®Tutor/s

Dra. Elisa Vallès Giménez
Departament de Ciència de Materials i Química Física

Dra. Ana Cristina Calpena Campmany
Facultat de Farmàcia i Ciències de l'Alimentació

Grau de Química

Treball Final de Grau

Biopharmaceutic study of an HIV-1 inhibitor peptide using nanosystems.

Estudi biofarmacèutic d'un pèptid inhibidor de l'HIV-1 formulat en nanosistemes.

Anna Paús Frutos

June 2017

UNIVERSITAT DE BARCELONA

B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

Primerament voldria agrair a la meva tutora, la Dra. Ana Cristina Calpena, tot el que he après durant aquests mesos, les seves correccions i els seus consells.

També agrair a la meva tutora de la Universitat, la Dra. Elisa Vallès Giménez, les seves correccions i el bon tracte.

Un especial agraïment a la Dra. Isabel Haro i la Dra. Maria José Gómara, per tots els seus consells i sobretot per que la finalització d'aquest treball, i per tant, d'aquesta etapa hagués sigut molt més difícil sense elles.

Agrair als meus companys de laboratori Martha, Cristina, Carlota i Nico que sempre "hagin sumat".

Voldria agrair-li a la Dra. Aimee Vasconcelos la paciència que ha tingut aquests mesos (i anys), gràcies pe compartir els teus coneixements amb mi i permetre'm parlar exclusivament de zeta potencials i nanopartícules durant aquests mesos .

Agrair a la meva família per la seva paciència i sobretot a la meva germana Laura, pel gran interès mostrat i la seva dedicació, moltes gràcies germaneta.

Per últim, un especial agraïment a l'Iván per les seves valuoses aportacions i consells, però sobretot, pel seu suport i les seves paraules d'ànim, que han sigut tan importants com la resta.

REPORT

CONTENTS

1. SUMMARY 3
2. RESUM 5
3. INTRODUCTION 7
3.1. Human immunodeficiency virus (HIV) 8
3.2. Vaginal transmission of HIV 9
3.3 Microbicide, active pharmaceutical ingredients (APIs) 10
3.4. Nanocarries for vaginal drug delivery and studies on drug delivery permeation 12
4. OBJECTIVES 15
5. EXPERIMENTAL 16
5.1. Reagents and solvents 16
5.2. Instrumentation 17
5.3. Preparation of formulations 18
5.3.1. Preparation of liposomes E1P47 18
5.3.2. Preparation of nanoparticles (NPs) E1P47 18
5.4. Characterization of formulations 19
5.4.1. Particle size, zeta potential (Zpot) and polidispersion index (PdI) 19
5.4.2. Entrapment efficiency 19
5.4.3. Stability studies 20
5.5. Stability of E1P47 20
5.6. *In vitro* release study 20
5.7. *Ex vivo* permeation study 21
5.8. Quantification of E1P47 by UPLC-MS/MS 22
5.9. Data analysis 22
6. RESULTS AND DISCUSSION 23
6.1. Physicochemical study parameters, entrapment efficiency and stability of particles 23
6.2. Optimization of UPLC-MS/MS for E1P47 quantification 27

6.3. *In vitro* release and stability study 30
6.4. *Ex vivo* permeation study 34
7. CONCLUSIONS 38
8. REFERENCES AND NOTES 39
9. ACRONYMS 40

1. SUMMARY

Women in Africa are disproportionately affected by human immunodeficiency virus type 1 (HIV-1) and many are unable or unwilling to negotiate condom use due to cultural/traditional behaviour or simply wants to conceive. For that reason, the development of a drug delivery system loaded with vaginal microbicide would be of greater benefit to inhibit the spread of HIV-1 not only in disadvantaged areas, like Africa, but also at global scale.

In this study, different formulations containing a fusion inhibitor peptide as a microbicide have been prepared. These two preparations consist of two nanosystems, poly(lactic-co-glycolid acid (PLGA) coated with polyvinyl alcohol (PVA) nanoparticles (NPs) and 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) large unillamelar vesicles (LUVs), that improve tissue absorption, penetration and targeting for peptide anti-HIV-1 activity.

PLGA-PVA NPs were formulated via solvent displacement and POPC LUVs via the hydration of thin film method, both formulations loaded with the peptide. Size, polidispersion index (PdI), zeta potential (Zpot) and entrapment efficiency (EE) were determined to evaluate their properties. It was necessary to evaluate the stability of the liposomes and nanoparticles at 4 ° C by measuring their size and polydispersity over a period of one month and a half.

Both formulations were characterized to determine peptide release rate using a synthetic membrane (*in vitro* study*)* and vaginal mucosa permeation *(ex vivo* study*)*. Previously studies had assayed the stability of the peptide using the same conditions.

Keywords: HIV-1, microbicides, drug delivery system, liposome, nanoparticle, fusion inhibitor peptide, vaginal mucosa.

2. RESUM

A l'Àfrica, el nombre de dones afectades pel virus de la immunodeficiència humana del tipus 1 (HIV-1) és molt superior al nombre d'homes. Les dones, per diversos motius, es veuen amb la impossibilitat de fer us dels preservatius. Així que, el desenvolupament d'un sistema d'administració que incorpori un microbicida vaginal seria molt beneficiós per inhibir la propagació del VIH-1, no només per zones desfavorides com l'Àfrica, sinó també a nivell mundial.

En aquest estudi, s'han preparat diverses formulacions que contenen un pèptid inhibidor de la fusió cel·lular que actua com a microbicida. Aquestes dues preparacions consisteixen en dos nanosistemes, el primer format per nanopartícules (NPs) de poli (àcid làctic-co-glicólido (PLGA) recobertes amb alcohol de polivinil (PVA), i el segon consisteix en liposomes de 1-palmitoil-2-oleoil-sn-glicero-3-fosfocolina (POPC). Aquest sistemes milloren l'absorció als teixits, la permeació i l'orientació del pèptid amb activitat anti-HIV-1.

Les NPs de PLGA-PVA es van preparar mitjançant el mètode de desplaçament de solvent i els liposomes de POPC, mitjançant la resuspensió del lípid seguit de cicles de congelació i descongelació, formant vesícules unil·lamelars grans (LUVs), ambdues formulacions carregades amb el pèptid. Per avaluar les seves propietats es va determinar la mida de la partícula, l'índex de polidispersió (PdI), el potencial zeta (Zpot) i l'eficàcia d'encapsulació (EE). Per determinar l'estabilitat de les formulacions va ser necessari mesurar la mida de partícula i el PdI a 4 °C durant un mes i mig.

Els dos nanosistemes i el pèptid lliure es van avaluar mitjançant estudis *in vitro* i *ex vivo* per determinar la velocitat d'alliberament del pèptid en una membrana sintètica i la seva permeació en la mucosa vaginal. Per altra banda, es va realitzar l'estabilitat del pèptid usant les mateixes condicions.

Paraules clau: HIV-1, microbicides, Sistema d'administració de fàrmacs, liposoma, nanopartícula, pèptid inhibidor de la fusió, mucosa vaginal.

3. INTRODUCTION

Cure and prevention of human immunodeficiency virus infection (HIV) pandemic has been a major challenge for the scientific community for the last thirty years. Women in the world are notably more vulnerable to HIV than men. This fact is supported by several studies in developed countries, which prove that women up to 24 years old are HIV-positives by 4:1 compared against men in the same range of age [1]. In 2015, there were more than 2.1 million of new HIV infections around the world. However, these new infections were mainly based in Africa and most of them were young women [2].

Most experts indicate that "treatment as prevention" is an important approach to eradicate the HIV epidemic around the world. The main method of prevention in most of the countries of the world is the use of condoms. The use of condoms must be agreed by both and unfortunately, in most of the countries, due to the different cultures or religions, it always depends on men. For that reason, women are unprotected against possible infections. At the same time, condoms are the main contraceptive, so they cannot be used by women who want to conceive, increasing the risk of HIV. Not only the cultural or the religion issue affects the use of the condoms, in the poorest regions of the world, the use of the condoms is dramatically reduced because of their cost, forcing the people to have unprotected sex.

Regarding prevention is one of the most common recommendations to reduce the HIV epidemic, multiple approaches are required to mitigate the growth of infections and the microbicides have been approved as suitable solution for it. Microbicides have an unprecedented potential for mitigating the global burden from HIV infection by heterosexual contact. In the present study, different formulations of anti-HIV vaginal delivery systems that act as microbicide have been developed, being one of the best alternatives for women to be able to protect themselves.

3.1. HUMAN IMMUNODEFIENCY VIRUS (HIV)

The HIV is the infectious agent that causes acquired immune deficiency syndrome (AIDS). The virus causes the disease by attacking CD4+ lymphocytes destroying the immune system.

There are two types of HIV: HIV-1 and HIV-2. HIV-1 is more common, virulent and infective than HIV-2. The way HIV-1 HIV infects is through direct contact with certain body fluids such as blood, semen and pre-seminal fluid, vaginal fluids, rectal fluids and breast milk.

HIV-1 is an RNA virus of the *Retroviridae* family and belongs to the *Lentivirus* genus. HIV-1 (figure 1) is composed of a lipid membrane in which glycoproteins 120 and 41 (gp120 and gp41 are inserted. Inside the viral capsid two copies of RNA are located and stabilized by protein complexes which include the nucleocapsid protein p7 (NU). In the interior of the capside there are found the enzymes required by the viral replication (reverse transcriptase (TI), protease (PR) and integrase (IN)), as well as regulatory proteins.

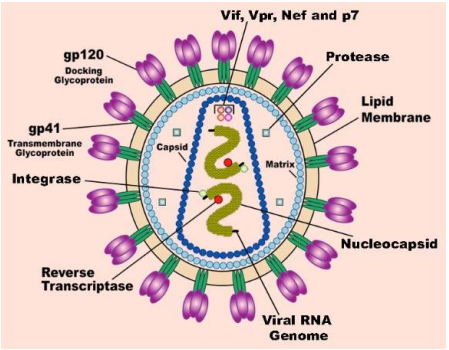

Figure 1. HIV-1 virion. (*Figure reproduced from US National Institute of Health).*

The binding and fusion of HIV-1 to the cell, as shown in figure 2, is given by the interaction of glycoprotein 120 (gp120) with receptor CD4+ of the host cell. This binding triggers a conformational change in the gp120/CD4+ complex, leading to the interaction of gp120 with the coreceptors CCR5 and CXCR4. Then, another conformational change occurs, but this time in gp41. The hydrophobic sequence of amino acids on the N-terminus of gp41, which is also known as fusion peptide (FP), generates the union and fusion of the membranes of the virion and the host cell [3]

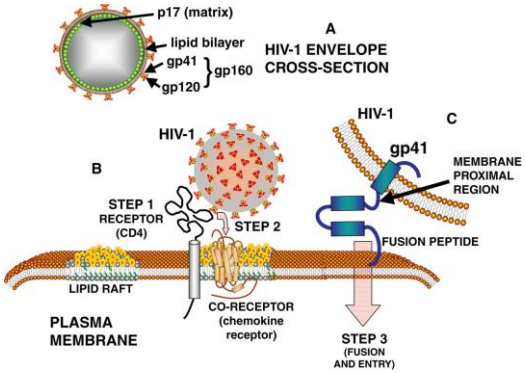

Figure 2. Interactions of HIV-1 envelope proteins with cellular membrane lipids during target cell binding (A) and fusion steps (B). (*Figure reproduced from Ref 3).*

3.2. VAGINAL TRANSMISSION OF HIV

To try to develop a possible microbicide, it is important to understand the stages involving transmission of HIV through the vaginal mucosa surface (figure 3).

HIV present in semen or by donor HIV-infected leukocytes requires to firstly cross mucus fluids covering the mucosa. Then, virus can enter through the stratified vaginal epithelium by different pathways. One is by direct access to the lamina propria across gaps in the epithelium (a). Another mechanism involves Langerhans cells (LCs): these cells are a type of dendritic cells present in the mucosa that trap the virus and transport it through the epithelium (b). It may be the case that some of intraepithelial CD4+ T cells (or other leukocytes) infected by virus penetrate lamina propria (c). In the last case, by transcytosis of virions across the epithelial cell line barrier (d). Once the HIV has passed through the epithelium it can infect the target cells (e), which are CD4+ T lymphocytes, CD4+ cells of the macrophage lineage and the Dendritic cells (DCs). Eventually, free virus (f), infected cells (g) or non-productively infected DCs (h) are transferred to lymph nodes, where it will stablish a systemic infection (f). To try to develop a possible microbicide, it is important to understand the stages involving transmission of HIV through the vaginal mucosa surface [4].

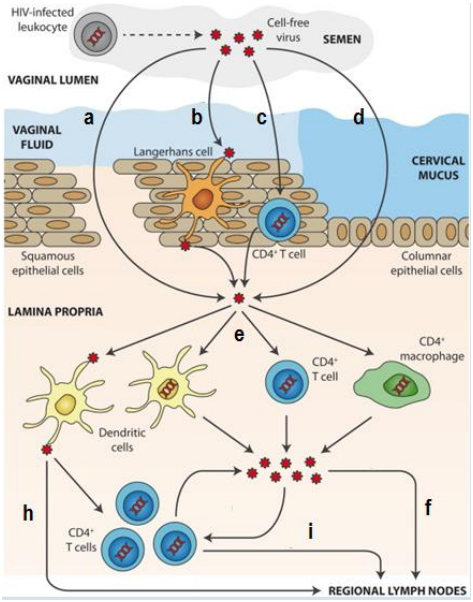

Figure 3. Mechanisms of transmission of cell-free HIV through the vaginal mucosa. (*Figure reproduced from Ref 4).*

3.3 MICROBICIDE, ACTIVE PHARMACEUTICAL INGREDIENTS (APIS)

Microbicides are novel topical products containing APIs to block the infection by pathogens. These APIs may be released intravaginally, using different delivery systems as gels, tablets, suppositories, emulsion type formulations and microparticulate or nanoparticulate systems [5].

Microbicides are classified in two types: physical barriers (physically blocking virus entry) and chemical barriers (APIs). Chemical barrier microbicides block HIV replication before the integration of the virus into the host cells. Numerous classes of APIs have been tested as vaginal microbicides, including virucidal agents (detergents), CCR5 co-receptor antagonist, lectins and neutralizing monoclonal antibodies, entry/fusion inhibitors, nucleoside reverse transcriptase inhibitors (NRTIs) or non-nucleosides reverse transcriptase inhibitors (NNRTIs), as Tenofovir (TFV), Dapivirine (DPV), and the viral integrase inhibitors [5].

There are various types of microbicides which have been developed in pre-clinical and clinical studies. The first break-through in the field of microbicidal research was the outcome of the CAPRISA 004 (Centre for the AIDS Program of Research in South Africa) trial, using a 1%

vaginal Tenofovir gel which reduced the transmission of HIV by 39%. However, the VOICE (Vaginal and Oral Interventions to Control the Epidemic) study halted the oral Tenofovir and Tenofovir gel, because data analysis showed that the results were not so promising [5].

This study is focused on peptides microbicides that inhibit the entry of HIV-1 into the host cells. These compounds have been shown to be successful in the inhibition of early events of the infection, so they have benefits in front of microbicides such as Tenofovir or Dapivirine due to peptides are able to target the HIV-1 in the lumen of the vagina, before genital tissue penetration and dissemination towards the lymph nodes [5]. In the literature, several peptides have been reported as fusion inhibitors of gp41 (T20, T1249, C34, L'644 and Sifuvirtide): they have been pre-clinically evaluated as putative microbicides. Fusion inhibitors targets different domains of the gp41 blocking the formation of the post fusion hairpin state [6].

Our microbicide candidate is a fusion inhibitor peptide named E1P47, that can act as preventive or therapeutic drug due the fact it can act before the virus enters into target cells.

This synthetic peptide is an 18-mer that comes from the GB virus C (GBV-C) E1 protein. GBV-C is a non-pathogenic virus that is usual in HIV infected individual due to its transmission through the same routes. The studies carried out in 1998 discovered the patients co-infected with GBV-C and HIV had an unexpected benefit [7]. Co-infected patients showed a lower progression of the disease and a higher survival once AIDS had already been developed [8].

E1P47 has a high anti-HIV activity because it interacts with the fusion peptide (figure 4), and this is essential for viral entry and it does not allow the union and fusion of the membranes of the virion and the host cell [9].

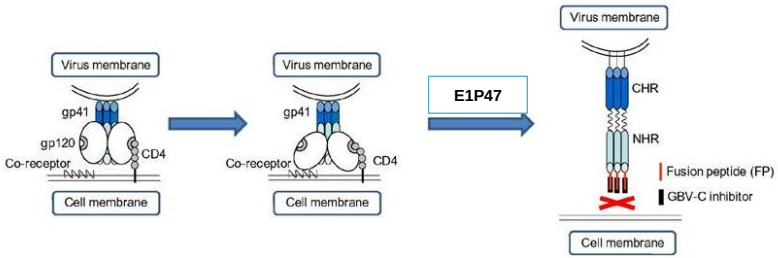

Figure 4. Schematic mechanism of a GBV-inhibitor peptide (E1P47) with fusion peptide (*Figure reproduced from Ref 9.)*

3.4 NANOCARRIERS FOR VAGINAL DRUG DELIVERY AND STUDIES ON DRUG DELIVERY PERMEATION

Developing an active microbicide product not only requires a potent anti-HIV drug compound, but also a suitable formulation strategy which will affect both safety and bioavaility. There are proved several vaginal drug delivery systems such as gels, tablets, suppositories/pessaries, emulsion-type formulation, microparticulate systems and nanoparticulate systems have been good candidates [5].

Nanosystems are defined as vehicles with particles size 10-300 nm, which compounds can be dissolved in, encapsulated in, or attached to for delivery [4]. The advantages of using nanocarriers in microbicides are related mostly with the protection provided to active agents (when they are entrapped in the nanosystem) and to the capability of nanocarriers to facilitate APIs to approach the target cells in the vaginal. Carriers are particularly attractive since they provide protection to the drug, increase drug efficacy, permeate physiological barriers and decrease toxicity [4].

In this study two nanosystem formulations have been assayed, nanoparticles (NPs) and liposomes.

First one, NPs (figure 5), are defined as particulate dispersions. The drug is dissolved, entrapped, encapsulated or attached to a nanoparticle matrix. Depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Nanocapsules are systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. In this study, the nanoparticle formation method was solvent displacement due the hydrophobic character of our peptide [10]. Solvent displacement is a method that allows obtaining nanospheres [5].

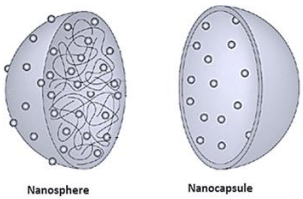

Figure 5. A schematic illustration of (a) nanosphere and (b) nanocapsule. (*Figure reproduced from Ref 10).*

Controlled release and particle degradation characteristics can be readily modulated by the choice of matrix constituents. For our experiments poly(lactic-co-glycolid acid) (PLGA) have been chosen because of their biodegradability and biocompatibility and has been approved by U.S Food and Drug Administration (FDA). PLGA copolymers are widely investigated for the vaginal controlled release because their mucoadhesive properties.

Liposomes (figure 6) are the second nanocarrier formulation, lipid-based drug delivery system are usually composed of polar lipids such as phospholipids, triglycerides and fatty acids which are biocompatible and biodegradable, making them ideal drug delivery vehicles. Liposomes are concentric bilayered vesicles in which an aqueous volume, containing the peptide drug, is entirely enclosed by a membranous lipid bilayer. They can be classified according to their size and lamellar forms. Figure 6 shows various forms of lamellar and sizes [10].

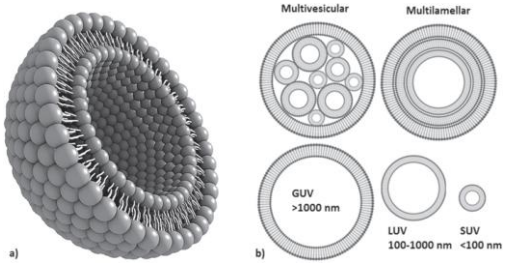

Figure 6. A schematic illustration of a liposome (a) various forms of lamellar and sizes; small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), giant unilamellar vesicles (GUVs), multilamellar and multivesicular. (*Figure reproduced from Ref 10)*

The type of phospholipid used is 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). POPC has a phosphocholine (PC) head group. Since the lipid PC is the most abundant in the cellular membranes, this has been chosen so that the interaction with the target cells could be very strong.

There are several methodologies that led researchers to characterize and predict drug permeation. In this studied, there are carried out two permeation tests. First of all, an *in vitro* study has been conducted to characterize the release of free drug and encapsulated drug in the two formulations using a synthetic, inert and highly permeable membrane. The second one, an ex vivo methodology has been used to predict drug permeation through the vaginal barrier using vaginal tissue. Franz cells are used as a permeation system to carry out the two experiments.

This model constitutes the methodology of reference in The United States Pharmacopeia (USP) for drugs products that institution defines the performance tests for vaginal administration, which are focused on the assessment of in vitro drug release [11].

4. OBJECTIVES

The main objective of this work is the design of delivery systems of an HIV-1 entry inhibitor as a microbicide and to study peptide permeation on porcine vaginal mucosa. To attain this general objective, the specific ones are:

- Preparation and characterization of the two formulations: nanoparticles (NPs) and liposomes loaded with E1P47 peptide.
- Study of stability of both nanosystems at 4ºC.
- Study of stability of E1P47 at 37ºC.
- *In vitro* and *ex vivo* assays to study the release and permeation of two formulations and free peptide.

5. EXPERIMENTAL SECTION

5.1 REAGENTS AND SOLVENTS

Preparations of LUVs

Entry	Supplier	Reagents
1	Avanti Polar Lipids	POPC
2	Merck	Chloroform, methanol, KH_2PO_4, Na_2HPO_4, KCl
3	Sigma-Aldrich	DMSO
4	Mill-Q	H_2O
5	Carlo Erbo	NaCl

Preparation of NPs

Entry	Supplier	Reagents
1	BASF	PVA
2	Aldrich	PLGA Mw: 7000-17000
3	Mill-Q	H_2O
4	Carlo Erbo	Acetona, NaCl

In vitro and *ex vivo* studies

Entry	Supplier	Reagents
1	Gattefossé	Transcutol
2	Fischer	ACN
3	Mill-Q	H_2O
4	Carlo Erbo	Acetone

5.2 INSTRUMENTATION

Entry	Supplier	Instrument
1	Agilent 1260 Infinity	HPLC
2	Agilent	Column HPLC C18 3.5 µm, 4.6 x 100 mm
3	Gilson	Pipetes
4	Heidolph	Rotavapor
5	Lipex TM Extruder	Extruder
6	AQQUITY WATERS	UPLC-MS/MS
7	Agilent	Column UPLC Zorbax C8 1.8 µm, 2.1 mm × 50 mm
8	Milipore	Milli Q water equip
9	Metller Toledo	Balance
10	Selecta	Water Bath
11	Selecta	Ultrasonic Bath
12	Lioalfa, Telstar	Lyophilazer
13	Lipex TM Extruder	Extruder
14	Whatman	Polycarbonate filters (200nm and 100nm)
16	Medicell membranes LTD	Synthetic Membrane (3500 Da)
17	Z sizer Nano ZS, Malvern instruments	Zetasizer
18	Beckman	Ultracentrifuge
19	Amicon Ultra	Centrifugal filters 0.5 mL (3K)
20	Eppendorf	Centrifuge
21	Hanson Research	Cell Manual diffusion test system
22	Vidra Foc	Franz cell

5.3 PREPARATION OF FORMULATIONS

To perform the release and permeation studies two different formulations were chosen, PLGA-PVA NPs and POPC LUVs, both loaded with E1P47. E1P47 was synthesized manually in the solid phase following the Fmoc/tBu protection strategy in the Unit of Synthesis and Biomedical Applications of peptides (IQAC-CSIC). Crude peptides extracts were purified using semipreparative HPLC to >95% purity.

5.3.1. Preparation LUVs with E1P47

LUVs of POPC containing E1P47 were prepared at 4 mM concentration. POPC was dissolved in 1 mL of chloroform/methanol 2:1 (v/v) mixt, after that the lipid solution was dried by evaporation under vacuum. A thin film was formed and finally the film was lyophilized overnight. The film was resuspended in PBS buffer with E1P47 at 0.5 mg/mL and 3% of DMSO. The mixture was sonicated until a film disappear obtaining a white suspension, formed by multilamellar vesicles (MLVs) and they were frozen and thawn ten times. After that step, freeze and thaw multilamellar vesicles (FTMLV) were obtained. To prepare LUVs, FTMLV was extruded (figure 7) in 2 times through 200nm and 5 times through 100 nm pore-size polycarbonate filters. In practice, blank liposomes were prepared by the method described previously.

Figure 7. Extruder

5.3.2. Preparation of NPs with E1P47

PLGA-PVA NPs containing E1P47 peptide were prepared by the solvent displacement technic described [7] (figure 8). 24 mg of PLGA polymer and 2 mg of E1P47 peptide were weighed and then dissolved in 2 mL of acetone. The organic solution was poured into 6 mL of

aqueous solution containing 10 mg/mL of PVA under moderate magnetic stirring. PVA was used as a stabilizer to produce NPs. Acetone was evaporated under reduced pressure. The NPs were centrifuged at 14000 rpm at 4ºC 4 times for 45 minutes. Then, the NPs pellet was washed with deionized water. The final colloidal suspension was concentrated to the desired volume. Blank PLGA-PVA NPs were prepared by solvent displacement method described previously.

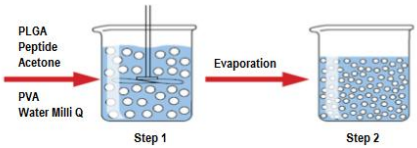

Figure 8. Elaboration of nanoparticles by the method of displacement of the solvent (*Figure reproduced from Ref 15).*

5.4. CHARACTERIZATION OF FORMULATIONS

5.4.1. Particle size, zeta potential and polidispersity (PdI)

Particle size, polydispersity and zeta potential were determined by dynamic light scattering using a Zetasizer nano ZS at 25ºC and scattering angle of 90º. In order to do the measurements, the samples were previously diluted 1:10 (v/v) in water. The assays were carried out in triplicate.

5.4.2. Entrapment efficiency (EE)

The amount of E1P47 entrapped in the NPs and liposomes was calculated by the difference between the total amount of peptide in the formulation and the amount of non-entrapped peptide remaining in the aqueous supernatant. The nanosystems were diluted 1:10 (v/v) in water, later was determined following the separation of free E1P47 from E1P47-loaded nanosystems by centrifugation of the suspensions in centrifugal device (3 kDa MWCO) at 12000 rpm for 30 min. The amount of free E1P47 in the supernatant was determined by high-performance liquid chromatography (HPLC) analysis at 220 nm using a C18 column, using linear gradient of 95% to 0% A in B over 20 minutes at flow rate 1 mL/min using 0.05% TFA in water (A) and 0.05% TFA in ACN (B) as the eluting system. E1P47 entrapment efficiency was calculated using the equation below (equation 1).

$$EE\% = \frac{Total\ amount\ of\ E1P47 - Free\ E1P47}{Total\ amount\ of\ peptide} x100 \qquad \textit{Equation 1}$$

5.4.3 Stability at 4ºC

Particles were storage at 4ºC for 40 days to evaluate their stability, determining their size and PdI.

5.5. STABILITY OF E1P47

The stability of E1P47 was evaluated using an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method. A known concentration of E1P47 in transcutol ®/H_2O 1:1 (v/v) was incubated at room temperature and 37ºC. A 100 µL aliquot was removed several times for 48 hours.

5.6. *IN VITRO* RELEASE STUDY

The release study was carried out for 2 days using Franz-type diffusion cells (figure 9) with dialysis cellulose membranes. This cell consists of two compartments, a donor compartment and a receptor compartment. The cellulose membrane is placed between de donor and the receptor compartments. The effective diffusional area was 0.632 cm^2 and the receptor compartment capacity was 5 mL, which was filled with transcutol®/H_2O 1:1 (v/v). In the donor compartment 0.4 mL of the formulations were placed in duplicated (E1P47 dissolved in transcutol®/H_2O 1:1 (v/v), POPC LUVs E1P47 and PLGA-PVA E1P47). The system was kept at 37ºC and stirred continuously at 700 rpm. Samples (0.3 mL) were withdrawn at selected time intervals and were replaced with the same volume of receptor medium. All samples were analysed by UPLC-MS/MS.

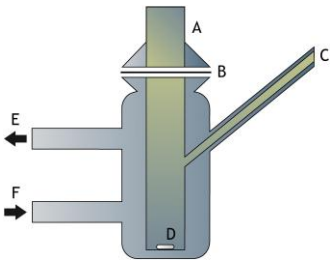

Figure 9. Schematic representation of a Franz cell. (A) donor compartment; (B) membrane; (C) sampling port; (D) stirbar; (E) water outlet; (F) water inlet. (*Figure reproduced from Ref 11).*

To determine the peptide remaining in the cellulose membrane, E1P47 was extracted from the membrane with ACN/H_2O 1:1 (v/v) medium for 30 minutes under sonication in an ultrasound bath. The supernatant was analysed by UPLC-MS/MS.

5.7. *EX VIVO* PERMEATION STUDY

The experiment was carried out under the protocol approved by the Animal Experimentation Ethics Committee of the University of Barcelona (Spain) and the Committee of Animal Experimentation of the regional autonomous of Catalonia (Spain). The vaginal mucosa was provided by the Animal Facility at Bellvitge Campus University (University of Barcelona, Spain). The study was carried out with fresh porcine vaginal mucosa from two animals. The fresh vaginal tissues were transported to the laboratory in containers filled with Hank's solution.

Each of the tissues was trimmed with surgical scissor in three adequate pieces. Then, the tissues were mounted on specially designed membrane holders with a permeation hold diameters of 1.2 cm which has a diffusion area of 0.632 cm^2 (S). For the permeation study, Franz -type diffusion cells were used. The membrane holder was mounted between the two compartments with the epithelium side facing the donor chamber and the connective tissue region facing the receptor chamber. Transcutol®/H_2O 1:1 (v/v) was used in the receptor compartment. In the donor compartment 0.4 mL of the formulations were placed (E1P47 dissolved in transcutol®/H_2O 1:1 (v/v), POPCLUVs E1P47 and PLGA-PVA E1P47 NPs). Samples from both animals were used as replicates for each formulation. All over the process the receptor compartment was incubated at 37ºC to mimic *in vivo* mucosa by insulating jacket, the receptor phase was constantly stirred by magnetic stirrer at 700 rpm. At the predetermined time (1, 2, 3, 4, 5 and 6 hours), 0.3 mL of acceptor solution was removed via syringes from the centre of the cell and immediately replaced by the fresh receptor solution. The withdrawn supernatant was analysed to determine the content of E1P47 by UPLC-MS/MS.

The tissues were frozen at 20ºC and the mucosa was trimmed by the contact area of donor compartment. The peptide retained at the vaginal mucosa was punctured. Then was extracted during 30 minutes under sonication in an ultrasound bath with 1 mL (V_P) of ACN/H_2O Milli Q 1:1 (v/v) medium. The medium was retired from de mucosa (extracted mucosa concentration; C_p) and the vaginal mucosa was weighted (mucosa weight; W_p). The supernatant was analysed by UPLC-MS/MS.

To determine the recovery of E1P47 in mucosa 1.2 mL (V_1) a solution of known (C_o) of E1P47 in transcutol®/H_2O 1:1 (v/v) was added to three previously weighted tissue sample (W_t). The samples were introduced into a 37 ° C bath for 6 hours, next the supernatant (non-permeabilized concentration; C_{trans}) was separated from the tissue. The three tissues were weighted (W_t'). Then 1 mL (V_2) of ACN/H_2O 1:1 (v/v) was added. The vaginal mucosa samples were sonicated in ultrasound for 30 min finally the supernatant was removed from the tissue (C_{acn}), the resulting supernatant was measured by UPLC-MS/MS.

To calculate the recovery percentage of the drug in the tissue applied the following equation 2:

$$\%\ Recovery = \frac{\frac{C_{trans} \times V_2}{W'_t}}{\frac{(C_0 - C_{acn}) \times V_1}{W_t}} \times 100 \qquad \textit{Equation 2}$$

To determinate the real amount of peptide that was retained inside the mucosa was applied the following equation 3:

$$Quantity\ of\ peptide = \frac{C_p \times V_P}{W_p \times S} \times \frac{100}{\%\ Recovery} \qquad \textit{Equation 3}$$

5.8. QUANTIFICATION OF E1P47 BY UPLC-MS/MS

All samples were analysed by UPLC-MS/MS. This system consisted of a triple quadrupole mass spectrometer (TQD) with an electrospray ionization (ESI) coupled to an UPLC. The autosampler was at room temperature during operation. The samples were analysed using a C8 column, under a gradient elution at a flow rate of 0.30 mL/min. Mobile phase A consisted of ACN 0.1% acid formic (FA) and mobile phase B consisted of water 0.1% FA. The gradient was initiated at 5% A for 30 seconds and increased to 100% A in 8 min. The capillary voltage (2.5 kV), collision energy (15 V), cone gas flow (30 L/h), cone voltage (40 V), desolvation gas flow (800 L/h), and desolvation temperature (350 °C) were optimised for the multiple reaction monitoring (MRM) intensity of E1P47.

5.8. DATA ANALYSIS

The stability study of peptide was analysed using GraphPad Prism 5 program.

The permeation parameters and the resultant of accumulative drug versus time for permeation studies were fitted by an appropriate model using the WinNonlin computer program (Pharsight 5.2, Mountain View, CA).

6. RESULTS AND DISCUSSION

6.1 PHYSICOCHEMICAL STUDY PARAMETERS AND ENTRAPMENT EFFICIENCY

NPs and liposomes are good candidates for their properties on vaginal delivery of microbicides. Mucins, through the establishment of intramolecular and intermolecular interactions, lead to the formation of an heterogeneous and intricate tridimensional aqueous channel network [12]. Particles might be retained along the network depending on their surface and size properties.

PLGA and PVA polymers are good candidates to produce NPs for their functional properties, biological behaviour and their biocompatibility and biodegradability. Surface hydrophobicity conferred by PLGA polymer contributes to adhesion, as it promotes the establishment of bonding hydrophobic regions of mucin. PVA is also used as a surface stabilizer for PLGA NPs and its ability to impair particle migration through cervicovaginal mucus due to PVA surface adsortion has been demonstrated [12].

Liposomes of POPC have a lot of qualities due the fact they are composed by a synthetic phospholipid endowing them completely biocompatible, biodegradables, non-toxic and non-immunogenic [1].

To understand the behaviour of our particles across the mucus, (mucoadhesion, drug release, drug loading and stability), it is necessary to characterize their physicochemical parameters such as particle size, PdI and zeta potential by dynamic light scattering technique.

As shown in table 1, peptide entrapment or association to PLGA-PVA NPs influenced their size, which increased slightly due to the interaction of PLGA-PVA NPs surface with E1P47. LUVs E1P47 did not exhibit and important variation in in size whether they were loaded with E1P47 or not. Both formulations had PdI values lower than 0.1, indicating narrow particles size distribution.

PLGA-PVA E1P47 NPs and POPC LUVs E1P47 tested in this study were smaller than 500 nm (table 1), then both formulations can distribute in cervicovaginal mucosa suggesting that steric obstruction should be minimal [5, 12].

Formulation	Size ± SD (nm)	PdI ± SD
PLGA-PVA NPs free	245 ± 2	0.094 ± 0.004
PLGA-PVA-E1P47 NPs	240 ± 2	0.07 ± 0.05
POPC LUVs free	242 ± 3	0.09 ± 0.01
POPC LUVs E1P47	250 ± 10	0.11 ± 0.01

Table 1. Size Distribution and PdI PLGA-PVA NPs, PLGA-PVA-E1P47 NPs and LUVs E1P47 (n=30).

Zeta potential parameter is used to characterize the surface of particles. This parameter defines the electrical potential of particles and depends on the composition, the pH medium and other factors in which NPs or liposomes are dispersed [13, 14].

Our zeta potential results show negative values for PLGA-PVA NPs and neutral values for PLGA-PVA-E1P47 NPs (table 2). PLGA NPs, as reported in many studies, have a negative charge that can be attributed to the presence of end carboxyl groups of the polymer on the nanoparticle surface [15]. However, there are some studies that tested PVA coating of PLGA NPs and found exhibited neutral zeta potential or decrease its negative charge [15]. The mucus layer itself is at a neutral pH value an anionic polyelectrolyte. The neutrality of PLGA-PVA-E1P47 NPs shown in table 1 should minimize particle electrostatic/ionic interactions with mucus.

LUVs are composed by the zwitterionic lipid POPC, meaning it has a neutral charge surface. Even though zeta potential is -13±1 for POPC LUVs, as shown in table 2, some studies have previously reported that some neutral liposomes exhibit non-zero zeta potential in an electric field even when they are dispersed at pH 7.4 due to the orientation of lipid head groups in the liposome surface. The direction of the lipid head group depends on temperature and ionic strength [13]. According to this study, the phosphatidyl groups are located at the outer portion of the head group region and choline groups hide behind the surface.

Zeta potential can also indicate if charged material is found adsorbed onto the surface of NPs. In one hand, in table 2 shows the increase in zeta potential of PLGA-PVA E1P47 NPs with respect to PLGA-PVA NPs, could be attributed to part of the peptide has not been encapsulated and is found on the surface of the nanoparticles varying its zeta potential. On the other hand, zeta potential measurement of E1P47 (table 2) revealed that the positive charge has influenced to surface of POPC LUVs decreasing their negativity until obtaining a zeta potential of -3.5 ± .1 for POPC LUVs E1P47. This fact could be attributed that part of E1P47 could be entrapment between lipidic bilayers.

Formulation	Z_{pot} ± SD (mV)
Free E1P47	10.6 ± 0.6
PLGA-PVA NPs	-13 ± 1
PLGA-PVA-E1P47 NPs	Neutral
POPC LUVs	-13 ± 1
POPC LUVs E1P47	-3.5 ± 0.1

Table 2. Zeta potencial values E1P47, PLGA-PVA NPs, PLGA-PVA-E1P47 NPs, POPC LUVs and POPC LUVs E1P47 (n=30).

According to the results obtained from particle stability studies (table 3 and 4), nanoparticles and liposomes sizes and PdI remained stable for almost a month and a half at 4ºC. Although in the later measure slight increase in particle size can be observed.

Day	Size ± SD (nm)	PdI ± SD
0	245 ± 2	0.094 ± 0.004
4	240 ± 2	0.07 ± 0.05
12	242 ± 3	0.09 ± 0.01
40	250 ± 10	0.11 ± 0.01

Table 3. Size and PdI values for PLGA-PVA E1P47 NPs samples at different days (n=30)

Day	Size ± SD (nm)	PdI ± SD
0	95 ± 2	0.07 ± 0.02
4	100 ± 1	0.09 ± 0.03
8	100.1 ± 0.8	0.12 ± 0.02
16	97 ± 1	0.08 ± 0.01
44	107 ± 3.	0.10 ± 0.04

Table 4. Size and PdI values for POPC LUVs samples at different days (n=30).

To calculate the amount of the E1P47 peptide loaded in LUVs and NPs the entrapment efficiency was assessed indirectly, determining the free E1P47 (non-encapsulated) by HPLC analytical method using a standard curve of E1P47 and applying the equation 1. The standard covered a range from 4 µg/mL to 63 µg/mL and included a total of eight standards

concentrations. The standards, LUVs E1P47 and NPs E1P47 were analysed by analytical reversed -phase HPLC as explained before.

The linearity study verified that the sample solutions were in a concentration range where analyte response was proportional to the concentration. Linearity was established by calculation of a regression line from the graphical plot of the chromatographic peak area versus E1P47 standard concentration obtaining a calibration curve. Linearity was studied by calculating the regression equation and the correlation coefficient (r^2) of the calibration curve. The calibration curve and correlation coefficient were y= 31990.2 x - 5.6 and 0.9998 respectively. The correlation curve is shown below in figure 10.

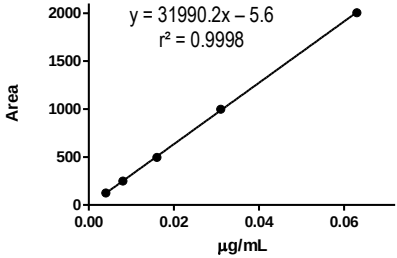

Figure 10. Linear regression from the standard curve generated with E1P47.

The E1P47 content of NPs and LUVs are listed in the table 5. The E1P47 content of the POPC LUVs E1P47 was 93%, larger than PLGA-PVA E1P47 NPs.

Formulation	EE (%)
PLGA-PVA E1P47 NPs	69%
POPC LUVs E1P47	93%

Table 5. PLGA-PVA E1P47 NPs, POPC LUVs E1P47 entrapment efficiency.

6.2 OPTIMIZATION OF UPLC-MS/MS FOR E1P47 QUANTIFICATION

Two standards curves were made using E1P47 in transcutol®/H_2O 1:1 (v/v) and other using ACN/H_2O 1:1 (v/v) to evaluate the concentrations of all samples in *ex vivo* and *in vitro*. The two curves contained fifteen standards of E1P47 from 0.02 µg/mL to 350 µg/mL.

This experiment used the selectivity of the triple quadrupole mass spectrometer by only allowing ions with specific mass, that of the intact peptide or parent ion, to be transmitted to the triple quadrupole detector (TQD) in the collision cell. The intact peptide ions are fragmented in the collision cell producing daughter ions. In a Multiple reaction monitoring (MRM) experiment, more than one daughter is transmitted to the third quadrupole. The mass spectra of E1P47 were acquired under a positive ESI at 0.3 mL/min flow rate. The average mass spectra of E1P47 are shown in figure 11. The mass spectrum of E1P47 shows a base peak at *m/z* 1184.3 and low-abundant peak at *m/z* 790.3, corresponding to the triply and doubly charged molecular ions of E1P47, respectively.

As shown in figure 11 the doubly charge ions of E1P47 (*m/z* 1184*)* were selected for the collision-induced dissociation (CID), since they were the most abundant ions. The cone voltage was optimized at 50 V to obtain the most intense sight for the peak.

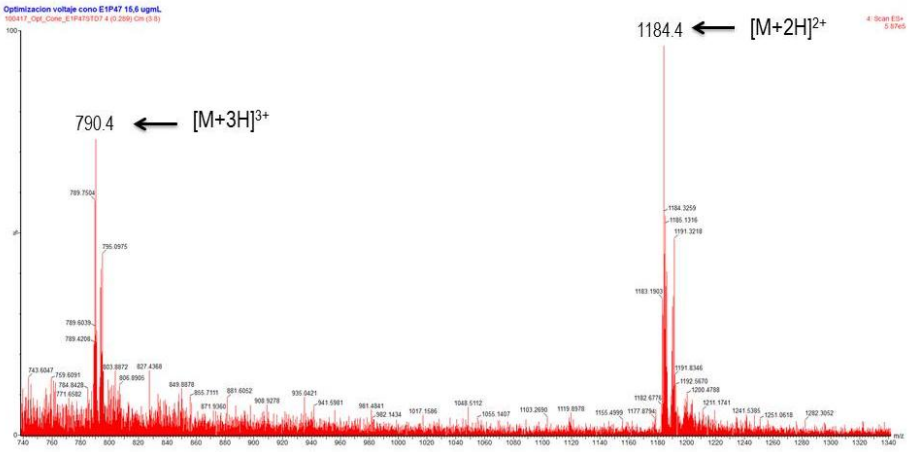

Figure 11. The average mass spectra from E1P47 under ESI at 50 V of cone voltage.

The CID spectrum of E1P47 (figure 12) shows two abundants peaks at 159 *m/z* and 272 *m/z*. Therefore, the precursor/parents ion was at *m/z* 1184/159-279 were selected in the MRM mode for quantification of E1P47. The collission energy was optimizated at 60 V.

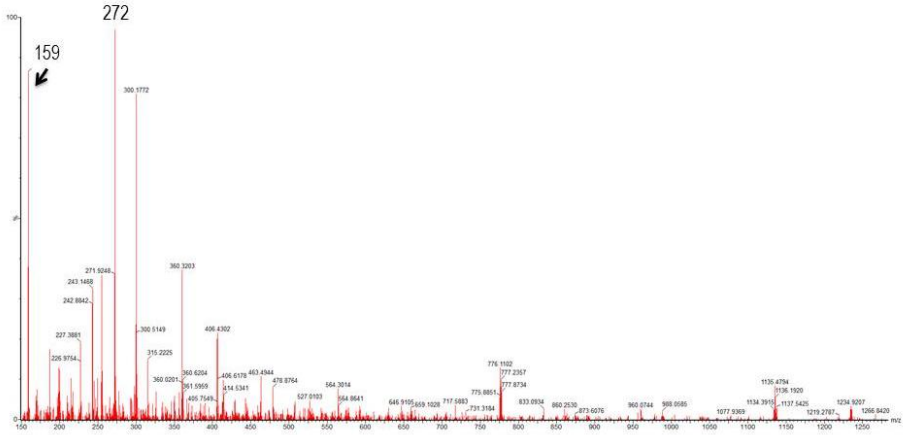

Figure 12. The CID mass spectra of doubly charged molecular ion of E1P47 at *m/z* 1184 with 60 V of collission energy showing 159 *m/z* and 279 *m/z* peaks

All standard were analyzed with the optimized parameters and using the conditions explained before. The obtained curve standards for E1P47 in transcutol®/H_2O 1:1 (v/v) and for ACN/H_2O 1:1 (v/v) were not lineal, as shown in figure 13a and figure 14a. Due to the two curves having polynomial distribution, they have been segmented in different sections, one for each rang, obtaining three linear regression for curve standards of E1P47 in transcutol®/H_2O 1:1 (v/v), (figure 13b, 13c and 13d) and two for E1P47 in ACN/H_2O 1:1 (v/v) (figure 14b, 14c and 14d).

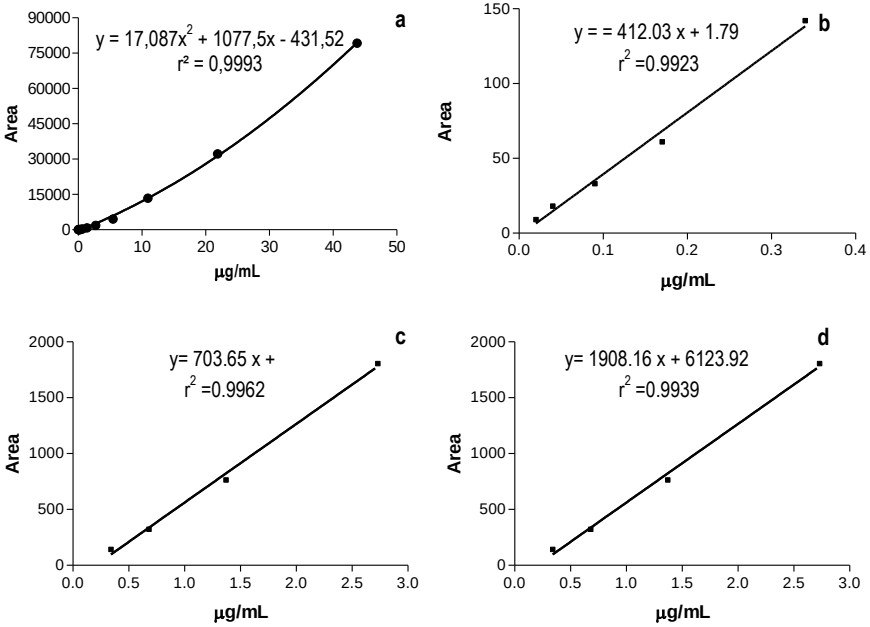

Figure 13. a) Polynominal regression from standard curve generated using E1P47 in Transcutol®/H_2O 1:1 (v/v). b) Liniar regression from 0.02 – 0.34 µg/mL of E1P47. c) Liniar regression from 0.34 – 2.73 µg/mL of E1P47. d) Liniar regression from 2.73 – 43.75 µg/mL E1P47

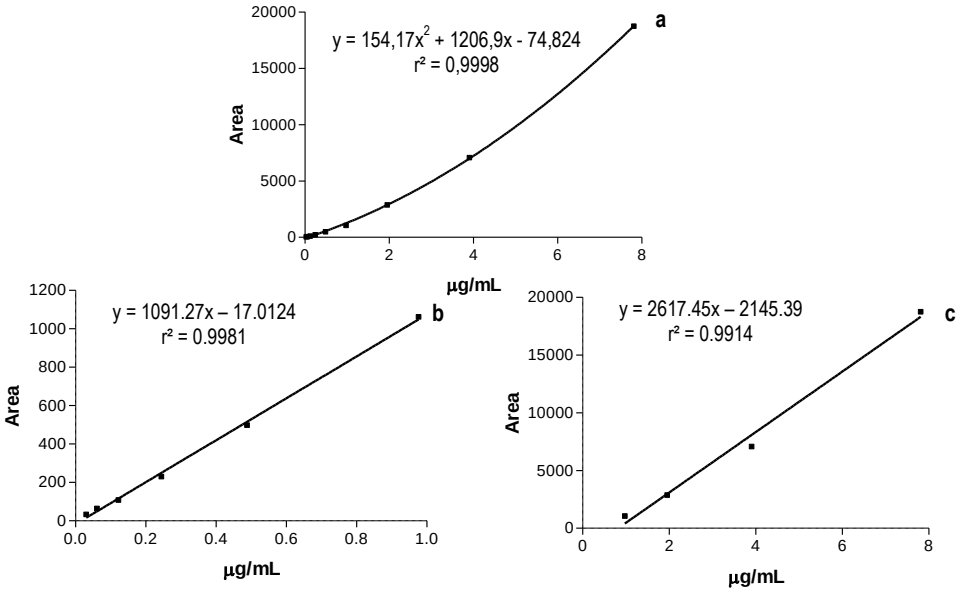

Figure14. a) Polynominal resgression from standard curve generated using ACN/H_2O 1:1 (v/v). b) Liniar regression from 0.03 – 0.98 µg/mL of E1P47. c) Liniar regression from 0.98 – 7.81 µg/mL of E1P47.

6.4 *IN VITRO* RELEASE AND STABILITY OF FREE E1P47 STUDIES

Release studies are mandatory to evaluate the efficacy of drug delivery of the two nanosytems (NPs and LUVs) with E1P47 that are developed in this study, and also for free E1P47.

In vitro release profiles of the three preparations were fitted to a monocompartmental model using WinNonlin v5.2. This profile corresponds to pharmacokinetic model, where two steps peptide entry and elimination occurs simultaneously. In our case, the peptide elimination could be attributed to possible peptide degradation in receptor medium. On the other hand, to carry out the release study 0.4 mL of each sample were added to the dialysis membrane. At the end of the study no liquid onto dialysis membranes was observed, indicating that the whole volume crossed the dialysis membrane, thus diluting the receptor phase. Finally, observing the elimination step on the release profile.

Figure 15 illustrates the different monocompartmental model parameters which are entry rate constant (K_a), elimination rate constant (K_{10}), maximal drug level contained (C_{max}), time at which C_{max} occurs (T_{max}) and time from to administration appear on medium (L_{tme}).

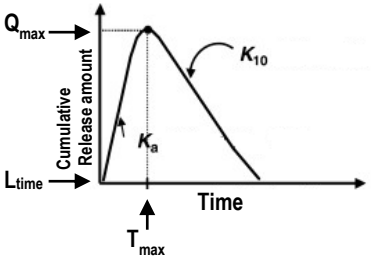

Figure 15. Monocompartmental model

Next, the observed release profile and the one set to a monocompartmental model are shown of the samples tested in the release assay.

Free E1P47 monocompartmental model and the observed release profile are show in figure 16 and table 6 respectively. The entry rate constant is (1.9 ± 0.3) 10^{-1} h^{-1} (Ka) and the elimination rate constant is (6 ± 3) · 10^{-2} h^{-1} (K_{10}). The entry of peptide starts at 1 ± 1 · 10^{-1} hours (L_{time}) and reaches maximum concentration of E1P47 is at 11 ± 2 hours (T_{max}) from this moment the amount of peptide begins to decrease.

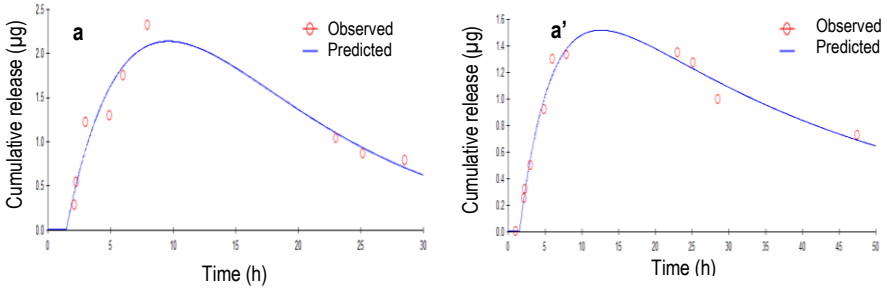

Figure 16. Duplicate *in vitro* release profile, monocompartmental fitted model of free E1P47 expressed by mean and standard deviation.

	K_a (h^{-1})	K_{10} (h^{-1})	L_{time} (h)	Q_{max} (µg)	T_{max} (h)
E1P47 (a)	$1.50 \cdot 10^{-1}$	$9.4 \cdot 10^{-2}$	1.3	2.1	9.6
E1P47 (a')	$2.31 \cdot 10^{-1}$	$2.5 \cdot 10^{-2}$	1.5	1.5	12.6
Mean ± SD	$(1.9 \pm 0.3)\ 10^{-1}$	$(6 \pm 3) \cdot 10^{-2}$	$1 \pm 1 \cdot 10^{-1}$	$2 \pm 3 \cdot 10^{-1}$	11 ± 2

Table 6. Pharmacokinetis parameters from free E1P47 release expressed by mean and standard deviation (n=2).

Figure 17 shows the release profiles of E1P47 from NPs and the pharmacokinetic parameters in table 7. As it can be observed, the entry constant (K_a) is quite pronounced and the elimination constant is $(2.8 \pm 0.3) \cdot 10^{-2}$ h^{-1} (K_{10}). The maximal drug contained (Q_{max}) is seen at 8 ± 1 hours and from this point the amount begins to decrease. The release of drug depends upon the nature of delivery system, in our case, peptide is uniformally distributed or dissolved in the matrix and the release occurs by diffusion or erosion of the matrix. Rapid initial release is to burst effect, attributed to the fraction of the drug which is adsorbed or weakly bound to the large surface area. of the NPs.

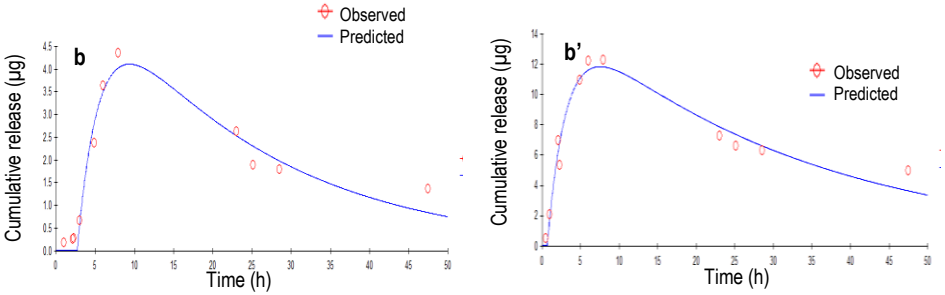

Figure 17. Duplicate i*n vitro* release profile and monocompartmental model fitted of PLGA-PVA-E1P47 NPs expressed by mean and standard deviation.

	K_a (h^{-1})	K_{10} (h^{-1})	L_{time} (h)	Q_{max} (µg)	T_{max} (h)
PLGA-PVA-E1P47 NPs (b)	$6.59 \cdot 10^{-1}$	$3.03 \cdot 10^{-2}$	2.9	4.1	9.4
PLGA-PVA-E1P47 NPs (b')	$5.41 \cdot 10^{-1}$	$2.50 \cdot 10^{-2}$	0.8	11.8	7.5
Mean ± SD	$(6.0 \pm 0.6) \cdot 10^{-1}$	$(2.8 \pm 0.3) \cdot 10^{-2}$	2 ± 1	8 ± 4	8 ± 1

Table 7. Pharmacokinetis parameters from PLGA PEG E1P47 NPs release (n=2).

Monocompartmental fitted and observed proflie from POPC LUVs E1P47 are shown below (figure 18 and table 8). It can be observed that the release profile is sustained over time with a

rate entry constant of $(1.1 \pm 0.3) \cdot 10^{-1}$ h^{-1}. The maximal E1P47 release level is at 23 ± 1 hours and lag time is at 3 ± 2 hours. These results describe a slow release of the peptide, which could be due to the peptide being trapped between the lipid bilayer.

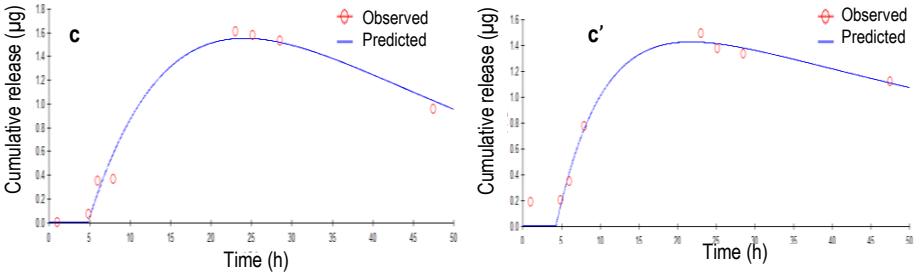

Figure 18. Duplicate *in vitro* release profile and monocompartmental model fitted of POPC LUVs E1P47 expressed by mean and standard deviation.

	K_a (h^{-1})	K_{10} (h^{-1})	L_{time}(h)	Q_{max} (µg)	T_{max} (h)
POPC LUVs E1P47 (c)	$1.42 \cdot 10^{-1}$	$1.81 \cdot 10^{-2}$	4.7	1.6	24.2
POPC LUVs E1P47 (c')	$7.72 \cdot 10^{-2}$	$9.21 \cdot 10^{-3}$	$7.48 \cdot 10^{-1}$	1.4	21.8
Mean ± SD	$(1.1 \pm 0.3) \cdot 10^{-1}$	$(1.4 \pm 0.4) \cdot 10^{-2}$	3 ± 2	$1.5 \pm 7 \cdot 10^{-2}$	23 ± 1

Table 8. Pharmacokinetis parameters from POPC LUVs E1P47 release (n=2).

In all three preparations, the release assay was performed in duplicate. Some parameters showed significant differences between them, such as the lag time in PLGA-PVA-E1P47 NPs (table 7) release or in POPC LUVs E1P47 (table 8) release. This could be due to the difficulty of quantifying the peptide found in the medium at such low concentrations.

The release profiles obtained for the free peptide and the NPs formulations were very similar, showing a rather pronounced release profile, somewhat greater in the case of the release of the peptide in the nanoparticles. In contrast, the loaded peptide in the liposomes showed slower release.

As explained before, 0.4 mL of each sample were added in duplicate at a 0.350 mg/mL, 0.379 mg/mL and 0.376 mg/mL concentration of free E1P47, POPC LUVs E1P47 and PLGA-PVA E1P47 NPs respectively. Thus, the amount of peptide used for each sample was 140.0 µg for free peptide, 150 for PLGA-PVA-E1P47 NPs and 151.6 µg for POPC LUVs E1P47. The maximal amount of peptide found in the medium (Q_{max}) was $1.8 \pm 3 \cdot 10^{-1}$ µg (table 6), 8 ± 4 µg

(table 7) and 1.5 ± 7· 10^{-2} µg (table 8) respectively for each preparation. This represents a very small amount of peptide founded in the receptor phase. Thus, a peptide extraction of each membranes was carried out to quantify the peptide that might have been retained in them.

Table 9 shows the retained peptide on each release membrane. The amount of entrapped peptide in the membrane remains very low in all cases.

Formulation	Peptide retained in the membrane (µg)
Free E1P47 (a)	1.07
Free E1P47 (a')	0.00
PLGA-PVA-E1P47 NPs (b)	1.17
PLGA-PVA-E1P47 NPs (b')	4.14
POPC LUVs E1P47 (c)	2.03
POPC LUVs E1P47 (c')	1.06

Table 9. Quantity of peptide retained in the release membranes.

Due to the low recovery rate of the peptide in the medium and the membrane, it was hypothesized that the peptide could have undergone a degradation process.

To corroborate the hypothesis of a possible degradation of the peptide in the receptor medium and low recovery rate of the peptide, a stability study of the peptide was performed in the same medium and temperature that study *in vitro*, which was in transcutol®/H_2O 1:1 (v/v) and 37ºC respectively for 48 hours. Peptide solution had a concentration of 0.072 mg/mL.

The samples and the standard curve of E1P47 were analysed with the following optimized parameters: the precursor/parents ion was at *m/z* 790/159-279 were selected in the MRM mode, the cone voltage was optimized at 20 V and collission energy of 159 *m/z* and 279 *m/z* peaks was 60 V and 30 V respectivly. The obtained standars curve of E1P47 in transcutol®/H_2O 1:1 (v/v) is shown at below at figure 19.

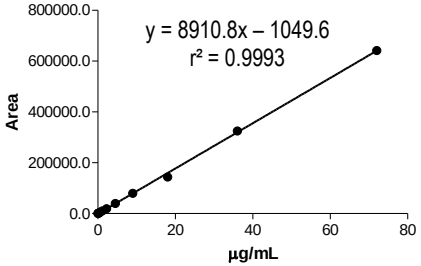

Figure 19. Lineal resgression from 0.018 to 72.00 µg/mL of E1P47 standard curve generated using transcutol®/H_2O 1:1 (v/v).

Figure 20 illustrates the results of the study and shows that peptide concentration decreases 17 % in a period of 2 days. The profile was fitted to exponential curve.

E1P47 shows a slight degradation. Another plausible reason for decrease E1P47 concentration would be possible to peptide aggregation due to the peptides hydrophobicity. Aggregation depends on several factors such as ionic strength, pH or peptide concentration. In this case, the addition in the receptor compartment was 5 times more concentrated than in the stability study, so a possible aggregation could have happened. More aggregation studies should be carried out at study concentration.

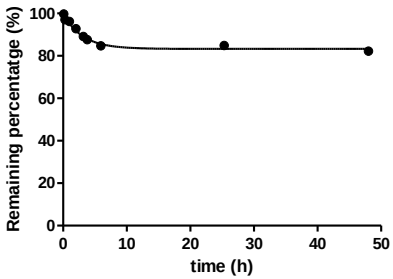

Figure 20. Degradation of E1P47 at 37ºC in transcutol®/H_2O 1:1 (v/v) for 48 hours.

6.4 *IN VITRO* PERMEATION STUDY

To develop the permeation experiment, 0.4 mL of each sample were added in duplicate at a 0.35 mg/mL, 0.39 mg/mL and 0.34 mg/mL concentration of free E1P47, POPC LUVs E1P47 and PLGA-PVA-E1P47 NPs respectively to the donor compartment. Thus, the amount of

peptide used for each sample was 140.0 µg for free peptide, 136.0 for PLGA-PVA-E1P47 NPs and 156.0 µg for POPC LUVs E1P47.

All samples were analysed by HPLC. In all receptor medium, none E1P47 peptide was found. Given this result, it can be considered that the whole amount of added peptide was retained in the mucosa.

First, the determination of the peptide retained in the mucosa was necessary to evaluate the recovery. Recovery was calculated applying the equation 2 described in *ex vivo* permeation study from the experimental section. In the assay 41 µg/mL (C_o) was added to each mucosa. All values can be observed in the table 9.

Mucosa	W_t (g)	$W_{t'}$ (g)	C_{trans} (µg/mL)	C_{acn} (µg/mL)	Recovery (%)
1	0.63130	0.53982	21.05	4.50	22
2	0.39300	0.29994	22.15	3.60	21
3	0.53416	0.40343	23.20	4.65	28

Table 9. Recovery results expressed by percent (n=3).

Mean and standard deviations for recovery values was 24 ± 3 %.

The quantity of peptide retained in the mucosa was calculated applying equation 3. The concentration of peptide extracted from the mucosa (C_p), weight of the mucosa (W_p) and the actual quantity of peptide are shown in table 10.

Formulation	C_p (µg/mL)	W_p (g)	Quantity of peptide retained (µg/g · cm^{-2})	Medium ± SD (µg/g · cm^{-2})
Free E1P47 (a)	0.23	0.50	3.03	10 ± 7
Free E1P47 (a')	1.05	0.39	17.75	
PLGA-PVA-E1P47 NPs (b)	0.27	0.51	3.49	3.6 ± 0.1
PLGA-PVA-E1P47 NPs (b')	0.16	0.29	3.64	
POPC LUVs E1P47 (c)	2.65	0.36	48.53	40 ± 8
POPC LUVs E1P47 (c')	2.33	0.48	32.00	

Table 10. Values of C_p, W_P and quantity of peptide retained of free E1P47, PLGA-PVA-E1P47 NPs and POPC LUVs E1P47 (n=2).

The most important question is whether this concentration of peptide found inside the mucosa would be sufficient to inhibit the entry of the virus to cell membrane. There is an *ex vivo* study performed with T-20, an HIV-1 inhibitory peptide, which have shown a significant inhibition of viral genomic integration in intraepithelial vaginal cells with a IC_{50} of 0.153 μM [16]. This value can give an idea of the order of concentration of peptide that is needed to inhibit a HIV-1.

In our case, the inhibition of replication of HIV-1 NL4-3 in cell cultures E1P47 showed a strong inhibitory activity, IC_{50} 3 μM [9]. As shown in table 11, of all developed formulation only POPC LUVs E1P47 showed a concentration greater than IC_{50}. Therefore, LUVs can be considered the best candidate to act as a possible microbicide with a concentration of 11 ± 2 μM.

Formulation	Concentration of peptide retained (μM)	Mean ± SD (μM)
Free E1P47 (a)	0.81	3 ± 2
Free E1P47 (a')	4.73	
PLGA-PVA-E1P47 NPs (b)	0.93	0.95 ± 0.02
PLGA-PVA-E1P47 NPs (b')	0.97	
POPC LUVs E1P47 (c)	12.95	11 ± 2
POPC LUVs E1P47 (c')	8.54	

Table 11. Values of concentration of peptide retained of free E1P47, PLGA-PVA-E1P47 NPs and POPC LUVs E1P47 (n=2).

However, an *ex vivo* study of microbicide efficacies for preventing HIV-1 genomic integration in intraepithelial vaginal cells should be carried out to confirm that these results are accurate.

7. CONCLUSIONS

PLGA-PVA-E1P47 and POPC LUVs E1P47 were correctly prepared. The two formulations showed a suitable mean size for the permeation through the vaginal mucosa (less than 500 nm) and polydispersity index (PdI) of less than 0.1 corresponding to monodisperse system. High encapsulation efficiency was observed.

Stability studies performed at 4 ° C of both formulations revealed that the particle size was constant but with a slight increase after a month and a half of their storage. The polydispersion was constant throughout the experiment with its value very close to 0.1.

The stability study of the peptide mimicking the conditions of the *in vitro* and *ex vivo* studies showed a degradation of 17% in 48 hours.

The formulation of PLGA-PVA-E1P47 did not slow down the release of E1P47, showing a very similar release profile to the free E1P47. The concentration of E1P47 retained in the vaginal mucosa of both preparation was lower than active concentration (IC_{50} = 3μM), which has been reported in other studies, showing that PLGA-PVA-E1P47 and free E1P47 are not an adequate choice to be a microbicide.

The developed formulation of LUVs showed a sustained release of E1P47. The concentration of E1P47 retained in the vaginal mucosa was also greater than IC_{50}, suggesting that this formulation could be a suitable candidate as a potential microbicide.

8. REFERENCES AND NOTES

1. Antimisiaris, S. G., & Mourtas, S. (2015). Recent advances on anti-HIV vaginal delivery systems development. *Advanced drug delivery reviews*, *92*, 123-145.
2. Joint United Nations Programme on HIV/AIDS (UNAIDS). AIDSinfo. http://aidsinfo.unaids.org/ (accessed May 5, 2017)
3. Pacheco, A. V. (2015). *Diseño y síntesis de nanosistemas derivatizados con péptidos y su aplicación en biomedicina* (Doctoral dissertation, Universitat de Barcelona).
4. das Neves, J., Nunes, R., Rodrigues, F., & Sarmento, B. (2016). Nanomedicine in the development of anti-HIV microbicides. *Advanced drug delivery reviews*, *103*, 57-75.
5. Caramella, C. M., Rossi, S., Ferrari, F., Bonferoni, M. C., & Sandri, G. (2015). Mucoadhesive and thermogelling systems for vaginal drug delivery. *Advanced drug delivery reviews*, *92*, 39-52.
6. Harman, S., Herrera, C., Armanasco, N., Nuttall, J., & Shattock, R. J. (2012). Preclinical evaluation of the HIV-1 fusion inhibitor L'644 as a potential candidate microbicide. *Antimicrobial agents and chemotherapy*, *56*(5), 2347-2356.
7. Heringlake, S., Ockenga, J., Tillmann, H. L., Trautwein, C., Meissner, D., Stoll, M., ... & Manns, M. P. (1998). GB virus C/hepatitis G virus infection: a favorable prognostic factor in human immunodeficiency virus-infected patients?. *Journal of infectious Diseases*, *177*(6), 1723-1726.
8. Alcalde, R., Nishiya, A., Casseb, J., Inocêncio, L., Fonseca, L. A., & Duarte, A. J. (2010). Prevalence and distribution of the GBV-C/HGV among HIV-1-infected patients under anti-retroviral therapy. *Virus research*, *151*(2), 148-152.
9. Gómara, M. J., Sánchez-Merino, V., Paús, A., Merino-Mansilla, A., Gatell, J. M., Yuste, E., & Haro, I. (2016). Definition of an 18-mer synthetic peptide derived from the GB virus C E1 protein as a new HIV-1 entry inhibitor. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1860*(6), 1139-1148.
10. Brako, F., Mahalingam, S., Rami-Abraham, B., Craig, D. Q., & Edirisinghe, M. (2016). Application of nanotechnology for the development of microbicides. *Nanotechnology*, *28*(5), 052001.
11. Machado, R. M., Palmeira-de-Oliveira, A., Gaspar, C., Martinez-de-Oliveira, J., & Palmeira-de-Oliveira, R. (2015). Studies and methodologies on vaginal drug permeation. *Advanced drug delivery reviews*, *92*, 14-26.
12. das Neves, J., Nunes, R., Machado, A., & Sarmento, B. (2015). Polymer-based nanocarriers for vaginal drug delivery. *Advanced drug delivery reviews*, *92*, 53-70.
13. Makino, K., Yamada, T., Kimura, M., Oka, T., Ohshima, H. and Kondo, T. (1991). Temperature- and ionic strength-induced conformational changes in the lipid head group region of liposomes as suggested by zeta potential data. *Biophysical Chemistry*, 41(2), pp.175-183.
14. Mohanraj, V. J., & Chen, Y. (2006). Nanoparticles-a review. *Tropical Journal of Pharmaceutical Research*, *5*(1), 561-573.
15. Yang, M., Lai, S. K., Yu, T., Wang, Y. Y., Happe, C., Zhong, W., ... & Fu, J. (2014). Nanoparticle penetration of human cervicovaginal mucus: The effect of polyvinyl alcohol. *Journal of Controlled Release*, *192*, 202-208.
16. McElrath, M. J., Ballweber, L., Terker, A., Kreger, A., Sakchalathorn, P., Robinson, B., ... & Hladik, F. (2010). Ex vivo comparison of microbicide efficacies for preventing HIV-1 genomic integration in intraepithelial vaginal cells. *Antimicrobial agents and chemotherapy*, *54*(2), 763-772.

9. ACRONYMS

ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
CAPRISA	Centre for AIDS Program of Research in South Africa
CDI	Collision- induced dissociation
DPV	Dapivirine
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EE	Entrapment efficiency
FA	Formic acid
GBV-C	GB virus C
Fmoc/tBu	9-fluorenylmethoxycarbonyl/tet-butyl
FP	Fusion peptide
FTMLV	Freeze and thaw multilamellar vesicles
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HPLC	High-performance liquid chromatography
LCs	Langerhan cells
LUVs	Large unilamelar vesicles
MLV	Multilamellar vesicles
MRM	Multiple reaction monitoring

MW	Molecular weight
NPs	Nanoparticle
NNRTIs	Non-nucleoside transcriptase inhibitors
NRTIs	Nucleoside transcriptase inhibitors
LUVs	Large unillamelar vesicles
PdI	Polidispersity index
PC	phosphocholine
PLGA	Poly(D,L-lactide-co-glycolide)
POPC	1-Palmitoyl-2-oleoyl-sn-glycero-3-posphocholine
PVA	Polivynilic alcohol
RNA	Ribonucleic acid
SD	Standard deviation
SPPS	Solid phase peptide synthesis
SUV	Small unillamelar vesicles
TFV	Tenefovir
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
USP	The United States Pharmacopeia
VOICE	Vaginal and Oral Interventions to Control the Epidemic
Zpot	Zeta potential