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Synthesis and *in vitro* evaluation of cyclodextrin hyaluronic acid conjugates as a new candidate for intestinal drug carrier for steroid hormones

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ABSTRACT

Steroid hormones became increasingly interesting as active pharmaceutical ingredients for the treatment of endocrine disorders. However, medical applications of many steroidal drugs are inhibited by their very low aqueous solubilities giving rise to low bioavailabilities. Therefore, the prioritized oral administration of steroidal drugs remains problematic. Cyclodextrins are promising candidates for the development of drug delivery systems for oral route applications, since they solubilize hydrophobic steroids and increase their rate of transport in aqueous environments. In this study, the synthesis and characterization of polymeric β-cyclodextrin derivates is described, which result from the attachment of a hydrophilic β-CD-thioether to hyaluronic acid. Host-guest complexes of the synthesized β-cyclodextrin hyaluronic acid conjugates were formed with two poorly soluble model steroids (β-estradiol, dexamethasone) and compared to monomeric β-cyclodextrin derivates regarding solubilization and complexation efficiency. The β-cyclodextrin-drug (host-guest) complexes were evaluated *in vitro* for their suitability (cytotoxicity and transport rate) as intestinal drug carriers for steroid hormones. In case of β-estradiol, higher solubilities could be achieved by complexation with both synthesized β-cyclodextrin derivates, leading to significantly higher intestinal transport rates *in vitro*. However, this success could not be shown for dexamethasone, which namely solubilized better, but could not enhance the transport rate significantly. Thus, this study demonstrates the biocompatibility of the synthesized and characterized β-cyclodextrin derivates and shows their potential as new candidate for intestinal drug carrier for steroid hormones like β-estradiol.

1. Introduction

Steroid hormones, natural derivates of cholesterol, became increasingly interesting as active pharmaceutical ingredients (APIs) for the treatment of endocrine disorders like erectile dysfunction, sarcopenia, depression or climacteric syndrome (Cauley et al., 1995; S et al., 1995; Zitzmann et al., 2006; Cohen et al., 2003). Steroidal drugs are derived through synthetic modification of natural steroids, for example dexamethasone for the treatment of inflammation, immune response and allergies (Meikle and Tyler, 1977). Medical applications of

Abbreviations: 1, Heptakis-6-(2,3-dihydroxypropyl)thio-6-deoxy-β-CD; 2, Mono-6-deoxy-6-(3-(((2-aminoethyl)carbamoyl)oxy)-2-hydroxypropyl)thio-hexa-6-deoxy-6-(2,3-dihydroxypropyl)thio-β-CD; 3, Monoaminoethylcarbamat-hepta-thioglyceryl-β-CD-hyaluronic acid conjugate; API, active pharmaceutical ingredients; Caco-2, human colon carcinoma cell line; CCM, cell culture medium; CD, cyclodextrin; CDI, carbonyldiimidazole; CDMT, 2-Chloro-4,6-dimethoxy-1,3,5-triazine; CD-P, cyclodextrin polymers; D, dexamethasone; DMEM, Dulbecco's Modified Eagle Medium; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DDS, drug delivery system; d.s., degree of substitution; E, β-estradiol; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GIT, gastrointestinal tract; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ^{1}H-NMR, ^{1}H-nuclear magnetic resonance; HP-β-CD, hydroxypropyl-β-CD; HPLC, high performance liquid chromatography; HSES, heptakis-6-sulfoethylsulfanyl-6-deoxy-β-CD; HT29-MTX-E12, mucus-secreting goblet cells; K_D, dissociation constant; K_S, equilibrium constant; NMM, N-Methylmorpholine; PES, polyethersulfone; PLGA, poly(lactide-glycolide); TEER, transepithelial electrical resistance; WST-1, water-soluble tetrazolium salt 1

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many steroidal APIs are inhibited by their very low aqueous solubilities giving rise to low bioavailabilities. If only a small part of an API is taken up into the body, the dosage of the API becomes uncertain. Excess of applied hydrophobic API might even cause over-dosage in case of accidental unfavorable conditions (Ahmed et al., 1988; Vom Saal et al., 1997). Since oral administration remains problematic, transdermal administration appears to be the better choice up to now. However, transdermal application might lead to skin irritation and shows low efficacy (An et al., 2003). Since a great part of steroidal API is taken up by the liver through the first pass effect, high doses of the API have to be employed. Again, administration of these high doses is accompanied by the risk of over-dosage, which might cause prostate or liver cancer (Ahmed et al., 1988; Vom Saal et al., 1997). The previously addressed aspects impede the oral application, which is considered to be the most user-friendly and usually prioritized method for application of drugs.

Drug carriers have to be developed for the necessary improvement of bioavailability of steroidal APIs to both solubilize a hydrophobic API and to protect it from excretion by the kidney and metabolization by the liver (Takakura and Hashida, 1996; Hariharan et al., 2006). Encapsulation of steroids into nanoparticles, e.g. nanoparticles from poly (lactide-glycolide) (PLGA), leads indeed to slow release of the steroid hormone estradiol within 2-7 days, but the relative fast release at the beginning (so-called burst release) remains as a disadvantage (Hariharan et al., 2006). Also nanoparticles may provoke side effects like inflammation and do not improve slow diffusion of a steroid to the target (De Jong and Borm, 2008).

A better choice for oral delivery of steroids might be the molecular encapsulation into cyclodextrins (CDs), because CDs solubilize steroids and increase their rate of transport in aqueous environments (Pitha et al., 1986). CDs are cyclic, α(1-4)-linked oligomers of glucose, obtainable by enzymatic degradation of starch (Cyclodextrins, 2000). Ring sizes of six, seven and eight glucose units (named α-, β-, γ-CDs) are produced in industrial scale in pharmaceutical grade (Angew, 1994). CDs are water-soluble, bio-degradable, non-toxic materials (Wenz, 1994; Irie and Uekama, 1997) and represent an option for improving the solubility and increasing the bioavailability in the body. Only β-CD is known to show adverse side effects at high concentrations (3 mM) caused by hemolysis triggered by extraction of cholesterol from the erythrocyte cell walls (Irie et al., 1982; Kiss et al., 2010). In general, CDs are known to complex smaller hydrophobic molecules, so-called guest molecules within their hydrophobic cavities (Wenz, 1994). The supramolecular structures formed are referred to as host-guest complexes. The complex and both constituents are in dynamic equilibrium in aqueous solution (Loftsson and Brewster, 1996). The stability of the complex is quantified by the equilibrium constant K_s or its reciprocal value the dissociation constant K_D according to the law of mass action (Connors, 1997; Connors and Pendergast, 1984; Higuchi and techniques, 1965). Values of the dissociation constant K_D range between 1 μM and 100 mM mostly between 100 μM and 1 mM (Houk et al., 2003; Rekharsky M and Inoue, 1998). The main driving force for complex formation in water is the hydrophobic interaction (Biedermann et al., 2014; Schneider, 2017).

Hydrophilic derivatives of CDs are often favorable to native CDs because solubility of steroid complexes of native CDs in water are often very low. Methyl-, hydroxypropyl- and sulfobutyl-derivatives of β-CD are the most commonly used statistically substituted CDs for the solubilization of steroids (Rajewski et al., 1995; Szente and Szejtli, 1999). Substitution of the secondary site of β-CD is known to reduce binding potential, while substitution at the primary site of β-CD can lead to an improvement (Wenz, 2012). Complete thioether substitution at all primary positions of β-CD gives rise to highly water-soluble hosts showing high binding potentials towards steroids and steroidal drugs. These host compounds had been synthesized by regio-selective nucleophilic displacement reactions of heptakis-6-bromo-6-deoxy-β-CD with hydrophophilic thiols (Wenz et al., 2008; Thiele et al., 2011).

Cyclodextrin polymers (CD-P) might be advantageous to monomeric CDs, because of slower renal clearance and lower toxicities (Bentsen et al., 1989; Necas et al., 2008; Auzély-Velty, 2011). The retention time of CD-Ps in the body would increase leading to an increased bioavailability of the incorporated active substance. Thus, CD-Ps seem to be promising drug delivery system (DDS) candidates for oral application of steroid hormones.

Furthermore, uptake of drug loaded CD-P into cells might be facilitated by endocytosis (Duncan et al., 1981; Gaspar and Duncan, 2009). CD-Ps can be synthesized by polymerization of CD monomers as well as by covalent linkage of monofunctional CDs, especially 6-mono-amino-6-deoxy-β-CD, to polymers (Auzély-Velty, 2011; Yamaguchi et al., 2011). Biodegradable polymer backbones, like starch or hyaluronic acid are especially advantageous, because drug carriers should not remain permanently in the human body (Auzély-Velty, 2011). Covalent linkage between CD and polymer was achieved by amide coupling (Aoki et al., 2003), reductive amination (Ramírez et al., 2006) and click-reaction (Nielsen et al., 2010).

The oral route always represents the prioritized route of application for therapeutic applications, but the limitation is a successful overcoming of the intestinal barrier. Therefore, the requirement for a DDS used for oral therapies is the ability to overcome the intestinal barrier, besides solubility, bioavailability and low toxicity. Promising DDS candidates have to be proven regarding their mode of action and toxic side effects. In a first step, *in vitro* studies with suitable models for the organ of interest are necessary tools for testing the functionality and suitability of unknown DDS for their further development.

In this study, the attachment of a hydrophilic β-CD-thioether to hyaluronic acid leading to a water-soluble CD-P and its *in vitro* evaluation as a new candidate for intestinal drug carrier for steroid hormones is described.

2. Materials and methods

The steroidal drugs β-estradiol and dexamethasone were purchased in pharmaceutical quality from Sigma-Aldrich (St. Louis, USA).

The cell line Caco-2, a human adenocarcinoma with epithelial morphology, was obtained by DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). HT29-MTX-E12 cells, representing mature mucus-secreting goblet cells by differentiation of HT29 cell line in presence of methotrexate, as well as fetal bovine serum were obtained by Sigma-Aldrich, Steinheim, Germany.

Cell culture medium (DMEM high glucose, 4.5 g/L), penicillin/streptomycin and all other solvents, buffer and supplements were purchased from ThermoFisher Scientific, Darmstadt, Germany.

Cell culture consumables were purchased from VWR, Darmstadt, Germany. Transwell® inserts with a pore size of 3.0 μm and a growth area of 1.12 cm^2 were purchased from Corning®, Amsterdam, Netherlands. WST-1 cell proliferation assay reagent was purchased from Roche, Mannheim, Germany.

2.1. Synthesis of CD derivates

2.1.1. Heptakis-6-(2,3-dihydroxypropyl)thio-6-deoxy-β-CD **1**

The synthesis of 1 was performed as previously described (Schwarz et al., 2017). 10.00 g (6.35 mmol, 1 eq.) Heptakis (6-deoxy-6-bromo)-β-CD, which was synthesized according to Chmurski et al. (Chmurski and Defaye, 2000), were dissolved in 80 mL DMF under nitrogen and 9.6 mL (111 mmol, 2.5 eq./glucose) 1-thioglycerol and 15.4 mL (111 mmol, 2,5 eq./Glucose) triethylamine were added. The solution was stirred at 60°C for 3 days. After cooling to room temperature the solution was concentrated down to 40 mL. The crude product was precipitated from 600 mL cold acetone, filtered and recrystallized from H_2O/acetone 1:1 (v/v). The product was dried in vacuum. Yield: 7.37 g (4.18 mmol, 66 %), TLC: R_f = 0.72 (isopropanol/NH_4Ac 2:1), ^{1}H-NMR: δ/ppm (DMSO-d_6, 400 MHz) = 5.89 (d, 7H,

^3J = 6.4 Hz, OH-2), 5.80 (s, 7H, OH-3), 4.87 (br s, 7H, H-1), 4.68 (d, 7H, ^3J = 4.8 Hz, OH-8), 4.51 (t, 7H, ^3J = 5.6 Hz, OH-9), 3.80 (br s, 7H, H-5), 3.62-3.57 (m, 14H, H-3/8), 3.46-3.40 (m, 7H, H-4), 3.38-3.30 (m, 21H, H-2/9), 3.04-3.01 (m, 7H, H-6a), 2.91-2.89 (m, 7H, H-6b), 2.73-2.66 (m, 7H, H-7a), 2.60-2.53 (m, 7H, H-7b). ^{13}C-NMR: δ/ppm (DMSO-d^6, 100 MHz) = 102.1 (C-1), 84.2 (C-4), 72.6 (C-3), 72.3 (C-2), 71.4 (C-5), 71.3 (C-8), 64.6 (C-9), 36.7 (C-7), 34.0 (C-6).

Mono-6-deoxy-6-(3-(((2-aminoethyl)carbamoyl)oxy)-2-hydroxypropyl)thio-hexa-6-deoxy-6-(2,3-dihydroxypropyl)thio-β-CD **2**

7.50 g (4.25 mmol, 1 eq.) **1** and 1.03 g (6.38 mmol, 1.5 eq.) carbonyldiimidazole (CDI) were dissolved under nitrogen in 50 mL DMF. The solution was stirred at 60°C for 90 min and 1.02 g (17.00 mmol, 4 eq.) ethylendiamine were added. Stirring was continued for 68 h at 60°C. After cooling to room temperature the solution was concentrated down to 30 mL. The crude product was precipitated from 500 mL cold ethanol, filtered and washed. The product was dried under vacuum. Yield: 7.11 g (3.84 mmol, 90 %), DC: R_f = 0.49 (isopropanol/NH_4Ac 2:1), ^{1}H-NMR:δ/ppm (DMSO-d^6, 400 MHz) = 5.90 (br s, 7 H, OH-2), 5.81 (br s, 7 H, OH-3), 4.87 (s, 7H, H-1), 4.70 (br s, 6 H, OH-8), 4.53 (br s, 6 H, OH-9), 3.81 (br s, 7H, H-5), 3.62-3.58 (m, 14H, H-3/8), 3.47-3.43 (m, 7H, H-4), 3.34 (br s, 23 H, H-2/9/11/12), 3.05-3.02 (m, 7H, H-6a), 2.91-2.87 (m, 7H, H-6b), 2.73-2.66 (m, 7H, H-7a), 2.60-2.54 (m, 7H, H-7b)., ^{13}C-NMR: δ/ppm (DMSO-d^6, 100 MHz) = 145.0 (C-10), 102.1 (C-1), 84.2 (C-4), 72.6 (C-9), 72.3 (C-8), 71.4 (C-5), 71.3 (C-3), 64.7 (C-2), 64.6 (C-11/12), 36.8 (C-7), 33.9 (C-6); MALDI-TOF-MS: m/z_{exp} = 1873.77 [M+Na^+] (1-time modified), m/z_{theo} = 1873.52 [M+Na^+] (1-time modified), m/z_{exp} = 1959.82 [M+Na^+] (2 times modified), m/z_{theo} = 1959.56 [M+Na^+] (2 times modified).

2.1.2. Monoaminoethylcarbamat-hepta-thioglyceryl-β-CD-hyaluronic acid conjugate **3**

600 mg (1.58 mmol, 1 eq.) hyaluronic acid (27 kDa) were dissolved in 25 mL H_2O. 696 mg (3.96 mmol 2.5 eq.) 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and 1.2 mL (6.34 mmol, 4 eq.) NMM were dissolved in 3 mL acetonitrile and 120 µL H_2O. The mixture was added to the hyaluronic acid solution. After stirring at room temperature for 2 h 5.54 g (2.38 mmol, 1.5 eq.) **2** were added. Stirring was continued for 42 h at room temperature. Then 50 mL H_2O and cation exchanger (Amberlyst 15 hydrogen form) was added and the solution was shaked for 30 min. After prefiltration the solution was first purified by cross-flow filtration against a 10 kDa polyethersulfon membrane with 0.05 M NaCl solution. Then the cross flow filtration was continued with water and the retentate was lyophilized. Yield: 415 mg (0.74 mmol, 46 %), ^{1}H-NMR: δ/ppm (D_2O, 400 MHz) = 4.97 (br s, 0.72H, H-1/1‘), 4.38-4.29 (m, 2H, H-01/01‘), 3.87-2.62 (m, 18.9H, H-2/2‘ - H-9/9‘, H-11‘/12‘,H-02/02‘ – H-06), 1.86 (s, 3H, H-08).

2.2. Characterization of CD derivates

For the characterization of CD derivates, NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer at 298 K using the solvent peaks as internal references.

MALDI-TOF-MS analysis were performed on a 4800 TOF/TOF Analyzer mass spectrometer (Applied Biosystems) in positive reflector mode using a pulsed 200 Hz solid state Nd:YAG laser with a wavelength of 355 nm.

2.3. Solubility measurements

To generate the phase solubility isotherm solutions of **1** or **3** (0-1000 µM, 1 mL) in cell culture medium (CCM) without fetal bovine serum (FBS) were stirred with an excess of steroidal drug (β-estradiol or dexamethasone) at 25°C for 18 hours. The resulting solutions were filtered through a mixed cellulose syringe filter (Kinesis group, 0.45 µm). The steroid was extracted from the aqueous phase three times with tert-butylmethylether. The combined organic phases were concentrated by using a SpeedVac (Thermo Electron Corporation, 35°C, 0.1 bar, 30 min). The resulting residues were dissolved in eluent (methanol/phosphate buffer 70/30 (0.5 % triethylamine, pH 6.38) (Patel and Meshram, 2015). A LiChrospher 100 RP-18 5 µm 125×4 mm column with a Spectraflow 501 UV-detector (flow: 1 mL/min, injection volume: 20 µL) were used to determine the concentration of the steroids via HPLC measurements.

2.4. Drug carrier formation

For the transport studies complexes of CD derivates **1** or **3** and the steroids (β-estradiol or dexamethasone) were dissolved in 10 mM HEPES buffer (0.154 M NaCl, pH 7.4). The amount of the steroids were dependent of the slope of the corresponding solubility isotherms. The complexes were stirred at 25°C for 20 hours and lyophilized. The freeze dried DDS were dissolved in DMEM cell-medium to generate a total steroid concentration of 1 mg/mL (stock solution). After incubation at room temperature for one hour the stock solution was diluted with DMEM (CCM) to a steroid concentration of 10 µg/mL.

2.5. Quantification of steroids via HPLC

After the 3h-incubation of the DDS at the apical side of the intestinal *in vitro* barrier, the steroids in the cell medium (apical and basolateral side) and inside the harvested cells were extracted three times with tert-butylmethylether. The combined organic phases were concentrated by using a SpeedVac (35°C, 0.1 bar, 30 min). The resulting residues were dissolved in eluent and the concentration of the steroids were determined via HPLC measurements as previously described (Schwarz et al., 2017).

2.6. Cell culture

Both cell lines, Caco-2 and HT29-MTX-E12 were cultured in DMEM high glucose (4.5 g/L) supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-Glutamine, 1 % (v/v) penicillin/streptomycin, 1 % (v/v) non-essential amino acids in a humidified incubator at 37°C with 5 % CO_2 atmosphere.

2.7. Preparation of CD complexes for in vitro studies

The lyophilized CD drug complexes and the steroidal drug representative without CD complexation were diluted in CCM, solubilized on the shaker for one hour at room temperature and filtrated with a 0.2 µm polyethersulfone (PES) filter to remove insoluble remains. The solutions were further diluted in fresh CCM to an estimated steroid concentration of 0.4, 10 and 100 µg/ml. These solutions were used for the *in vitro* cell viability assay and the *in vitro* transport studies. Additionally, the concentration of each applied solution were measured by HPLC to determine the solubility of the steroid.

2.8. Cell viability assay

To determine the biocompatibility of the synthesized CD drug complexes, *in vitro* co-cultures of Caco-2 and HT29-MTX-E12 cells (seeding ratio 9:1) were seeded in 96 well plates at a density of 10.000 cells/well. After 24 hours, the co-culture model was incubated with 0.4, 10 and 100 µg/ml of the CD drug complexes or the free steroids for three hours. As untreated control, fresh CCM was used. After the incubation time the viability of the cells was determined by WST-1 cell proliferation reagent according to manufacturer's operating instruction. The absorption was determined at 450 nm (reference wavelength 690 nm). CCM containing the WST-1 reagent was measured as background for blank correction. Viability of the untreated control was set as 100 %.

Transport studies and measurement of the transepithelial electrical

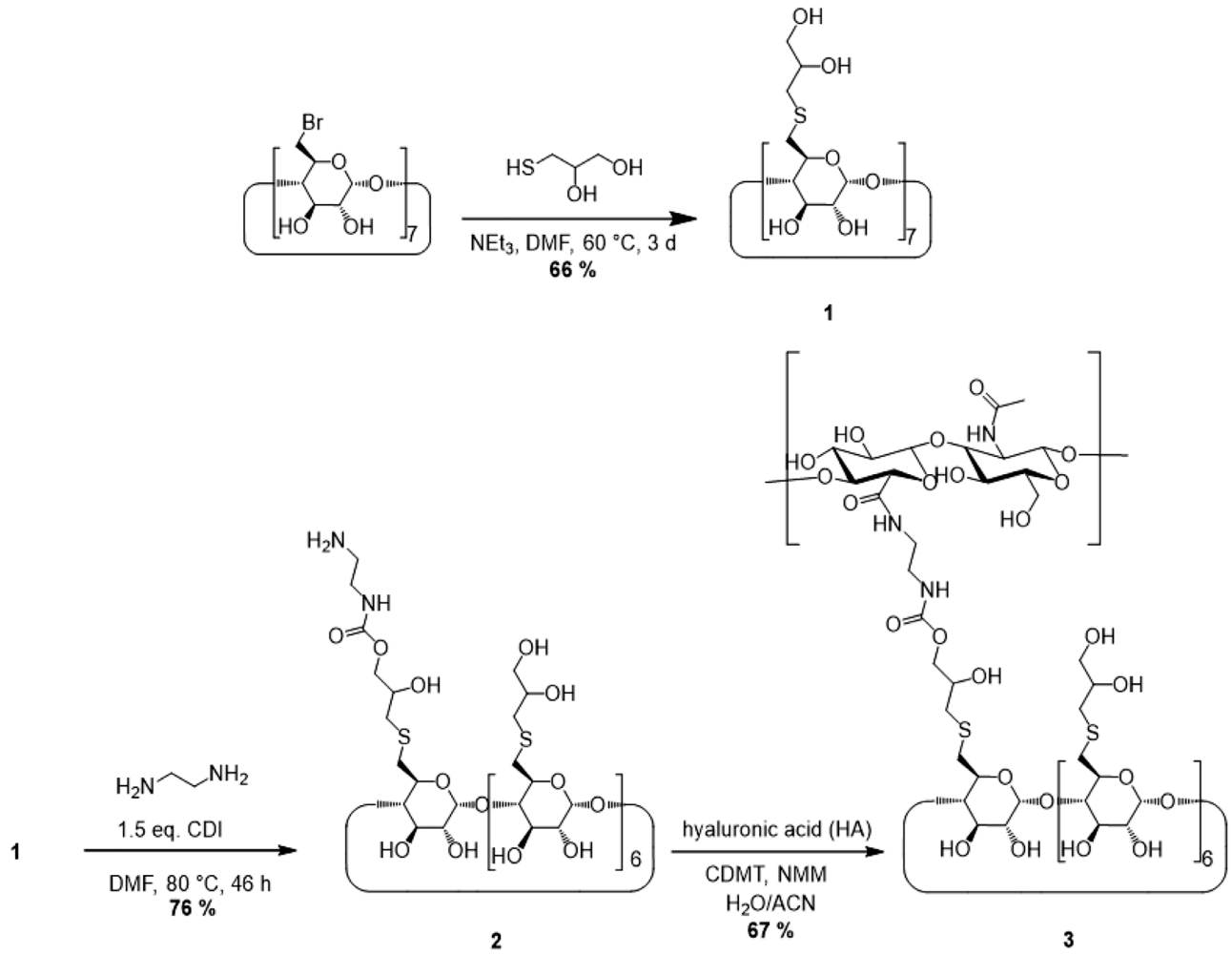

Fig. 1. Reaction scheme for the synthesis of the β-CD hyaluronic acid polymer 3.

resistance

For the *in vitro* transport studies of the CD drug complexes over the gastrointestinal (GIT) barrier, co-cultures of Caco-2 and HT29-MTX-E12 were seeded on porous cell culture inserts at a density of 1.0×10^5 cells/insert (seeding ratio 9:1) and cultured for 21 days. Medium was exchanged every second day (apical volume 0.5 ml, basolateral volume 1.5 ml). The transepithelial electrical resistance (TEER) of the *in vitro* co-culture was measured to monitor the development and maintenance of an intact epithelial cell barrier. Measurements were performed every 24 hours over the growth period of 21 days and during the transport process once an hour with the cellZscope® device (nanoAnalytics, Münster, Germany) at 37°C. Before starting the CD exposure, the flux of the paracellular marker fluorescein sodium salt was determined. 5 µM fluorescein sodium salt in cell culture medium was applied on top of the culture and incubated for 3 hours at 37°C. Samples were collected in the basolateral supernatant and the fluorescence intensity (ex/em) was measured at 485 nm/535 nm with an Infinite F200 multi-plate reader (Tecan).

On day 21, the co-culture model was exposed on the apical side with the CD drug complexes or free steroids (10 µg/ml). For a better solubility of the CD drug complexes after a successful transport, 42.4 µg/ml heptakis-6-sulfoethylsulfanyl-6-deoxy-β-CD (HSES) was applied on the basolateral side. After three hours, the apical and basolateral supernatants were collected for HPLC analysis. The cells were washed with phosphate buffered saline and detached from the membrane by adding 0.05% Trypsin-EDTA into the apical and basolateral compartment for 30 min at 37°C. The cells were resuspended in cell lysis buffer (150 mM sodium chloride, 1% NP-40 (Tergitol), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM TRIS) and lysed in a ultrasonic water bath (Elmasonic, Singen, Germany) for 10 seconds.

2.9. Light microscopic investigation of in vitro intestinal co-culture

After 21 days, *in vitro* intestinal co-cultures were prefixed overnight in modified Karnovsky medium at 4°C. Punch biopsies (ø 3mm) were taken and washed three times with 0.2 mol/L sodium cacodylate buffer for 10 min, and postfixed with 1% (wt/vol) OsO_4 in aqua bidest for 45 min. Samples were dehydrated in ethanol and embedded in Epoxy resin (Plano, Wetzlar, Germany). Polymerization was carried out overnight at 60°C according to Luft (Luft, 1961). Semi-thin sections (300nm) were cut with a Ultracut S microtome (Leica, Wetzlar, Germany) with a 35° diamond knife (Diatome, Nidau, Switzerland). Sections were transferred with a platin loop to microscope slides and dried. Staining of the sections was carried out with Löfflers Methylen blue solution (Carl Roth, Karlsruhe, Germany) for 8 min at 40°C. After rinsing with tap water, samples were dried and embedded with Roti-Histomount (Carl Roth, Karlsruhe, Germany). After hardening overnight the samples were investigated by a light microscope (Leica DM-LS, Leica, Wetzlar, Germany) and images were taken with a coupled CCD camera (Tucsen H-Series, Tucsen Photonics, Fuijan China).

2.10. Statistical analysis

The data analysis was performed with Microsoft Excel and the

results were illustrated using OriginPro 2016 G. The data were statistically analyzed by unpaired Student´s t-test. A value of $p \leq 0.05$ was accepted as statistically significant. Non-significant values were not visualized by any sign.

3. Results and discussion

3.1. Synthesis of CD derivates

Due to the better binding constants of thioether β-CD derivatives compared to commercially available β-CD derivatives (e.g. HP-β-CD) (Schwarz et al., 2017), a thioether-β-CD polymer was synthesized in this study. The use of hyaluronic acid as a biopolymer prevents an uncontrolled uptake of the CD derivatives in the organism. The binding constants of steroids with thioether β-CD derivatives (e.g. Heptakis-6-(2,3-dihydroxypropyl)thio-6-deoxy-β-CD **1**) was described previously (Schwarz et al., 2017). The conjugation of **1** to hyaluronic acid is illustrated in Fig. 1. Thioether **1** is obtained from per-6-bromo-6-deoxy-β-CDs by nucleophilic displacement reaction of thioglycerol. To achieve a covalent binding of **1** on the carboxylic acid group of hyaluronic acid, an amino group on the primary side of **1** is necessary (Markenstein et al., 2014). Thus, the amino group was introduced in a mono functionalization, ethylenediamine was bound on the hydroxyl group of the thioglycerol spacer of **1** with 1.5 eq. CDI. In the ^{1}H-NMR-spectrum of **2,** the ethyl group of the introduced aminoethylcarbamat group is visible on 3.34 ppm (Fig. S3). Additionally, a MALDI-TOF-MS analysis of **2** showed, that the product obtained is mostly a mono-substituted derivate. Only a small amount of disubstituted product could be found. In the last step of the synthesis, **2** was bound to hyaluronic acid via amid coupling reaction with CDMT. For the isolation of CD-hyaluronic acid polymer **3** from unbound CD derivative **2**, the crude product was purified with a 10 kDa cross-flow PES membrane via cross-flow-nanofiltration.

3.2. Characterization of the CD hyaluronic acid polymer *3*

The degree of substitution (d.s.) of CD hyaluronic acid polymer **3** was determined via ^{1}H-NMR-spectroscopy (Fig. 2). The characteristic signals in the NMR spectrum are the anomeric proton of the CD (H-1/1') at 4.97 ppm, the anomeric proton of the hyaluronic acid (H-01/01') at 4.38-4.29 ppm and the three methyl protons of the acetamide group of the hyaluronic acid (H-08) at 4.97 ppm. The integral of the acetamid protons was normalized to three, so the number of CD derivatives per repeat unit of the hyaluronic acid is shown on the integral of the anomeric proton of the CD. The integration of 0.62 indicates a d.s. = 0.09.

Solubility isotherms were measured to quantify the differences in solubilization of the tested CD-steroid complexes. In contrast to the CD-β-estradiol complex (**1** + E), the solubility isotherm of the CD-dexamethasone complex (**1** + D) linearly increased in solubility of dexamethasone with increasing CD concentration (Fig. 3). CD-β-estradiol complexes showed a saturation effect with increasing CD concentrations. A plateau of solubility was reached at 500 µM, in case of **1** + E ($[G]_{max}$ = 0.31 mM) as well as in case of **3** + E ($[G]_{max}$ = 0.34 mM) (Fig. 3A and B). The slope of the solubility isotherms of the CD-β-estradiol complexes is much higher than the slope of the CD-dexamethasone complexes. The CD polymer **3** forms a higher number of complexes with β-estradiol molecules than with dexamethasone molecules.

Due to the difference in solubility, β-estradiol (7.3 µM) and dexamethasone (183.8 µM) were used as test steroids for studying the complexation efficiency and the binding constant in cell culture medium (CCM) (Table 1). Compared to dexamethasone (0.45 and 0.35), the complexation efficiency obtained by **1** and **3** were higher for β-estradiol (1.56 and 2.45) (Table 1). Also the binding constant was increased for β-estradiol. Compared to dexamethasone the binding constant of β-estradiol is 88-times higher for **1** and 175-times higher for **3** (Table 1). Thus, CD hyaluronic acid polymer was expected to be the more efficient DDS for the transport of β-estradiol than for dexamethasone.

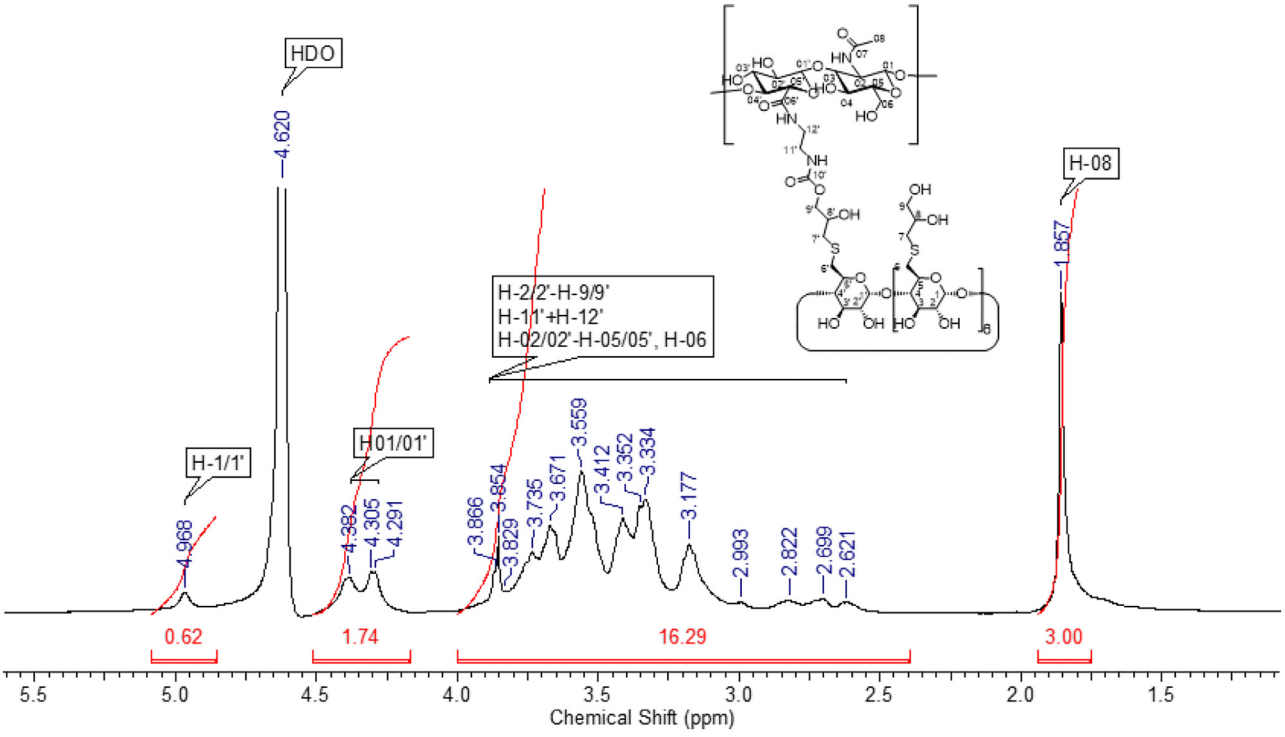

Fig. 2. ^{1}H-NMR-spectrum of 3 (D2O, 400 MHz).

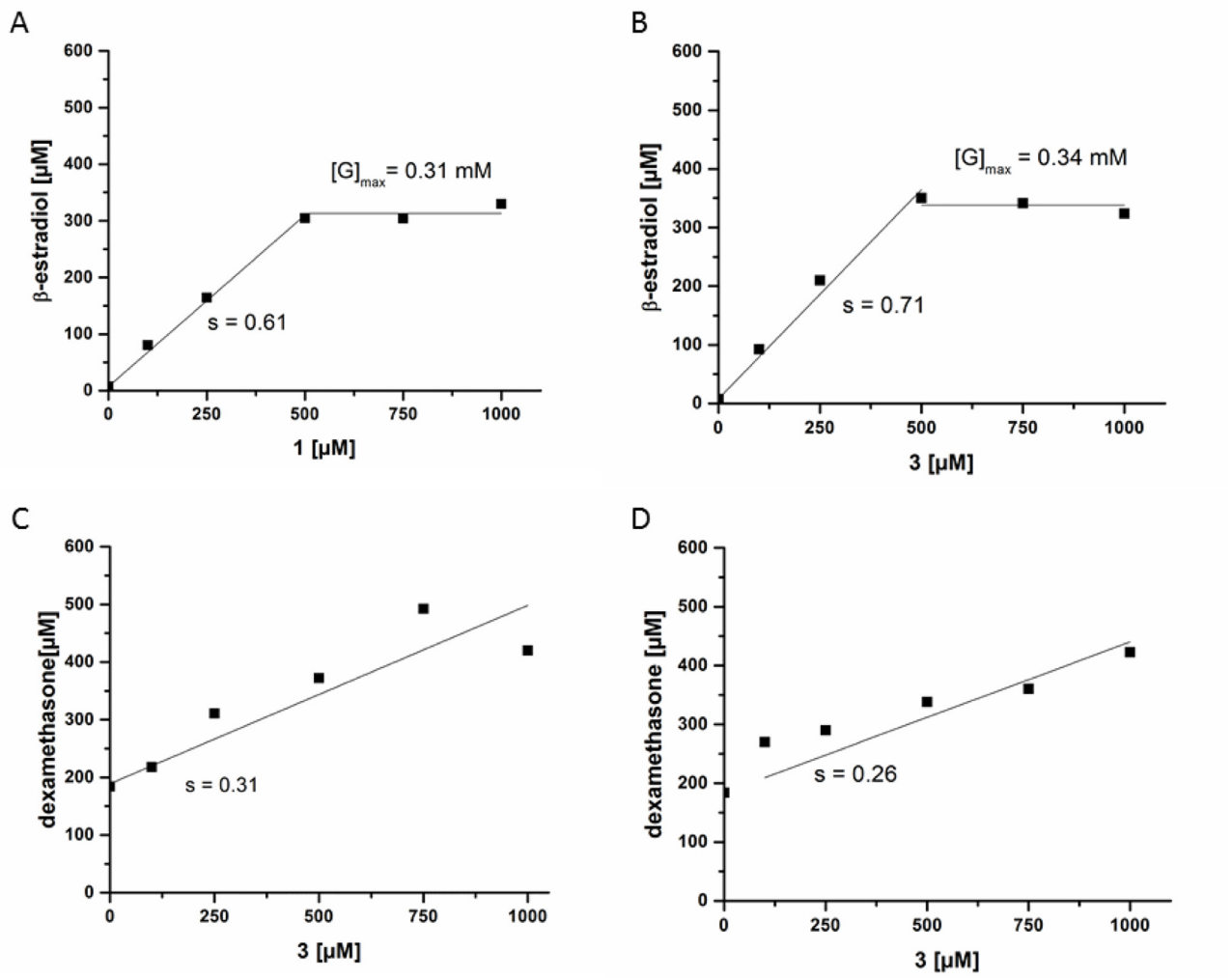

Fig. 3. Solubility isotherms of the steroids β-estradiol (E) and dexamethasone (D) in cell culture medium (without FBS) with CD monomer 1 and CD hyaluronic acid polymer 3. A: Monomeric CD β-estradiol complex (1 + E); B: Polymeric CD β-estradiol complex (3 + E); C: Monomeric CD dexamethasone complex (1 + D), D: Polymeric CD dexamethasone complex (3 + D).

3.3. In vitro evaluation of the CD derivates

*3.3.1. Biocompatibility of β-estradiol loaded CD derivates **1** and **3***

The biocompatibility of the β-estradiol-CD derivates as well as the free β-estradiol was investigated in an *in vitro* intestinal co-culture model (Caco-2 and HT29-MTX-E12 in a physiological ratio of 9:1), representing the barrier forming enterocytes as well as the mucus-secreting goblet cells. The cells were exposed to three concentrations (0.4, 10 and 100 µg/ml) for three hours, according to the average GIT passage time *in vivo* after oral administration. The results of the WST1-assay verify that the viability of the cells is not affected by any of the tested compounds in any of the applied concentrations (Fig. 4). No change of relative viability, based on the metabolic activity of the cells, was induced by free β-estradiol or the β-estradiol-CD complexes **1** + E and **3** + E up to 100 µg/ml. Based on these results, the transport studies could be performed with a therapeutically relevant steroid concentration of 10 µg/ml excluding toxic effects induced by the test compounds. In the study of Markenstein et al. 2014, the monomeric CD **1** was cytotoxic to Caco-2 cells after two hours in a concentration of 25 mg/ml and the methylated CDs conjugated to the HES backbone (polymeric) exhibited significantly lower cytotoxicity than the corresponding monomeric CD derivatives (Markenstein et al., 2014). This difference can be explained by noticeable lower test concentrations in this study (2500-fold lower concentration) and the use of a co-culture model, in which the produced mucus of the HT29-MTX-E12 cells has a protective function for the enterocytes, reflecting the situation *in vivo*.

3.3.2. Measurement of the transepithelial electrical resistance (TEER) during the growth phase of 21 days

The intestinal co-culture consisting of 90% Caco-2 and 10% HT29-

Table 1
Solubility of the tested steroids β-estradiol (E) and dexamethasone (D), complexation efficiencies and binding constant with the CD derivatives 1 and 3 in cell culture medium without FBS.

	solubility [µM]	complexation efficiency		binding constant [M^{-1}]	
		1	**3**	**1**	**3**
β-estradiol (E)	7.3	1.56	2.45	214260	335380
dexamethasone (D)	183.8	0.45	0.35	2444	1912

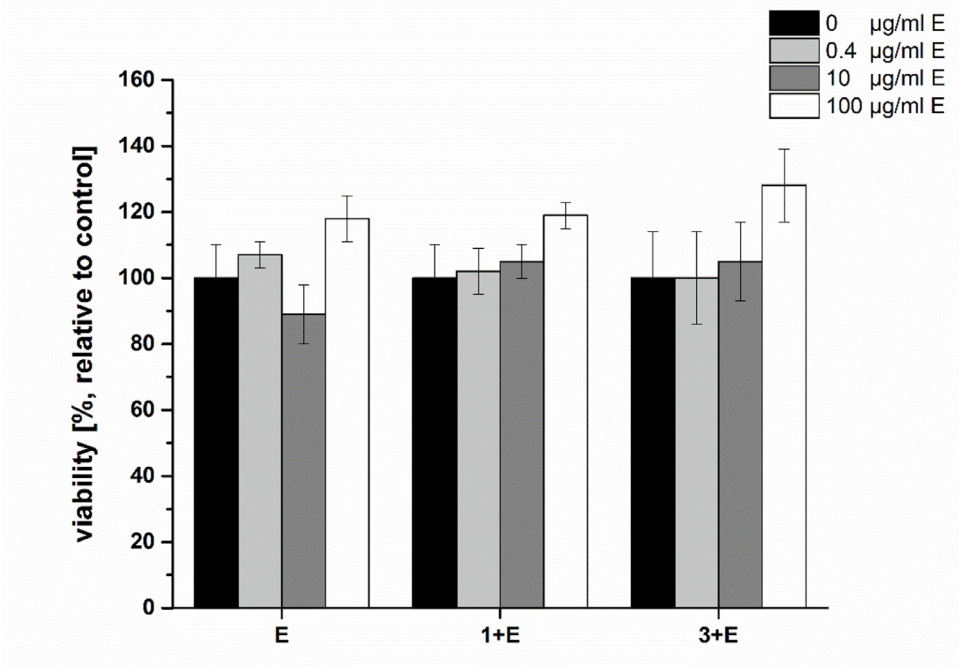

Fig. 4. Effect of free β-estradiol (E) and β-estradiol loaded CD derivates (1 + E, 3 + E) on cell viability of an intestinal *in vitro* co-culture model (90% Caco-2 cells/10% HT29-MTX-E12 cells). The co-culture was exposed for three hours with the test substances and analyzed via WST-1 assay. Untreated cells are set as 100% viability. The viability is presented as relative value to the untreated control (mean of three experiments ± SD).

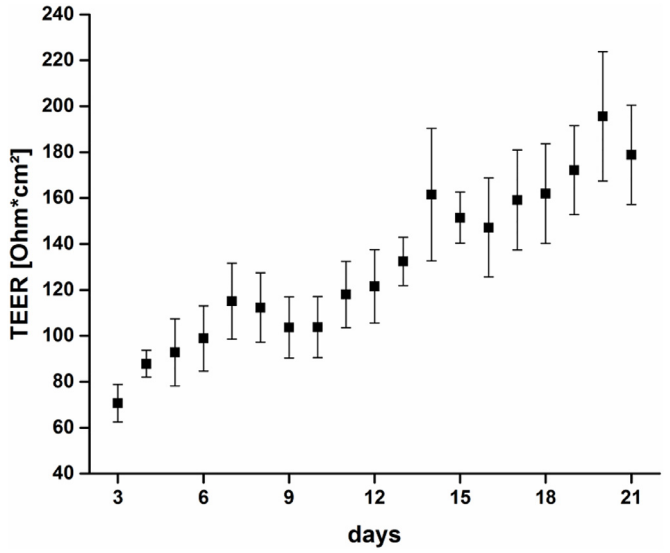

Fig. 5. Increase of the transepithelial electrical resistance (TEER) of the intestinal *in vitro* co-culture barrier model (90% Caco-2 cells/10% HT29-MTX-E12 cells) during 21 days cultivation. The *in vitro* model was cultivated for 21 days for the formation of tight junctions (intracellular connections) and of brush borders as well as for mucus production. The TEER values are presented as mean of three experiments ± SD.

MTX-E12 cells that was used to determine the transport of free β-estradiol, dexamethasone and the steroid loaded CD derivates **1** and **3** was prior cultured for 21 days on porous cell culture inserts to ensure the development of a cell layer with distinct barrier properties. Caco-2 cells representing the enterocytes, differentiate over that period of time and form apical brush borders (microvilli) on their cell surface and intracellular cell connections (tight junctions) even to adjacent HT29-MTX-E12 cells, resulting in an increase of transepithelial electrical resistance (TEER) from 70.7 ± 8.2 Ohm*cm^2 on day three to 178.8 ± 21.6 Ohm*cm^2 on day 21 (Fig. 5). In this differentiation period, HT29-MTX-E12 cells, mucus secreting goblet cells, form a closed mucus layer, which protects the cells from external factors and is essential for the barrier properties of the intestine (Cone, 2009). The TEER values of the co-culture in this study correspond with other studies in literature (Béduneau et al., 2014; Hilgendorf et al., 2000; Bazes et al., 2011). The transported amount of the paracellular marker sodium fluorescein after 3 hours at 37°C was negligible in comparison to empty inserts (Fig. S5). This result verifies the absolute tightness of the cellular barrier on day 21. To confirm the ratio 9:1 after the 21 days culture period, the co-culture was investigated by light microscopy, which enables the discrimination of the different cell types (Fig. S6). Caco-2 cells cultured alone, show a typical appearance. On the basal side, a dense stained region, where nuclei are located, is visible. On the apical side, a very weak stained region filled with granular matrix is visible (Fig.S6A). HT29-MTX-E12 cells culture alone, show a more dense stained cytoplasm, with some less dense stained areas (Fig.S6B). In the 90:10 co-culture of both cell types a discrimination between the seeded cell types is possible because of the specific morphological appearance of the HT29-MTX-E12 cells. The explicit dense stained cytoplasm, especially in the apical region of the cells, acts as a marker to identify this cell type in the co-culture model, indicated by red arrows and confirming the correct ratio of cells (Fig.S6C).

*3.3.3. Measurement of the transepithelial electrical resistance during exposure with β-estradiol loaded CD derivates **1** and **3***

To confirm the tightness of the barrier in the *in vitro* co-culture model, TEER was measured before and during the CD derivate exposure. The free steroid β-estradiol without CD derivates as well as the CD complexes **1** + E and **3** + E did not affect the integrity of the intestinal *in vitro* barrier within three hours in comparison to untreated cells. Therefore, a transport of the test compounds over an intact barrier is guaranteed (Fig. 6). The maintenance of barrier integrity are in line with the cytotoxicity results, showing that E, **1** + E and **3** + E are not cytotoxic to the intestinal co-culture and do not affect the tight junctions and thus do not lead to a damage of the intact cell barrier.

CD-based DDS candidates were already tested in several *in vitro* studies such as series of anticancer drugs, which had a better solubility (Cai et al., 2010), stability (Mognetti et al., 2012) and permeation through the intestinal barrier (Yavuz et al., 2010) after complexation with different CD derivates. Usually, for the determination of the CD DDS efficiency, monolayer cell cultures existing of single cell types, such as HeLa cervical carcinoma cells (Bilensoy et al., 2007; Barata et al., 2015), MCF-7 breast cancer cells (Bilensoy et al., 2007;

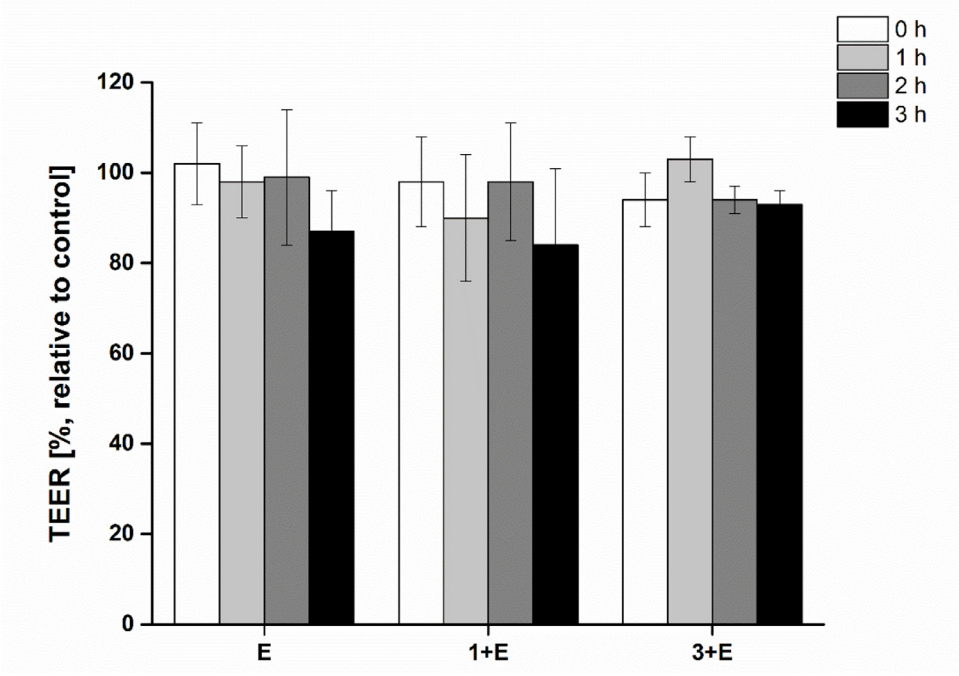

Fig. 6. TEER measurements of the intestinal *in vitro* co-culture barrier model (90% Caco-2 cells/10% HT29-MTX-E12 cells) during transport studies with free β-estradiol (E) and the drug-loaded CD derivates 1 and 3. The co-culture was exposed from zero to three hours with the formulations. Untreated cells are set as 100 % viability. The TEER values are presented as relative value to the untreated control (mean of three experiments ± SD).

Eid et al., 2011; Dhule et al., 2012) and hepatocellular carcinoma cells HepG2 (Lau et al., 2011; Cao et al., 2017) are used. In case of intestinal permeability studies, Ussing Chamber experiments with primary tissue (Agüeros et al., 2009; Mendes et al., 2018) or Parallel Artificial Membrane Permeability Assay (Delrivo et al., 2018; Paczkowska et al., 2018; Beig et al., 2013) have been performed to investigate the transport of CD drug complexes across the intestinal barrier. However, the most common approach to determine the permeability of drugs is the Caco-2 permeability assay, which has been performed in some studies with CD DDS (Mendes et al., 2018; Beig et al., 2013; Markenstein et al., 2014; Song et al., 2018; Malapert et al., 2018; Stappaerts et al., 2018). Nevertheless, this model has several limitations in comparison to the normal intestinal epithelium, which contains more than one cell type, not only enterocytes and Caco-2 cells do not produce mucus, which strongly influences the uptake of substances *in vivo* (Lea, 2015). For these reasons, a more physiological *in vitro* model for the intestinal barrier, consisting of enterocytes (Caco-2) and mucus-secreting goblet cells (HT29-MTX-E12) was used to determine the biological effectiveness of the synthesized CD complexes.

Drug delivery of β-estradiol via CD derivates **1** and **3** in presence and absence of FBS

To determine the solubility and permeation of free β-estradiol and β-estradiol via CD complexes, transport studies across the intestinal *in vitro* barrier were performed.

To investigate whether serum proteins contained in FBS have influences on the solubility and transport efficiency of CD complexes, the experiments were performed in presence and absence of FBS in the CCM (Fig. 7).

The transported amount of β-estradiol is significantly increased in CCM without FBS. In case of the free substance, the transported amount of steroid could be slightly enhanced from 0.23 µg to 0.34 µg in absence of FBS. In case of **1** + E, the transported amount of E was significantly increased, almost quadrupled (+286 %), without FBS from 0.21 µg to 0.81 µg. The amount of E transported by CD **3** was significantly increased by 79.2 % from 0.48 µg to 0.86 µg in CCM without FBS.

The presence of serum proteins such as bovine serum albumin contained in FBS, which may compete with β-estradiol molecules for free binding sites of CDs (Sideris et al., 1994), could be a reasonable explanation for this result. In absence of these proteins, more binding

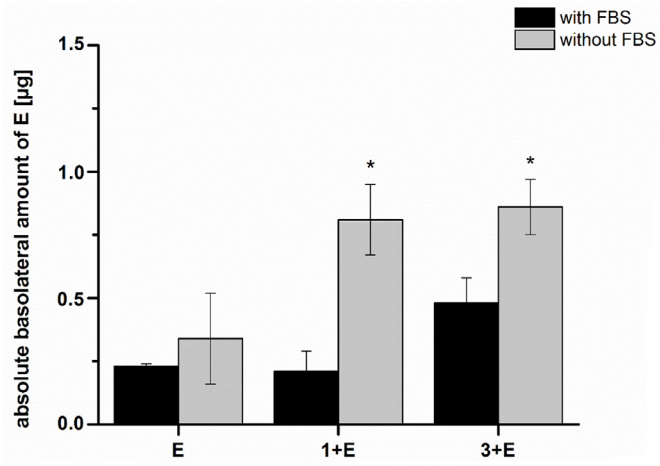

Fig. 7. Transport of β-estradiol (E) over the intestinal *in vitro* co-culture barrier model (90% Caco-2 cells/10% HT29-MTX-E12 cells) with drug carrier 1 or 3 in presence and absence of FBS after three hours. The amount of transported β-estradiol is presented as absolute values (µg) of three independent experiments (mean ± SD); $p \leq 0.05$ compared to samples with FBS.

sites are available, resulting in a better solubility and thus a higher starting concentration of the steroid. Because of the better transport efficiency of E, **1** + E and **3** + E in absence of FBS (Fig. 7), the following transport studies were performed with CCM without FBS (over a time period of three hours) (Fig. 8).

*3.3.4. Transport studies with β-estradiol incorporated in CD derivates **1** and **3***

To determine the suitability of the CD derivates **1** and **3** to transport substantial amounts of the hardly soluble steroid β-estradiol (E) across the intestinal barrier after oral application, *in vitro* studies with a physiological relevant co-culture model consisting of 90 % Caco-2 and 10 % HT-29-MTX-E12 cells were performed. E was applied to CCM without FBS in a concentration of 10 µg/ml as free substance or as CD complex **1** + E or **3** + E. The absolute distribution of E was determined after three hours on the apical, basolateral and cell compartment.

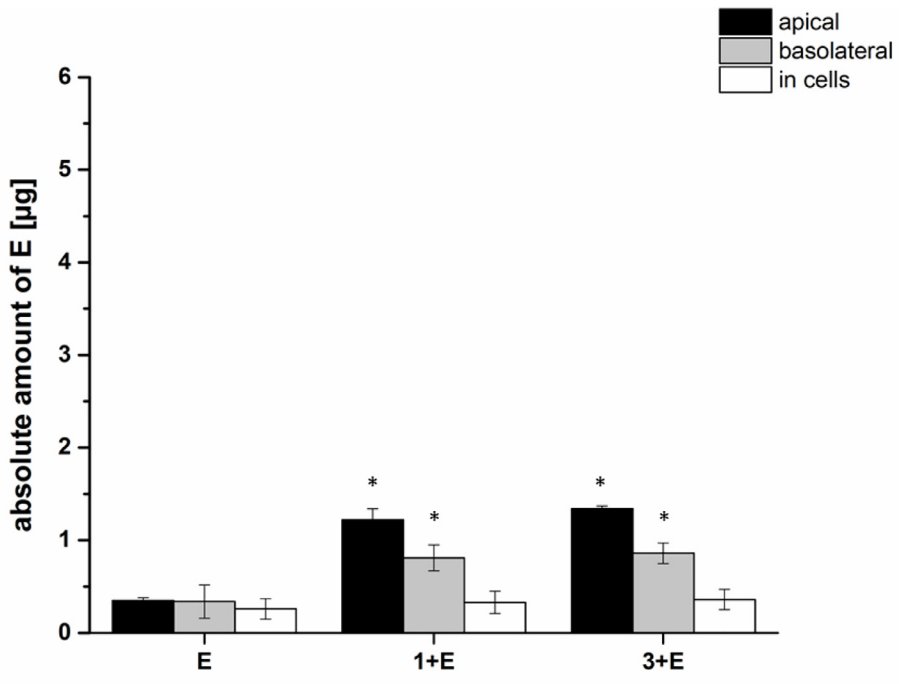

Fig. 8. Transport of β-estradiol (E) over the intestinal *in vitro* co-culture barrier model (90% Caco-2 cells/10% HT29-MTX-E12 cells) with drug carrier 1 or 3 after three hours. The amount of transported β-estradiol is presented as absolute values (µg) of three independent experiments (mean ± SD); *p ≤ 0.05 compared to free E.

Table 2
Absolute [µg] and relative [%] solubility of β-estradiol (E) as free substance and in complex with CD derivates 1 and 3 in cell culture medium without FBS.

solubility	E	1 + E	3 + E
µg	0.96	2.36	2.56
%	19.2	47.2	51.2

The dissolution of E in CCM distinguished between the different solutions as shown in Table 2. Only 19.2 % of free E dissolved in CCM, thus 0.96 µg of E was applied to the intestinal cells, whereas 47.2 % and 51.2 % of E dissolved in CCM in case of the CD complexes 1 and 3, respectively. Thus, 2.36 µg E and 2.56 µg E were applied to the intestinal cells in case of **1** + E and **3** + E.

E could be detected in all three compartments after three hours. For the free substance, 0.35 ± 0.03 µg E (37 %) remained apical, 0.34 ± 0.18 µg E (36 %) were transported and 0.26 ± 0.11 µg E (27 %) stayed inside of the cell layer (Fig. 8). In the case of **1** + E and **3** + E, significantly higher amounts of E could be found in the apical and basolateral compartments, due to the better solubility of the CD complex. CD complex **1** transported 0.81 ± 0.14 µg E (35 %), whereas 1.22 ± 0.12 µg E remained apical (53 %) and 0.27 ± 0.02 µg E (12 %) stayed inside of the cells (Fig. 8). CD complex **3** transported 0.86 ± 0.11 µg E (34 %) across the intestinal cells, whereas 1.34 ± 0.03 µg E (53 %) remained apical and 0.31 ± 0.05 µg E in cells (12 %). Based on the amounts found in the basolateral compartments meaning a successful transport of β-estradiol across the intestinal cell barrier, it can be summarized that the drug carriers **1** + E and **3** + E have a significantly higher transport efficiency due to a better solubility in comparison to E without CD complexation. After the successful demonstration of the CD **1** and **3** ability to transport water-insoluble steroid hormones across the intestinal *in vitro* barrier using the steroid β-estradiol, a second model steroid was used to verify the transport efficiency of the CDs. The complexation of dexamethasone, a corticosteroid with anti-inflammatory effects, with CDs has been demonstrated earlier and improved the solubility significantly (Dilova et al., 2019). In this study, dexamethasone was complexed with the CD **1** and **3** and the transfer efficiency was investigated according to the same principle as for β-estradiol.

*3.3.5. Measurement of the transepithelial electrical resistance during exposure with dexamethasone loaded CD derivates **1** and **3***

As demonstrated for β-estradiol, also the free steroid dexamethasone without CD derivates as well as the CD complexes do not affect the integrity of the intestinal *in vitro* barrier within three hours in comparison to untreated cells. Therefore, a transport of the test compounds over an intact barrier is guaranteed (Fig. 9).

3.3.6. Transport studies with dexamethasone incorporated in CD derivates 1 and 3

To determine the absolute amount of free dexamethasone (D) and dexamethasone transported via CD complexes (**1** + D; **3** + D) across the intestinal *in vitro* barrier as another steroidal model compound, the *in vitro* transport studies were repeated. D was applied to CCM without

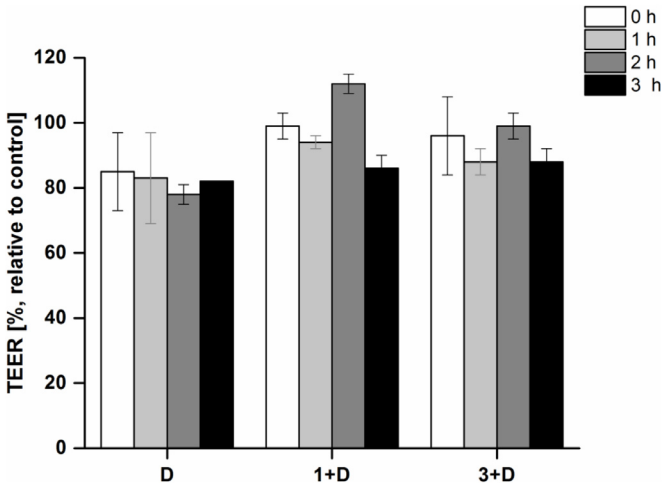

Fig. 9. TEER measurements of the intestinal *in vitro* co-culture barrier model (90% Caco-2 cells/10% HT29-MTX-E12 cells) during transport studies with free dexamethasone (D) and the drug-loaded CD derivates 1 and 3. The co-culture was exposed from zero to three hours with the formulations. Untreated cells are set as 100 % viability. The TEER value is presented as relative value to the untreated control (mean of three experiments ± SD).

Table 3
Absolute [μg] and relative [%] solubility of dexamethasone (D) as free substance and in complex with CD derivates 1 and 3 in cell culture medium without FBS.

solubility	D	1 + D	3 + D
μg	3.97	6.06	1.81
%	79.4	100	36.2

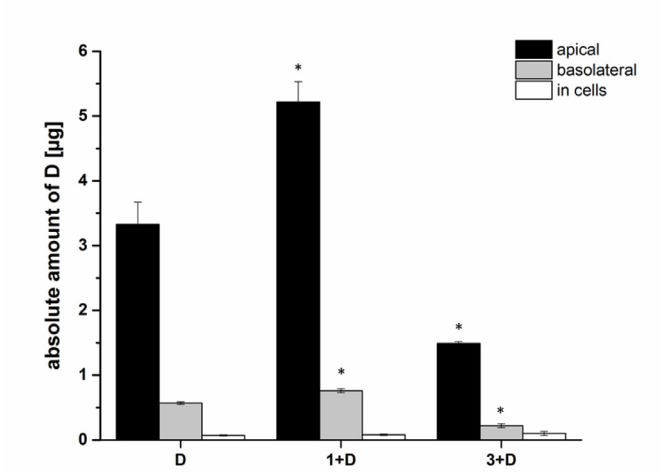

Fig. 10. Transport of dexamethasone (D) over the intestinal *in vitro* co-culture barrier model (90% Caco-2 cells/10% HT29-MTX-E12 cells) with drug carrier 1 or 3 after three hours. The amount of transported dexamethasone is presented as absolute values (μg) of three independent experiments (mean ± SD; *p ≤ 0.05 compared to free D).

FBS in a concentration of 10 μg/ml as free substance or as CD complex **1** + D or **3** + D. The absolute distribution of D was determined after three hours on the apical, basolateral and cell compartment.

The dissolution of D in CCM distinguished between the different solutions as shown in Table 3. Surprisingly, D which is usually considered as a hardly soluble compound, dissolved well in CCM without FBS and CDs (79.4 %). D as a complex with CD **1** has completely been dissolved and D as complex with CD **3** only dissolved partially (36.2 %). Thus, 3.97 μg D, 6.06 μg D and 1.81 μg D were applied to the intestinal cells, respectively.

After three hours, D could be detected in all three compartments (Fig. 10). For the free substance, 3.33 ± 0.34 μg D (84 %) remained apical, whereas 0.57 ± 0.02 μg D (14 %) were transported to the basolateral compartment and 0.07 ± 0.01 μg D (2 %) was detected inside the cells.

In the case of the CD complex **1** + D, a significantly higher amount of D (5.22 ± 0.31 μg, 86 %) stayed apical and was not transported or absorbed by cells. 13 % of the applied D (0.76 ± 0.03 μg) was transported and a small amount 0.08 ± 0.01 μg (1 %) was detected inside the cells. Also in case of **3** + D a very high amount of the applied D remained apical (1.49 ± 0.03 μg, 82 %), 0.22 ± 0.03 μg (12 %) have been transported and 0.1 ± 0.03 μg (6 %) stayed inside the cells. This is a significantly lower amount of D found in the apical and basolateral compartment in comparison to the free substance, which can be explained by the poor solubility of D in case of CD **3** and thus a lower amount of D which was applied to cells (Fig. 10).

By the means of these values, although the solubility of **1** + D was significantly higher, it can be summarized that the CD derivates **1** and **3** could not bring a clear added value for an efficient transport of D across the intestinal *in vitro* barrier. This could be a result of the lower complexation efficiency and binding constants in comparison to β-estradiol, which is shown in Table 1.

4. Conclusion

In this study a water-soluble Cyclodextrin polymer (CD-P) was synthesized by the attachment of a hydrophilic β-CD-thioether to hyaluronic acid, as a new candidate for intestinal drug carrier for steroid hormones. Additionally, a CD monomer was synthesized to evaluate and compare the results between monomeric and polymeric CD derivates. Host-guest complexes of the synthesized β-cyclodextrin derivates were formed with the two poorly soluble steroids β-estradiol and dexamethasone. Both CDs were able to enhance the solubility of the steroids in aqueous environment in comparison to the free substance. Solubility isotherms of the monomeric and polymeric CD complexes with β-estradiol showed a saturation effect at 500 μM, whereas the solubility isotherms of both CD complexes with dexamethasone increased linearly. The complexation efficiencies and binding constants for both CDs were higher for β-estradiol than for dexamethasone. Thus, the CDs were expected to be the more efficient as DDS for the transport of β-estradiol than for dexamethasone.

After excluding cytotoxic effects of the CDs in the used concentration, the transport rate of steroids was evaluated *in vitro*. Due to the enhanced solubility of β-estradiol by the CDs, the transported amount of drug could be significantly increased (monomeric and polymeric), which could finally increase the transport efficiency in comparison to the free β-estradiol. In case of dexamethasone, the second model steroid in this study, the monomeric drug carrier could significantly improve the solubility in comparison to the free substance but only slightly enhanced the transport over the intestinal *in vitro* barrier. The CD polymer could not bring a clear added value for the solubility and transport of dexamethasone.

Thus, limitations such as a low aqueous solubility and a low bioavailability of β-estradiol are clearly improved by the developed CD-based DDS, nevertheless further improvements of the β-cyclodextrin hyaluronic acid conjugates are needed.

Additionally, a modification of CD complexes with cell specific receptor target proteins are a conceivable possibility to enhance the interaction of CD complexes and cells, potentially leading to a higher transport efficiency across the intestinal cell barrier.

In conclusion, the synthesized CD complexes might be suitable candidates for oral administration of steroids to enhance the bioavailability and to solubilize steroids like β-estradiol in order to increase the serum level to reach clinical relevant concentrations.

Declaration of competing interest

none.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2019.105181.

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