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

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35 **Abstract**

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

52

53 **Keywords**

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

55 **1. Introduction**

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

70 Acyclovir (ACV), a synthetic nucleoside analogue derived from guanosine, is the drug of choice for
71 the treatment of epidermal, ocular, genital or systemic herpetic infections (O'Brien et al., 1989).
72 Infections caused by HSV are incurable and episodic and suppressive ACV treatment is aimed at
73 reducing the severity, duration and recurrence of symptoms and at avoiding transmission (Groves,
74 2016). The 2015 CDC Sexually Transmitted Diseases Treatment Guidelines recommended oral
75 regimens with ACV 400 mg three times a day for seven to ten days for first clinical episode of genital
76 herpes and episodic or suppressive treatment with ACV 400 mg orally three times or twice per day
77 for recurrent genital herpes (www.cdc.gov/std/tg2015/default.htm). Intravenous ACV therapy should
78 be provided for patients who have severe HSV disease or complications that necessitate
79 hospitalization (e.g., disseminated infection, pneumonitis, or hepatitis) or central nervous system
80 (CNS) complications (e.g., meningoencephalitis).

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

89 Furthermore, patients treated with commercially available topical ACV products often complain of
90 stinging and burning sensation (Kaur et al., 2018).

91 Nanotechnology is an advanced approach for the delivery of currently approved antiviral drugs
92 modifying their physico-chemical, pharmacokinetics and pharmacodynamics properties, reducing the
93 side effects and achieving effective drug concentrations in the sites of infection (Lembo et al., 2018).
94 Polymer-based nanocarriers have been extensively investigated for the vaginal delivery of active
95 molecules taking into account the advantages to which they could lead, such as controlled release,
96 enhanced residence time, safety profile and efficacy, modulation of mucoadhesion or mucosal tissue
97 penetration (das Neves et al., 2015, Iqbal and Dilnawaz, 2019, Ullah et al., 2019). Interestingly,
98 mucoadhesive materials, such as chitosan, alginate, carrageenan, pectin, cellulose derivative,
99 thiolated polymers, polyacrylate and carbomer, have been explored in order to improve nanoparticle
100 retention in vaginal tract and permeation through mucus and epithelium (Wong et al., 2014, Ijaz et
101 al, 2016, Cazorla-Luna et al., 2020, Tentor et al., 2020).

102 In particular, chitosan presents a high mucoadhesion capability, mainly thanks to the electrostatic
103 interactions between positively charged chitosan amino groups and negatively charged mucus layer,
104 and penetration enhancing properties (Kumar et al., 2016). In addition, chitosan can play the role of
105 penetration enhancer for transmucosal absorption of drugs (Sandri et al., 2004, Maher et al., 2019).

106 Chitosan-based vaginal delivery systems of ACV have been developed for the good binding capacity
107 of the polysaccharide with mucosal tissues (Sanchez et al., 2015, Al-Subaie et al., 2015).

108 Chitosan-shelled nanodroplets (NDs) are spherical nanostructures comprising a core filled by liquid
109 or vaporizable compounds (i.e. perfluorocarbons) and stabilized by a polysaccharide shell (Cavalli et
110 al., 2016). A growing number of studies underline their potential as versatile multifunctional platform
111 for the delivery of drugs, gases and genetic materials (Cavalli et al., 2015, Khadjavi et al, 2015, Yu
112 et al., 2018, Zhou et al, 2020). Chitosan NDs have been already proposed for the topical treatment of
113 skin infections as such (Banche et al., 2015) or loaded with antimicrobial drugs, such as vancomycin
114 (Argenziano et al, 2017).

115 The aim of this study was to investigate a next generation platform of NDs with a hybrid
116 polysaccharide shell containing chitosan and an anionic cyclodextrin. For this purpose, we designed
117 sulfobutyl ether- β -cyclodextrin decorated chitosan NDs for the acyclovir delivery, in order to provide
118 sustained release and improved antiviral efficacy of the drug for the local treatment of herpetic genital
119 infections. Here, we reported the preparation and *in vitro* characterization of ACV-loaded
120 nanodroplets. Biological assays were performed to assess the effect of ACV-loaded nanodroplets on
121 cell viability and their potential to inhibit the HSV-2 infection *in vitro*.

122

123

124

125 **2. Materials and Methods**

126 All materials used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA),
127 unless otherwise stated. Epikuron 200[®] was kindly supplied by Cargill (Hamburg, Germany).
128 Captisol[®] was a gift from Ligand (San Diego, CA, USA).

129

130 *2.1. Preparation of Acyclovir inclusion complex*

131 The ACV inclusion complex with SBE- β -CD (Captisol[®], average molecular weight 2163 Da, average
132 degree of substitution 6.5) was prepared in a 1:1 molar ratio. Briefly, a weighted amount of ACV was
133 dispersed in 5 mL of ultrapure water containing an equimolar amount of SBE- β -CD and stirred for
134 24 h at room temperature. After equilibrium reaching, the mixture was filtered through a membrane
135 filter of 0.45-mm size and the filtrate was freeze-dried for 16 hours using a Modulyo freeze-drier
136 (Edwards, Burgess Hill, UK) at -40 °C under 0.1 mbar. To evaluate the ACV content in the inclusion
137 complex, a weighted amount of freeze-dried ACV-SBE- β -CD complex was dispersed in water and
138 sonicated for 15 min. The sample was then centrifuged (15000 rpm, 5 min) and the properly diluted
139 supernatant was analyzed by High-Performance Liquid Chromatography (HPLC), using the method
140 reported in section 2.3.

141

142 *2.2 Characterization of Acyclovir inclusion complex*

143 *2.2.1 Phase solubility studies*

144 Phase solubility experiments were performed according to the method described by Higuchi and
145 Connors (Higuchi and Connors, 1965). An excess amount of ACV (25 mg) was added to 5 mL of
146 aqueous solutions containing increasing concentrations of SBE- β -CD, ranging from 0 to 10 mM. The
147 mixtures were magnetically stirred at room temperature until equilibrium (24 h). The suspensions
148 were then centrifuged and the ACV concentration in the supernatant was determined by HPLC
149 analysis (see section 2.3).

150 The phase solubility diagram was constructed by plotting the apparent molar concentration of ACV
151 against the molar concentration of SBE- β -CD. The apparent stability constant (K_{st}) was calculated
152 from the initial straight-line portion of the phase-solubility diagram according to following equation:

$$153 K_{st} = \frac{\text{slope}}{[S_0(1-\text{slope})]} \quad (1)$$

154 where S_0 is the solubility of ACV in the absence of CD.

155 The complexation efficiency (CE) was calculated from the phase-solubility diagram, using the
156 equation:

157 $CE = K_{st}S_0$ (2)

158

159 2.2.2 Differential Scanning Calorimetry analysis

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

166

167 2.2.3 FTIR spectroscopy analysis

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

172

173 2.3 Quantitative Determination of Acyclovir

174 The quantitative determination of ACV was carried out by HPLC analysis using a PerkinElmer
175 PUMP 250B, equipped with a Flexar UV/Vis LC spectrophotometer detector (PerkinElmer,
176 Waltham, MA, USA). A reversed-phase Agilent TC C18 column (25 cm \times 4.6 mm, 5 μ m, Agilent
177 Technologies, Santa Clara, CA, USA) was used. Elution was performed isocratically at a flow rate of
178 1 mL/min, using a mobile phase consisting of acetonitrile and ammonium acetate buffer (20 mM, pH
179 = 3.5) at the 12:88 (v/v) ratio. The UV detector was set to 250 nm. The ACV concentration was
180 determined using an external standard method. An ACV calibration curve linear in the concentration
181 range between 0.5–20 μ g/mL with r^2 of 0.999 was obtained.

182

183 2.4 Preparation of Acyclovir-loaded sulfobutyl ether- β -cyclodextrin decorated chitosan nanodroplets

184 Blank chitosan nanodroplets (NDs) were prepared according to a preparation method previously
185 reported (Cavalli et al., 2012b), using decafluoropentane for the inner core and chitosan low
186 molecular weight (degree of deacetylation > 75%) for the shell. Briefly, an ethanol solution of
187 Epikuron 200[®] and palmitic acid (1% w/v) was added to decafluoropentane, obtaining a pre-emulsion.
188 Then, phosphate buffered saline (PBS) at pH 7.4 was added to the mixture and the system was
189 homogenized (2 min, 24000 rpm) using an Ultra-Turrax[®] homogenizer (IKA, Königswinter,
190 Germany). Finally, a 2% w/v chitosan solution at pH 5.0 was dropwise added to the nanoemulsion

191 under magnetic stirring for the polymer shell deposition. To obtain ACV loaded NDs the freeze-dried
192 ACV-SBE- β -CD inclusion complex was dissolved in distilled water (2% w/v) and then added to the
193 preformed chitosan NDs under magnetic stirring. As control, SBE- β -CD decorated chitosan NDs
194 were prepared in the absence of ACV.

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

197

198 *2.5 Characterization of Acyclovir-loaded sulfobutyl ether- β -cyclodextrin decorated chitosan* 199 *nanodroplets*

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

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

212

213 *2.6 Determination of loading capacity and encapsulation efficiency of Acyclovir loaded nanodroplets*

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

217 The encapsulation efficiency and loading capacity of ACV-loaded NDs were determined using Eqs.
218 3 and 4, respectively.

$$219 \text{ Encapsulation efficiency} = \frac{\text{amount of ACV in NDs}}{\text{initial ACV amount}} \times 100 \quad (3)$$

220

$$221 \text{ Loading capacity} = \frac{\text{amount of ACV in NDs}}{\text{Mass of NDs}} \times 100 \quad (4)$$

222

223 *2.7 In vitro release studies*

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

233

234 *2.8 Evaluation of mucoadhesion of nanodroplet formulation*

235 Mucoadhesive properties of ND formulations were evaluated by *in vitro* mucin adhesion assay. The
236 interaction between mucin and NDs was determined by turbidimetric analysis. Briefly, a volume of
237 each ND sample was mixed with a mucin solution (1 mg/mL) at a 1:1 (v/v) ratio. The mixture was
238 incubated under magnetic stirring for 30 minutes. Then, the sample was centrifuged for 5 minutes at
239 10000 rpm and the transmittance of the supernatant, containing the amount of mucin that did not
240 interact with NDs, was measured at 500 nm with an UV spectrophotometer (Du730
241 spectrophotometer, Beckman, Coulter, Fullerton, CA, USA). The concentration of mucin was
242 determined using an external standard method, from a mucin calibration curve obtained in the
243 concentration range between 0.1 to 10 mg/mL. The amount of mucin adhesive to NDs was calculated
244 as the difference between the total amount of added mucin and the free mucin content in the
245 supernatant. The percentage of mucoadhesion was calculated using the following equation:

$$246 \text{ Mucoadhesion (\%)} = \frac{(\text{mucin adhesive to NDs})}{(\text{total mucin})} \times 100$$

247

248 *2.9 Biological Assays*

249 *2.9.1 Cells*

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

255

256 *2.9.2 Virus*

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

263

264 *2.9.3 Cell Viability Assay*

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

274

275 *2.9.4 Anti-HSV-2 inhibition assays*

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

284 The virus yield reduction assay was performed infecting pre-seeded cells with HSV-2 at a MOI of
285 0.01 PFU/cell. Following adsorption at 37 °C for 2 hr, the virus inocula were removed, and cultures
286 were grown in medium in the presence of nanodroplets or ACV until control cultures displayed
287 extensive cytopathology. Then, supernatants were harvested, pooled and cell-free virus infectivity
288 titers were determined by plaque assay.

289 The end-point of these assays was the inhibitory concentration of nanodroplets that reduced viral
290 plaques or virus yield by 50% (IC50), respectively, compared to untreated virus controls. The IC50

291 values for inhibition curves were calculated by using the program PRISM 4 (GraphPad Software, San
292 Diego, California, USA) to fit a variable slope-sigmoidal dose–response curve. Three independent
293 experiments for both assays were performed in duplicate.

294

295 *2.9.5 Evaluation of Cellular Uptake by Confocal Laser Microscopy*

296 Vero cells were seeded in 24-well plates at a density of 3×10^4 cells/well on glass coverslips. The day
297 after, cell monolayers were incubated with 100 μM of the fluorescent-labelled ND for 5 minutes, 30
298 minutes, 1 hour and 3 hours, respectively; then cells were extensively washed with PBS 1X. Confocal
299 sections of green living cells were taken on Confocal laser microscope (LSM800, Carl Zeiss, Jena,
300 Germany).

301

302 *2.9.6 Determination of Acyclovir concentration in Vero cells*

303 Vero cells were seeded in 60 mm culture dishes at a density of 40×10^4 cells/well. The day after, cells
304 were treated with 1 μM , 10 μM and 50 μM of ACV-ND and ACV for two hours at 37°C. Then, the
305 cells were washed three times with PBS 1X, lysed with a saturated solution of ammonium sulphate
306 at 4°C and centrifuged at 4°C for 10 min at 13000 rpm. Cell lysates were frozen and stored at –80
307 °C. Immediately prior to the analysis, cell lysates were thawed and centrifuged (13000 rpm, 10 min,
308 10 °C). The supernatants were diluted with the mobile phase and analyzed by HPLC, as described
309 above, to determine the amount of ACV inside the cells. The experiment was performed in triplicate.

310

311 *2.10 Statistical Analysis*

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

315

316 **3. Results and Discussion**

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

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

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

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

342 Here, the electrostatic interaction between chitosan and SBE- β -CD was exploited with the aim to
343 increase the ACV loading capability of nanodroplet system. SBE- β -CD was integrated within the
344 shell to encapsulate ACV and allow the loading of the complexed drug into ND nanostructure.
345 Interestingly, the combination of cyclodextrin derivatives with chitosan have been previously studied
346 to enhance the permeation of mucosal tissues and the oral bioavailability of drugs (Maestrelli et al.,
347 2011). This formulation strategy might be exploited to increase the paracellular absorption of
348 acyclovir after vaginal administration.

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

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

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

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

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

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

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

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

372 The calculated apparent stability constant (K_{st}) of ACV-SBE- β -CD inclusion complex was $266.99 \pm$
373 10.2 M^{-1} and the complexation efficiency (CE) was 2.36.

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

376 These results were consistent with the phase solubility studies of ACV with HP- β -CD performed by
377 Nair et al. (Nair et al., 2014). Other studies reported the formation of stable ACV inclusion complex
378 with thiolated β -CD, showing improved drug dissolution and mucoadhesive properties (Ijaz et al.,
379 2016). Furthermore, ACV incorporation in a semi-synthetic biopolymer complex prepared from
380 cross-linking of hyaluronic acid with poly(acrylic acid) (PAA) and conjugated with 2-hydroxypropyl-
381 β -cyclodextrin (HP- β -CD) resulted in an improvement of its solubility and permeation (Sithole et al.,
382 2018). The formation of an inclusion complex between ACV and SBE- β -CD was studied in the solid
383 state to evaluate the interactions of the drug with the CD.

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

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

387 FTIR spectrum of pure ACV showed the characteristic bands for N-H and O-H stretching between
388 3150 and 3450 and peaks for C=O stretching at around 1700 cm^{-1} .

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

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

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

395

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

396 **Table 1.** Physico-chemical characteristics of ND formulations

397

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

405 The TEM image (Figure 3) showed the spherical morphology and the core shell structure of NDs.
 406 NDs were able to load ACV in a good extent with an encapsulation efficiency of 96.6%. The loading
 407 capacity of ACV-loaded NDs was of 2%. The physical stability of ACV-loaded NDs stored at 4 °C
 408 was confirmed up to 2 months. Indeed, no significant changes in their physico-chemical parameters
 409 and in the ND ACV concentration (99.90 % of the initial ACV content) were observed.

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

413 The ACV complexation with SBE- β -CD resulted in an increase of its apparent solubility and favored
 414 the drug diffusion in the receiving phase. A prolonged *in vitro* release profile with no initial burst
 415 effect was observed for ACV from NDs, indicating that it was not weakly adsorbed on the ND surface
 416 but the complex is incorporated within the chitosan chains of the shell. About 34 % of ACV was
 417 released from the NDs after 6 hours. This prolonged *in vitro* release kinetics was also observed for

418 others ACV loaded nanoparticulate systems. For example, ACV encapsulation in carboxylated
419 cyclodextrin-based nanosponges, thanks to the presence of carboxylic groups besides the cyclodextrin
420 cavities in the polymer matrix, provided the sustained release of the drug over time (Lembo et al.,
421 2013).

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

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

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

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

449 The antiviral effect of ACV-loaded ND was further confirmed by the yield reduction assay, a stringent
450 test that allows multiple cycles of viral replication to occur before measuring the production of
451 infectious viruses. As reported in Figure 5B, 14.8 μ M and 3.7 μ M concentrations of ACV-loaded ND

452 totally inhibited the viral titers. Furthermore, this assay confirmed the higher antiviral potency of
453 ACV-loaded ND than that of free Acyclovir against HSV-2 infection. In particular, IC₅₀ values were
454 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
455 μM) for free acyclovir. The IC₅₀ values for plain acyclovir was similar to previously reported values
456 (Visalli et al., 2015). No antiviral activity was exerted by the blank ND at tested doses (data not
457 shown).

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

462

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

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

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

480 **Conclusions**

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

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

488

489

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494

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689 **Figure legends**

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

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

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

696 **Figure 4.** *In vitro* release kinetics of acyclovir from ACV-loaded NDs. The results represent the mean
697 \pm SD (n = 3).

698 **Figure 5. Panel A.** Antiviral activity of acyclovir (ACV), acyclovir loaded nanodroplets (ACV-ND)
699 and plain nanodroplets (ND) against HSV2, as determined by plaque reduction assay. ACV-loaded
700 ND was active against HSV-2 infection in a dose-response manner with IC50 value significantly
701 lower than free ACV (0.32 μ M and 0.89 μ M, respectively; p < 0.01, as determined by F-test). Notably,
702 blank ND exhibited weak antiviral activity only at high doses, and its IC50 value was not assessable.

703 **Panel B.** Effects of acyclovir-loaded ND on multiple replicative cycles of HSV-2, as determined by
704 virus yield reduction assay. Vero cells were infected at a multiplicity of infection (MOI) of 0.01 and
705 then exposed to serial drug concentrations. Virus titers in the supernatants of cell cultures were
706 determined by standard plaque assay. Viral titers (expressed as plaque-forming units, PFU/mL) are
707 shown as mean plus standard error of the mean for three independent experiments (* p<0.05; ***
708 p<0.001, Student's t-test). (UT, untreated).

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

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

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