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Acyclovir-loaded sulfobutyl ether-β-cyclodextrin decorated chitosan nanodroplets for the local treatment of HSV-2 infections

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Abstract

Acyclovir is the gold standard drug for herpes simplex virus type 2 (HSV-2) infection treatment. Vaginal topical therapy with acyclovir is hampered due to its poor bioavailability, low retention at the vaginal mucosa, thus requiring high doses and frequent administrations. Nanocarriers have been proposed to overcome the challenges associated with antiviral delivery. This work aims at developing a novel formulation consisting of sulfobutyl ether-β-cyclodextrin decorated nanodroplets for acyclovir topical delivery to improve its antiviral effectiveness. To obtain acyclovir-loaded nanodroplets, the drug was previously complexed with sulfobutyl ether-β-cyclodextrin, and then incorporated in the nanodroplet chitosan shell via electrostatic interaction. The acyclovir-cyclodextrin inclusion complex was characterized by phase solubility, DSC, FTIR studies. The nanodroplets showed an average diameter of about 400 nm and positive surface charge. Acyclovir was efficiently incorporated in the nanodroplets (about 97% of encapsulation efficiency) and slowly released over time. The acyclovir-loaded nanodroplets exhibited an enhanced antiviral activity compared to the free drug against HSV-2 in cell cultures, which might be ascribed to a higher intracellular accumulation of the drug in nanodroplet-treated cells than in free acyclovir-treated cells. Based on these results, this new nanoformulation paves the way for the development of a future nanomicrobicide for the HSV-2 infections.

Keywords

Acyclovir, chitosan nanodroplets, sulfobutyl ether-β-cyclodextrin, HSV-2 infection

1. Introduction

Herpes simplex virus (HSV) type 2 is widespread throughout the world and is almost exclusively sexually transmitted, causing genital herpes. World health organization estimates approximately 417 million people worldwide were living with HSV-2 infection (www.who.int/en). More women are infected with HSV-2 than men and the prevalence of infection was estimated to be highest in Africa. Genital herpes infections often have no symptoms or are characterized by painful ulcerative or vesicular lesions or genital ulcers (Gupta et al., 2007 Hofstetter et al., 2014). The asymptomatic shedding increases the risk of HSV transmission. Following the primary infection, the virus persists in a latent state within neurons of the sensory sacral ganglia and periodical reactivations can occur (Roizman et al., 2007). In recent years the incidence of HIV/HSV-2 co-infections has been significantly increasing: HSV-2 infection increases the risk of acquiring a new HIV infection by approximately three-fold and it occurs in 60-90% of HIV-infected persons (Schiffer et al., 2017; Patel et al., 2016; www.who.int/en). Furthermore, HSV-2 infection can be transmitted from mother to infant during delivery. Neonatal herpes occurs in an estimated 10 out of every 100,000 births globally, and the risk is greater when a mother acquires HSV infection for the first time in late pregnancy.

Acyclovir (ACV), a synthetic nucleoside analogue derived from guanosine, is the drug of choice for the treatment of epidermal, ocular, genital or systemic herpetic infections (O’Brien et al., 1989). Infections caused by HSV are incurable and episodic and suppressive ACV treatment is aimed at reducing the severity, duration and recurrence of symptoms and at avoiding transmission (Groves, 2016). The 2015 CDC Sexually Transmitted Diseases Treatment Guidelines recommended oral regimes with ACV 400 mg three times a day for seven to ten days for first clinical episode of genital herpes and episodic or suppressive treatment with ACV 400 mg orally three times or twice per day for recurrent genital herpes (www.cdc.gov/std/tg2015/default.htm). Intravenous ACV therapy should be provided for patients who have severe HSV disease or complications that necessitate hospitalization (e.g., disseminated infection, pneumonitis, or hepatitis) or central nervous system (CNS) complications (e.g., meningoencephalitis).

The management of herpetic genital infections by applying local formulations with antiviral agent potentially provide some advantages over oral and intravenous administration: specific drug targeting, increased drug levels at the site of infection, reduced side effects, as the high systemic toxicity of ACV (nausea, vomiting, diarrhea, renal insufficiency) and the improvement of the patient compliance (Sharma et al., 2017, Szymańska et al., 2018). Unfortunately, topical therapy with ACV offers minimal clinical benefit and is discouraged since its bioavailability is low and highly variable, associated with low retention rate at the vaginal mucosa, and requires frequent administrations to reach high drug concentrations in genital tissues (Kinghorn et al., 1983, Donalisio et al., 2018).

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2.2.2 Differential Scanning Calorimetry analysis

Differential Scanning Calorimetry (DSC) analysis was carried out using a Perkin Elmer DSC/7 differential scanning calorimeter (Perkin-Elmer, CT-USA), equipped with a TAC 7 /DX instrument controller. The instrument was calibrated with indium. The analysis was performed in the temperature range of 25-350°C using 10°C/min heating rate. Standard aluminium sample pans (Perkin-Elmer) were used; and an empty pan was used as a reference standard. Analyses were done in triplicate on about 3 mg of freeze-dried samples under nitrogen purge.

2.2.3 FTIR spectroscopy analysis

FTIR spectra of free Acyclovir (ACV), sulfobutyl ether-β-cyclodextrin (SBE-β-CD) and Acyclovir inclusion complex (ACV- SBE-β-CD) were recorded on a Perkin Elmer Spectrum 100 FT-IR in the region of 4000- 650 cm-1. Data acquisition was performed using spectrum software version 10.03.05 Perkin Elmer Corporation.

2.3 Quantitative Determination of Acyclovir

The quantitative determination of ACV was carried out by HPLC analysis using a PerkinElmer PUMP 250B, equipped with a Flexar UV/Vis LC spectrophotometer detector (PerkinElmer, Waltham, MA, USA). A reversed-phase Agilent TC C18 column (25 cm × 4.6 mm, 5 µm, Agilent Technologies, Santa Clara, CA, USA) was used. Elution was performed isocratically at a flow rate of 1 mL/min, using a mobile phase consisting of acetonitrile and ammonium acetate buffer (20 mM, pH = 3.5) at the 12:88 (v/v) ratio. The UV detector was set to 250 nm. The ACV concentration was determined using an external standard method. An ACV calibration curve linear in the concentration range between 0.5–20 µg/mL with r^2 of 0.999 was obtained.

2.4 Preparation of Acyclovir-loaded sulfobutyl ether-β-cyclodextrin decorated chitosan nanodroplets

Blank chitosan nanodroplets (NDs) were prepared according to a preparation method previously reported (Cavalli et al., 2012b), using decafluoropentane for the inner core and chitosan low molecular weight (degree of deacetylation > 75%) for the shell. Briefly, an ethanol solution of Epikuron 200® and palmitic acid (1% w/v) was added to decafluoropentane, obtaining a pre-emulsion. Then, phosphate buffered saline (PBS) at pH 7.4 was added to the mixture and the system was homogenized (2 min, 24000 rpm) using an Ultra-Turrax® homogenizer (IKA, Konigswinter, Germany). Finally, a 2% w/v chitosan solution at pH 5.0 was dropwise added to the nanoemulsion under magnetic stirring for the polymer shell deposition. To obtain ACV loaded NDs the freeze-dried ACV-SBE-β-CD inclusion complex was dissolved in distilled water (2% w/v) and then added to the preformed chitosan NDs under magnetic stirring. As control, SBE-β-CD decorated chitosan NDs were prepared in the absence of ACV.

Fluorescent NDs were prepared by adding 6-coumarin (0.01% w/v) as fluorescent marker in the decafluoropentane core of NDs.

2.5 Characterization of Acyclovir-loaded sulfobutyl ether-β-cyclodextrin decorated chitosan nanodroplets

The average diameter, polydispersity index and zeta potential of sulfobutyl ether-β-cyclodextrin decorated chitosan nanodroplets, either blank or ACV loaded, were determined by dynamic light scattering (DLS) using a 90Plus particle size analyzer (Brookhaven Instrument Co., Holtsville, NY) at a fixed scattering angle of 90° and at 25 ± 1 °C. For zeta potential determination the sample was placed in the electrophoretic cell and an approximately 15 V/cm electric field was applied. The ND samples were diluted (1:30 v/v) using distilled water before analysis. All measurements were performed in triplicate. The morphological analysis of the ND was performed by transmission electron microscopy (TEM) using a Philips CM10 (Eindhoven, NL) instrument. For sample preparation, a drop of the ND samples diluted 100-fold with ultrapure water was placed on a copper grid and air dried prior to examination.

The physical stability of ND formulations was investigated over time on samples stored at 4 °C, evaluating their physico-chemical characteristics and the ACV content up to 2 months.

2.6 Determination of loading capacity and encapsulation efficiency of Acyclovir loaded nanodroplets

A freeze-dried sample of ACV-loaded NDs was precisely weighed, suspended in filtered water and sonicated for 15 minutes. After centrifugation (15000 rpm, 5 min), the supernatant was diluted with mobile phase and analyzed by HPLC, to determine ACV concentration in the ND sample.

The encapsulation efficiency and loading capacity of ACV-loaded NDs were determined using Eqs. 3 and 4, respectively.

[illegible] (3)

[illegible] (4)

2.7 In vitro release studies

In vitro release studies were carried out by dialysis bag technique (Spetra/Por cellulose dialysis membrane, with a molecular weight cut-off of 14,000 Da; Spectrum Laboratories (Rancho Dominguez, CA) in phosphate buffered saline (PBS) at pH 4.2 to simulated vaginal fluid. The ACV-loaded ND formulation (3 mL) was placed into a dialysis bag and immersed in 50 mL of receiving phase maintained at 37°C under magnetic stirring. At predetermined time intervals, 1 mL of the release medium was withdrawn and the same volume of fresh PBS was added to maintain sink conditions. The ACV concentration in the receiving phase was quantified by HPLC analysis. The results were expressed as % of ACV released over time and they represented the mean ± standard deviation (SD) based on three independent experiments.

2.8 Evaluation of mucoadhesion of nanodroplet formulation

Mucoadhesive properties of ND formulations were evaluated by in vitro mucin adhesion assay. The interaction between mucin and NDs was determined by turbidimetric analysis. Briefly, a volume of each ND sample was mixed with a mucin solution (1 mg/mL) at a 1:1 (v/v) ratio. The mixture was incubated under magnetic stirring for 30 minutes. Then, the sample was centrifuged for 5 minutes at 10000 rpm and the transmittance of the supernatant, containing the amount of mucin that did not interact with NDs, was measured at 500 nm with an UV spectrophotometer (Du730 spectrophotometer, Beckman, Coulter, Fullerton, CA, USA). The concentration of mucin was determined using an external standard method, from a mucin calibration curve obtained in the concentration range between 0.1 to 10 mg/mL. The amount of mucin adhesive to NDs was calculated as the difference between the total amount of added mucin and the free mucin content in the supernatant. The percentage of mucoadhesion was calculated using the following equation:

[illegible]

2.9 Biological Assays

2.9.1 Cells

African green monkey kidney cells (Vero) (ATCC CCL-81) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown as monolayers in Eagle’s minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD, USA) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco/BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany).

2.9.2 Virus

The MS strain (ATCC VR-540) of HSV-2 was used for in vitro antiviral experiments. The virus was propagated in Vero cells by infecting a freshly prepared confluent monolayer grown in MEM supplemented with 2% of FCS. When the cytopathic effect involved the whole monolayer, the infected cell suspension was collected and the viral supernatant was clarified. The virus stocks were aliquoted and stored at -80°C. The infectivity of virus stocks was determined on Vero cell monolayers by standard plaque assay.

2.9.3 Cell Viability Assay

Cell viability was measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay, as described by Cavalli et al., 2012a. Vero cell cultures, seeded in 96-well plates, were incubated with different concentrations of ACV, ACV-loaded ND and blank ND in triplicate under the same experimental conditions used for the virus plaque reduction assay. Absorbances were measured using a Microplate Reader (Model 680, BIORAD, Hercules, CA, USA) at 490 nm. The effect on cell viability at different concentrations was expressed as a percentage, by comparing absorbances of treated cells with those of cells incubated with culture medium alone. The 50% cytotoxic concentrations (CC50) and 95% confidence intervals (CIs) were determined using Prism software (Graph-Pad Software, San Diego, CA, USA).

2.9.4 Anti-HSV-2 inhibition assays

The effect of ACV-loaded ND, blank-ND and ACV on HSV-2 infection was evaluated by two antiviral activity assays on Vero cells: the plaque reduction assay and the virus yield reduction assay according to Donalisio et al. with modifications (2016). Briefly, the plaque reduction assay was carried out seeding Vero cells in 24-well plates at a density of 10 x10^4 cells/well and infected at a MOI of 0.001 PFU/cell for 2 h at 37°C. After incubation, cells were washed with fresh medium and overlaid with 1.2% methylcellulose MEM in presence of nanodroplets or ACV. After 24 h of incubation at 37°C, cells were fixed and stained with 0.1% crystal violet in 20% ethanol and viral plaques were microscopically counted.

The virus yield reduction assay was performed infecting pre-seeded cells with HSV-2 at a MOI of 0.01 PFU/cell. Following adsorption at 37 °C for 2 hr, the virus inocula were removed, and cultures were grown in medium in the presence of nanodroplets or ACV until control cultures displayed extensive cytopathology. Then, supernatants were harvested, pooled and cell-free virus infectivity titers were determined by plaque assay.

The end-point of these assays was the inhibitory concentration of nanodroplets that reduced viral plaques or virus yield by 50% (IC50), respectively, compared to untreated virus controls. The IC50 values for inhibition curves were calculated by using the program PRISM 4 (GraphPad Software, San Diego, California, USA) to fit a variable slope-sigmoidal dose–response curve. Three independent experiments for both assays were performed in duplicate.

2.9.5 Evaluation of Cellular Uptake by Confocal Laser Microscopy

Vero cells were seeded in 24-well plates at a density of $3x10^4$ cells/well on glass coverslips. The day after, cell monolayers were incubated with 100 μM of the fluorescent-labelled ND for 5 minutes, 30 minutes, 1 hour and 3 hours, respectively; then cells were extensively washed with PBS 1X. Confocal sections of green living cells were taken on Confocal laser microscope (LSM800, Carl Zeiss, Jena, Germany).

2.9.6 Determination of Acyclovir concentration in Vero cells

Vero cells were seeded in 60 mm culture dishes at a density of $40x10^4$ cells/well. The day after, cells were treated with 1 μM, 10 μM and 50 μM of ACV-ND and ACV for two hours at 37°C. Then, the cells were washed three times with PBS 1X, lysed with a saturated solution of ammonium sulphate at 4°C and centrifuged at 4°C for 10 min at 13000 rpm. Cell lysates were frozen and stored at −80 °C. Immediately prior to the analysis, cell lysates were thawed and centrifuged (13000 rpm, 10 min, 10 °C). The supernatants were diluted with the mobile phase and analyzed by HPLC, as described above, to determine the amount of ACV inside the cells. The experiment was performed in triplicate.

2.10 Statistical Analysis

The results are expressed as mean ± SD. Statistical analyses were performed using unpaired Student’s t-test, and Extra sum-of-square F test by GraphPad on GraphPad Prism version 4.00 software, as appropriate. Significance was reported for p-value <0.05.

3. Results and Discussion

A new formulation strategy for the vaginal delivery of acyclovir has been designed. ACV is a slightly soluble in water molecule and according to the Biopharmaceutics Classification System (BCS) it is classified as class III drug up to 400 mg dose, whereas in class IV at higher doses (800 mg) (Ates et al., 2016). After topical application ACV bioavailability is low and highly variable, associated with poor permeability and low retention at the vaginal mucosa. For these reasons high doses and frequent administrations are required to assure ACV therapeutic concentrations. The ACV incorporation in a nanodelivery system could be promising to overcome these limitations and to increase its antiviral efficacy.

In fact nanoparticles enable the antiviral drug delivery at the target sites and their penetration through biological barriers (Lembo et al., 2018, Cojocaru et al., 2020). Nanotechology approach has been advantageously exploited for the vaginal administration of antivirals to improve their distribution and retention in vaginal tract. In particular, chitosan-based nanoparticles have been widely proposed for their interesting mucoadhesive and penetration enhancement properties, enabling mucosal site-specific delivery.

Chitosan is a linear cationic polysaccharide largely investigated because of its biocompatibility, biodegradability, non-antigenic nature. Moreover, the presence of primary amino groups and hydroxyl groups facilitates direct derivatization of the polymer and conjugation with specific ligands for suitable pharmaceutical applications (Rajitha et al., 2016).

Previously, chitosan nanoparticles have been successfully prepared by ionic cross-linking between positively charged amino groups of chitosan and negatively charged sulfonate groups of SBE-β-CD (Fülöp et al., 2015). SBE-β-CD is a polyanionic beta-cyclodextrin derivative with sulfobutyl ether groups, that shows much greater solubility in water and inclusion ability than the parent β-CD. It is FDA-approved for both oral and parenteral administration (EMA 2017). The use of the anionic SBE-β-CD as both chitosan crosslinker and drug solubilizing agent was studied for the development of several nanoformulations (Liu et al., 2016 a,b, Zhang et al., 2016).

Here, the electrostatic interaction between chitosan and SBE-β-CD was exploited with the aim to increase the ACV loading capability of nanodroplet system. SBE-β-CD was integrated within the shell to encapsulate ACV and allow the loading of the complexed drug into ND nanostructure. Interestingly, the combination of cyclodextrin derivatives with chitosan have been previously studied to enhance the permeation of mucosal tissues and the oral bioavailability of drugs (Maestrelli et al., 2011). This formulation strategy might be exploited to increase the paracellular absorption of acyclovir after vaginal administration.

The hybrid polysaccharide-CD shell composition was optimized by studying the influence of several key parameters, including chitosan/CD ratio.

The association of a drug-cyclodextrin inclusion complex with its subsequent incorporation into a nanocarrier was explored to simultaneously benefit from the CD solubilizing and stabilizing properties and the nanocarrier features. This dual approach has been recently investigated by different authors to overcome the drawbacks associated with each separate delivery system, with the goal to improve their effectiveness. Indeed, this strategy enables to combine their respective advantages in a single delivery system (Mura, 2020).

To obtain acyclovir-loaded NDs, the drug was previously complexed (1:1 molar ratio) with SBE-β-CD. The acyclovir-CD complex was then incorporated into the chitosan surface of the preformed

NDs, exploiting electrostatic interactions. The same strategy was previously used to formulate vancomycin loaded NDs for the management of chronic ulcers (Mazzaccaro et al, 2020).

At first, the inclusion complex of ACV with SBE-β-CD was characterized performing phase solubility studies according to Higuchi and Connors method. The phase solubility diagram shown in Figure 1 was obtained by plotting the apparent concentration of ACV against the concentration of SBE-β-CD.

The diagram showed that the aqueous solubility of ACV linearly increased in a concentration dependent manner as a function of CD concentration, due to the formation of inclusion complex between the drug and CD.

The phase solubility curve can be classified as an A_L-type diagram, according to the Higuchi and Connors classification (Higuchi and Connors, 1965).

The slope of the solubility diagram (0.70) was lower than 1, indicating the formation of 1:1 (mol/mol) ACV-SBE-β-CD inclusion complex.

The calculated apparent stability constant (K_{st}) of ACV-SBE-β-CD inclusion complex was 266.99 ± 10.2 M^{-1} and the complexation efficiency (CE) was 2.36.

The inclusion ability of SBE-β-CD is generally greater than that of β-CD due to the hydrophobic butyl side arms that extend from the hydrophobic cavity of the CD (Saokham et al., 2018).

These results were consistent with the phase solubility studies of ACV with HP-β-CD performed by Nair et al. (Nair et al., 2014). Other studies reported the formation of stable ACV inclusion complex with thiolated β-CD, showing improved drug dissolution and mucoadhesive properties (Ijaz et al., 2016). Furthermore, ACV incorporation in a semi-synthetic biopolymer complex prepared from cross-linking of hyaluronic acid with poly(acrylic acid) (PAA) and conjugated with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) resulted in an improvement of its solubility and permeation (Sithole et al., 2018). The formation of an inclusion complex between ACV and SBE-β-CD was studied in the solid state to evaluate the interactions of the drug with the CD.

DSC thermogram of ACV-SBE-β-CD did not present the endothermic peak at about 260 °C related to ACV melting indicating the molecular dispersion of ACV in the CD cavity (data not shown).

Moreover, FTIR analysis (Figure 2) confirmed the complexation of ACV with SBE-β-CD.

FTIR spectrum of pure ACV showed the characteristic bands for N-H and O-H stretching between 3150 and 3450 and peaks for C=O stretching at around 1700 cm^{-1}.

The peak modification and the reduction of absorption band intensity in the inclusion complex spectrum indicated the formation of ACV- SBE-β-CD complex.

Acyclovir loaded NDs were then prepared by the incorporation of the ACV inclusion complex in the preformed chitosan NDs. The complex is efficiently loaded among the polymer chains of the ND shell, due to electrostatic interactions.

The results of the physico-chemical characterization of ND formulations were reported in Table 1.

Formulation	Average diameter ± SD (nm)	PDI ± SD	Zeta potential ± SD (mV)
Chitosan NDs	405.3 ± 20.5	0.21 ± 0.02	31.25 ± 2.79
Blank SBE-β-CD chitosan NDs	396.6 ± 15.2	0.22 ± 0.01	20.16 ± 1.94
Fluorescent SBE-β-CD chitosan NDs	398.2 ± 13.7	0.21 ± 0.01	20.23 ± 2.15
Acyclovir-loaded NDs	395.4 ± 12.6	0.20 ± 0.02	19.98 ± 3.02

Table 1. Physico-chemical characteristics of ND formulations

The chitosan NDs, prepared as control without the addition of SBE-β-CD, showed sizes of about 400 nm and a positive surface charge. A reduction of zeta potential values of about 35% was observed after the incorporation of SBE-β-CD in the ND shell. However, their surface charge remains high enough to assure the stability of the ND nanosuspension. The shell modification did not alter the physical stability of the nanostructure. Indeed, no precipitation or aggregation phenomena were observed. Moreover, the presence of ACV in the CD complex did not affect the physico-chemical parameters of NDs.

The TEM image (Figure 3) showed the spherical morphology and the core shell structure of NDs.

NDs were able to load ACV in a good extent with an encapsulation efficiency of 96.6%. The loading capacity of ACV-loaded NDs was of 2%. The physical stability of ACV-loaded NDs stored at 4 °C was confirmed up to 2 months. Indeed, no significant changes in their physico-chemical parameters and in the ND ACV concentration (99.90 % of the initial ACV content) were observed.

The in vitro release kinetics of ACV from ACV-loaded NDs was evaluated at pH 4.2 to simulate vaginal fluids (Figure 4). For comparison the diffusion of ACV from the inclusion complex was investigated.

The ACV complexation with SBE-β-CD resulted in an increase of its apparent solubility and favored the drug diffusion in the receiving phase. A prolonged in vitro release profile with no initial burst effect was observed for ACV from NDs, indicating that it was not weakly adsorbed on the ND surface but the complex is incorporated within the chitosan chains of the shell. About 34 % of ACV was released from the NDs after 6 hours. This prolonged in vitro release kinetics was also observed for others ACV loaded nanoparticulate systems. For example, ACV encapsulation in carboxylated cyclodextrin-based nanosponges, thanks to the presence of carboxylic groups besides the cyclodextrin cavities in the polymer matrix, provided the sustained release of the drug over time (Lembo et al., 2013).

The mucoadhesion capability of chitosan was maintained for the ND formulations and also after its complexation with SBE-β-CD. Mucoadhesive property is a key parameter to take into account in the development of vaginal nanoformulations to prolong the residence time and retention on mucosal tissue improving their efficacy (Caramella et al, 2015).

Regarding the biological experiments, two antiviral assays were performed in vitro to compare the inhibition activity of ACV-loaded ND and ACV against HSV-2 infection on Vero cells. The plaque reduction assay is finalized to quantify the antiviral effect of a formulation evaluating its ability to reduce the number of viral plaques on cell monolayer. To generate dose–response curves, cells were treated with decreasing concentrations of ND-ACV, ACV or blank ND in 1.2% methylcellulose medium after viral infection. Twenty four hours post infection, the IC50 was determined by comparing the number of viral plaques in treated and untreated wells, as described in Materials and Methods section. As shown in Figure 5A, ACV-loaded ND was active against HSV-2 infection in a dose-response manner with an IC50 value of 0.32 μM (95% CI: 0.16 - 0.63 μM . Of note, the antiviral activity of ACV-loaded ND was significantly higher than free ACV which displayed an IC50 value of 0.89 μM (95% CI: 0.56 -1.42 μM; $p<0.05$ By contrast, blank ND showed a weak antiviral activity only at high doses, and its IC50 value was not assessable.

The antiviral activity of blank NDs could be ascribable to the presence of SBE-β-CD in the ND chitosan shell. Previously, cyclodextrin derivatives have been investigated as antiviral agents. A mechanism exploited to inhibit virus infections might be related to their capability to extract cholesterol from membranes leading to the block of viral penetration (Nishijo et al., 2003).

Indeed, some authors have been reported that sulfonated cyclodextrin derivatives, displayed antiviral activity against a number of viruses (Goncharova et al., 2019, Moriya et al., 1993, Mori et al., 1999). Recently, Jones et al. (2020) synthetized modified CDs linking highly sulfonated groups to CD scaffold. The functionalized CDs exhibited a broad-spectrum virucidal activity against several HS-dependent viruses. Of note, the authors evidenced an inhibition of the growth of HSV-2 when CDs were added to cells after removal of the virus (post-infection assay), similarly to our experimental procedure.

The antiviral effect of ACV-loaded ND was further confirmed by the yield reduction assay, a stringent test that allows multiple cycles of viral replication to occur before measuring the production of infectious viruses. As reported in Figure 5B, 14.8 μM and 3.7 μM concentrations of ACV-loaded ND totally inhibited the viral titers. Furthermore, this assay confirmed the higher antiviral potency of ACV-loaded ND than that of free Acyclovir against HSV-2 infection. In particular, IC50 values were found to be 0.10 μM (95% CI: 0.04 - 0.26) for ACV-loaded ND and 0.40 μM (95% CI: 0.30 - 0.54 μM) for free acyclovir. The IC50 values for plain acyclovir was similar to previously reported values (Visalli et al., 2015). No antiviral activity was exerted by the blank ND at tested doses (data not shown).

Results of viability assays indicated that the inhibitory activities of ACV-loaded ND were not a consequence of cellular alterations because the CC50 value (57.50 μM) on Vero cells was much higher than the IC50 value 0.32 μM. In particular, a reduction of cell viability by ACV-loaded ND and ND alone was observed only at high doses (Figure 6).

On the basis of the reported results, we speculated that the higher antiviral activity of ACV-loaded ND than that of free ACV might be related to a higher intracellular concentration of the drug delivered by the nanodroplets. Therefore, firstly, we investigated whether NDs could enter inside cells. To this aim, cells were treated with fluorescent NDs and observed at different time points by confocal laser microscopy. The assay was carried out on living unfixed cells to avoid misleading due to the cell fixation protocols. As reported in Figure 7, nanodroplets were able to bind Vero cells after 5 minutes of exposure. After 1 h of treatment several cells revealed a cytoplasmic distribution of fluorescent NDs and after 3 hours of exposure all cells appeared homogenously green on coverslip. No intracellular fluorescence was detected in control cells unexposed to the labelled compounds (data not shown).

Furthermore, the cellular uptake of ACV-loaded NDs was investigated by HPLC quantitative determination of intracellular ACV concentration in Vero cells (Figure 8).

The experiments finalized to determine the intracellular ACV concentration evidenced a considerable higher intracellular accumulation of the drug in ACV-ND treated cells than in free ACV-treated cells. A statistically significant increase in the intracellular accumulation of ACV in Vero cells was observed at all the doses tested. In particular, for the cells treated with 50 M formulations a 2.56 fold enhancement of ACV concentration was found.

Conclusions

The feasibility to produce NDs with a hybrid polysaccharide-cyclodextrin shell was demonstrated. Sulfobutyl ether-β-cyclodextrin decorated chitosan nanodroplets were able to incorporate and release ACV in a sustained manner. ACV loaded into nanodroplets showed a higher antiviral activity against HSV-2 in cell cultures compared to the free drug. Future investigation will concern the incorporation of acyclovir-loaded NDs in a hydrogel suitable for vaginal application.

To conclude, ACV-loaded nanodroplets might open new strategies for developing a future nanomicrobicide for the local treatment of herpetic genital infections.

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Figure legends

Figure 1. Phase solubility diagram of acyclovir-sulfobutyl ether-β-cyclodextrin (ACV-SBE-β-CD) inclusion complex

Figure 2. FTIR spectra of free Acyclovir (A), sulfobutyl ether-β-cyclodextrin (B) and Acyclovir inclusion complex (C)

Figure 3. TEM image of acyclovir-loaded NDs (scale bar 200 nm)

Figure 4. In vitro release kinetics of acyclovir from ACV-loaded NDs. The results represent the mean ± SD ($n = 3$).

Figure 5. Panel A. Antiviral activity of acyclovir (ACV), acyclovir loaded nanodroplets (ACV-ND) and plain nanodroplets (ND) against HSV2, as determined by plaque reduction assay. ACV-loaded ND was active against HSV-2 infection in a dose-response manner with IC50 value significantly lower than free ACV (0.32 µM and 0.89 µM, respectively; $p < 0.01$, as determined by F-test). Notably, blank ND exhibited weak antiviral activity only at high doses, and its IC50 value was not assessable. **Panel B.** Effects of acyclovir-loaded ND on multiple replicative cycles of HSV-2, as determined by virus yield reduction assay. Vero cells were infected at a multiplicity of infection (MOI) of 0.01 and then exposed to serial drug concentrations. Virus titers in the supernatants of cell cultures were determined by standard plaque assay. Viral titers (expressed as plaque-forming units, PFU/mL) are shown as mean plus standard error of the mean for three independent experiments (* $p<0.05$; *** $p<0.001$, Student's t-test). (UT, untreated).

Figure 6. Effect of acyclovir (ACV), acyclovir-loaded nanodroplets (ACV-ND), and nanodroplets alone (ND) on the viability of non-infected Vero cells as a function of the drug concentration at 24 hours. X axis: ND concentration; Y axis: cell viability (% of untreated control). Each point represents the mean ± SD ($n = 3$).

Figure 7. Cell uptake of fluorescent NDs. Vero cells were incubated with the ND formulation for 5 minutes, 30 minutes, 1 and 3 hours, and then analyzed by confocal laser microscopy without fixation. The first picture on the left shows the control cells, which were not incubated with the ND formulation (untreated). Scale bar, 5 µm.

Figure 8. Intracellular acyclovir concentration (M) in Vero cells. Vero cells were incubated with 1 µM, 10 µM and 50 µM of ACV-ND and ACV for two hours at 37°C. Then, the cells were lysed and cell lysates were analyzed by HPLC. The results represent the mean ± SD ($n = 3$; * $p<0.05$; *** $p<0.001$, unpaired t-test).