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Thermosensitive Gel Containing Cellulose Acetate Phthalate-Efavirenz Combination Nanoparticles for Prevention of HIV-1 Infection

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Abstract

The objective of this investigation was to develop and evaluate a nano-microbicide containing a combination of cellulose acetate phthalate (HIV-1 entry inhibitor) and efavirenz (anti-HIV agent) for HIV prophylaxis. Cellulose acetate phthalate-efavirenz combination nanoparticles (CAP-EFV-NPs) were fabricated by the nanoprecipitation method and were characterized for particle size, zeta potential and encapsulation efficiency of efavirenz. CAP-EFV-NPs were incorporated into a thermosensitive gel (CAP-EFV-NP-Gel). CAP-EFV-NPs, CAP-EFV-NP-Gel and efavirenz solution were evaluated for cytotoxicity to HeLa cells and for *in vitro* short-term (1-day) and long-term (3-day) prophylaxis against HIV-1 infection in TZM-bl cells. CAP-EFV-NPs had size < 100 nm, negative surface charge and encapsulation efficiency of efavirenz was > 98%. CAP-EFV-NPs and CAP-EFV-NP-Gel were significantly less toxic (P < 0 01) to HeLa cells as compared to efavirenz solution. CAP-EFV-NPs showed significantly higher prophylactic activity (P < 0 01) against HIV-1 infection to TZM-bl cells as compared to efavirenz solution and blank CAP nanoparticles. CAP-EFV-NP-Gel can be a promising nano-microbicide for long-term HIV prophylaxis.

Keywords

Cellulose Acetate Phthalate; Nanoparticles; Efavirenz; HIV Prophylaxis; Pluronic; Thermosensitive Gel

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INTRODUCTION

HIV infection is a serious global issue. At the moment, more than 34 million people are infected with HIV worldwide and 2.5 million people were newly infected with HIV in 2011.[1] Prevention as well as treatment of HIV infection has become a world-wide priority in order to reach the international goal of reducing HIV-1 infection by 50% in 2015.[2] As sexual transmission is the most common mode of HIV infection, researchers have focused on developing modalities that can inhibit HIV-1 at the site of infection and prevent acquisition of HIV. Physical barriers such as condoms and male circumcision are currently used for HIV prophylaxis and have shown only moderate success.[3] Hence, 'use of topical (vaginal or rectal) microbicides' is considered to be an important strategy for HIV prophylaxis. Topical microbicides are able to inactivate HIV-1, block HIV-1 attachment, and/or disrupt intracellular HIV replication.[4,5]

Macromolecular HIV-1 entry inhibitors like cellulose sulfate, PRO-2000 and carrageenan sulfate were evaluated as vaginal microbicides but they failed to show any significant protective effect in clinical trials.[6] Hence, antiretroviral agents such as tenofovir are also being evaluated as a vaginal microbicide. In CAPRISA 004 trial, pre-and post-coital application of 1% tenofovir gel showed some degree of success in preventing acquisition of HIV infection.[7] However, coitus-independent, once daily application of 1% tenofovir gel did not show any efficacy in VOICE trial.[8] These results suggest that development of combination vaginal microbicides with coitus independent activity is of great importance. Recently, a combination of carrageenan, zinc acetate and MIV-150 was found to give one day protection from HIV infection in macaques.[9] At the moment, a vaginal ring containing a combination of dapivirine (a non-nucleoside reverse transcriptase inhibitor or NNRTI) and maraviroc (an entry inhibitor that blocks CCR5 receptor) is being evaluated in clinical trials.[10] Thus, combination of an entry inhibitor and a NNRTI may provide needed HIV prophylaxis.

Cellulose acetate phthalate (CAP) is a low-cost pharmaceutical excipient that is widely used for the enteric coating of pharmaceutical formulations.[11] CAP is pH sensitive polymer that dissolves at pH higher than 6.2.[12] Interestingly, CAP was found to be active against viruses such as HIV-1, HSV-1 and HSV-2 indicating its potential as a topical microbicide.[13] CAP acts as a HIV-1 entry inhibitor (for R4 and R5 tropic viruses) by binding to gp 120 and by formation of gp 41 six-helix bundles.[14,15] CAP can also result in virus disintegration and stripping of envelope glycoproteins causing loss of viral infectivity.[14–16] Due to pH sensitive nature, CAP can maintain its integrity in the acidic environment of vagina. CAP was found to be active against HIV-1 in soluble as well as insoluble form indicating that CAP can prevent HIV infection in vaginal lumen (cervicovaginal mucus) as well as vaginal mucosa.[16] Vaginal application of a gel containing micronized CAP (13% w/v) did not alter vaginal pH, vaginal microflora or integrity of vaginal epithelium in macaques.[17–19] CAP gel successfully prevented macaques from SHIV infection even after repeated viral exposure.[17–19] In a recent phase 1 clinical trial, CAP gel was found to cause unacceptable vulvo-vaginal side effects due to very high osmolarity of gel.[20] Thus, there is a need for an alternative formulation approach for CAP and continued evaluation of the prophylactic properties of CAP alone or in combination with other anti-retroviral drugs.

Nanotechnology has garnered considerable interest in the field of HIV prophylaxis. Various types of nanocarriers such as dendrimers, liposomes, polymeric nanoparticles and nanosuspensions are being evaluated for this purpose.[21,22] Currently, a gel containing dendrimers (VivaGel[®], Starpharma, Australia) is being evaluated in the clinical trials for prophylactic activity against HIV and other sexually transmitted diseases.[23,24] In view of this, formulating CAP as nanoparticles is a promising approach. Until today, there are no reports on fabrication of CAP nanoparticles for HIV prophylaxis. In the present investigation, we developed a vaginal nano-microbicide containing a combination of a HIV-1 entry inhibitor, CAP and a NNRTI, efavirenz. The objective of this investigation was to establish a proof-of-concept for use of CAP as a microbicide as well as a (nano)carrier for delivering efavirenz (EFV). The CAP nanoparticles and CAP-EFV nanoparticles (CAP-EFV-NPs) were evaluated for cytotoxicity and antiviral efficacy. Finally, CAP-EFV-NPs were incorporated into a thermosensitive gel to enable their effective vaginal delivery.

MATERIALS AND METHODS

Materials

Cellulose acetate phthalate (CAP), polysorbate 80 (Tween 80), polysorbate 20 (Tween 20), polyoxyethylene-40-stearate (Myrj 52) were purchased from Sigma Chemicals (St. Louis, MO). Efavirenz (EFV) was purchased from Sequoia Research Ltd. (Pangbourne, UK). Potassium dihydrogen phosphate (HPLC grade), acetonitrile (HPLC grade), dimethyl sulfoxide (DMSO, AR Grade), acetone (AR grade), citric acid (AR grade), trisodium citrate (AR grade) and Rhodamine 6G were purchased from Fischer Scientific Ltd (Pittsburg, PA, USA). Pluronic F127, Pluronic F68, Solutol HS 15 and Kolliphor TPGS (BASF Corp., Edison, NJ, USA) were received as gift samples. Ultrapure water was used for all the experiments.

Cell Culture

Human cervical (HeLa) cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA). TZM-bl cells were procured through NIH AIDS Research and Reference Reagent Program. HeLa and TZM-bl cells were maintained in Dulbecco's Modified Eagle Media (DMEM, MediaTech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Hyclone Inc., Utah), 4 mM L-glutamine, 100 U/ml penicillin and 100μg/ml streptomycin (MP Biomedical Inc., Solon, OH) and maintained in a logarithmic growth phase. All cells were grown at 37°C and 5% CO_2.

Preparation of Blank CAP Nanoparticles

Blank CAP nanoparticles were prepared using a nanoprecipitation method. Various stabilizers such as Tween 80, Tween 20, Myrj 52, Solutol HS 15, Kolliphor TPGS, Pluronic F68 and Pluronic F127 were evaluated for the fabrication of blank CAP nanoparticles. Briefly, CAP (50 mg) was dissolved in 10 ml of acetone (organic phase) by heating at 40°C in an incubating shaker bath. A selected stabilizer (200 mg) was dissolved in 10 ml of ultra-pure water (aqueous phase) by heating at 40°C in an incubating shaker bath. Aqueous phase (10 ml) was transferred to a 50 ml beaker and the beaker was kept on a magnetic stirrer. The aqueous phase was stirred at 700 rpm, the organic phase was added drop-wise under stirring

to an aqueous phase (10 ml) and stirring was continued for 3 h to remove acetone. The particle size of the resulting CAP nanoparticle dispersion was measured. All experiments were carried out in triplicate.

In Vitro Cytotoxicity of CAP Nanoparticles

In vitro cytotoxicity of CAP nanoparticles was evaluated in HeLa cells. Briefly, cells were seeded in 12 well plates at a density of 1×10^6 cells/well in triplicate and allowed to attach to wells overnight. CAP nanoparticles were added to obtain a CAP concentration of $500 \mu g/ml$, $250 \mu g/ml$, $100 \mu g/ml$, $50 \mu g/ml$, and $5 \mu g/ml$ in each well. Cell viability was assessed after 24 and 48 h as per MTT method described in our earlier paper.[25] The absorbance obtained for CAP nanoparticles was compared to control cells (no treatment) to calculate% cell viability.

Preparation of EFV Loaded CAP Nanoparticles

Briefly, CAP (50 mg), EFV (5 mg) were dissolved in 10 ml of acetone (organic phase) by heating at 40°C in an incubating shaker bath. Pluronic F127 (200 mg) was dissolved in 10 ml of ultra-pure water (aqueous phase) by heating at 40°C in an incubating shaker bath. The rest of the procedure was same as described for the blank CAP nanoparticles. The resulting CAP nanoparticle dispersion containing EFV (CAP-EFV-NPs) was used for further studies. Particle size, polydispersity index, and surface charge of resulting CAP-EFV-NPs were measured using dynamic light scattering (ZetaPlus instrument, Brookhaven Instruments Corp, NY, USA) as previously described.[26] All experiments were carried out in triplicate. CAP-EFV-NPs were filtered through a $0.22 \mu m$ filter and used for further studies. All the experiments were carried out in triplicate.

For fabrication of fluorescent CAP nanoparticles, EFV was replaced with Rhodamine 6G (1 mg) and nanoparticles were prepared as described above.

Entrapment Efficiency

CAP-EFV NPs (0.4 ml) were transferred to a centrifugal filter (Amicon Ultra, Sigma–Aldrich, MO, USA). The nanoparticle dispersion was centrifuged at 14000 rpm (Eppendorf centrifuge 5417R) and at 4°C for 20 min to obtain a filtrate.

The entrapment efficiency was calculated by the following equation:

$$\%EE = [(M_{initial} - M_{free})/M_{initial}] \times 100$$

where 'M$_{initial}$' is the amount of EFV/ml of nanoparticle dispersion and M$_{free}$ is amount of EFV/ml of filtrate obtained by centrifugation of nanoparticles. All experiments were performed in triplicate. The amount of the EFV in the nanoparticles and filtrate was analyzed by using a reverse phase-HPLC method described in our earlier paper.[26]

High-Pressure Liquid Chromatography (HPLC)

The amount of the EFV in the nanoparticles and filtrate was analyzed by using a reverse phase-HPLC method described in our earlier paper.[26] The HPLC apparatus consisted of a

pump (LC-10ATvp), system controller (SIL-10ADvp), degasser unit (DGU-14A), refrigerated auto-sampler (SIL-10ADvp), a UV-Vis detector (SPD-10ADvp) and a column heater (Shimadzu Corporation, Columbia, MD). Samples were run through a C_{18} pre-column and a Gemini C_{18} reverse-phase [150 × 4 5 mm (I.D.)] with 5 μm particle size packing (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile and 25 mM KH_2PO_4 solution (55:45). For HPLC analysis, the flow rate of the mobile phase was at 0.9 ml/min, column oven was set at 35°C, injection volume was 20 μl and detector was set at 212 nm. The retention time for EFV was 10.4 min. For standard curve, EFV stock solution (1 mg/ml) was prepared in acetonitrile. The stock solutions were diluted with acetonitrile to obtain solutions of various concentrations. Standard curve was obtained by injecting 0.1–10 μg/ml of EFV. Limit of detection for EFV was 50 ng/ml. All the experiments were performed in triplicate. The inter-day and intra-day variability for the standard curve was always < 10%.

Imaging of CAP-EFV Nanoparticles by SEM

About 30 μl of CAP-EFV NPs were placed onto a conductive tape (PELCO Tabs, 12 mm OD, TED PELLA, Inc, Redding, CA) mounted on a aluminum stub. The NP samples were air-dried for > 24 h and sputter-coated with palladium under an argon gas atmosphere using a Denton Desk V HP TSC Sputter Coater (Denton Vacuum, LLC-USA, Moorestown, NJ). The coated the NPs were examined using a Hitachi S4700 field-emission Scanning Electron Microscope (SEM).

Development of a Thermosensitive Vaginal Gel Containing CAP-EFV-NPs

A thermosensitive vaginal gel containing CAP-EFV-NPs was developed as per our earlier publication with suitable modifications.[26] Briefly, CAP-EFV-NPs were prepared as described earlier and the pH was adjusted to 4.5 using a mixture of citric acid and sodium citrate. Glycerol (0.225 gm) was added to CAP-EFV-NPs (10 ml) to adjust the osmolarity of nanoparticles. CAP-EFV-NPs were transferred to a screw-capped bottle and Pluronic F127 (2 gm) and Pluronic F68 (100 mg) were added to CAP-EFV-NPs with intermittent stirring. The screw-capped bottle containing CAP-EFV-NPs and Pluronics was stored overnight in the refrigerator to dissolve Pluronics. On the next day, the dispersions were gently stirred to obtain a homogenous translucent solution. The solution was observed for signs of nanoparticle aggregation and/or phase separation. Thermosensitivity of the gel was confirmed by incubating the gel in the water bath maintained at 37°C.

Characterization of Transfer of Fluorescent CAP Nanoparticles from Thermosensitive Gel Using Transwell® Permeable Supports

HeLa cells were plated at 0 5 × 10^6 cells/ml on Poly-D Lysine pre-coated 12 mm BD BioCoat coverslips (BD Bio-sciences, San Jose, CA). Cells were cultured overnight in DMEM plus 10% fetal calf serum at 37°C, 5% CO_2. Thermosensitive gel (100 μl) containing Rhodamine 6G labeled fluorescent CAP nanoparticles was placed on 0.2 μm Transwell® permeable supports (Corning Inc. Life Sci., MA, USA) at 37°C for 10 min to allow solidification. Transwells® were placed above HeLa cells for 30 min, 2 h, 24 h, 48 h, 96 h. At each time point, Transwells® were removed and cells were fixed by adding 100 μl of 37% formaldehyde in culture media for 15 min at 37°C. Fixed cells were rinsed in 1 ×

PBS and incubated with 300 ng/ml of DAPI for 15 min in $1 \times$ PBS. Cells were rinsed three times in $1 \times$ PBS, mounted in Permount (Fisher) and viewed with 40X/63X objective on a Leica DMIL inverted fluorescent microscope. The images were captured without software enhancement. The schematic representation of this experiment is given in Figure 1.

In Vitro Cytotoxicity of EFV Solution, CAP-EFV-NPs and CAP-EFV-NP-Gel

In vitro cytotoxicity of EFV solution, CAP-EFV-NPs and thermosensitive gel containing CAP-EFV-NPs (CAP-EFV-NP-Gel) was evaluated to establish ability of CAP nanoparticles to reduce cytotoxicity of efavirenz. Briefly, HeLa cells were seeded in 12 well plates at a density of 1×10^5 cells/well in triplicate and allowed to attach to wells overnight. EFV solution, CAP-EFV-NPs and CAP-EFV-NP-Gel were added to obtain EFV concentration of 5 μg/ml in each well. Cell viability was assessed after 24, 48 and 96 h as per MTT method described in our earlier papers.[25,26] HeLa cells grown in culture without any treatment served as control cells. The absorbance obtained for different treatment groups was compared to control cells (no treatment) to calculate% cell viability.

Short-Term *In Vitro* Prophylaxis of CAP Nanoparticles, EFV Solution and CAP-EFV-NPs

Short-term (1 day pre-treatment) *in vitro* prophylaxis of CAP nanoparticles, EFV solution and CAP-EFV-NPs against HIV-1$_{NL4-3}$ was determined using TZM-bl HIV indicator cells as reported in our earlier publication.[26] Briefly, TZM-bl cells were seeded in 96-well plates at a density of 1×10^4 cells per well. After 24 h, the cells were treated with different concentrations of CAP nanoparticles, EFV solution and CAP-EFV-NPs (concentration range: 50 μg/ml to 5 pg/ml). After 24 h, media from all the wells was removed to get rid of treatment and replaced with fresh media. On the following day, the cells were inoculated with HIV-1$_{NL4-3}$ virus (25 μl) for 4 h. The cells were washed after the 4 h and incubated for 48 h. Bright-GLO (Promega, Madison, WI) assay was used to measure the luminescence obtained with different treatments. TZM-bl cells with no treatment and HIV infection and HIV infected TZM-bl cells with no treatment were used as controls. The schematic representation of this experiment is given in Figure 1. The % antiviral activity was calculated using following formula

$$\% \text{ antiviral activity} = [(L_{untreated} - L_{treated})/L_{untreated}] \times 100$$

where $L_{untreated}$ is the luminescence of HIV infected TZM-bl cells with no treatment and $L_{treated}$ is the luminescence of HIV infected TZM-bl cells treated with CAP nanoparticles, EFV solution or CAP-EFV-NPs.

Short-Term *In Vitro* Prophylaxis of CAP Nanoparticles in Gel (CAP-NP-Gel) and CAP-EFV-NP-Gel

Short-term *in vitro* experiment was repeated to study influence of thermosensitive gel and its components on the antiviral activity of CAP-EFV-NPs. The experimental design was similar to the earlier experiment. In this experiment, CAP nanoparticles incorporated into a gel and CAP-EFV-NP-Gel were used instead of CAP nanoparticles and CAP-EFV-NPs. Blank Pluronic gel was used as a control.

Long-Term *In Vitro* Prophylaxis Using CAP Nanoparticles, EFV Solution and CAP-EFV-NPs

Long-term (3 days) *in vitro* prophylactic activity of CAP nanoparticles, EFV solution and CAP-EFV-NPs was evaluated using TZM-bl assay described earlier. Briefly, TZM-bl cells were seeded in 96-well plates at a density of 1×10^4 cells per well. After 24 h, the cells were treated with EFV solution and CAP-EFV-NPs (2 different batches) equivalent to 50 ng/ml and 5 ng/ml of EFV. After 24 h, media from all the wells was removed to get rid of treatment and replaced with fresh media. After 3 days, the cells were inoculated with HIV-1_{NL4-3} virus (25 μl) for 4 h. The rest of the procedure is similar to the earlier experiment. The schematic representation of this experiment is given in Figure 1.

Immunohistochemistry

TZM-bl cells were pre-treated with CAP-NP and CAP-EFV-NPs for 24 h and incubated with HIV-1_{NL4-3} for 4 h prior to washing and culturing for 48 h. For all nanoparticles, CAP was at concentration of 0.5, 5.0 and 50 μg/ml and EFV concentrations were 0.05, 0.5 and 5.0 μg/ml. HeLa cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature (RT) then washed with warm PBS. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes, washed, and blocked for 1 hour at room temperature in PBS, 0.2% BSA, and 0.2% Triton X-100. Cells were incubated with primary antibody directed against β-galactosidase (Thermo Fisher, 1:1000) overnight at 4°C in PBS, 0.2% BSA, and 0.2% Triton X-100. Cells were washed in PBS and secondary antibodies were applied at a concentration of 1:500 for 1 h at room temperature for goat anti-rabbit IgG (H+L) rhodamine conjugate. Qualitative and quantitative analysis of immunocytochemistry was performed by acquiring images with a Leica DMI4000B inverted microscope with a cooled CCD camera (Q Imaging) and fluorescent capabilities. Images were analyzed with ImageProPlus software (MediaCybernetics). For image data, 3 field views of at least 100 cells from 3 separate experiments were analyzed for each condition.

Statistical Analysis

Results are reported as mean + SEM for all experiments. Statistical significance was evaluated using Student's *t*-test or analysis of variance (ANOVA, GraphPad Prism). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Preparation of Blank CAP Nanoparticles

For preparation of CAP nanoparticles, various biocompatible non-ionic surfactants such as Tween 80, Tween 20, Solutol HS 15, Myrj 52, Vitamin E TPGS, Pluronic F68 and Pluronic F127 were evaluated. These surfactants have different chemical structures and HLB values. Particle size and polydispersity index of CAP nanoparticles stabilized by various surfactants are shown in Figure 2. All surfactants were able to form CAP nanoparticles with size less than 150 nm. Pluronic F127 showed lowest particle size and polydispersity index amongst all the surfactants. Moreover, CAP nanoparticles stabilized with Tween 80, Tween 20, Solutol HS 15, Myrj 52 and Vitamin E TPGS showed signs of colloidal instability after 24 h

whereas Pluronic stabilized CAP nanoparticles exhibited good colloidal stability. Pluronic F127 stabilized CAP nanoparticles were selected for further studies.

In Vitro Cytotoxicity of CAP Nanoparticles

Cytotoxicity of CAP nanoparticles to HeLa cells was evaluated using MTT method. CAP nanoparticles did not show signs of cytotoxicity to HeLa cells even at a concentration equivalent to 500 μg/ml of CAP after 48 h (Fig. 3). These observations are in accordance with the *in vitro* cytotoxicity data reported for CAP.[27]

Preparation and Characterization of EFV Loaded CAP Nanoparticles (CAP-EFV-NPs)

EFV could be successfully incorporated into CAP nanoparticles. No drug precipitation or nanoparticle aggregation was observed after incorporation of EFV into CAP nanoparticles after 24 h. The particle size, polydispersity index and zeta potential of CAP-EFV-NPs are listed in Table I. The encapsulation efficiency of EFV in CAP nanoparticles was greater than 98% (Table I). SEM image showed that CAP-EFV-NPs were spherical and less than 100 nm (Fig. 4).

Development of a Thermosensitive Vaginal Gel Containing CAP-EFV-NPs (CAP-EFV-NP-Gel)

A thermosensitive vaginal gel containing CAP-EFV-NPs was developed using a mixture of Pluronic F127 (20% w/v) and Pluronic F68 (1% w/v). Incorporation of CAP-EFV-NPs into a thermosensitive gel did not show any visible signs of aggregation of CAP-EFV-NPs after 48 h at room temperature.

Characterization of Transfer of Fluorescent CAP Nanoparticles from Thermosensitive Gel Using Transwell® Permeable Supports

We wanted to have an estimate of duration required by the thermosensitive gel to enable release (or translocation) of CAP nanoparticles. Hence, thermosensitive gel containing fluorescent CAP nanoparticles was placed on 0.2 μm Transwell® permeable supports and allowed to solidify at 37°C. We hypothesized that CAP nanoparticles released from the gel would translocate through the Transwell® membrane and be taken up by HeLa cells cultured below the permeable support. The results of these experiments are shown in Figure 5. Fluorescent nanoparticles were able to transfer through Transwell® permeable supports and were taken up by HeLa cells within 30 min. Rhodamine fluorescence intensity in the cells grew stronger until 24 h and slowly diminished over a sustained time frame. Rhodamine fluorescence could be observed in HeLa cells even after 4 days (Fig. 5).

In Vitro Cytotoxicity of EFV Solution, CAP-EFV-NPs and CAP-EFV-NP-Gel

In vitro cytotoxicity of EFV solution, CAP-EFV-NPs and CAP-EFV-NP-Gel to HeLa was evaluated to check whether nanoparticles can reduce the cytotoxicity of efavirenz. CAP-EFV-NPs and CAP-EFV-NP-Gel showed significantly higher cell viability ($P < 0.01$) as compared to EFV solution at 48 h and 96 h (Fig. 6). This clearly indicated the advantage of CAP-EFV-NPs and CAP-EFV-NPs in thermosensitive gel.

Short-Term *In Vitro* Prophylaxis of CAP Nanoparticles, EFV Solution and CAP-EFV-NPs

To evaluate whether combination of CAP and EFV in the form of nanoparticles provide enhanced prophylactic activity, short-term *in vitro* prophylaxis experiments were carried out as per our earlier report.[26] CAP-EFV-NPs showed significantly higher anti-HIV activity ($P <$ 0.01) as compared to EFV solution at concentrations 50 ng/ml by significantly reducing HIV infection in pretreated TZM-bl indicator cells (Fig. 6). Interestingly, CAP-EFV-NPs showed more than 50% antiviral activity even at a concentration equivalent to 500 pg/ml of EFV whereas EFV solution did not show any inhibition at this concentration. CAP nanoparticles showed antiviral activity at concentration of 50 μg/ml confirming previous reports demonstrating anti-HIV activity of CAP.[27] However, CAP nanoparticles showed negligible antiviral activity at concentrations 5 μg/ml (Fig. 7). CAP-EFV-NPs showed significantly higher anti-HIV activity ($P < 0.01$) as compared to CAP nanoparticles at all concentrations 5 μg/ml. This study clearly demonstrated the advantage of CAP-EFV-NPs over CAP nanoparticles as well as EFV solution for prophylaxis.

Short-Term *In Vitro* Prophylaxis of CAP-NP-Gel and CAP-EFV-NP-Gel

Short-term *in vitro* prophylaxis experiment was also performed using CAP nanoparticles in gel and CAP-EFV-NP-Gel. Blank Pluronic gel served as a control. CAP-EFV-NP-Gel showed significantly higher anti-HIV activity at all concentrations starting from 5 μg/ml (Fig. 8). Blank gel showed negligible anti-HIV activity at all concentrations. CAP-EFV-NP-Gel showed similar anti-HIV activity profile as that of CAP-EFV-NP. This indicated that components of gel did not have any positive or negative effect on anti-HIV activity of CAP-EFV-NPs.

Long-Term *In Vitro* Prophylaxis Using CAP Nanoparticles, EFV Solution and CAP-EFV-NPs

Long-term *in vitro* HIV prophylaxis experiments were performed to ascertain whether combination of CAP and EFV in the form of nanoparticles can prevent HIV infection for 3 days after 1 day pre-treatment of TZM-bl cells with CAP nanoparticles, CAP-EFV-NPs and EFV solution. CAP-EFV-NPs and EFV solution equivalent to 50 ng/ml and 5 ng/ml of EFV were selected for this study. At a concentration equivalent to 50 ng/ml of EFV, CAP-EFV-NPs as well as EFV solution did not show any significant difference in anti-HIV activity (Fig. 9). However, CAP-EFV-NPs showed significantly higher antiviral activity ($P < 0.01$) as compared to EFV solution at a concentration equivalent to 5 ng/ml of EFV (Fig. 9). CAP nanoparticles were used as a control to account for anti-HIV activity of CAP in CAP-EFV-NPs. CAP nanoparticles showed significantly lower anti-HIV activity as compared to CAP-EFV-NPs.

Immunohistochemistry

Immunohistochemistry was performed to validate results of *in vitro* prophylaxis experiments using TZM-bl cells. TZM-bl cells contain a β-galactosidase promoter which is activated upon HIV infection. We evaluated β-galactosidase expression in HIV infected TZM-bl cells with no treatment, TZM-bl cells treated with CAP nanoparticles and TZM-bl cells treated with CAP-EFV-NPs by using antibody against β-galactosidase. HIV infected TZM-bl cells with no treatment showed considerable β-galactosidase expression as indicated by the

presence of red fluorescence in the cells (Fig. 10). TZM-bl cells treated with CAP nanoparticles showed negligible red fluorescence indicating little β-galactosidase expression at a concentration of 50 μg/ml. β-galactosidase immunofluorescence was higher at lower concentrations of CAP nanoparticles (5 μg/ml and 0.5 μg/ml). Cells incubated with CAP-EFV-NPs did not express β-galactosidase at all concentrations tested and are unlikely to be infected with HIV as indicated by undetectable levels of β-galactosidase immunofluorescence. This study validated results of *in vitro* prophylaxis experiments. We also counted at least 100 cells from 3 different locations to determine number of β-galactosidase+ cells and calculated% infection in untreated, CAP NP treated and CAP-EFV-NP treated cells (Table II).

DISCUSSION

Development of various modalities for prevention of HIV has received a great impetus in recent years. As sexual transmission is the most common mode of HIV infection, strategies to prevent/inhibit HIV at its route of entry (vaginal or rectal) are considered to be important for HIV prophylaxis. In view of this, vaginal or rectal delivery of natural or semi-synthetic polymers and antiretroviral agents has been attempted for HIV prophylaxis.[5,21] Although many natural or semi-synthetic polymers can act as HIV-1 entry inhibitors, they were not successful in clinical trials.[6] Moreover, vaginal delivery of antiretroviral agents like tenofovir has also shown a moderate success in HIV prevention.[7] Hence, novel approaches are required for improving HIV prophylaxis. Nanotechnology has the potential to bring a paradigm shift in the field of HIV prophylaxis and a nanotechnology-based product is already being evaluated in clinical trials for HIV prophylaxis.

In the present investigation, we aimed at developing a nano-microbicide containing a combination of HIV-1 entry inhibitor (cellulose acetate phthalate; CAP) and NNRTI (efavirenz) for establishing the proof-of-concept. CAP is a FDA approved semi-synthetic cellulose.[11] Due to its pH sensitive nature, CAP is commonly used for enteric coating of tablets to prevent drug release in acidic environment of stomach. Interestingly, CAP also acts as a HIV-1 entry inhibitor in soluble as well as insoluble form.[16] Safety of CAP for vaginal delivery and its efficacy in preventing HIV infection (in macaques) has been proven.[17–19] Additionally, low-cost and ease of availability of CAP are also important criteria for its application as a modality for HIV prevention. Efavirenz is a FDA-approved potent NNRTI with longer half-life and maximum stability at vaginal pH.[28,29] Our earlier studies indicate that nano-encapsulated efavirenz can reside in cells for as long as 14 days which indicated that efavirenz may have potential for long-term HIV prophylaxis.[26] In this investigation, we used CAP nanoparticles as a modality for HIV prophylaxis as well as a modality to deliver efavirenz. Until today, there are very few reports on the development of CAP nanoparticles. [30,31] These investigations employ polyvinyl alcohol as a stabilizer for CAP nanoparticles. However, it has been shown that polyvinyl alcohol stabilized nanoparticles are trapped in the cervicovaginal mucus and will not reach vaginal epithelium (which are one of the main target cells for HIV prophylaxis).[32] Hence, we screened various FDA approved non-ionic surfactants for fabrication of CAP nanoparticles. We employed simple, low energy and scalable nanoprecipitation method for fabrication of CAP nanoparticles. Various nonionic surfactants such as Tween 80, Tween 20, Solutol HS 15, Myrj 52, Vitamin E TPGS (or

Kolliphor TPGS), Pluronic F68 and Pluronic F127 were screened for fabrication of CAP nanoparticles with good colloidal stability. Interestingly, CAP nanoparticle stabilized with Pluronic F127 showed lowest particle size and good colloidal stability (Fig. 2). It was also observed that concentration of Pluronic F127 and ratio of organic solvent to aqueous phase had considerable effect on the size and colloidal stability of the nanoparticles (data not shown). CAP nanoparticles stabilized with 2% Pluronic F127 had size less than 100 nm and good colloidal stability (Table I). It has been demonstrated that Pluronic F127 stabilized nanoparticles can rapidly penetrate through cervicovaginal mucus and can reach vaginal epithelium.[32,33] Hence, Pluronic F127 stabilized CAP nanoparticles were selected for the further studies. Although *in vitro* and *in vivo* safety of CAP has been demonstrated, we wanted to assess effect of nano-scale CAP on cell viability. CAP nanoparticles did not show any signs of cytotoxicity in HeLa cells at a concentration of 500 μg/ml after 48 h indicating a good safety profile (Fig. 4). Efavirenz could be incorporated into CAP nanoparticles with high encapsulation efficiency (encapsulation efficiency: > 98% and drug loading: ~10% w/w of CAP). Encapsulation of efavirenz had negligible effect on the size of CAP nanoparticles. CAP-EFV nanoparticles were incorporated into the thermosensitive gel reported in our earlier publication.[26] Incorporation of CAP-EFV nanoparticles did not have any effect on the colloidal stability of nanoparticles and thermogelation temperature of gel. Although we have shown in our earlier publication that thermosensitive gel allows rapid translocation (< 30 min) of PLGA nanoparticles[26], we wanted to ensure that CAP nanoparticles can also rapidly translocate through the thermosensitive gel. Furthermore, we also wanted to monitor residence time of the CAP nanoparticles in the HeLa cells after translocation through transwell membrane. We did not carry out this study in our earlier investigation. We hypothesized that if the fluorescently labeled CAP nanoparticles can remain in the HeLa cells for longer duration, then it might be possible to achieve long-term prophylaxis. Interestingly, CAP nanoparticles showed fluorescence in HeLa cells up to 4 days (Fig. 5).

The ability of CAP nanoparticles to improve selectivity index of efavirenz was evaluated by monitoring cytotoxicity of efavirenz, CAP-EFV NPs and CAP-EFV-NP gel to HeLa cells over a period of 4 days. Based on our earlier publication,[26] we selected EFV concentration of 5 μg/ml for cytotoxicity studies. We also used 2 different batches of CAP-EFV-NPs and CAP-EFV-NP gel in order to evaluate whether there is batch-to-batch variation in results. Interestingly, both batches of CAP-EFV-NPs and CAP-EFV-NP gel were significantly less toxic to HeLa cells ($P < 0.01$) than EFV solution after 48 and 96 h of treatment (Fig. 6) and there was negligible batch to batch variation. Encapsulation of EFV into CAP nanoparticles may reduce its interaction with cell organelles such as mitochondria which can ultimately lead to reduced cytotoxicity. Thus, CAP nanoparticles can improve selectivity index of efavirenz. These results also established that the components of thermosensitive gel did not have any cytotoxic effect on the cells. The antiviral efficacy of various concentrations of CAP nanoparticles, CAP-EFV-NPs and EFV solution (concentration range: 50 μg/ml to 5 pg/ml) was determined using a modified protocol that mimics prophylaxis. We also wanted to evaluate whether delivering a combination of CAP and EFV in the form of nanoparticles has any advantage over individual treatments. It should be noted that EFV solution and CAP-EFV-NPs were not studied at a concentration of 50 μg/ml because of the cytotoxicity issues. In order to establish that concentrations < 50 μg/ml are non-toxic to cells, we

evaluated cytotoxicity of CAP-EFV-NPs from concentration of 5 μg/ml to 5 pg/ml using CellTiterGLO^{TM} assay. We observed that the selected concentrations are non-toxic to cells. Hence, the reduction in the luminescence (corresponding to % antiviral activity) was not due to the cytotoxicity. It is evident that CAP nanoparticles showed considerable antiviral efficacy only at the concentration of 50 μg/ml (Fig. 7) and at lower concentrations the antiviral activity of CAP nanoparticles was negligible. EFV solution showed considerable antiviral activity (more than 50%) up to concentration of 50 ng/ml whereas CAP-EFV-NPs showed more than 50% antiviral activity even at the concentration of 500 pg/ml (Fig. 7). It is noteworthy that CAP-EFV-NPs showed significantly higher antiviral activity ($P < 0.01$) as compared to EFV solution at all the concentrations after 50 ng/ml. This clearly indicated the advantage of delivering a combination of CAP and EFV as nanoparticles. The dramatically higher antiviral activity of CAP-EFV-NPs as compared to CAP nanoparticles and EFV solution alone also indicated the possibility of synergistic interaction between CAP and EFV. Further, we evaluated concentration dependent antiviral activity of CAP nanoparticles and CAP-EFV-NPs after their incorporation into the thermosensitive gel. We also evaluated thermosensitive gel alone for antiviral activity. Interestingly, antiviral activity of CAP nanoparticles and CAP-EFVNPs did not change even after incorporation into thermosensitive gel (Fig. 8). Moreover, blank gel did not show any antiviral activity (Fig. 8). Thus, thermosensitive gel had neither positive nor negative effect on the antiviral activity of CAP-EFV-NPs.

In order to establish the potential of CAP-EFV-NPs for the long-term HIV prophylaxis, we further modified the antiviral efficacy protocol. For this purpose, we pre-treated TZM-bl cells for 1 day with EFV solution, CAP-EFV-NPs (equivalent to 50 ng/ml and 5 ng/ml of EFV) or CAP nanoparticles (at CAP concentrations equivalent to that present in CAP-EFV-NPs) and all the samples were infected with HIV-1 after 3 days of EFV solution, CAP-EFV-NPs and CAP nanoparticles pre-treatment of TZM-bl cells. We also evaluated two different batches of all the samples to confirm batch-to-batch reproducibility of the results. Interestingly, CAP-EFV-NPs showed significantly higher ($P < 0.01$) antiviral activity only at a concentration equivalent to 5 ng/ml of EFV (Fig. 9). The lack of difference in antiviral activity between EFV solution and CAP-EFV-NPs at a concentration of 50 ng/ml indicates that EFV (even as a free drug) can reside inside HeLa cells for a longer duration. It is known that HeLa cells do not express CYP450 enzymes required for efavirenz metabolism.[34] As TZM-bl cells are engineered from HeLa cells, they are unlikely to have these CYP450 enzymes. This could be the reason for longer residence of efavirenz in the TZM-bl cells. It is reported that the EC$_{90}$ value of EFV (free drug) is around 0.1–1.6 ng/ml.[35,36] If EFV (as a free drug) can reside in the TZM-bl cells for a longer duration then pre-treatment of cells with EFV solution with a concentration of 50 ng/ml (almost 50-fold higher than EC$_{90}$ may still maintain a concentration greater than EC$_{90}$ value of efavirenz in the cells even after 3 days. In such a situation, EFV solution and CAP-EFV-NPs will show similar antiviral activity. Due to limitations of our HPLC assay, we could not determine whether pre-treatment of TZM-bl cells with 50 ng/ml of EFV solution yield measurable intracellular concentrations of EFV for 3 days. Nevertheless, pre-treatment of cells with a 10-fold lower concentration of EFV showed superiority of CAP-EFV-NPs. Finally, we also validated the functionality of *in vitro* HIV prophylaxis experiments by immunohistochemistry studies. β-

galactosidase expression assays demonstrated that 24 hour pre-treatment with CAP-EFV-NPs completely inhibits HIV-1 infection of TZM-bl indicator cells for 96 hours following exposure to HIV-1 (Fig. 10). Quantitative evaluation was done by measuring β-galactosidase + cells to determine level of HIV infection in the different treatments. It was observed that none of the cells treated with CAP-EFV-NPs showed HIV infection indicating a good correlation was observed between qualitative and quantitative analysis.

Recently, Huang et al., have reported potential of CAP nanofibers either alone or in combination with for antiretroviral drugs (TMC 125 and tenofovir) for HIV prophylaxis by *in vitro* studies.[37] Although the results were promising, Huang et al., performed short term (1 h) HIV neutralization studies which involved incubation of CAP nanofibers with or without tenofovir with HIV-1. Huang et al., noted that CAP nanofibers have synergistic effect with antiretroviral drugs (which corroborate our results with CAP-EFV-NPs). Our investigation is quite different from Huang et al., as we focused on a different nano-architecture (nanoparticles instead of nanofibers) and different delivery vehicle (thermosensitive gel). Moreover, we focused on short-term and long-term pre-treatment of cells with CAP nanoparticles and CAP-EFV-NPs followed by HIV-1 infection as it mimics clinical situation and also gives some hint about long-term HIV prophylaxis.

CONCLUSION

Cellulose acetate phthalate nanoparticles can be used as a modality for HIV prevention and for delivering antiretroviral drugs such as efavirenz. Cellulose acetate phthalate-efavirenz combination nanoparticles showed significantly higher antiviral activity and significantly less cytotoxicity as compared to efavirenz alone. *In vitro* studies suggest that CAP-EFV-NPs can have potential for long-term HIV prophylaxis and thermosensitive gel containing CAP-EFV-NPs could be a clinically viable nano-scale modality for HIV prophylaxis. Further studies will be focused on establishing *in vivo* proof of long-term HIV prophylaxis using humanized BLT mouse model for HIV-1 infection.

Acknowledgments

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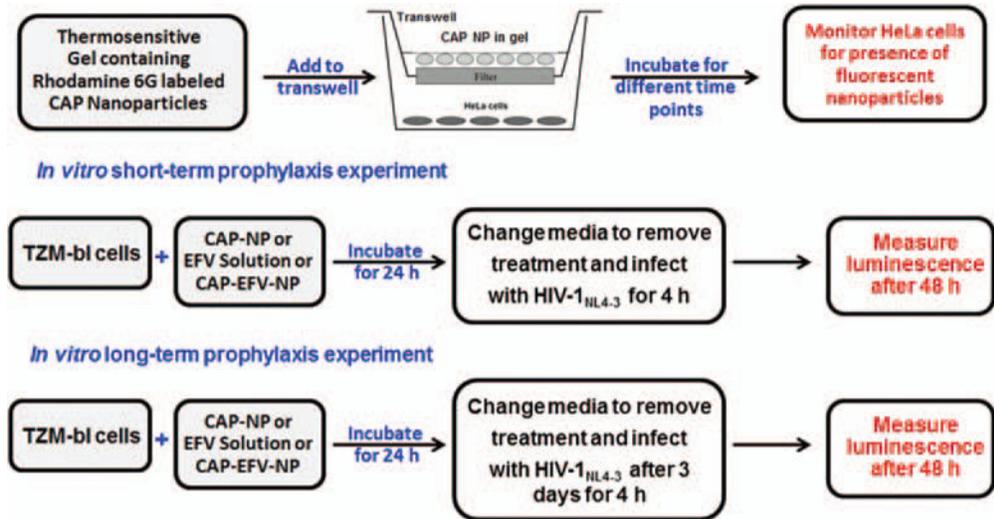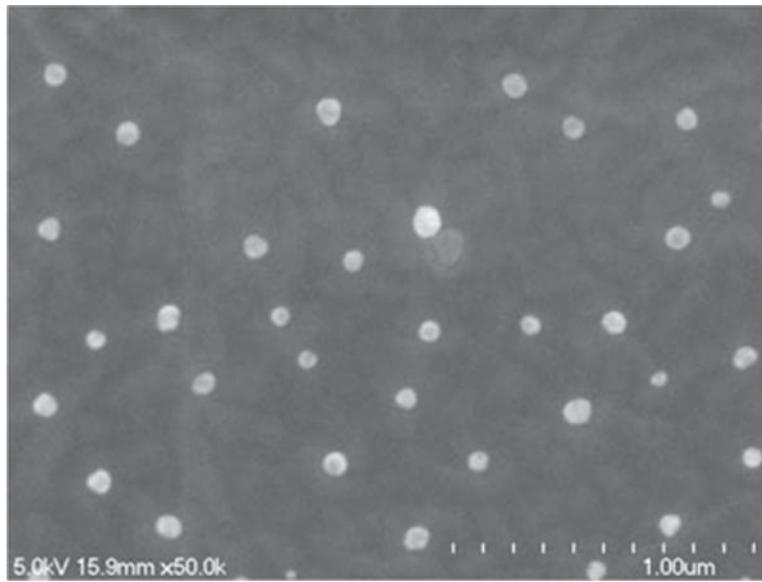
Figure 1.
Schematic representation of thranslocation through transwell permeable support experiment and *in vitro* short-term and long-term prophylaxis experiments.
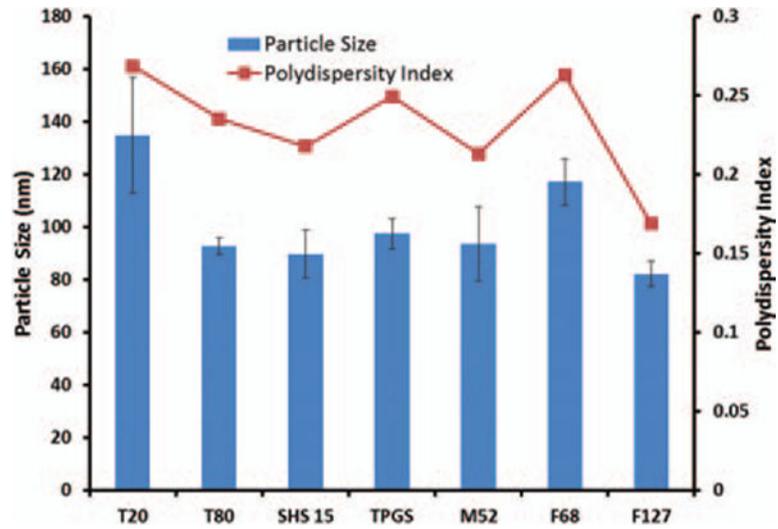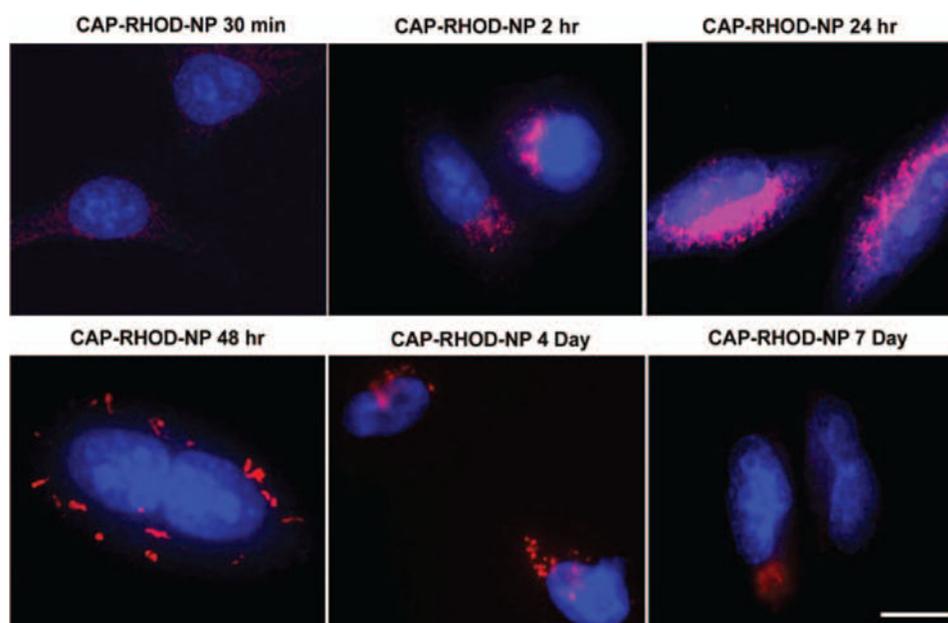
Figure 2.
Effect of various nonionic surfactants on the particle size of CAP nanoparticles ($n = 3$); T20: Tween 20, T80: Tween 80, SHS 15: Solutol HS 15, TPGS: Vitamin E TPGS, M52: Myrj 52, F68: Pluronic F68, F127: Pluronic F127.
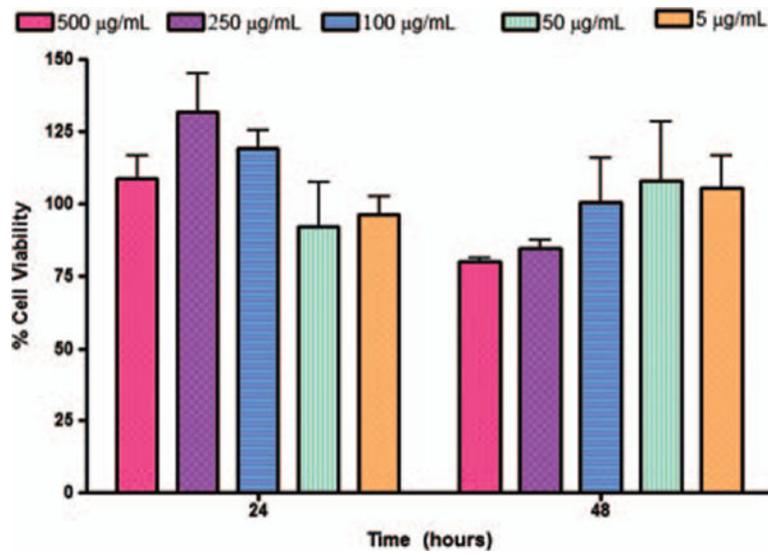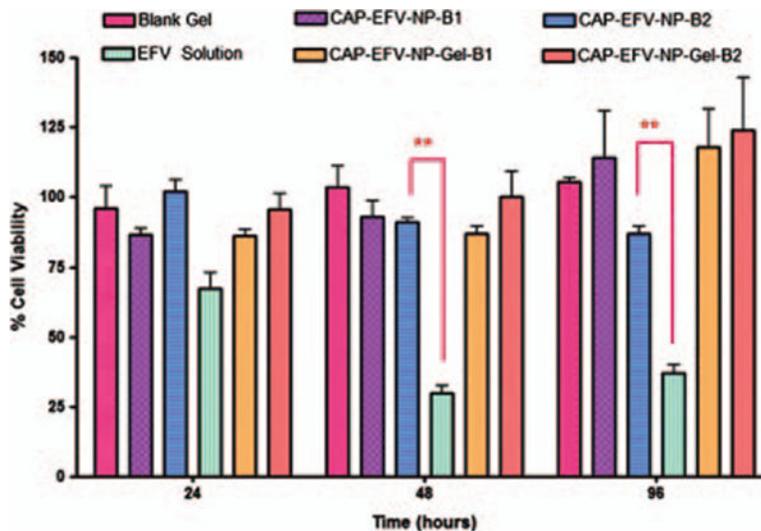
Figure 3.
In vitro cytotoxicity of CAP nanoparticles to HeLa cells (*n* = 6).

Figure 4.
SEM image of CAP-EFV-NPs.

Figure 5.
Time dependent fate of Rhodamine-6G labeled CAP nanoparticles (embedded in thermosensitive gel) in HeLa cells after translocation through transwell permeable supports ($n = 3$).
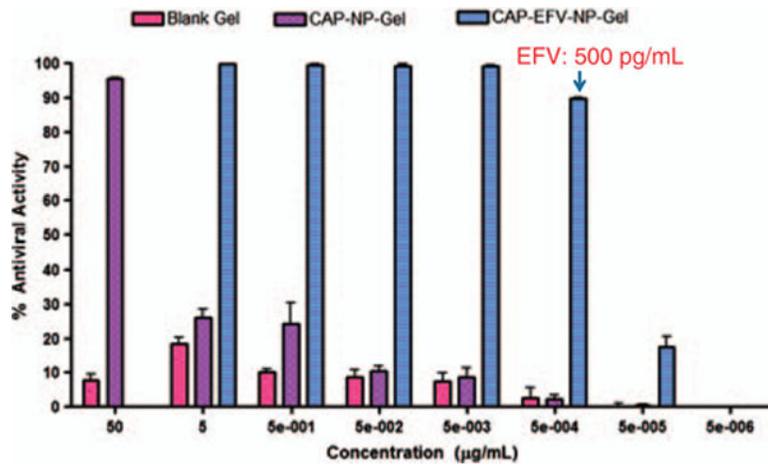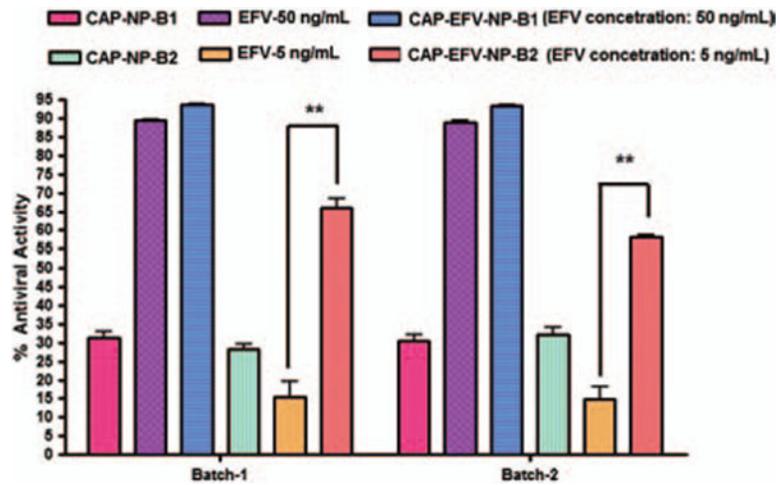
Figure 6.
In vitro cytotoxicity of EFV solution, blank gel, CAP-EFV-NPs and CAP-EFV-NP-Gel to HeLa cells ($n = 6$); **$P < 0.01$; B1 and B2: Batch 1 and Batch 2; CAP-EFV-NP: Cellulose acetate phthalate combination nanoparticles; CAP-EFV-NP-Gel: Thermosensitive gel containing CAP-EFV-NPs; EFV concentration in EFV solution, CAP-FEV-NPs (both batches) and CAP-FEV-NP-Gel (both batches) was 5 μg/ml.
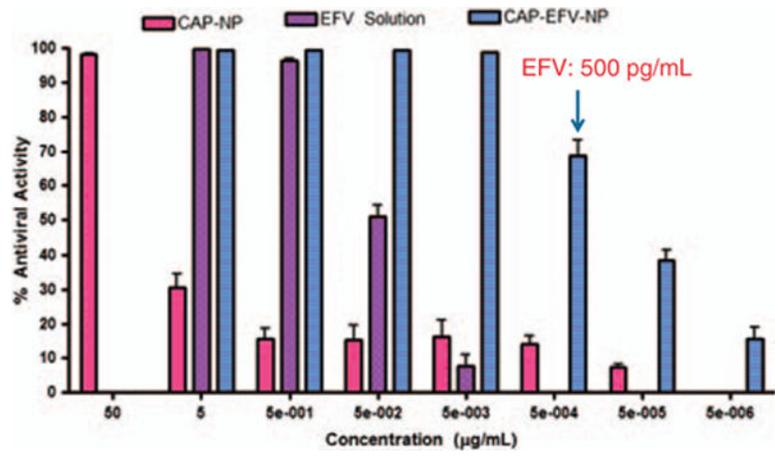
Figure 7.
Results of *in vitro* short-term HIV prophylaxis study of CAP nanoparticles, EFV solution and CAP-EFV-NPs (*n* = 6).

Figure 8.
Results of short-term *in vitro* prophylaxis study of thermosensitive gel containing CAP nanoparticles or CAP-EFV-NPs ($n = 6$).

Figure 9.
Results of long-term *in vitro* HIV prophylaxis study ($n = 6$); [**]P < 0.01; B1 and B2: Batch 1 and Batch 2; CAP-NP: Cellulose acetate phthalate nanoparticles; CAP-EFV-NP: Cellulose acetate phthalate combination nanoparticles; EFV-50 ng/ml: Solution containing 50 ng/ml of EFV; EFV-5 ng/ml: Solution containing 5 ng/ml of EFV.
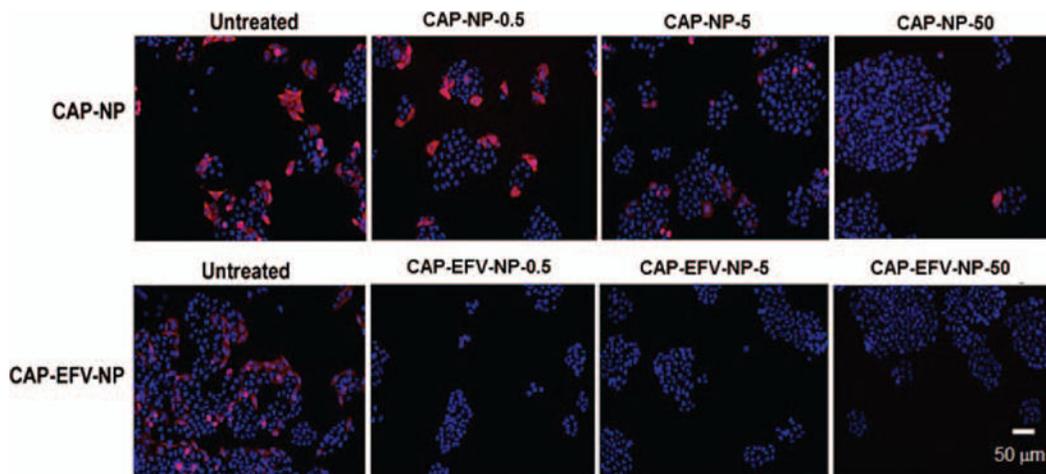
Figure 10.
β-galactosidase expression in TZM-bl cells treated with CAP nanoparticles or CAP-EFV NPs; Red color indicates β-galactosidase + HIV infected cells and blue color indicates β-galactosidase negative cells (no HIV infection); CAP-NP-0.5: NPs with 0.5 μg/ml of CAP; CAP-NP-5: NPs with 5 μg/ml of CAP; CAP-NP-50: NPs with 50 μg/ml of CAP; CAP-EFV-NP-0.5: NPs with 5 μg/ml of CAP and 50 ng/ml of EFV; CAP-EFV-NP-5: NPs with 5 μg/ml of CAP and 0.5 μg/ml of EFV; CAP-EFV-NP-50: NPs with 50 μg/ml of CAP and 5 μg/ml of EFV.

Table I

Physicochemical characteristics of CAP nanoparticles and CAP-EFV-NPs.

Parameter	CAP Nanoparticles	CAP-EFV-NPs
Particle size	82 2 ± 4 7 nm	96 9 ± 11 3 nm
Polydispersity index	0.16 ± 0.02	0.17 ± 0.02
Zeta potential	−22 47 ± 5 5 mV	−17.08 ± 2.68 m
Encapsulation efficiency of EFV	–	98.1 ± 1.2%

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