

Advanced Mucus Infiltrating Nanoparticles for Microbicide Delivery

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Abstract

Advanced mucus infiltrating nanoparticles (NPs) based on ES-100 have been developed for the intravaginal delivery of Dapivirine against HIV-1. They were further surface engineered with poly ethylene glycol (PEG) that helps them overcome the mucosal barrier via enhanced pH-mediated infiltration properties. PEG coated NPs (PCN) loaded with Dapivirine was characterized for particle size distribution, morphology, loading efficiency and mucus infiltration properties. The cellular uptake profiles of PCN by human vaginal epithelial cells (VK2 E6/E7) were examined using confocal microscopy. The cytotoxicity of PCN was assessed using MTS assay as well as Annexin V-FITC/PI assays. The mucus infiltration rates of PCN were examined on the in-vitro simulated cervico-vaginal mucus system and ex vivo porcine vaginal tissues. PCN loaded with Dapivirine possessed physicochemical properties, readily traversing through mucus layer. The IR spectra of both NPs (i.e., NP uncoated but loaded with Dapivirine and PCN that were loaded with Dapivirine) did not display any additional peaks representing new functional groups, indicating there were no significant interactions among drug, PEG₂₀₀₀ and formulations components. The percentage yield, entrapment efficiency and loading efficacy of Dapivirine in PCN were around 75%, 64.6% and 2.03%, respectively. The micrographs of freeze dried NPs analyzed by SEM displayed smooth surface spherical particles that were previously demonstrated from the DLS study. TEM images confirmed the presence of coated PEG₂₀₀₀ that is supported by the difference in the zeta potential values. The results of MTS assay as well as Annexin V-FITC/PI assay demonstrated that PCN loaded with Dapivirine maintained 85% viability of human vaginal epithelial cells (VK2 E6/E7) upon exposure to the concentrations up to 0.1 mg/ml of Dapivirine in PCN. Alamar blue assay also demonstrated that cells exposed to PCN at the concentration up to 500 µg/ml were viable, indicating that PCN did not exert any cytotoxicity. The data from ensemble-averaged geometric mean square displacements confirmed that PEG₂₀₀₀ coating significantly enhanced the uptake rates as well as mucus penetration rates of PCN. PCN mimics two basic features of HIV-1 (i.e. capability to stay unreactive/unresponsive at acidic pH and exerting its action at neutral pH) and has achieved the enhanced mucus penetration rate. This PCN can serve as an ideal platform for vaginal delivery of Dapivirine against HIV-1.

Keywords: Eudragit S-100 (ES-100) nanoparticles; Anti HIV-1 Microbicides; Mucus infiltrating property

Introduction

Microbicides can be spread over mucosal surface lining vaginal or rectal cavity for the protection of women from sexually transmitted infections (STIs) including acquired immune deficiency syndrome (AIDS) [1]. Various features including potential barriers against human immunodeficiency virus (HIV-1) offer female controlled microbicide delivery the most viable option for women's protection from sexually transmitted diseases (STD) [2]. Especially, in the absence of an effective vaccine, the intravaginal delivery of microbicides that do not require partner's consent can become the most suitable choice as an alternative to condom against HIV-1 [3,4].

Topical delivery of microbicides through vaginal cavity for the protection of women from HIV-1 infection is limited by various obstacles, such as potential degradation of drugs at vaginal lumen due to lower pH and presence of various enzymatic and mucosal barriers [5]. The barrier function of mucus layer stems from the presence of gel layer at the apical side of mucosal epithelia [6]. Mucus is highly viscoelastic and adhesive complex hydrogel made of mucin, carbohydrates, proteins salts and cellular debris [7]. The human mucus displays the net negative charge due to the presence of sialic and sulfonic acid [8].

Mucus contains various innate and adaptive immunity components that serve as a physical barrier [9,10]. As a first line of defense, mucus traps and limits the access of foreign particles, toxins and pathogens, eventually eradicating them via physiological mucus clearance mechanism [11]. Disruption of the integrity of vaginal mucosa allows a

direct contact with the HIV-1 particles with intraepithelial Langerhans cells and $\gamma\delta$ CD4⁺ cells or virus to reach supra-basal or basal epithelial cells that are more susceptible to viral transcytosis [12].

The mucosal barrier against drug penetration has been overcome by various approaches: mucoadhesive [13], mucolytic agents that either dissolve or breakdown the mucus [14,15] and mucus penetration systems [16,17]. Even though mucoadhesive systems offer numerous advantages, such as extended drug residence time of the dosage forms and prolonged drug performance in the vaginal cavity [7,18], they also suffer from the drawbacks of not being able to infiltrate into submucosal layers, getting shed through normal mucus clearance, and patient's compliance issues [13,19,20].

Mucus penetration or infiltrating systems intended for mucosal delivery has been rapidly evolved in past several years due to their capability of avoiding fast clearance from topical sites and facilitating the loaded therapeutics to reach the underlying epithelia [20,21]. In addition, nanoparticles were engineered for the mucus infiltrating

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properties using numerous coating resources, such as low molecular weight polyethylene glycol (PEG) [11,22-24] polyvinyl alcohol [25] and dextran protamine [26]. The use of various conventional analytical technologies as well as scientometric or informetric methodologies offer real-time basis image guided analysis of NPs and subsequently provide world-wide scientists with variety of impending needs for development of advanced NPs [27,28].

Polyethylene glycol (PEG) is an uncharged hydrophilic polymer and PEG coated NPs maintain their stability and did not significantly interact with mucus components [29,30]. PEG has been widely used to minimize the degradation rates of exogenous compounds, such as microbicides, proteins and genes, and enhance their bioavailability [31-34]. Due to these beneficial properties, PEG was selected as a surface engineered infiltrating polymer in this study.

At present, several microbicides, such as Tenofovir, Dapivirine and Maraviroc, are commercially available in the market and frequently evaluated for their potential usage as a topical pre-exposure prophylaxis (PrEP) agent [35]. Dapivirine is a noncompetitive, non-nucleoside inhibitor, binding to hydrophobic binding pocket on HIV-1 reverse transcriptase enzyme with high affinity and blocking HIV-1 replication [36,37]. Nanoparticles (NPs) have been explored for the delivery of Dapivirine to the vaginal mucosa based on numerous polymers, such as poly-ε-caprolactone (PCL) [38], Poly ethylene oxide coated PCL (PEO-PCL) [39-41] and poly (d,l-lactic-co-glycolic acid) (PLGA) [42]. Recently, phase III safety and effectiveness trial of Dapivirine released from the vaginal matrix ring made of silicone elastomer against HIV-1 infection has been successfully completed and no cytotoxicity issues were reported in this trial [43].

In the present study, we have developed advanced NPs loaded with Dapivirine based on ES-100. NP was further surface engineered with a low molecular weight of polyethylene glycol (PEG). PEG coated NPs (PCN) loaded with Dapivirine were characterized for the particle size distribution, morphology, loading efficiency and mucus infiltration capacity. The cellular uptake profiles of PCN by human vaginal epithelial cells (VK2 E6/E7) were examined using confocal microscopy, whereas the cytotoxicity of PCN was assessed using cell viability assays. The mucus infiltration rates of PCN were determined on the in-vitro simulated cervico-vaginal mucus system and *ex vivo* in porcine vaginal tissues.

Materials and Methods

Micronized Dapivirine was obtained from International Partnership for Microbicides (IPM, Silver Spring, MD, USA). Eudragit S-100 (ES-100: molar mass 125,000 g/mol) was a gift from Röhm Pharma-Industrie GmbH (Weiterstadt, Germany). Poly ethylene glycol (MW 2000 Da) was purchased from Ruger chemical Co., Inc. (Irvington, NJ, USA). All other chemicals, reagents and solvents were purchased from Sigma Aldrich (St. Louise, MO, USA) and were of analytical grade.

Preparation of PEG₂₀₀₀ coated NPs (PCN)

NPs based on ES-100 were loaded with Dapivirine using single oil/water emulsion solvent evaporation technique with minor modifications [44]. Briefly, Dapivirine (0.179% w/v) was dissolved in 2 mL of chloroform containing ES-100 (5% w/v). The resulting polymer/drug mixture solution was added dropwise to external phase (10 mL) containing 0.5% poly vinyl alcohol (PVA, acidified, pH <5) under sonication. The polymeric solution-containing drug was the internal phase of the emulsion. The sonication of the mixture was performed at output watt of 15 and amplitude of 100 using probe sonicator (Ultrasonic processor, GE-130) for one min while stirring at 600 rpm.

A mixture of dispersed drug and polymer solution immediately transformed into emulsion with very fine droplets. This single o/w emulsion was slowly stirred for 4 h to evaporate the residual chloroform under the chemical hood. Then, the concentration of polymer increased and reached to the critical point where polymer concentration exceeded its solubility in the organic phase, leading to precipitation to produce nanoparticles [45]. The suspension containing nanoparticles was centrifuged at 20,000xg (Avanti J-25 centrifuge, Beckman Coulter Inc., Fullerton, CA) for 20 min. NPs suspension was washed multiple times to get rid of any residual chloroform. The obtained NPs suspension was then further freeze dried over night to obtain the dried nanoparticles, which further eliminates any chloroform, if there are still any remained. The concentrations of polymer, drug and PVA used for fabrication of NPs were shown in Table 1.

The surface of freeze dried NPs was physically modified by adsorbing PEG₂₀₀₀ onto the particle's surface via hydrophobic or electrostatic interaction [36,46,47]. A simple non covalent coating method with slight modification [47] was used for surface coating of NPs with PEG₂₀₀₀. Briefly, water was replaced with 0.1% PEG aqueous (acidic) solution during the washing step and NPs were re-suspended in 1 mL of 1% PEG solution. NPs suspension was further centrifuged at 92xg for 2 min to remove any large aggregates. The supernatant containing ES-100 NPs coated with PEG₂₀₀₀ was lyophilized (FreeZone Freeze Dry systems, LABCONCO, MO, USA) for 24 h and kept at -20°C until further use.

The NPs suspension was frozen at -20°C for more than 3 h before being placed in the lyophilizer. The vials containing frozen suspension of NPs were placed in the glass flask that was attached to freezer port. The lyophilization was carried out at or below -40°C and at $\leq 133 \times 10^{-3}$ mBar for 24 h. The vials were removed from the flask after 24 h and freeze-dried samples were stored at -20°C until further use. No cryoprotectant was used.

NPs either blank or loaded with Fluorescent Nile Red (NR) were also prepared and coated using the same methods described above. The size and Zeta (ζ)-potential of NPs were examined by dynamic light scattering technique (DLS: Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA)).

Formulation	Mean diameter ± Std. Dev. (nm)		PDI		Yield (%)	Entrapment Efficiency (EE) (%)	Drug Loading (DL) (%)
	Un-coated	Coated	Un-coated	Coated			
Dapivirine NPs	392 ± 6.1	480.8 ± 6.7	0.16 ± 0.07	0.784 ± 0.24	75.05 ± 1.92	64.68	2.23
NR-NPs	278.7 ± 0.8	365.3 ± 16.6	0.014 ± 0.01	0.524 ± 0.08	35.35 ± 2.5	-	-
Blank NPs	290.5 ± 2.1	410.4 ± 6.5	0.04 ± 0.03	0.281 ± 0.035	-	-	-

All were expressed as mean ± S.D. (n=3).

Table 1: Characterization of NPs loaded with Dapivirine.

Characterization of Surface Morphology of NPs

Morphological features of Dapivirine loaded NPs either coated with PEG (PCN) or uncoated controls were assessed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM study, specimens were sputter coated with Gold-Palladium for 3 min, and then attached to aluminum mounting stubs with double-sided sticky carbon tape. They were examined with Field-Emission SEM (FEG ESEM XL30 Hillsboro, OR) at 5 kV accelerating voltage. For TEM study, the aqueous suspension of NPs was mounted on copper grids incorporated with carbon film and air-dried, then size distribution and morphology were examined using TEM CM12 (FEI, Hillsboro, OR) at 80 kV accelerating voltage.

Determination of entrapment and drug loading efficiency in NPs

PEG coated NPs (PCN) loaded with Dapivirine were dissolved in sodium hydroxide solution (1 M) and the mixture was sonicated for 10 min in bath sonicator. The quantification of Dapivirine in supernatant was performed spectrophotometrically using UV VIS spectrophotometer (Genesys TM 10UV, ThermoFisher Scientific, MA, USA) at 290 nm. The standards used for calibration curve were 2, 5, 10, 15, 20, 30, 40, 50 µg/ml. The controls were prepared with the solution containing blank NPs. Samples were prepared and analyzed in triplicates. Entrapment and drug loading efficiency were determined using the following equations,

$$\text{Entrapment Efficiency (EE) \%} = \frac{\text{Amount of drug in Nanoparticles}}{\text{Amount of initially added drug}} \times 100 \quad (1)$$

$$\text{Drug Loading (DL) \%} = \frac{\text{Amount of drug in Nanoparticles}}{\text{Amount of Nanoparticles}} \times 100 \quad (2)$$

Determination of the particle size, zeta potential, and polydispersity index of NPs

The particle size, zeta potential and polydispersity index (PDI) of NPs samples, such as freeze dried blank NPs, NPs uncoated but loaded with Dapivirine, and NPs coated with PEG₂₀₀₀ (PCN) and loaded with Dapivirine, were examined using differential light scattering (DLS) technique (Zetasizer Nano ZSP, Malvern, Worcestershire, United Kingdom). Freeze dried NP formulations were dispersed in citrate buffer solution and subsequently diluted (10 µg/ml) with distilled water.

Assessment of the potential chemical interactions among NP components

To determine whether there are any potential interactions among Dapivirine, ES-100 and PCN, the distribution homogeneity of Dapivirine in NPs during the fabrication process were investigated using ATR-FTIR spectroscopy analysis (IRAffinity-1S, Shimadzu). The FTIR spectrum of Dapivirine and 2 formulations (i.e., NPs uncoated but loaded with Dapivirine and PCN loaded with Dapivirine) were recorded at the spectral range of 400-4000 cm⁻¹ with the spectral resolution value of 4.0 cm⁻¹.

In vitro Dapivirine release studies from NPs

Dapivirine loaded NPs (10 mg) either uncoated or coated with PEG were dispersed in citrate buffer (100 mM) and subsequently transferred to a dialysis bag (14000 MWCO BioDesign Dialysis Tubing™, Carmel, New York) that was placed in 10 mL of release media. At pre-determined time intervals of 0.5, 1, 2, 4, 6, and 8 h, the test bags were

removed and transferred to new tubes containing the fresh media. The release studies were conducted at 37°C and at two different pHs; phosphate buffer (pH=7.4 representing physiological/intracellular pH) and citrate buffer (pH=4.2, representing vaginal cavity pH).

The amount of Dapivirine present in the release samples was determined using the previously reported method with minor modifications [48-50]. For chromatographic separation LC-MS system, an EC 125/2 Nucleodur 100-3 C8 EC (Macherey Nagel) column was used with a gradient of 100% of solvent A (HPLC water) to 100% B (70:30 acetonitrile and water) in 10 min, changing to 100% Solvent C (100% acetonitrile) in 3 min, changing back to 100% solvent A in 1 min followed by post equilibration stage for 4-5 min with 100% solvent A at a flow rate of 0.3 ml/min. All the solvents had 10 µM Formic acid. The retention time of Dapivirine was at 6.83 min. A linear calibration curve of Dapivirine was constructed in the range of 0.01 µg/mL to 30 µg/mL (r²>0.99) at the detection wavelength of 290 nm.

Assessment of cell viability upon exposure to NPs

The potential cytotoxicity of formulations (i.e., blank NPs, NPs uncoated but loaded with Dapivirine, and PCN loaded with Dapivirine) was assessed using cell proliferation assay. The cell viability was examined using CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation assay Kit (Promega Corporation, Madison, WI) according to manufacturer protocols. The reagent contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (Phenazine Ethosulfate; PES). PES is highly chemically stable, and thus forms a long-standing solution when combined with MTS. The MTS tetrazolium compound is converted by cells into a colored formazan product that is soluble in tissue culture medium. This bio-conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells.

Briefly, VK2/E6E7 (human vaginal epithelial cells: 20,000 cells/well), were seeded onto separate 96 well plates. After the cells in wells reached to 70% confluence, the media was replaced with the fresh media containing each formulation. After 24 h incubation, 20 µl of CellTiter 96[®] AQueous One Solution Reagent was added into each well of the 96-well assay plate containing the samples in culture medium (100 µl). The plates were then incubated at 37°C for 2 h in a humidified 5% CO₂ atmosphere. The absorbance was recorded at 490 nm using a plate reader.

The effect of varying concentrations of Dapivirine (i.e., raw drug) as well as PCN loaded with Dapivirine on cell viability was assessed using Alamar blue assay. Briefly, VK2/E6E7 cells were cultured in a 96 well plate (20,000 cells/well). After cells in the wells reached to 70% confluence, the media was replaced with the fresh media containing either Dapivirine (0.01 µm to 200 µm) or PCN loaded with Dapivirine (1 µg/mL to 500 µg/mL). After 24 h incubation, each well was added with 20 µL of Alamar Blue[®] reagent and incubated at 37°C for 1 h under the protection from light. After incubation, the fluorescence intensity level was recorded for each sample (Ext: 540, Ems: 585 nm).

The effects of PCN containing Dapivirine on the degree of cell apoptosis were evaluated using Annexin-V assay (Annexin-V-FITC assay kit, Cayman chemical Co., MI) based on the manufacturer's protocol [51]. Briefly, cells (3 × 10⁵ cells/well) were seeded onto a 24-well plate and incubated for 24 h to allow cells for complete attachment to the plate. Fresh media was added to the wells. Then, uncoated or PCNs loaded with three concentrations (100, 200 and 500 µg/mL) of Dapivirine were added to the well. The plate was incubated for

24 h at 37°C. The cells were harvested using Trypsin (1 mM, Gibco TrypLE Express, Grand Island, NY) and stained with Propidium Iodide (PI) solutions in conjunction with Annexin-V-FITC for 10 min. Cell apoptosis were analyzed using fluorescence-assisted cell sorting technique (FACS) (BD FACS Canto™ II, BD Biosciences, San Jose, CA) at Ex-488 nm, and Em-525 and EM* 655-730 nm for FITC and PI, respectively.

Assessment of the cellular uptake profiles of NPs

The uptake profile of either uncoated but loaded with fluorescent dye or PCN loaded with fluorescent dye by VK2/E6/E7 cells were examined using both confocal microscopy and fluorescence assisted cell sorting technique. For confocal microscopy, VK2 E6/E7 cells were cultured on poly-L-Lysine coated coverslips in sterile, flat bottom TPP[®] 12 wells tissue culture plates made of polystyrene, and allowed to grow until they reached 70% confluence. The media was replaced with the fresh buffer solution (pH~6-6.5). Either NPs uncoated but loaded with fluorescent dye or PCN loaded with fluorescent dye were added to the wells and incubated for 24 h at 37°C. After incubation, the wells whose cover slips were removed were washed thrice with PBS. After fixing the cells with 4% paraformaldehyde for 10 min at 4°C, the coverslips were placed on glass slides using mounting media and sealed permanently with sealant. They were stored at 4°C in a dark place until the confocal images were taken using Leica TCS SP5 II.

The cellular uptake profiles were examined using fluorescence-assisted cell sorting technique. Briefly, VK2 E6/E7 cells were cultured in sterile (30,000 cells/well) TPP[®] 12 well tissue culture plates and allowed to grow until they reached 70% confluence. The media was replaced with fresh media containing NPs uncoated but loaded with NR or PCN loaded with NR (100, 200 and 500 µg/mL) and incubated at 37°C for 24 h. The cells were then harvested and analyzed for uptake rates of NPs using flow cytometry technique.

Assessment of mucus penetration rates of NPs

The mucus penetration properties and localization profiles of PCN in the intracellular compartment were evaluated using an ex vivo mucus simulated system. The porcine vaginal tissue was selected as a cell line, as it closely resembles human vagina in terms of physiological morphology and functionality [52]. The porcine tissues were collected from the slaughter house, transported to the laboratory in hanks buffer and used within 3 h of death to preserve mucosal barrier function.

Porcine vagina tissues were cut into the disk shapes having a diameter of 8-10 mm and mounted on Franz diffusion cells between donor and receptor chambers with the mucosa side facing donor chamber. Phosphate buffer (pH 7.4) was used as a receptor solution. NP solutions (100 µg/ml each of either blank NPs, NPs uncoated but loaded with Nile Red, or PCN loaded with Nile Red) were placed on top of the tissue in the donor chamber. Samples were drawn from the receptor compartment at specific time intervals and the NP concentration was determined using a multimode detector (DTX 880 Beckman Coulter Inc. CA.).

Statistical analysis

The experiments were replicated at least 3 times for drug release studies (n=3) and cell culture based studies (n=6). All data were expressed as a mean ± S.D. For multiple-group comparison, one-way ANOVA and Bonferroni tests were used. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Characterization of PEG coated NPs loaded with Dapivirine

PEG coated NPs (PCN) prepared using the O/W emulsion solvent evaporation technique were characterized for particle size, polydispersity index, zeta potential, entrapment efficiency and drug loading efficacy of PCN. As shown in Table 1, the mean diameters were around 290 nm and 365 nm for NPs uncoated and NP coated with PEG₂₀₀₀, respectively. The mean diameters were about 320 and 370 nm for Dapivirine loaded NPs uncoated and coated with PEG₂₀₀₀, respectively, whereas they were 280 and 345 nm for Nile Red loaded NPs.

The polydispersity index (PDI) values that reflect the relative variance in the particle size distribution profiles were within an acceptable range of less than 0.5 for the uncoated NPs, whereas those of PCN were greater than 0.5, even though the difference is not statistically significant. The difference is mainly attributable to agglomeration or aggregation of NPs during the PEG coating process.

The particles size distribution and zeta potential curves for NPs uncoated but loaded with Dapivirine and PCN loaded with Dapivirine were examined using SEM and TEM image analysis (Figure 1). The zeta (ζ) potential represents the magnitude of the electrostatic or charge repulsion/attraction between NPs, defining the stability of NPs. As shown in Table 2, uncoated NPs had a highly negative surface charge with Zeta potential in the range of 17 to 22, whereas PCN had a near neutral surface charge in the range of 0 to 3.

ES100 from Evonik Röhm GmbH (Darmstadt, Germany) is insoluble at low pHs, but dissolves freely at pH above 7 [53,54]. NPs based on ES100 can slower the drug release rate in the low pH environment (i.e., the vaginal cavity), whereas encapsulated drug readily releases at the higher pH environment (neutralization of acidic pH due to interaction with semen from the male partners).

The yield values, entrapment efficiency and Dapivirine loading efficacy of NPs were around 75%, 64.6% and 2.03%, respectively. The micrographs of freeze dried NPs from SEM image analysis displayed such smooth surface spherical particles as previously demonstrated from the DLS study. TEM images confirmed the presence of coating material (i.e., PEG₂₀₀₀) that is supported by difference in the zeta potential values.

PEG is a hydrophilic substance and confers the particles a near neutral surface charge with an enhanced penetration capability through the mucus [11]. PEG coated NPs can reduce their affinity to mucus constituents [42,47-58] and are able to readily penetrate through even chronic rhinosinusitis mucus (CRSM, highly viscoelastic) [59] or slip through the mucus [11]. Thus, PEGylation or covalent coating of high density of low molecular weight (MW) of conventional/polymeric particles has been explored for various biomedical applications, such as penetration to brain parenchyma [56], a biophysical tool, a means for drug and gene delivery [20], topical delivery of microbicides [47,55], and nano-rods for phototherapy [56]. The results of this study along with the previous studies further guarantee the potential application of PEG to surface modification of NPs intended for mucosal drug delivery.

Assessment of the potential chemical interactions among NP components

The potential chemical interactions among formulation components (i.e., ES 100, Dapivirine and PEG₂₀₀₀) and the incorporated

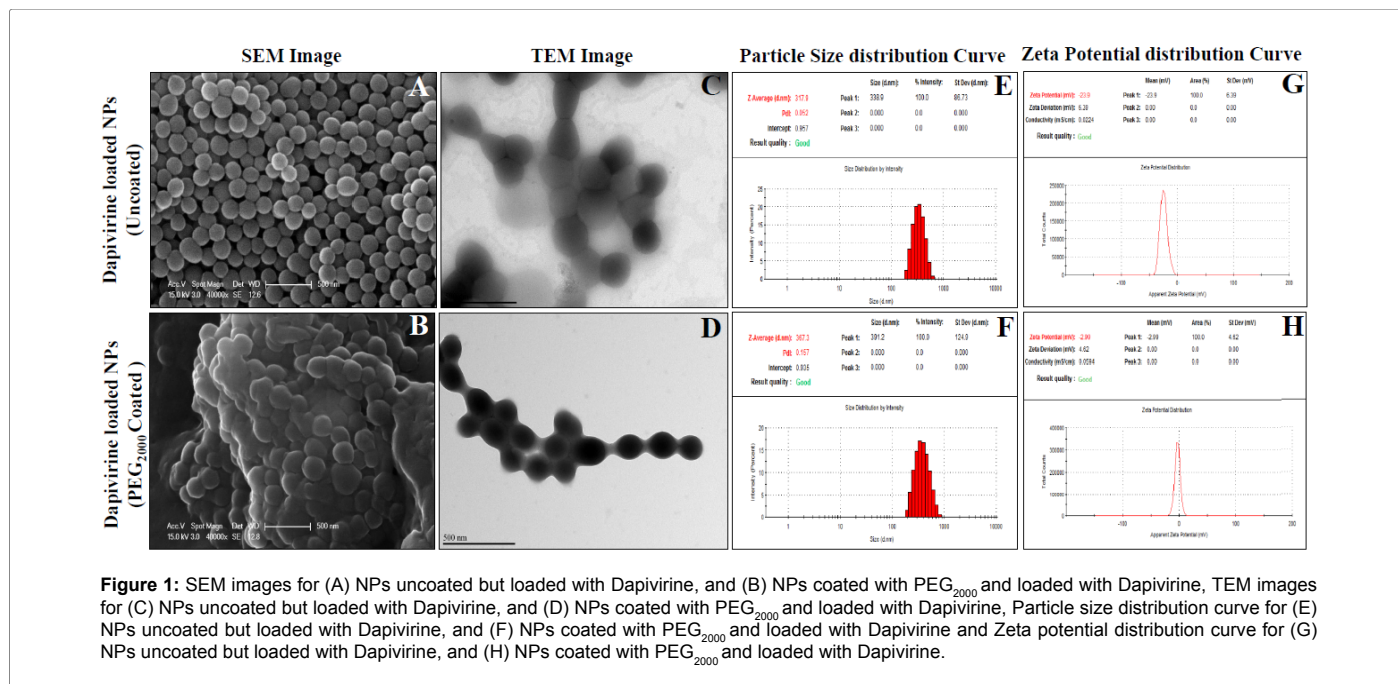


Figure 1: SEM images for (A) NPs uncoated but loaded with Dapivirine, and (B) NPs coated with PEG₂₀₀₀ and loaded with Dapivirine, TEM images for (C) NPs uncoated but loaded with Dapivirine, and (D) NPs coated with PEG₂₀₀₀ and loaded with Dapivirine, Particle size distribution curve for (E) NPs uncoated but loaded with Dapivirine, and (F) NPs coated with PEG₂₀₀₀ and loaded with Dapivirine, and Zeta potential distribution curve for (G) NPs uncoated but loaded with Dapivirine, and (H) NPs coated with PEG₂₀₀₀ and loaded with Dapivirine.

Formulation	Mean ζ-Potential ± Std. Dev. (mV)	
	Un-coated	PEG ₂₀₀₀ Coated
Dapivirine NPs	-23.9 ± 0.70	-2.99 ± 0.09
NR-NPs	-22.6 ± 1.68	-3.25 ± 0.62
Blank NPs	-22.6 ± 0.92	-3.38 ± 0.19

All were expressed as mean ± S.D. (n=3).

Table 2: Zeta (ζ) potential values of A) NPs uncoated but loaded with Dapivirine and B) NPs coated with PEG₂₀₀₀ and loaded with Dapivirine.

distribution profiles of Dapivirine within NPs were examined using Fourier Transform Infrared Spectroscopy (ATR-FTIR) analysis. The IR spectra for Dapivirine, NPs uncoated but loaded with Dapivirine, and PCN loaded with Dapivirine are shown in Figure 2. The IR spectra of Dapivirine or Eudragit S-100 were also examined for the comparison purpose. The IR spectrum of Dapivirine displayed the absorption peaks of N-H group at 3287 cm⁻¹ and C=N group at 2221 cm⁻¹ [60].

The presence of the same peaks in both spectra of NPs (i.e., NP uncoated but loaded with Dapivirine and PCN that were loaded with Dapivirine) confirmed that the drug inside the NPs was not affected by PEG coating. For PCN, there were distinctive absorption bands displayed for hydroxyl (O-H) groups at 3421 cm⁻¹, a C-H stretching at 2888 cm⁻¹ and the ether linkage (C-O-C group) at 1096 cm⁻¹, which are same as those from the previous reports [61]. The spectra of Eudragit-S 100 showed the carbonyl vibrations of the ester group at around 1735 cm⁻¹ [62]. The IR spectra of both NPs (i.e., NP uncoated but loaded with Dapivirine and PCN that were loaded with Dapivirine) did not show any additional peaks for new functional groups, indicating there were no significant interactions among drug, PEG₂₀₀₀ and formulations components.

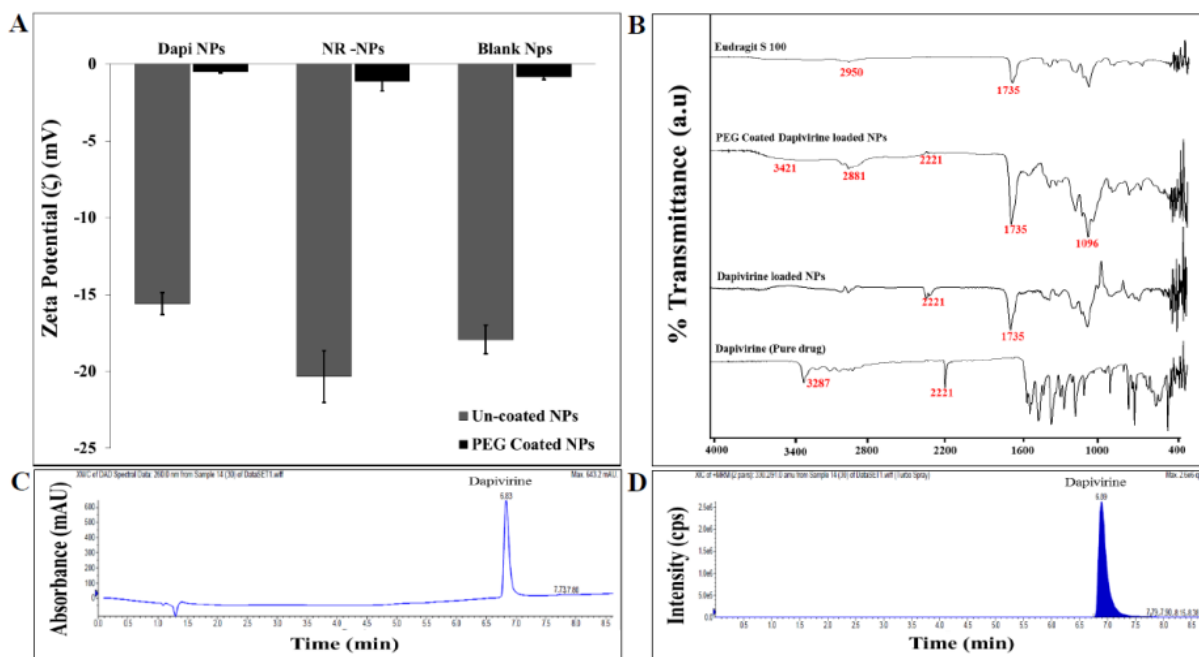
Assessment of cell viability against NPs

Cell viability against either (i) NPs uncoated but loaded with Dapivirine or (ii) PCN loaded with Dapivirine was assessed using Cell Proliferation Assay (CellTiter[®] 96 AQueous One Solution) that is a colorimetric method to determine the number of viable cells upon exposure to test formulations. The quantitative amount of a formazan

product based on the absorbance level at 490 nm is directly proportional to the number of living cells in the cell culture system. As shown in Figure 3A, the results of MTS assay revealed the non-cytotoxicity of PCN loaded with Dapivirine, maintaining 85% viability upon exposure to the concentrations of Dapivirine up to 0.1 mg/ml, beyond which cell viability gradually decreased. The results of MTS assay indicated that PCN loaded with Dapivirine were biocompatible and non-toxic as a delivery carrier for microbicides.

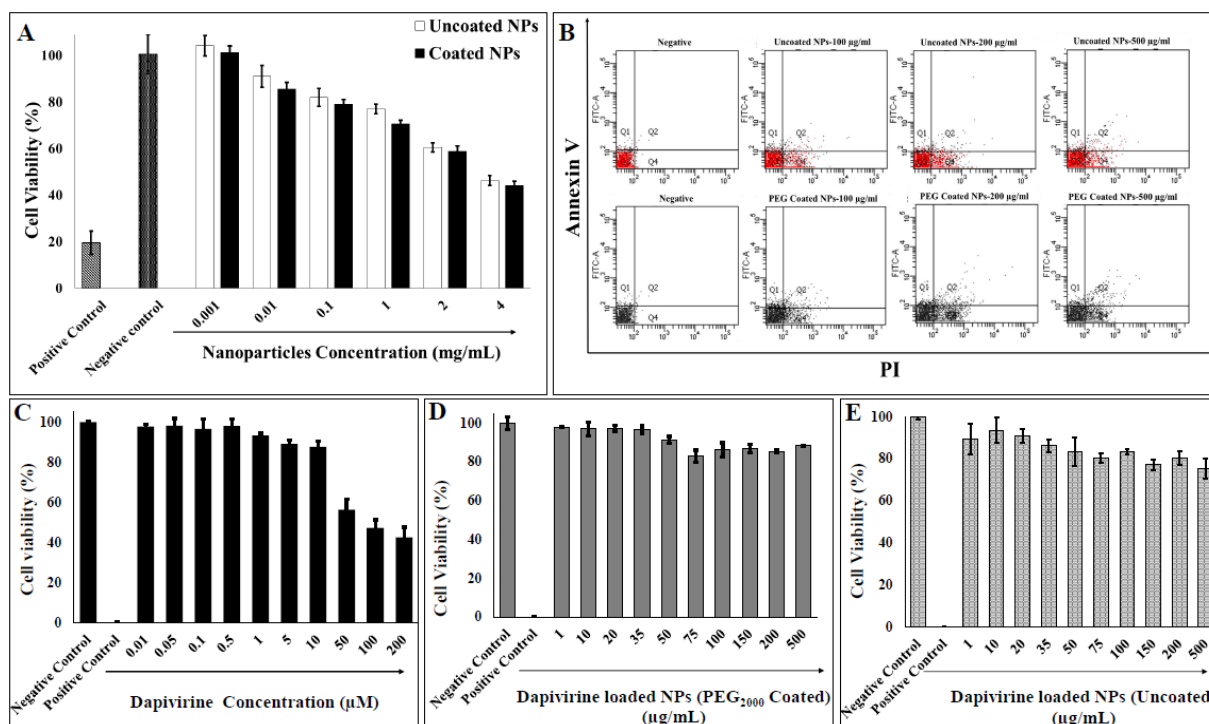
Annexin V-FITC/PI assay was used to determine whether NPs loaded with Dapivirine induced apoptosis in VK2 E6/E7 cells. Annexin V was used to detect phosphatidylserine (PS) externalization that is a hallmark of the early apoptosis, whereas Propidium iodide (PI) was used to label DNA fragments, a symbol for cell death. Q3 area (Annexin V and PI negative) represents the intact and healthy cells, Q1 contains cells in early apoptotic stage, Q2 represents dead/apoptotic cells, and Q4 contains damaged and necrotic cells. As shown in Figure 3B, Q3 areas in cells for NPs treatment of 100, 200 and 500 µg/ml were 91.3%, 89%, and 91.8%, respectively, for uncoated particles, whereas the percentage of cells gathered at Q3 area for NPs treatment of 100, 200 and 500 µg/ml were 88.8, 84.1 and 86.4%, respectively, for PEG coated NPs.

Alamar blue assay was used to assess the cell viability of human vaginal epithelial cells (VL2 E6/E7) upon exposure to Dapivirine (a raw drug) vs. NPs loaded with Dapivirine (both coated and uncoated with PEG₂₀₀₀). As shown in Figure 3C-3E, both cells exposed to either Dapivirine at the concentrations up to 50 µM or NPs (both uncoated and coated with PEG₂₀₀₀) at the concentrations up to 500 µg/ml were



*(A) All were expressed as mean \pm S.D. (n=3).

Figure 2: (A) Change in Zeta potential values of the blank, NPs coated with PEG₂₀₀₀ and loaded with either Nile red or Dapivirine. (B) Fourier transform infrared spectra for Endragit S 100, Dapivirine, NPs uncoated but loaded with Dapivirine, and NPs coated with PEG₂₀₀₀ and loaded with Dapivirine, (C) LC-MS chromatogram for Dapivirine, (D) Mass spectroscopy intensity signal for Dapivirine.



*(C, D and E, All were expressed as mean \pm S.D. (n=6).

Figure 3: (A) Cell viability assessment of VK2 E6/E7 against uncoated NPs loaded with Dapivirine, and NPs coated with PEG₂₀₀₀ and loaded with Dapivirine, (B) Bivariate Annexin V/PI analysis of VK2 E6/E7 cells after 24 hr incubation of NPs uncoated loaded with Dapivirine, and NPs coated with PEG₂₀₀₀ and loaded with Dapivirine. Cell viability assessment of VK2 E6/E7 against different concentration of (C) Dapivirine, (D) NPs coated with PEG₂₀₀₀ and loaded with Dapivirine, (E) NPs uncoated but loaded with Dapivirine.

viable, indicative of non-cytotoxicity and biocompatibility of raw drugs and PEG coated NPs.

In vitro drug release studies

The *in vitro* release profiles of Dapivirine from PCN were investigated under the simulated vaginal conditions (i.e., acidic pH as well as neutral pH (Figure 4A). The results of this study demonstrated that there was a gradual release profile of Dapivirine from NPs (both uncoated and coated with PEG₂₀₀₀) for the period of 8 h. However, there were differences in the release rates due to surface coating conditions at neutral pH.

At acidic environment (pH 4.2), the drug released amount closely compatible with each other for coated and uncoated NPs, indicating that PEG surface coating did not affect the release profiles of Dapivirine from NPs at acidic pHs. At neutral pH, about 85% of the loaded Dapivirine were released from NPs in 8 h, whereas only 62% of the loaded dose of Dapivirine were released from PEG coated NPs in 8 h.

Even though it was previously reported that PEG chains did not directly affect the drug release process at lower pHs [63-65], PEG surface coating significantly alter properties of the polymer shell (i.e., ES-100 used as the polymer base) at neutral environmental pH and subsequently the release profiles of Dapivirine from ES-100 NPs.

Assessment of cellular uptake profiles of NPs

The uptake rate of PCN (100, 200 and 500 µg/ml) incubated for 24 h in human VK2 E6/E7 was examined using Flow cytometry technique [65]. The uptake profiles of uncoated NPs and PCN are shown in Figure 4B. The uptake rate of PEGylated NPs by VK2 E6/E7 cells was lower than that of uncoated one. PEG coating partially impeded the cellular uptake rates of PEGylate particles, which may extend the duration for exerting its pharmacological activity in vaginal epithelial cells.

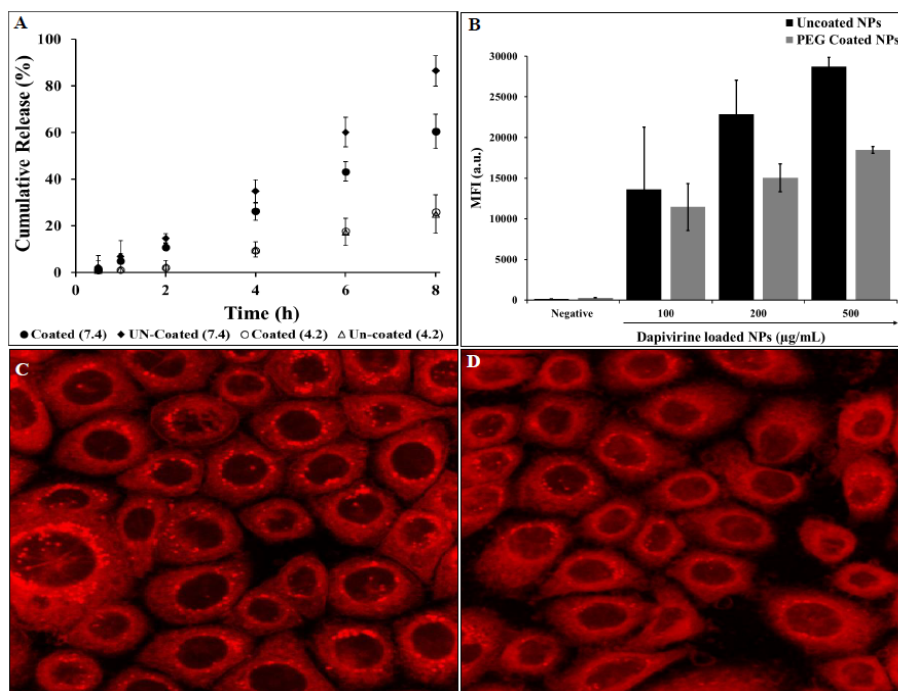
The similar studies were conducted for assessment of the cellular uptake profiles of loaded drugs using confocal microscopy. As shown in Figure 4C and 4D, the cellular uptake profile was closely correlated with the intracellular level of drugs [38,66]. Although, the same amount of Nile Red was loaded in both NPs (uncoated and PEG₂₀₀₀ coated NPs), there was a difference in florescent intensity, which may be attributed to PEG surface coating. The strong fluorescent signal in the intracellular compartment confirmed the cytoplasmic localization of Nile red loaded in NPs upon being uptaken by cells.

The uptake rate of NPs by VK2 E6/E7 cells was qualitatively assessed using confocal microscopy technique. The cells were incubated with Nile Red loaded NPs (both uncoated and PEG₂₀₀₀ coated nanoparticles) for 24 h. As shown in Figure 4C and 4D, there was a noticeable difference in the uptake rates between them, suggesting that PEG₂₀₀₀ coating significantly affected the uptake profile of NPs by VK2 E6/E7 cells.

Ex vivo mucus penetration rates of NPs

To elucidate the mechanism behind mucus penetration of loaded drugs released from NPs, ensemble-averaged geometric mean square displacements of NPs in porcine vagina tissues were defined as a function of time for such samples as the blank NPs, NPs uncoated but loaded with Nile Red, and PCN loaded with Nile Red [20,67]. As depicted in Figure 4A, the penetration rates reflected by differences in ensemble mean square displacements were examined using a quasi-experimental design [19,68].

The transport rates of PCN were significantly greater than those of uncoated NPs having an equivalent size (Figure 4B). The transport rates of PCN containing larger doses (i.e., 200 and 500 µg/ml) also displayed the same trend as that of 100 µg/ml. PEG surface coating not only facilitated mucus penetration rates for NPs but also enhanced



*(A) All were expressed as mean ± S.D. (n=3).

Figure 4: (A) *In vitro* release profile of Dapivirine from uncoated NPs and PEG₂₀₀₀ coated NPs. (B) *In vitro* cellular uptake profile of Nile red from uncoated NPs and PEG₂₀₀₀ coated NPs. Confocal microscopy images for the uptake of Nile Red from (C) uncoated NPs and (D) PEG₂₀₀₀ coated NPs.

the uniform distribution profiles within the mucus as compared with uncoated NPs having an equivalent size (Figure 5), as was previously demonstrated [69].

To assess the dynamic transport profiles and the mechanisms of NPs through vaginal mucus, fluorescent microscopy techniques, such as fluorescence recovery after photo-bleaching and high resolution multiple particle tracking, have been widely used [19,70]. Fluorescence recovery can provide ensemble-averaged diffusion rates but not quantified transport rates of NPs, whereas multiple particle tracking technique allows for quantification of NP fractions that are adherent to mucus in a time dependent manner. The results of the study with

multiple particles tracking technique demonstrated that PCN could rapidly penetrate human mucus and offer the potential usage as an efficient carrier for mucoadhesive drug delivery.

Conclusion

Surface modified pH responsive NPs based on ES-100 were developed for the vaginal delivery of Dapivirine against HIV-1 prophylaxis. PEG coated NPs (PCN) possessed physiochemical properties that help them overcome the mucosal barrier and readily penetrate through mucus layer. PCN did not exert any significant cytotoxicity. Dapivirine loaded NPs that are mimicking two basic

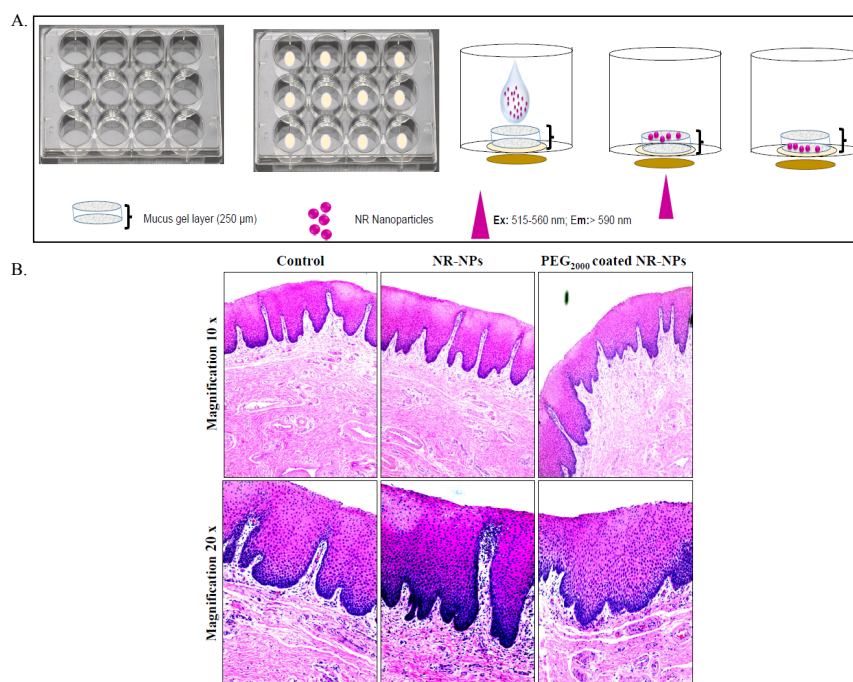


Figure 5: Schematic diagram for *ex vivo* mucus penetration studies and the fate of nanoparticles: (A) Schematic drawing of a diffusion chamber. Porcine vaginal mucus is sandwiched between donor and receptor solutions. (B) The fate of surface coated and uncoated NPs. NPs readily penetrate the luminal mucus layer and permeate through the underlying adherent mucus layer.

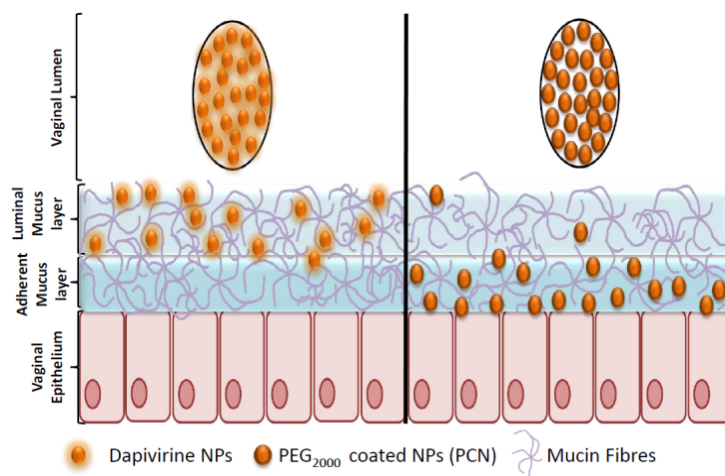


Figure 6: PEG coated nanoparticles mimicking two basic features of HIV viral particles (i.e. ability to stay unreactive/unresponsive at acidic pH and exerting its action only at neutral pH) along with enhanced mucus penetration capability may serve as an ideal platform for vaginal delivery of Dapivirine against HIV-1.

features of HIV-1 (i.e. capability to stay unreactive/unresponsive at acidic pH and exerting its action at neutral pH as shown in Figure 6) has achieved the enhanced mucus penetration rates. Thus, PCN can serve as an ideal platform for vaginal delivery of microbicides against HIV-1.

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Compliance with ethical standards

The authors declare that they have no conflict of interest.

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