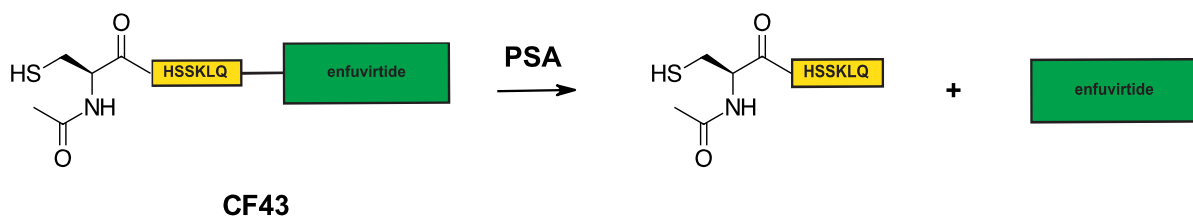


Figure 34. Hydrolysis of CF43 by PSA to its product Enfuvirtide.

CF43 was (0.2 mM, 200 μ L) was dissolved in Sodium carbonate (pH 8.6) buffer and mixed with PSA (0.87 μ M) in a HPLC vial. The hydrolysis was monitored using HPLC after 4 hours. HPLC chromatogram confirms the hydrolysis of CF43 to enfuvirtide as shown in Figure 35.

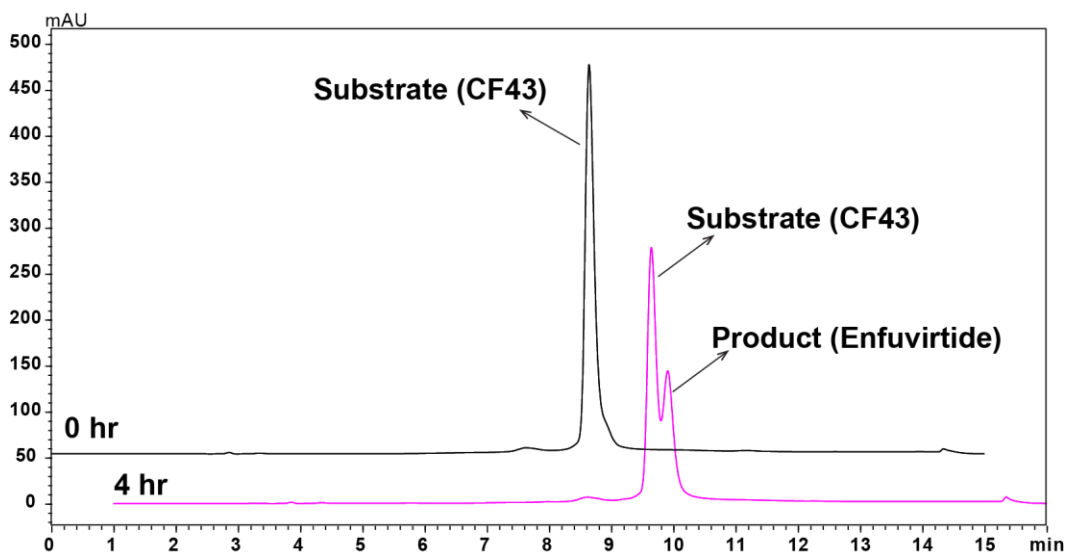


Figure 35. Chromatogram shows decrease in substrate (CF43) and increase in product (Enfuvirtide) after 4 hours of proteolysis by PSA.

As CF43 gets hydrolyzed by PSA as expected, its linkage with PEG-DBCO was attempted using previously described cysteine-cyclooctyne conjugation ¹⁶⁹. Several problems were faced while performing the PEG-DBCO and CF43 conjugation reaction. This reaction requires high concentration of substrates and products in phosphate buffer (pH 8.0). CF43 was found to be very insoluble at pH 8 and the mixture of PEG-DBCO and CF43 in 0.5 mM to 2 mM concentration range formed a lump when added to phosphate buffer. Analysis of this mixture in NMR did not provide conclusive results as it contained all reactants and products. The same reaction was attempted in low concentrations of reactant to allow dissolution of PEG-DBCO and CF43. However, gel formation was not observed upon addition of PEG-

azide to the mixture. This could be due to low crosslinking between polymers. This method was not pursued further due to lack of confirmation of product formation and inability to form hydrogel.

Attempt 2 using DBCO-azide (from CF43) click chemistry

Thiol end group of CF43 was modified to have azide end group as azide reacts spontaneously with DBCO groups of PEG polymer. As depicted by the reaction scheme in Figure 36, 3 μ moles of CF43 dissolved in DMF was mixed with 60 μ moles of Azido-PEG3-Maleimide to form azide functionalized CF43 which is termed as Azide-CF43. Purification of Azide-CF43 was performed on HPLC and azide functionalization was confirmed by infusing isolated peak in mass spectrometer. Figure 37 shows the reconstructed mass spectrum of the Azide-CF43 confirming functionalization of CF43 with azide.

Hydrolysis of Azide-CF43 to Enfuvirtide was studied before attempting its conjugation with PEG-DBCO. As can be seen in Figure 39, very low proteolysis was observed even after 24 hours of exposure with PSA. This can be due to the side chain functionalization with Azido-PEG3-Maleimide which could have led to loss of enzyme recognition. Therefore, side chain functionalization deemed unsuitable and Azide-CF43 was not used further.

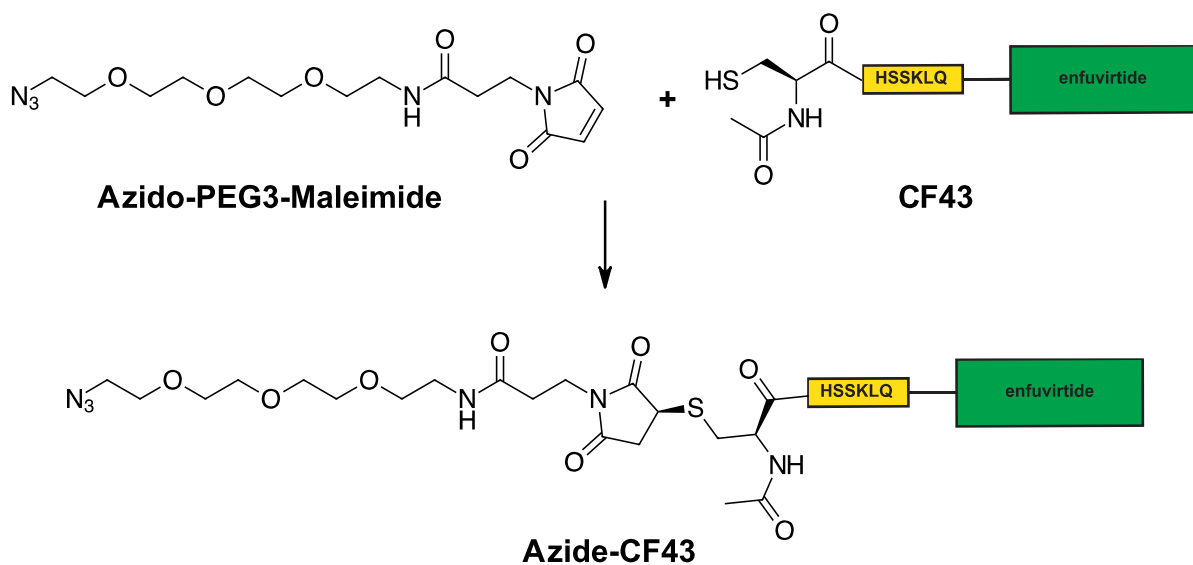


Figure 36. Synthesis of Azide-CF43 from CF43 and Azido-PEG3-Maleimide using thiol-maleimide click reaction.

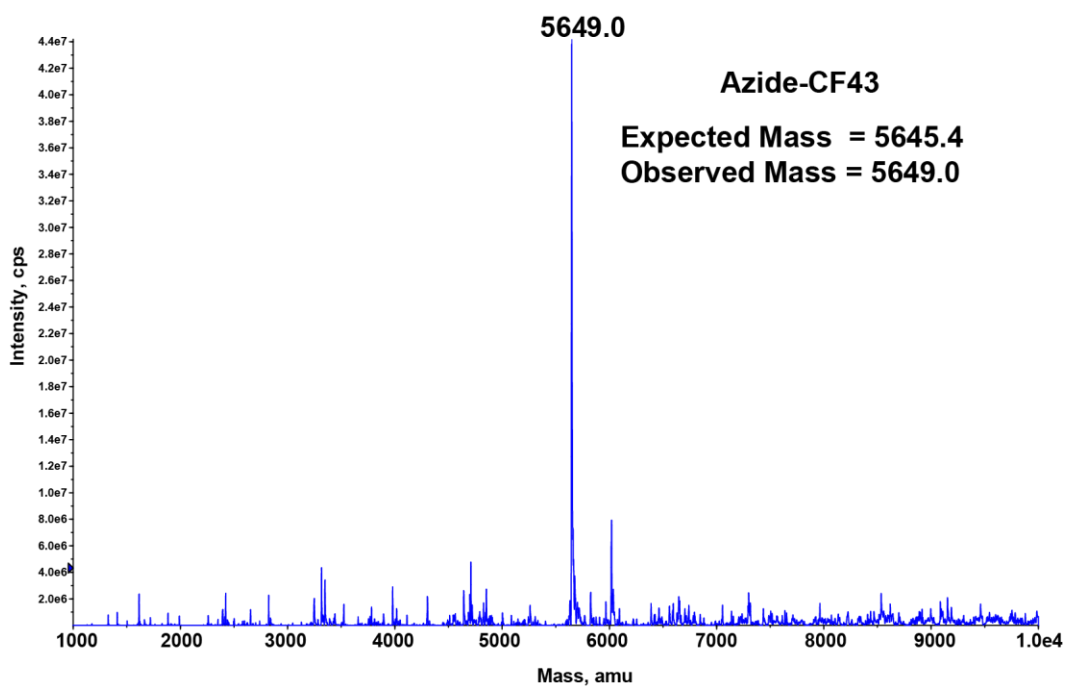


Figure 37. Reconstructed mass of Azide-CF43.

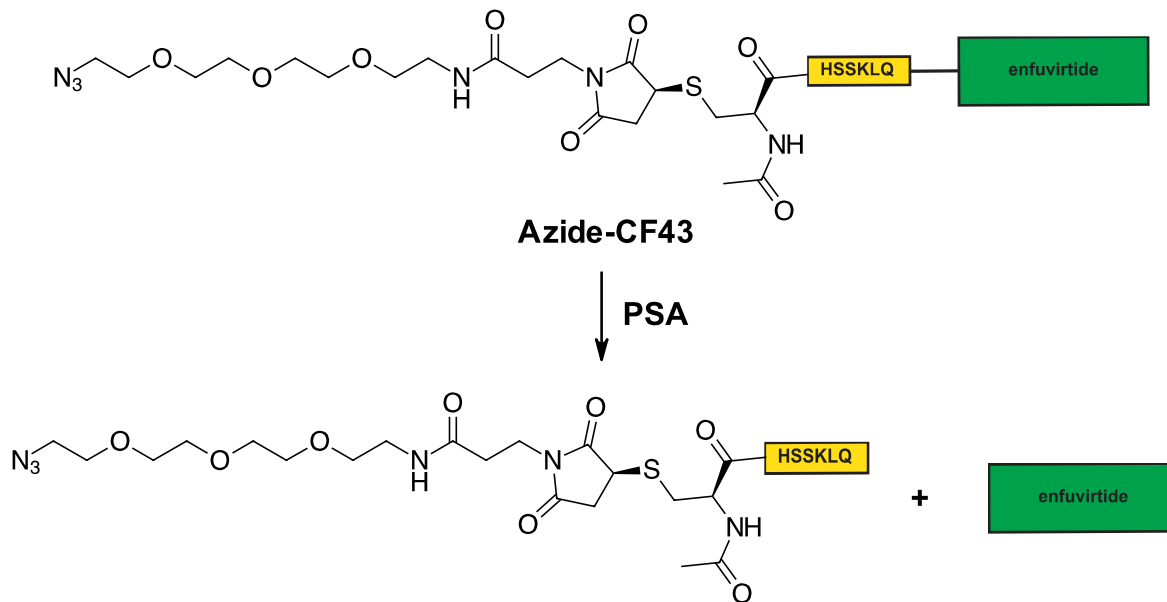


Figure 38. Hydrolysis of Azide-CF43 by PSA to its product Enfuvirtide.

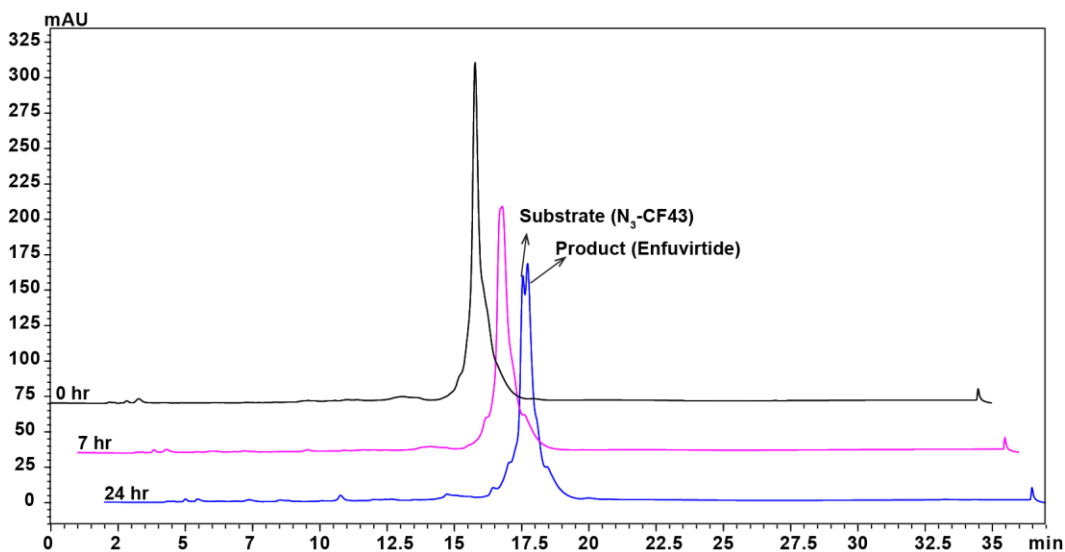


Figure 39. Chromatogram shows decrease in substrate (Azide-CF43) and increase in product (Enfuvirtide) over 24 hours of proteolysis by PSA.

Attempt 3 using DBCO-azide (from HF42) click chemistry

N3-HSSKLQ-Enfuvirtide (HF42) that was custom synthesized to obtain Azide end functionalization in the backbone of the peptide was used as side chain functionalization led to low proteolysis rate. Hydrolysis rate of HF42 by PSA was assessed before attempting the PEG-DBCO conjugation with HF42.

Assessment of HF42 hydrolysis

A solution (0.29 mM, 200 μ l) of N3-HSSKLQ-Enfuvirtide (HF42) was prepared in sodium bicarbonate buffer (100 mM, pH 8.6). PSA (the final concentration of 4.35 μ M) was homogenously added to the solution. The hydrolysis rates were determined at 2-hr intervals on HPLC for 24 hr. The HPLC method was optimized to resolve the substrate at retention time (RT) of 17.27 min and product peaks at RT of 17.78 min. Product (i.e., Enfuvirtide) is quantitated by calculating the percentage changes of area under the curve (AUC) of the substrate peak. The percentage of product formed was plotted against time.

It was found that about 70% of HF42 was hydrolyzed into Enfuvirtide in the presence of 0.13 mg/ml of PSA. The release profile of Enfuvirtide followed a linear function as shown in Figure 40. As the amount of PSA used in this assay is much lower than the mean concentration in seminal fluid ¹⁵⁵, and pH in this experiment was pH 8.6 (performed in 100 mM sodium carbonate buffer), the rate and the amount of

enfuvirtide released is expected to be higher in seminal fluid pH and clinical PSA concentration.

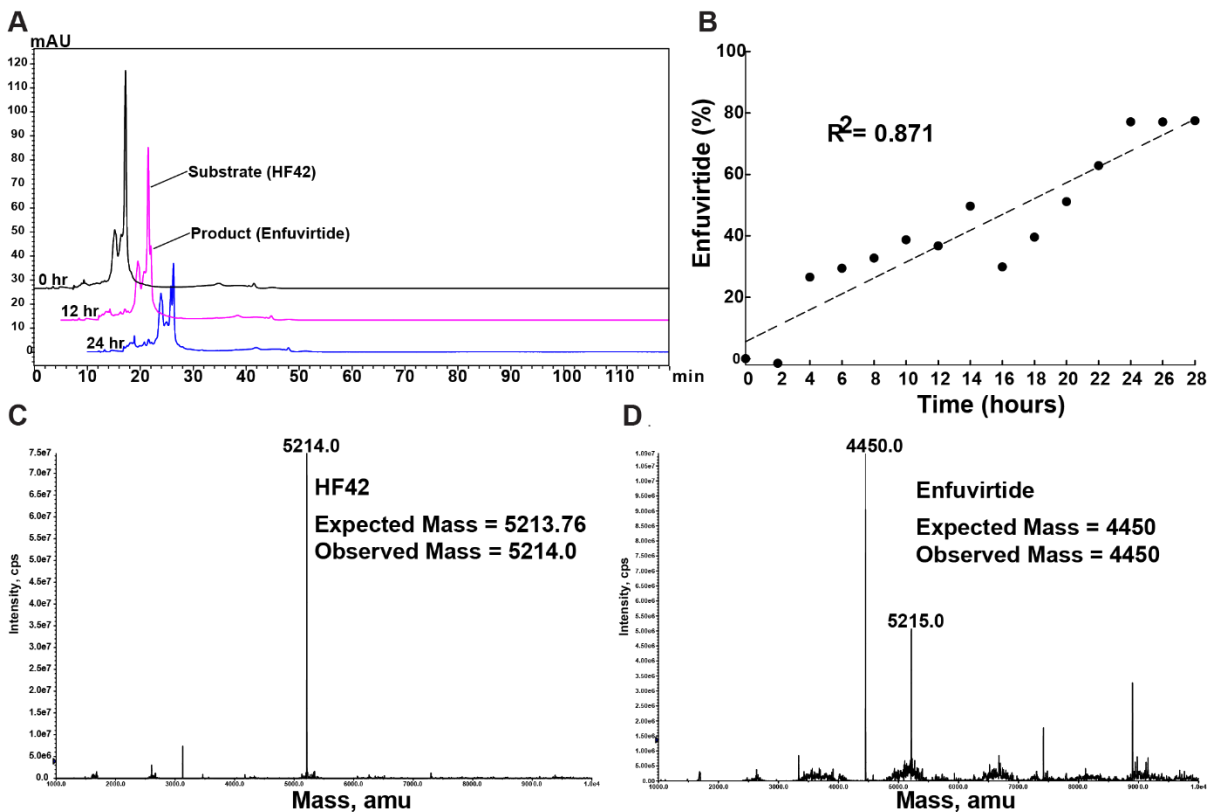
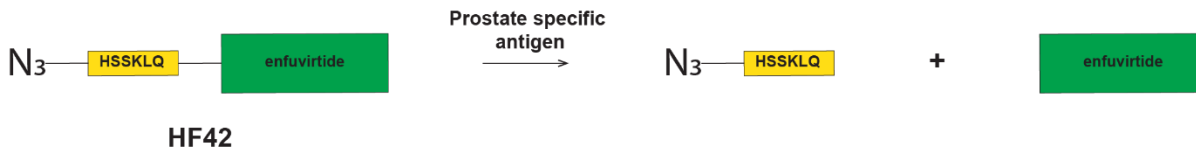


Figure 40. Proteolysis of HF42 by PSA in sodium carbonate buffer over 24 hr. A. Chromatograms show decrease in substrate (HF42) and increase in product (Enfuvirtide). B. Enfuvirtide was released from the formulation in a linear fashion. C. Reconstructed mass of substrate (HF42) D. Reconstructed mass of Enfuvirtide.

Assessment of HF42 linkage to PEG-DBCO

HF42 conjugation with PEG-DBCO was attempted using copper free click chemistry as it displayed significant hydrolysis upon exposure to PSA unlike Azide-CF43. 0.2 μ moles of HF42 was added to 0.15 μ moles of PEG DBCO in DMSO. Reaction was incubated at 37 °C for 18 hrs. The mixture was dialyzed against deionized water and freeze dried. Freeze dried powder was analyzed using Nicolet iS10 FTIR Spectrometer using Smart iTR base accessory with a ZnSe crystal plate (Thermo Scientific, Waltham, MA).

As shown in Figure 41, there was no azide peak, indicating triazole formation and the presence of peptide in the material was confirmed by the appearance of signals at 1543 cm^{-1} corresponding to N-H bending and 3286 cm^{-1} corresponding to N-H stretching of amide. These peaks confirm the conjugation of HF42 i.e., PLM linked Enfuvirtide to PEG-DBCO. Thus, HF42 was use for further development of hydrogel and subsequent studies.

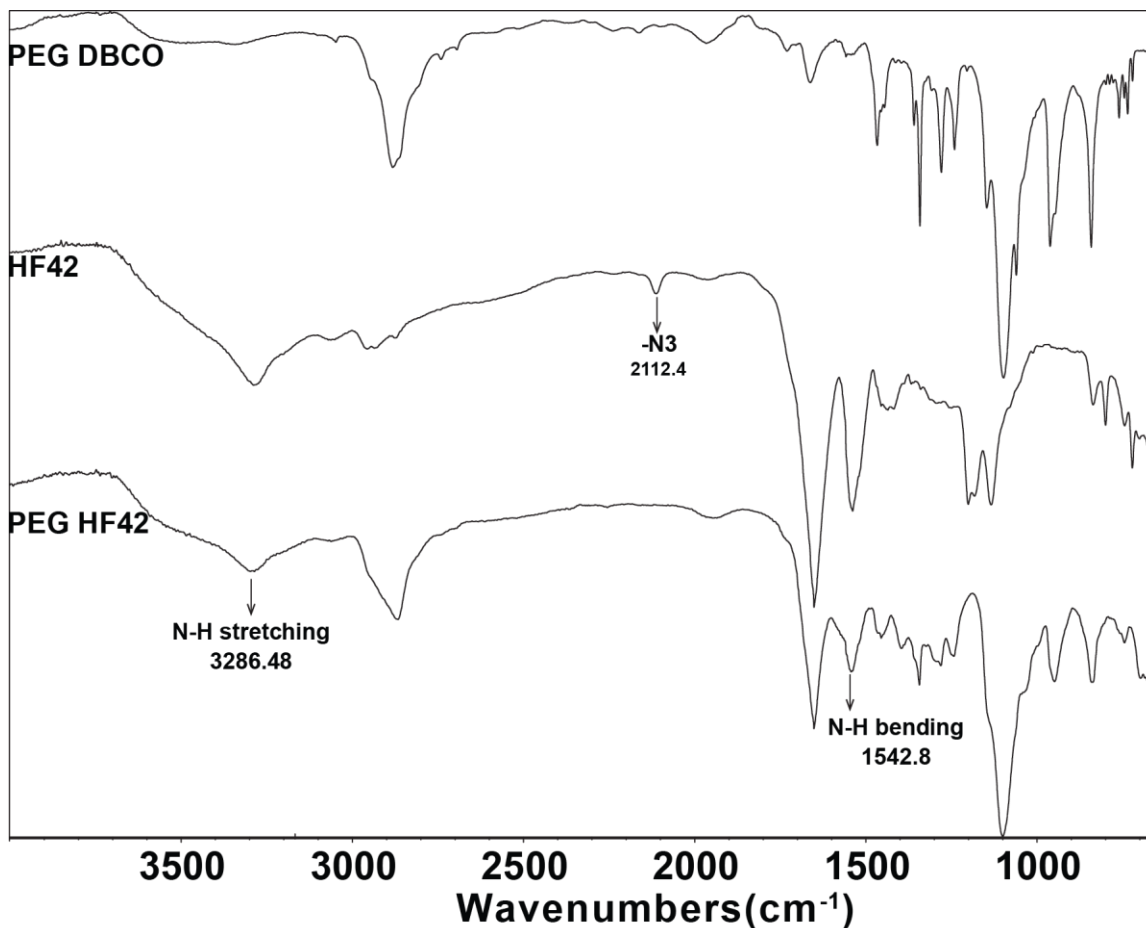


Figure 41. FTIR spectra of PEG-DBCO, HF42 and the conjugated product. The azide peak was not detected as a result of triazole formation between HF42 and PEG DBCO.

Synthesis of PSA labile PEG based hydrogel

N3-HSSKLQ-Enfuvirtide (HF42) (0.214 μ moles, 10 μ l) was added to 1.52 μ moles of PEG-DBCO (the final concentration of 12.7 mM in 110 μ l) in DMSO. The mixture was incubated for 18 hr. at 37 $^{\circ}$ C. The mixture (20 μ l) was combined with PEG-azide (20 μ l of 12 mM) and incubated overnight. Using the Strain-promoted azide-alkyne cycloaddition (SPAAC) method, HF42 could conjugate with PEG-DBCO

and PEG-azide in aqueous conditions within 5 min of the mixture. Due to low solubility of HF42 in water, the study was performed in DMSO which requires approximately 16 hours for complete reaction. The hydrogels were extensively washed with deionized water for one day, followed by washes with drug release buffer i.e., Tris buffer (pH 7.4) or sodium bicarbonate buffer (100 mM, pH 8.6) for two days to remove any unreacted HF42. 10 mM each of PEG-DBCO and PEG-azide was added to produce robust hydrogel, as the concentration below 5 mM did not yield any products. Whereas the concentrations between 5-10 mM produced a hydrogel that did not have sufficient rheological property to maintain the shape. It was also observed that Linkage of HF42 with PEG-DBCO and subsequent conjugation with PEG-azide did not occur, when HF42 was applied at >5% by weight. This could be due to the lowered number of DBCO moieties available for crosslinking with PEG-azide. Steric hindrance from the conjugated HF42 may also limit the availability of adjacent DBCO to PEG-azide. Thus, PEG-based hydrogel was loaded with 4% HF42 by weight for further studies.

4.4. Physicochemical Characterization of PEG-Based Smart Hydrogel

Analysis of PEG-based hydrogel porosity

Scanning Electron Microscopy (SEM) technique was used to examine the surface topography and porosity of the hydrogel synthesized in a semi-hydrated state from 10 mM each of PEG-DBCO and PEG-azide. Images were taken using Field-

Emission Environmental Scanning Electron Microscope Philips XL30 (FEI (Hillsboro, OR)) by placing the gel in the hydrated state on a microscopic slide. The hydrogel has a highly porous structure, as seen in the SEM images (Figure 42). The macro and micro pores of the hydrogel facilitate free diffusion of both the enzyme into the gel to trigger drug release and allows the movement of drug out of the hydrogel matrix.

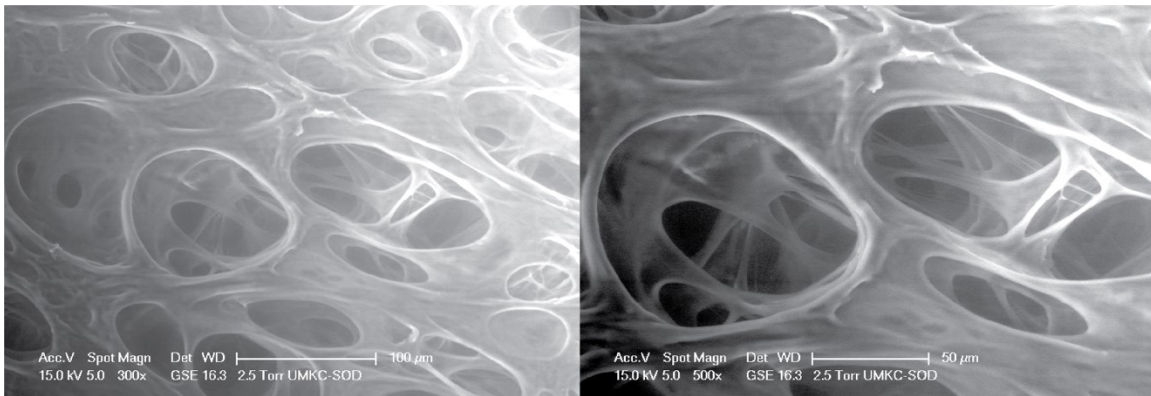


Figure 42. SEM images of hydrogel showed highly porous structure with interconnecting micro and macropores.

Assessment of Rheological Properties of PEG-based hydrogel

The rheological property of the hydrogel was examined to define its performance in the vaginal lumen. The sample was loaded on 20 mm parallel Peltier plate at a gap of 1000 μm at 25 °C to study rheological Properties of PEG-based Hydrogel. Amplitude sweep test was performed at a frequency of 1 Hz to determine

linear viscoelastic region, whereas frequency sweep test was performed at 15% strain using Discovery Hybrid Rheometer HR-3 (TA instruments, New Castle, DE). Upward flow sweep was performed from a shear rate of 0.01 to 100 s⁻¹ and downward from 100 to 0.01 s⁻¹. Creep test was also performed to determine the elastic-viscous balance of the PEG-based hydrogel at a stress of 10 Pa ¹⁷⁰.

It was shown from the frequency sweep test (Figure 43A) that hydrogel has maintained stability independent of storage modulus, suggesting that chemical crosslinking occurred between PEG-DBCO and PEG-azide. A high storage modulus as compared with loss modulus ($\tan \delta < 0.1$) at low frequencies suggests that hydrogel behaves like an elastic material and maintains longer retention time in the female organ. An increase in loss modulus with an increase in frequency indicates the propensity of the hydrogel to spreading without leakage once it is released from applicator, as it had enhanced viscosity yet high elasticity ($\tan \delta < 1$).

In creep recovery test (Figure 43B) at a stress of 10 Pa, hydrogel shows 59% elastic recovery and 32% of plasticity, demonstrating the elastic-viscous balance of the PEG-based hydrogel. To characterize rheological behavior at biologically relevant shear rates, the flow sweep test (Figure 43C) was performed at the shear range of 0.01 s⁻¹ to 100 s⁻¹¹⁷¹. Passive seeping between vaginal epithelial surface was reported to be less than 0.1 s⁻¹, whereas it was 100 s⁻¹¹⁷⁰ and higher during coitus and squeezing from

vaginal applicator. The results of the shear rate vs viscosity demonstrate that hydrogel follows pseudoplastic behavior, as viscosity decreases at the higher shear rate.

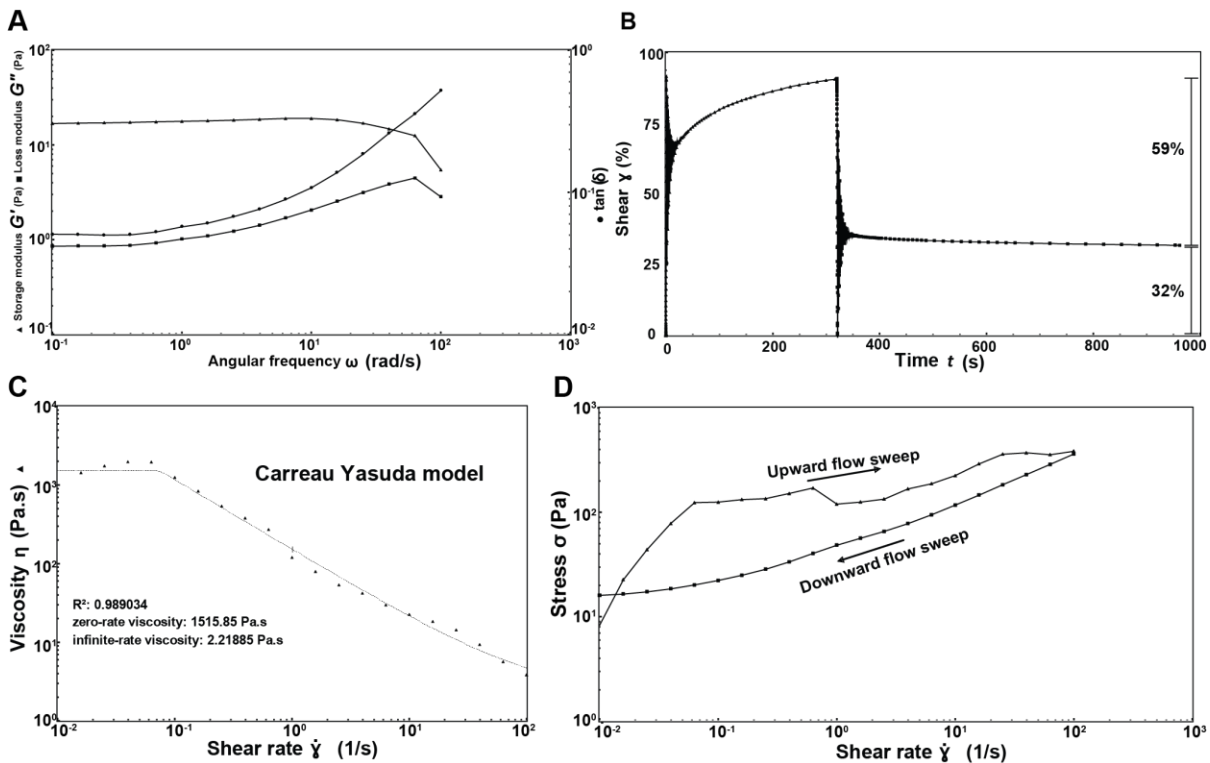


Figure 43. Rheological properties of the PEG hydrogel. A. Shear modulus, loss modulus and $\tan \delta$ as a function of frequency at 15% strain, B. Creep at a stress of 10 Pa for 320 sec. followed by recovery upon unloading, C. Flow rheogram shows pseudoplastic flow with zero shear rate and infinite shear viscosities, D. Hysteresis in upwards/downward flow sweep of hydrogel.

The data closely fit the Carreau-Yasuda model as shown in Figure 10C with a coefficient of determination of 0.99. PEG-based hydrogel had zero shear rate viscosity of 1515.8 Pa. s and infinite shear rate viscosity of 2.2 Pa. s. Based on these results, it

was expected that the hydrogel would retain its status without leakage at low shear rates. At the higher shear rates, PEG-based hydrogel will spread across the vaginal walls but still being retained, as it exhibits a viscosity of 2.218 Pa. s which is 2200 times viscosity of water. Furthermore, a thixotropic behavior was observed as shown in Figure 43D in flow rheogram of up/down shear sweep, indicating that the structural integrity of the hydrogel could be maintained without being leaked after cessation of the shear in post-application or post-coitus.

4.5. Assessment of Efficacy and Safety of Hydrogel

Assessment of PSA responsive release of Enfuvirtide from PEG-based smart hydrogel

The drug release amount from the PEG-based hydrogel was examined at pH 7.4 and 8.6 as the pH of the seminal fluid ranges from 7.2 to 9.0. To determine the available amount of Enfuvirtide in the vaginal lumen, testing HF42-hydrogel samples were incubated at 37 °C for 24 hr. with 300 µl each of sodium carbonate buffer (100 mM, pH 8.6) or Tris-buffer (50 mM Tris, 150 mM NaCl, 0.01% Tween 80 (pH 7.4)), both of which are containing PSA (2.61 µM, 23.49 µg) ^{172,173}. The HF42-hydrogel samples were also incubated with 300 µl each of the Sodium carbonate or Tris buffer in the absence of PSA and they are considered as the control. The release rates of Enfuvirtide were determined by quantifying the collected supernatants.

Briefly, the PEG-based hydrogels were washed with 300 µl of sodium carbonate or Tris buffer twice after 24 hr. incubation and all supernatants were

collected. The collected supernatants were pooled and lyophilized. The samples were reconstituted in 40 μ l each of deionized water and analyzed using HPLC. The drug release was determined in triplicate using Enfuvirtide standard curve. The amount of Enfuvirtide released from the test samples upon being exposed to PSA was compared with that of the control samples in which PSA was absent.

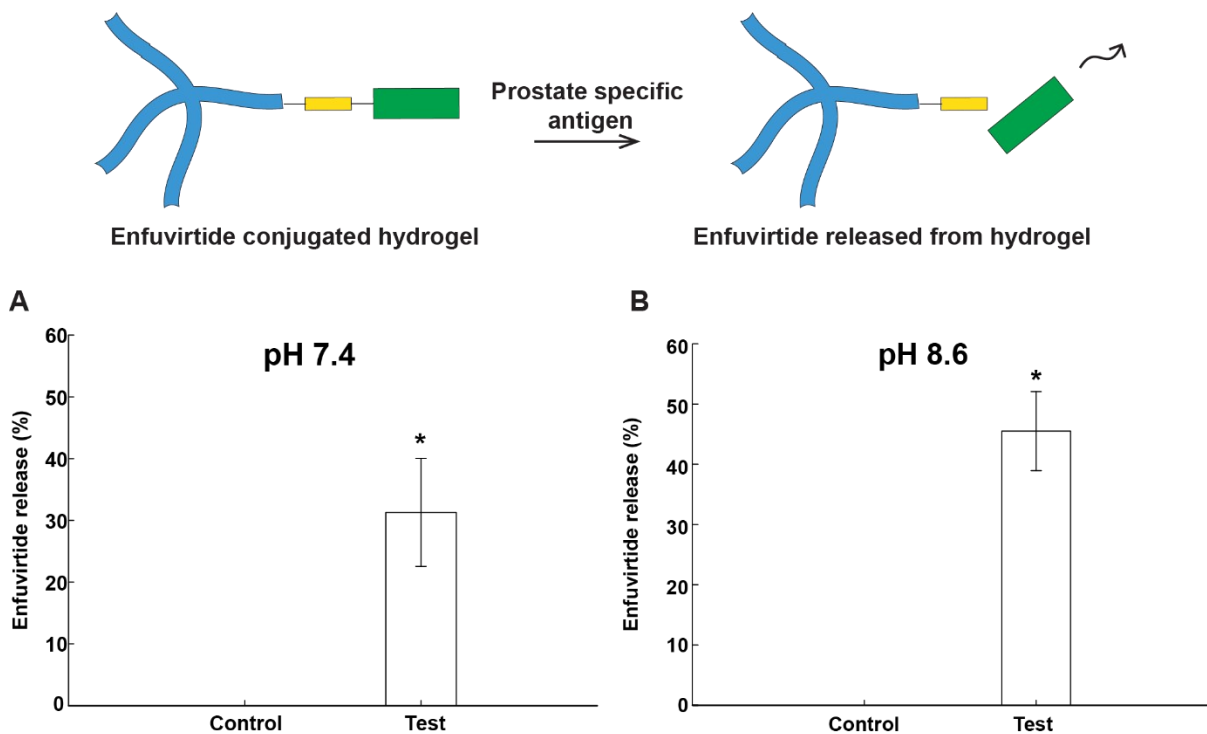


Figure 44. The amount of enfuvirtide released from hydrogel at pH 7.4 (A) and pH 8.6 (B). The control represents the drug loaded hydrogel that is not treated with PSA, and the Test represents drug loaded hydrogel treated with PSA. Data shows mean \pm SD (n=3). T-test on the data shows a significant difference between the means of the groups ($p < 0.001$).

When the drug loaded hydrogel (4% HF42 by weight) was treated with 0.08 mg/ml of PSA at pH 7.4 (50 mM Tris, 150 mM NaCl, 0.01% Tween 80), about $31.3 \pm 8.7\%$ of the drug was released over a 24-hour period as shown in Figure 44A. This corresponds to an amount of $54 \pm 1.4 \mu\text{g}$ of Enfuvirtide released from 5 mg of hydrogel, and an extrapolated amount of about 54 mg from 5 g of hydrogel which is an amount that is generally applied in a clinical setting. This concentration is significantly higher than the IC₅₀ of Enfuvirtide (1.56-1680 nM or 7-7530 ng/mL), exerting sufficient microbicidal activity to prevent women from infection ¹⁷².

The drug release particles examined at pH 8.6 (100 mM sodium carbonate bicarbonate buffer) had a release amount of $45.5 \pm 6.5\%$ over a 24-hour period as shown in Figure 44B. This confirms PSA triggered release of Enfuvirtide from the hydrogel in the entire postcoital pH range. The difference in amounts released at pHs 7.4 and 8.6 can be attributed to the difference in solubility of the drug at these conditions as the optimal pH of PSA activity is 7.8. The amount of drug diffused into buffer is not just limited by the proteolysis rate, but also by its dissolution rate in the buffer. Since Enfuvirtide has low solubility in aqueous buffers, the amount of drug detected may be lesser than the amount released from the hydrogel *in vitro*. The higher *in vivo* proteolysis rate and drug release rate are expected due to the presence of

higher concentration of PSA present in seminal fluid and sink condition of the drug by vaginal epithelium.

in vitro assessment of hydrogel safety for vaginal application

Safety of microbicides is critical for vaginal application as vaginal irritation leads to increased acquisition of HIV. VK2/E6E7 and RAW 264.7 cells were used to determine safety of the hydrogel as they represent vaginal epithelium and intra epithelial immune cells respectively. VK2E6/E7 cells were cultured in keratinocyte serum free medium (SFM) supplemented with bovine pituitary extract (0.05 mg/ml), human recombinant EGF (0.1 ng/ml), calcium chloride (0.4 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). RAW 264.7 cells were cultured in DMEM: F-12 medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated in humidified environment at 37 °C and 5 % CO₂.

Statistical analysis: One-way ANOVA with Tukey's honest significant difference test was performed to determine significant difference between the treatment groups. Sample size for all groups was 3 (n=3) and p < 0.05 was considered as statistically significant.

Assessment of cell viability

Cells were seeded in a 96 – well plate at cell density of 4×10^4 cells/well and incubated for 24 hours. Cell culture medium was replaced with 200 µl of treatment

solutions prepared in fresh culture medium after cells were allowed to attach to the bottom of plate for 24 hours. SFM was used as negative control for VK2/E6E7 cells and DMEM as negative control for RAW 264.7 cells. 1% Triton X-100 was used as positive control for both the cell lines. Other treatment groups were enfuvirtide (T-20) (38 μ M), HF42 (38 μ M), blank hydrogel (20 mg) and HF42 loaded hydrogel (20 mg with 4 % HF42 loading equivalent to 38 μ M). Treatment solutions were removed after an incubation period of 24 hours and cells were gently washed with PBS. 200 μ l of resazurin sodium salt dissolved in culture medium (44 μ M) was added to cells and incubated for 3 hours. Fluorescent intensity of the dye was measured using SpectraMax® M5/M5e Multimode Plate Reader from molecular devices (San Jose, CA) at 560 nm excitation wavelength and 590 nm emission wavelength. Cell viability was determined by using below equation.

$$\% \text{ Cell viability} = \frac{(FI_{treatment} - FI_{background})}{(FI_{negative\ control} - FI_{background})}$$

FI: Fluorescence intensity

Resazurin assay was used to determine cell viability as resazurin is converted to resorufin by metabolically active cells. As shown in Figure 45A, drug loaded hydrogel did not cause significant loss of viability of VK2/E6E7 and RAW 264.7 cells compared to negative control. Similar trend can be observed for blank hydrogel, native enfuvirtide and HF42.

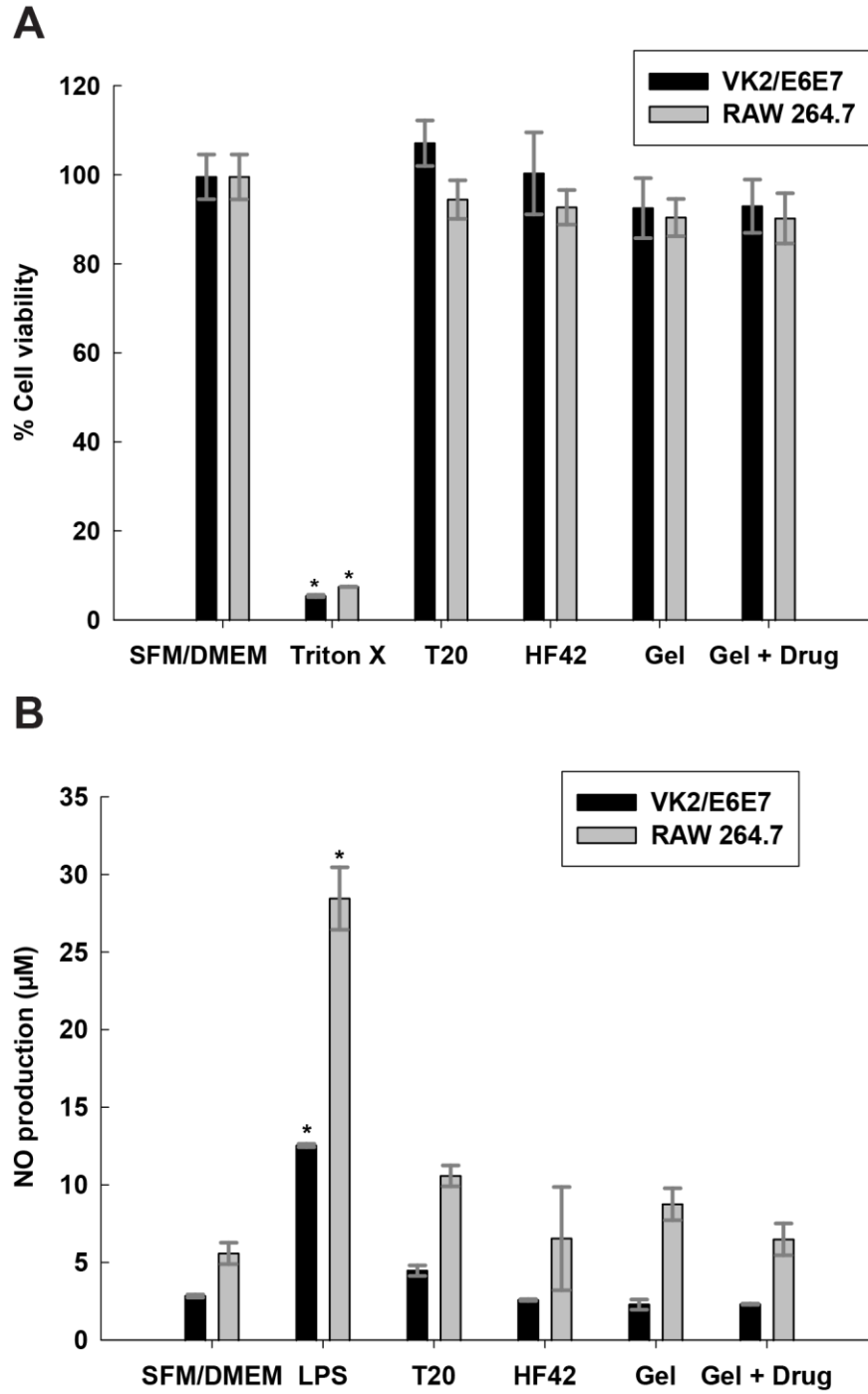


Figure 45. *in vitro* cytotoxicity studies showing A) cell viability and B) nitric oxide (NO) production of VK2/E6E7 and RAW 264.7 cells.

Assessment of nitric oxide (NO) production

Nitric oxide (NO) produced from VK2/E6E7 and RAW 264.7 cells upon treatment was determined using Griess assay. Cells were incubated with treatment groups as described for cell viability determination. LPS (10 µg/ml) was used as the positive control to induce nitric oxide production. After 24 hours of incubation, 100 µl of supernatant from each well was transferred to fresh wells. 100 µl of 1% sulfanilamide dissolved in 5% (v/v) phosphoric acid solution was added to each well and incubated for 5 min at room temperature. Then, 100 µl 0.1% of N-(1-Naphthyl) ethylenediamine dihydrochloride solution was added to each well and absorbance was measured using SpectraMax® M5/M5e Multimode Plate Reader from molecular devices (San Jose, CA) at 535 nm wavelength. Amount of NO was quantified using sodium nitrite standard curve.

Nitric oxide is an inflammatory mediator. Increased production of NO can trigger immune response and recruits HIV susceptible cells to the site of exposure. Therefore, NO produced from VK2/E6E7 and RAW 264.7 cells were measured to determine probable inflammation due to hydrogel treatment. Griess assay was used as an indirect measure of nitric oxide as it determined concentration of its stable breakdown product nitrite. As shown in Figure 45B, drug loaded hydrogel was non-inflammatory as it did not induce significant NO production compared to negative control. Native enfuvirtide, HF42 and blank hydrogel induced basal levels of NO.

Higher amount of NO was production was observed in macrophages compared to vaginal epithelial cells as immune cells are more reactive to inflammatory mediators. Overall, drug loaded hydrogel was non-cytotoxic and safe for vaginal application.

4.6. Conclusions

In this study, the enzyme-triggering linker and PEG-based hydrogel were developed and evaluated for the carrier of anti-retrovirals to protect women from HIV-1. PSA was chosen as the enzyme in the body to effectively trigger the release of peptide drugs, Enfuvirtide, from PEG-based hydrogel. Among the linker candidates, HSSKLQ has demonstrated its effectiveness in triggering Enfuvirtide in response to a proteolytic stimulus, PSA. The rheological properties of PEG-based hydrogels formulated with click chemistry and the release rates of Enfuvirtide were suitable for vaginal application and meet the patient's compliance. In vitro cytotoxicity studies revealed the safety of PEG-based hydrogel towards vaginal epithelial cells and murine macrophages. It could be concluded that PEG-based hydrogel is safe for vaginal application and would efficiently deliver peptide anti-retrovirals with pharmacologically active concentrations in response to a proteolytic stimulus.

CHAPTER 5

SUMMARY, SCOPE, AND RECOMMENDATIONS

5.1. Summary

Smart hydrogel which releases drug in response to an enzymatic stimulus was developed as a prevention method for heterosexual transmission of HIV-1 in women. Prostate specific antigen (PSA) was used as the enzymatic stimulus as it is present in high concentrations in seminal fluid, carrier of HIV-1 during male to female transmission. Smart hydrogel was designed by covalently attaching drug-linked PSA labile moiety (PLM) to the polymeric backbone of hydrogel. 4 arm PEG-DBCO and 4 arm PEG-azide were crosslinked through copper free click chemistry to form hydrogel backbone. Kinetics of PSA substrates were studied to identify ideal PLM for the hydrogel. HSSKLQ was chosen as the PLM as it can release native drug without significantly compromising on rate of release. Peptide based FDA approved entry inhibitor Enfuvirtide was used as the model drug for the study. Entry inhibitors inhibit HIV from entering the immune cells unlike NNRTIs and NRTIs that attack HIV replication cycle in the later stages of infection. In addition, it is easier to link peptide drug to the PLM moiety retaining enzyme recognition and enfuvirtide release kinetics.

PEG-based hydrogel formulated through click reaction of PEG DBCO and PEG azide has highly porous structure, demonstrating ideal rheological properties for

vaginal application with proper viscoelastic balance and safety towards vaginal epithelial cells and murine macrophages. In addition, it released more than IC_{50} values of enfuvirtide even at 10X lower PSA concentration observed clinically. PEG-based smart hydrogel has the potential to be used in clinical setting for effective prevention of HIV-1.

Hydrogels have multiple advantages over other stimuli sensitive vaginal delivery systems of anti-retroviral drugs. For example, nanoparticle-based microbicides such as mannose responsive and hyaluronidase response systems get taken up by the vaginal epithelial cells following application. Drug gets released or degraded inside the cytoplasm of vaginal epithelial cells irrespective of stimulus. Drug covalently linked to the hydrogel backbone retains in the vaginal lumen and gets released and crosses epithelial barrier only upon exposure to the seminal fluid. This reduces toxicity associated with continuous unwanted exposure of anti-retrovirals to the vaginal epithelium.

5.2. Scope

The developed hydrogel formulation is highly modular. Therefore, both the linker and peptide can be formulated with numerous possible sequences allowing delivery of any peptide based anti-retroviral. PEG backbone of the hydrogel can be replaced with hydrogel forming peptides, making whole formulation peptidic in nature and large-scale manufacture of the material can be performed using

recombinant DNA technology at a low cost. For the clinical application of the PEG-based hydrogel, there should be sufficient *in vivo* studies to avoid any vaginal irritation and maintain biocompatibility and efficacy.

The developed system is a prototype for ideal semen triggered microbicide delivery system. This can be applied to design enzyme responsive long-term delivery systems such as intravaginal rings and diaphragms. Figure 46 shows the design of such a system by covalently attaching PLM linked drug to the polymer backbone. These systems can have very high drug loading and act as a drug depot. Drug release from the depot can be controlled by careful selection of the PLM. This allows the depot to last from one day to one month based on the PLM incorporated in the system.

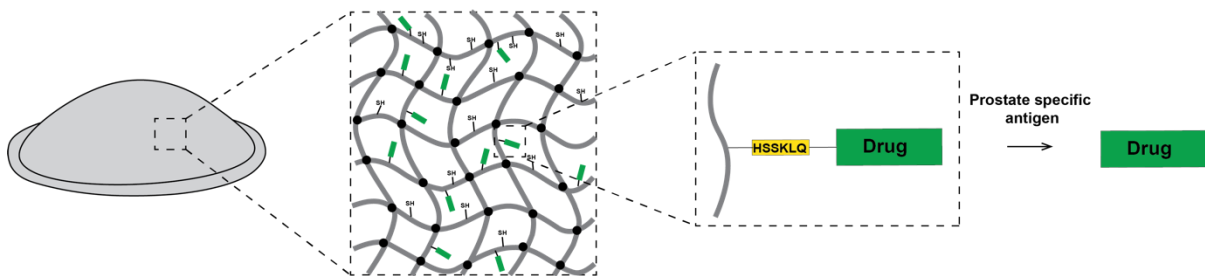


Figure 46. Design of long-term delivery system for PSA responsive delivery of anti-retrovirals.

5.3. Recommendations

Microbicides containing different classes of ARVs are more effective at preventing HIV and reducing drug resistance. Incorporating other ARV drugs such as tenofovir and darunavir along with enfuvirtide would be superior to the developed hydrogel system. Such system attacks HIV at different stages of infection and effectively inhibits transmission. The smart hydrogel developed in this study only allows incorporation of peptide-based drugs. However, majority of FDA approved anti-retrovirals are small molecules. Self-Immolative linkers can be used to develop enzyme triggered microbicide systems for small molecules. This allows incorporation of both peptide bases and small molecule drugs in one delivery system as a combination therapy.

Any stimuli sensitive formulation must be tested for tissue drug concentrations within few hours following stimulus. This is critical in prevention of HIV transmission as the virus crosses mucosal epithelium and established small founder population of infected cells within hours following viral exposure. Enzyme triggered systems should be formulated in different dosage forms such as gels, films, and rings to provide choice for women. This allows compliance and access to women based on their socioeconomic status.

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