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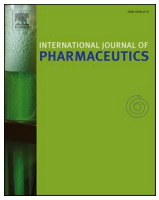

Thiolated cyclodextrins: A comparative study of their mucoadhesive properties

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ABSTRACT

Aim: The aim of this study was the comparison of the mucoadhesive properties of nonionic, negatively, and positively charged thiolated cyclodextrins (CDs), including α-, β-, and γ-CDs of low and high degree of thiolation.
Methods: Native α-, β-, and γ-CDs were thiolated with phosphorous pentasulfide in sulfolane (CD-SH) (i), via reductive amination with cysteamine after oxidative ring opening (CD-Cya) (ii), and via esterification with mercaptosuccinic acid (CD-MSA) (iii). These thiolated CDs were characterized via ^{1}H NMR and Ellman's test. Cytotoxicity was determined via resazurin and hemolysis assay. Mucoadhesive properties were evaluated via rheological studies with freshly isolated porcine mucus, as well as residence time studies on porcine small intestinal mucosa.
Results: The structure of thiolated CDs was confirmed via ^{1}H NMR. The degree of thiolation was in the range of 594–1034 μmol/g for low and 1360–3379 μmol/g for high CD-SH, whereas thiolated CD-Cya and thiolated CD-MSA exhibited a degree of thiolation of 1142–3242 μmol/g and 243–1227 μmol/g, respectively. Just cationic CDs showed cytotoxicity. Nonionic highly thiolated α-CD-SH, α-CD-Cya, and α-CD-MSA exhibited with mucus 5.6-, 15.7- and 2.8-fold improved dynamic viscosity, while improvement was 7.7-, 6.1-, and 5.4-fold for the corresponding thiolated β-CDs and 12.3-, 15.4- and 17.8-fold for the corresponding thiolated γ-CDs compared with native CDs, respectively. A prolonged mucosal residence time following the rank order $\gamma > \beta > \alpha$ was observed for all thiolated CDs, whereby γ-CD-Cya, nonionic highly thiolated β-CD-SH and α-CD-Cya showed the highest mucoadhesive properties.
Conclusion: A high degree of thiolation and the introduction of cationic charges are mainly responsible for high mucoadhesive properties of CDs.

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides composed of 6, 7, or 8 units of glucopyranose, named α-, β-, and γ-CDs, respectively (Haimhoffer, 2019). Due to their cyclic structure with a hydrophilic outer shell and hydrophobic inner cavity, CDs are widely used to complex and solubilize hydrophobic drugs. The large number of hydroxyl groups on CDs offers the opportunity for chemical modifications in order to introduce additional properties (Davis and Brewster, 2004). In contrast to most polysaccharides used for drug delivery such as cellulose derivatives, starch, hyaluronic acid or chitosan, these oligosaccharides do not exhibit any mucoadhesive properties. Due to thiolation of CDs, however, high mucoadhesive properties can be introduced making these excipients an even more powerful tool for mucosal drug delivery. Sulfhydryl groups are the most important bridging structures in nature involved in numerous physiological processes within the body. The chemical modification of CDs via attachment of sulfhydryl ligands leads to thiolated CDs, that can form disulfide bonds with cysteine-rich subunits of mucus glycoproteins. Several thiolated CDs have already been designed, exhibiting improved mucoadhesive properties and a prolonged residence time on the ocular and intestinal mucosa *in vivo* (Kali, 2023; Li, 2021; Grassiri, 2022). As these high mucoadhesive properties are based on thiol groups forming disulfide bonds with cysteine-rich mucus glycoproteins (Asim, 2020), the degree of thiolation seems to

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play an important role in this process (Hussain Asim, 2020; Brannigan and Khutoryanskiy, 2019). Another parameter influencing mucoadhesion are charged moieties on thiolated CDs. Cationic functionalities are beneficial for interactions with negatively charged substructures of mucus (Bernkop-Schnürch, 2018) additionally increasing the solubility of thiolated CDs in aqueous media. However, cationic CDs might get trapped already in the loose outer mucus that is not anymore properly connected with the firm mucus underneath and the cationic charge might cause toxic effects (Haimhoffer, 2019; Fabiano, 2020). In contrast, introducing anionic charges can also improve solubility and might enhance mucoadhesive properties via hydrogen bonding. To date, the mucoadhesive properties of different thiolated CDs have not been directly compared with each other, although such a comparison would provide fundamental knowledge for developing novel CDs of even more pronounced mucoadhesive properties.

It was therefore the aim of this study to synthesize nonionic, cationic, and anionic thiolated CDs and to directly compare their mucoadhesive properties with each other. In detail, α, β and γ-CDs were modified with two degrees of thiolation introducing anionic and cationic substructures. Solubility and cytotoxicity of these thiolated CDs were evaluated, followed by detailed mucoadhesion studies.

2. Material and methods

2.1. Materials

Alpha-cyclodextrin (α-CD), beta-cyclodextrin (β-CD), and gamma-cyclodextrin (γ-CD) were purchased from Cyclolab, Hungary. Cysteamine (Cya), mercaptosuccinic acid (MSA), phosphorus pentasulfide (P_2S_5, 99%), tetramethylene sulfone (sulfolane, 99%), dimethyl sulfoxide-d6 (DMSO-d_6, 99.9%), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), sodium borohydride, D-(+)-glucose anhydrous (Glc), resazurin sodium salt and Triton X-100 were received from Sigma-Aldrich, Austria. ZelluTrans dialysis tube MWCO 1 kDa and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) were obtained from Carl Roth, Austria. Fluorescein diacetate (FDA) was purchased from Acros Organics, Thermofisher Scientific, Austria. Porcine small intestinal mucosa was donated by a local slaughterhouse. Red blood cell concentrate was contributed by Tirol Kliniken GmbH and stored at 5 °C for further use.

2.2. Synthesis and purification of thiolated cyclodextrins

2.2.1. Synthesis and purification of nonionic thiolated cyclodextrins

Thiolated CDs were synthesized according to a method published by Kali et al. (Kali, 2023). α-CD (0.5 g, 0.51 mmol), β-CD (0.5 g, 0.44 mmol), or γ-CD (0.5 g, 0.39 mmol) and phosphorus pentasulfide (2.4 g, 5.3 mmol for β-CD) were dissolved in 15 mL of sulfolane. Triethylamine (1 mL, 13.5 mmol) was added to the solution, heated to 130 °C, and stirred for 24 h under N_2 atmosphere. Afterwards, the temperature was dropped to 80 °C and demineralized water was added dropwise to degrade the remaining P_2S_5. The precipitate was centrifuged at 4 °C at 12500 rpm, washed with ice cold water, and dried until constant weight. For CDs with low degree of thiolation (CD-SH low), a reaction time of 2 h instead of 24 h for CDs with high degree of thiolation (CD-SH high), was applied. The yields of thiolated CDs were 46.8% and 60.0% for α-CD-SH low and high, 61.5% and 86.0% for β-CD-SH low and high, and 75.4% and 67.2% for γ-CD-SH low and high, respectively.

2.2.2. Synthesis and purification of cationic thiolated cyclodextrins

Cysteamine-modified CDs were synthesized in a two-step reaction according to a previously established method (Ijaz, 2015; Ijaz, 2016; Ijaz, 2016). In the first step, CDs were oxidized. For that reason, 2.0 g of CDs, that is 2.0 mmol for α-, 1.8 mmol for β-, and 1.6 mmol for γ-CD, were dissolved in 180 mL of demineralized water, and 0.9, 0.8, and 0.7 g of $NaIO_4$ (4.27, 3.74, and 3.20 mmol), dissolved in 20 mL of demineralized water, was added, respectively. The reaction mixture was stirred for 2 h at room temperature in the dark, and 800 mL of ethylene glycol was added to consume the remaining unreacted $NaIO_4$. The reaction mixture was further stirred for 1 h at room temperature and then dialyzed against demineralized water. The purified product was freeze-dried.

In the second step, cysteamine was conjugated to the oxidized CDs. Briefly, 1.0 g of CD-CHO (1.0, 0.89, and 0.78 mmol for α-, β-, and γ-CD, respectively) was dissolved in 100 mL of demineralized water, then MES hydrate in a final concentration of 0.1 M was added to the solution. Afterwards, 0.5 g of cysteamine (6.48 mmol) was added, and the pH of the mixture was adjusted to 4. After 3 h of continuous stirring at room temperature, $NaCNBH_3$ (8.0 g, 0.13 mol) was added to the reaction mixture, which was further stirred for 72 h, at room temperature. The reaction mixture was dialyzed two times against demineralized water at pH 5 and two times against the same medium containing 1% NaCl at 10 °C in the dark. Finally, samples were dialyzed twice against demineralized water at pH 3 followed by freeze-drying. The yields of cationic thiolated CDs were 22.5%, 49.4%, and 54.4% for α-, β-, and γ-CD-Cya, respectively.

2.2.3. Synthesis and purification of anionic thiolated cyclodextrins

Mercaptosuccinic acid-modified CDs were synthesized by the reaction of S-acetyl-mercaptosuccinic anhydride and the corresponding CD. First, 15 g (0.1 mol) of mercaptosuccinic acid was refluxed with 24 mL of acetyl chloride for 3 h. The reaction mixture was concentrated by a rotary evaporator and precipitated into diethyl ether. The solid product was dried under reduced pressure until constant weight. In brief, CD (0.45 g, 0.46 mmol for α-, 0.40 mmol for β-, and 0.35 mmol for γ-CD) and S-acetyl-mercaptosuccinic anhydride (14.7 g, 84.4 mmol) were dissolved in 20 mL of dimethylacetamide and stirred at 90 °C temperature overnight. The reaction mixture was precipitated into diethyl ether cooled in ice bath, and the dried residue was dissolved in 0.5 M NaOH and dialyzed against demineralized water for 3 days at room temperature. The purified CDs were lyophilized for 36 h. The yields of anionic thiolated CDs were 60.2%, 76.7%, and 91.8% for α-, β-, and γ-CD-MSA, respectively.

2.3. Characterization of thiolated cyclodextrins

The amount of thiol groups attached to the CD backbone was quantified photometrically using Ellman's reagent, referring to an already established method (Bernkop-Schnürch et al., 1999), as well as after the reduction of disulfide content employing sodium borohydride determining the extent of disulfide bridges. The amount of free thiol groups was calculated employing a calibration curve of increasing concentrations of L-cysteine. Subsequently, absorbance was measured utilizing a microplate reader at a wavelength of 450 nm (Tecan Spark multimode microplate reader, Grödig, Austria).

^{1}H NMR measurements were performed on a "Mars" 400 MHz Avance 4 Neo spectrometer from Bruker Corporation (Billerica, MA, USA, 400 MHz) in dimethyl sulfoxide-d6 (DMSO-d_6) or deuterated water (D_2O) solution.

2.4. Solubility studies

Samples of 10 mg were dissolved in 0.5 mL of either demineralized water or 100 mM phosphate buffer pH 5, pH 6.8, and pH 8. An incubation time of 30 min at 25 °C and a shaking speed of 1000 rpm using a thermomixer (Eppendorf ThermoMixer® C, Eppendorf AG, Hamburg, Germany) was employed. After 30 min, samples were centrifuged at 13400 rpm for 10 min (MiniSpin®, Eppendorf AG, Hamburg, Germany). In the following, the supernatant was withdrawn, lyophilized (Freeze Dryer Christ, Gamma 1–16 LSC, Germany), and quantified gravimetrically.

2.5. Evaluation of cytotoxicity

2.5.1. Resazurin assay

To evaluate the cytotoxic potential of CDs in a concentration of 0.15% (m/v), a resazurin assay on a Caco-2 cell monolayer was conducted (O'Brien, 2000; Fürst, 2019). Briefly, 2.5×10^4 Caco-2 cells per well were seeded to 96-well plates in minimum essential medium (MEM) supplemented with penicillin/streptomycin solution (100 units/0.1 mg/L) and 10% (v/v) fetal calf serum (FCS). The cells were incubated for 6 days at 37 °C under 5% CO_2 and a 95% relative humidity environment. During the incubation period, the medium was replaced every 48 h. Test solutions of thiolated CDs were prepared in a concentration of 0.15% (m/v) in 25 mM HEPES 268 mM glucose buffer pH 7.4. For the experiment, cells were washed twice with preheated buffer at 37 °C. Test solutions were added in triplicate to the cell culture plate in the volume of 0.1 mL/well and incubated at 37 °C in a 5% CO_2 and 95% relative humidity environment for 4 h or 24 h. Cells treated with 0.15% (m/v) Triton X-100 served as positive control concerning cell lysis, while cells with 25 mM HEPES 268 mM glucose buffer pH 7.4 referred to cell viability as a negative control. After incubation, test solutions were removed, and cells were washed three times with phosphate-buffered saline followed by further incubation for 2 h with 150 µL of resazurin (44 µM) solution (phosphate-buffered saline and 25 mM HEPES 268 mM glucose buffer pH 7.4 1:20 v/v). Fluorescence was measured at 540 nm excitation and 590 nm emission wavelengths (Tecan Spark multimode microplate reader, Grödig, Austria). Cell viability was calculated by referring to the following equation:

$$\text{Cellviability}[\%] = \frac{\text{average fluorescence of samples}}{\text{average fluorescence of cells treated with 25 mM HEPES 268 mM glucose buffer pH 7.4}} \times 100$$

2.5.2. Hemolysis assay

In order to evaluate the toxic effect on human red blood cells, a hemolysis assay was performed (Evans, 2013; Akkuş-Dağdeviren, 2021). As the cell membrane of red blood cells is very sensitive and fragile, this assay gives more detailed information on cell toxicity as well as cell membrane damaging effects among CDs. The red blood cell concentrate was diluted using 25 mM HEPES 268 mM glucose buffer pH 7.4 in a ratio of 1:100 (v/v). After that, CDs were dissolved in 100 µL of 25 mM HEPES 268 mM glucose buffer pH 7.4 and 100 µL of already diluted blood in a final concentration of 0.15% (m/v) was added. Triton X-100, in a final concentration of 0.5% (m/v), served as positive control regarding total hemolysis, while 25 mM HEPES 268 mM glucose buffer pH 7.4 was used as a negative control, indicating 0% cell lysis, respectively. Samples were incubated on a thermomixer (Eppendorf ThermoMixer® C, Eppendorf AG, Hamburg, Germany) at 300 rpm and 37 °C for either 1 h or 3 h. Hemolysis was quenched by centrifugation at 8000 rpm for 10 min (MiniSpin®, Eppendorf AG, Hamburg, Germany). Subsequently, the supernatant was analyzed concerning hemoglobin content by measuring the absorbance at 415 nm (Tecan Spark multimode microplate reader, Grödig, Austria). The degree of hemolysis was calculated using the following equation:

$$\text{Hemolysis}[\%] = \frac{\text{average absorbance of samples} - \text{average absorbance of negative control}}{\text{average absorbance of positive control} - \text{average absorbance of negative control}} \times 100$$

2.6. Rheological investigations

Experiments were performed utilizing a cone-plate combination rheometer (Haake Mars Rheometer, 40/60, Thermo Electron GmbH, Karlsruhe, Germany; Rotor: C35/1°, D = 35 mm) while maintaining a constant temperature of 37 °C, as well as setting the gap between cone and plate to 0.052 mm. Oscillatory stress sweep measurements within the region of linear viscoelasticity were carried out with shear stress in the range of 0.01–50.0 Pa while keeping the frequency constant at 1 Hz. Parameters such as dynamic viscosity (η), elastic modulus (G'), and viscous modulus (G'') were measured (Perrone, 2018; Rossi, 2018).

In brief, freshly excised porcine small intestinal mucosa from a local abattoir was cut longitudinally in order to collect porcine mucus by scrapping it off from the underlying tissue. The mucus was purified by adding 5 mL of 0.1 M sodium chloride solution to 1 g of mucus, and this suspension was stirred for 1 h at 4 °C followed by centrifugation at 10400 g for 2 h at 10 °C. This procedure was repeated and the supernatant was discarded obtaining the purified mucus. The sample was stored at − 20 °C prior to use.

For the rheological measurement, CDs were dissolved in 100 µL of 100 mM phosphate buffer pH 6.8 in a concentration of 0.3% (m/v). CD solutions and porcine mucus were homogenized in a ratio of 1:5 (v/m). After incubation of 6 h at 37 °C, samples were analyzed, determining the viscoelastic characteristics.

2.7. Preparation of inclusion complexes employing FDA

To evaluate the mucoadhesive properties of thiolated CDs, fluorescein diacetate (FDA), a lipophilic fluorescent marker, was encapsulated within the hydrophobic cavity of CD derivatives following an established method (Wang and Cai, 2008; Ijaz, 2017). For this purpose, 1 mg of FDA was dissolved in 0.5 mL of ethanol and further dispersed in 6 mL of 100 mM phosphate buffer pH 6.8 containing 23 mg of CD. The dispersions were incubated for 24 h at 25 °C and 500 rpm utilizing a thermomixer. In order to separate the dissolved inclusion complexes from the remaining free undissolved FDA, a filter paper with particle retention from 8 to 12 µm was used to collect filtrates for lyophilization.

2.8. In vitro mucoadhesion studies on porcine small intestinal mucosa

Mucoadhesive properties of CDs were evaluated on freshly excised porcine small intestinal mucosa being cut longitudinally, obtaining pieces in a size of approximately 3 × 2 cm. Hence, the mucosal tissue was fixed on half-cut 50 mL falcon tubes being placed at an angle of 45 °C in a thermostatic chamber (Heratherm Oven, Thermofisher Scientific, Dreieich, Germany) at 37 °C and 100% relative humidity. Afterwards, employing 100 mM phosphate buffer pH 6.8 and a flow rate of 1 mL/min using a peristaltic pump (Ismatec, IPC, High Precision Multichannel Dispenser, Richmond Scientific, Lancashire, Great

Britain), the mucosa was rinsed for 15 min. Thereafter, 5 mg of CD inclusion complexes with FDA were applied on the mucosa and incubated for 15 min. Then, the mucosa was continuously rinsed with 100 mM phosphate buffer pH 6.8 and samples were collected at predetermined time points every 30 mins up to 180 min. As a control, 100 mM phosphate buffer pH 6.8 without any FDA inclusion complex was collected and 5 mg of CD inclusion complexes dissolved in 100 mM phosphate buffer pH 6.8 served as a 100% reference value for calculation. Collected samples were centrifuged at 13400 rpm for 10 min (MiniSpin®, Eppendorf AG, Hamburg, Germany), and aliquots were treated with an equal volume of 5 M NaOH in order to hydrolyze FDA to sodium fluorescein. Thereafter, samples were incubated for 30 min utilizing a thermomixer, maintaining a temperature of 37 °C and a shaking speed of 300 rpm. Fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm (Tecan Spark multimode microplate reader, Grödig, Austria) (Ijaz, 2017; Laquintana, 2019).

2.9. Statistical data analysis

Statistical data analysis was implemented by employing Student's t-test while setting $p < 0.05$ as the minimal level of significance for analyzing two groups in opposition to one-way ANOVA in the case of more than two groups. Furthermore, the Bonferroni test, a type of multiple comparison test, as post hoc analysis was conducted. Results were illustrated as means of at least triplicates ± SD.

3. Results

3.1. Synthesis and characterization of thiolated cyclodextrins

α-, β-, and γ-CDs were modified in three ways resulting in nonionic, cationic, and anionic CDs as illustrated in Fig. 1. The structure of all thiolated CDs was confirmed by ^{1}H NMR analyses. Examples of ^{1}H NMR spectra of the corresponding modified CDs are presented in the supplementary materials. In case of nonionic thiolated CDs having been analyzed in DMSO-d_6, the reduced intensity or even the elimination of the hydroxy peaks at 5.8–5.6 ppm (OH-2 and OH-3) and 4.5–4.4 ppm (OH-6), as well as the appearance of new peaks between 2.2 and 2.0 ppm (SH-2 and SH-3) and 1.2 ppm (SH-6), clearly indicated successful thiolation. For cationic cysteamine-modified CDs, a clear decrease of the OH-2 and 3 peaks was observed after the first step of the reaction. Furthermore, new peaks appeared at 2.1–3.1 ppm belonging to the CH_2 groups of the ligand and at 9.5 and 1.2 ppm, being related to the secondary amine salt and the thiol moiety, respectively. ^{1}H NMR spectra of

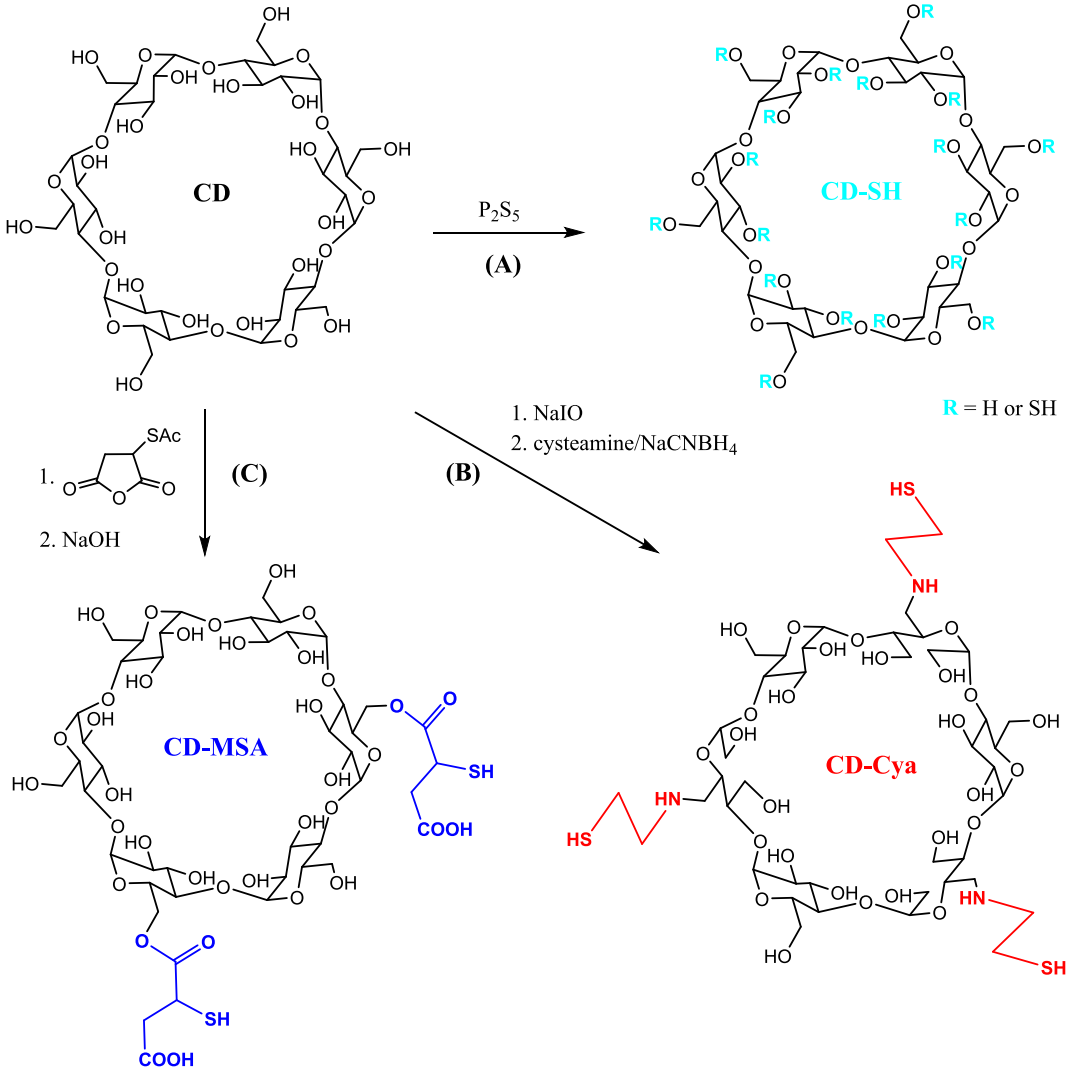

Fig. 1. Schematic representation of the synthesis routes of (A) nonionic (R = H or SH, cyan), (B) cationic (red), and (C) anionic (blue) thiolated α-cyclodextrins. Nonionic thiolated CDs were obtained via thiolation with phosphorus pentasulfide, whereas cationic and anionic CDs were generated by the covalent attachment of cysteamine and mercaptosuccinic acid, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Degree of thiolation of α-, β-, and γ-cyclodextrins. Indicated values are described as means ± SD (n ≥ 3).

	Free SH [μmol/g]	Total amount SH [μmol/g]	Yield [%]
α-CD-SH low	1034 ± 36	2012 ± 16	46.8
α-CD-SH high	1360 ± 25	2233 ± 65	60.0
β-CD-SH low	1594 ± 76	1952 ± 29	61.5
β-CD-SH high	3120 ± 21	3744 ± 26	86.0
γ-CD-SH low	1299 ± 25	1743 ± 85	75.4
γ-CD-SH high	3379 ± 20	4842 ± 29	67.2
	Free SH [μmol/g]	Total amount SH [μmol/g]	Yield [%]
α-CD-Cya	3242 ± 118	3380 ± 129	22.5
α-CD-MSA	243 ± 31	846 ± 27	60.2
β-CD-Cya	1142 ± 35	1324 ± 82	49.4
β-CD-MSA	1227 ± 59	1373 ± 30	76.7
γ-CD-Cya	1314 ± 38	1653 ± 84	54.4
γ-CD-MSA	365 ± 22	940 ± 6	91.8

the mercaptosuccinic acid-modified anionic CDs analyzed in D_2O showed chemical shifts of CH_2 and CH of the mercaptosuccinic ester in the range of 2.1–3.0 ppm, confirming the target structure.

Thiol groups were quantified before and after the reduction of disulfide bonds, determining free thiol groups and the total amount of thiol groups, respectively. The total amount of thiol groups includes the free thiols and the disulfides formed during the reaction or purification steps. Results are shown in Table 1. In terms of nonionic thiolated CDs, α-CDs showed a disulfide bond content of 19.5% and 24.3% for α-CD-SH low and high, respectively, while for β-CDs it is only 9.2% for the low and 8.3% for the high degree of thiolations. γ-CDs display a disulfide content of 12.7% and 15.1% for low and high thiolation, respectively. Cationic thiolated CDs show low disulfides, between 2.0% and 10.0%. The mercaptosuccinate-modified CDs display a higher disulfide content, up to 35.6%, most probably because of their purification at elevated pH, favoring disulfide bond formation.

3.2. Solubility studies

Generally, nonionic thiolated CDs showed lower solubility than the corresponding native CDs. Their solubility slightly increased at pH 8, due to the formation of thiolate anions. The solubility of ionic thiolated CDs showed a much higher pH dependency. Cysteamine ligands contributed to solubility at low pH due to cationic charges on the secondary amines. Mercaptosuccinic moieties had a higher effect at pH 8 due to the ionization of thiol and carboxylic acid groups resulting in anionic charges on the CDs. The most remarkable effect of the anionic ligand was observed in case of β-CD-MSA. The results of solubility studies are shown in Fig. 2.

Generally, higher solubility in buffer systems compared to demineralized water could be monitored. A pH of 5 was beneficial for the solubility of cationic thiolated CDs while pH 8 was advantageous for anionic thiolated CDs leading to either protonation or deprotonation of functional charged groups such as amino or carboxylic acid groups, respectively. The highest solubility for all thiolated CDs could be observed at pH 8. Furthermore, results demonstrated the overall beneficial effect of charges, either cationic or preferably anionic for the solubility of thiolated CDs.

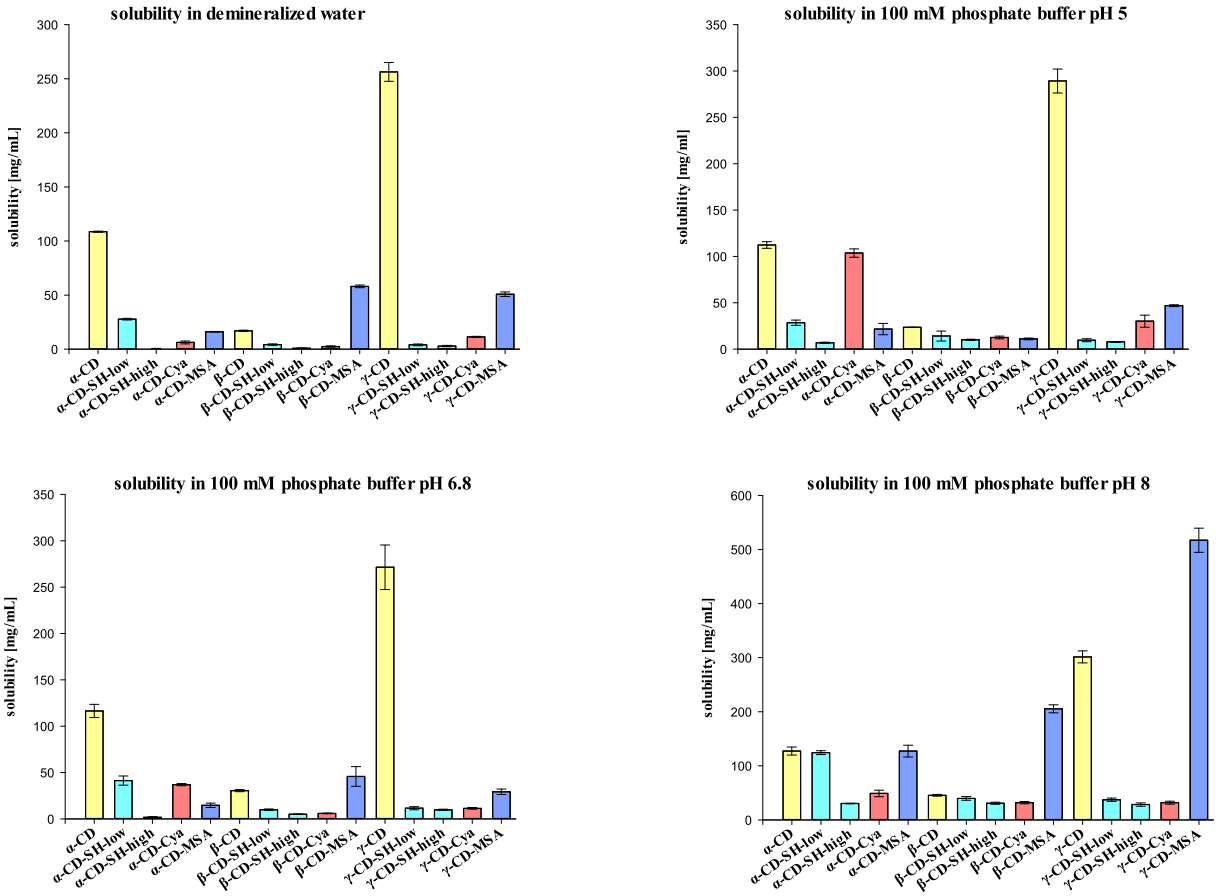

Fig. 2. Solubility of thiolated cyclodextrins in demineralized water, 100 mM phosphate buffer pH 5, pH 6.8, and pH 8 at 25 °C. Nonionic thiolated CDs = cyan bars with low degree of thiolation (CD-SH low) and high degree of thiolation (CD-SH high), cationic thiolated CDs = red bars, and anionic thiolated CDs = blue bars. Indicated values are presented as means ± SD (n ≥ 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

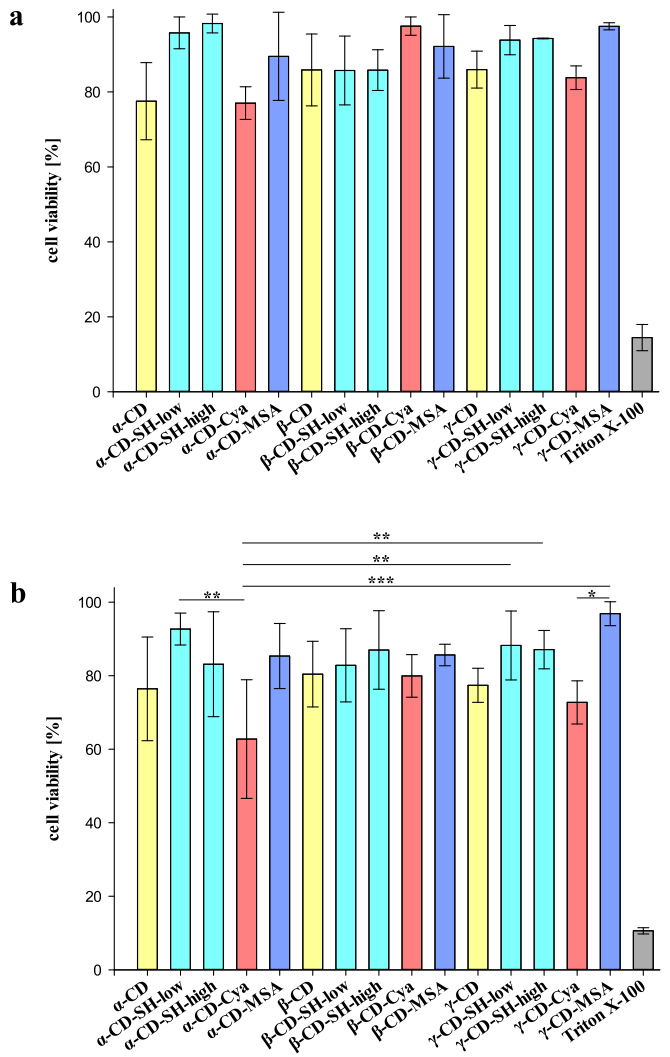

Fig. 3. Cell viability of Caco-2 cells after (a) 4 h and (b) 24 h of incubation at 37 °C in a concentration of 0.15% (m/v). Native CDs = yellow bars, nonionic thiolated CDs = cyan bars with low degree of thiolation (CD-SH low) and high degree of thiolation (CD-SH high), cationic thiolated CDs = red bars, anionic thiolated CDs = blue bars, and Triton X-100 = gray bars. Indicated values are illustrated as means ± SD ($n \geq 3$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

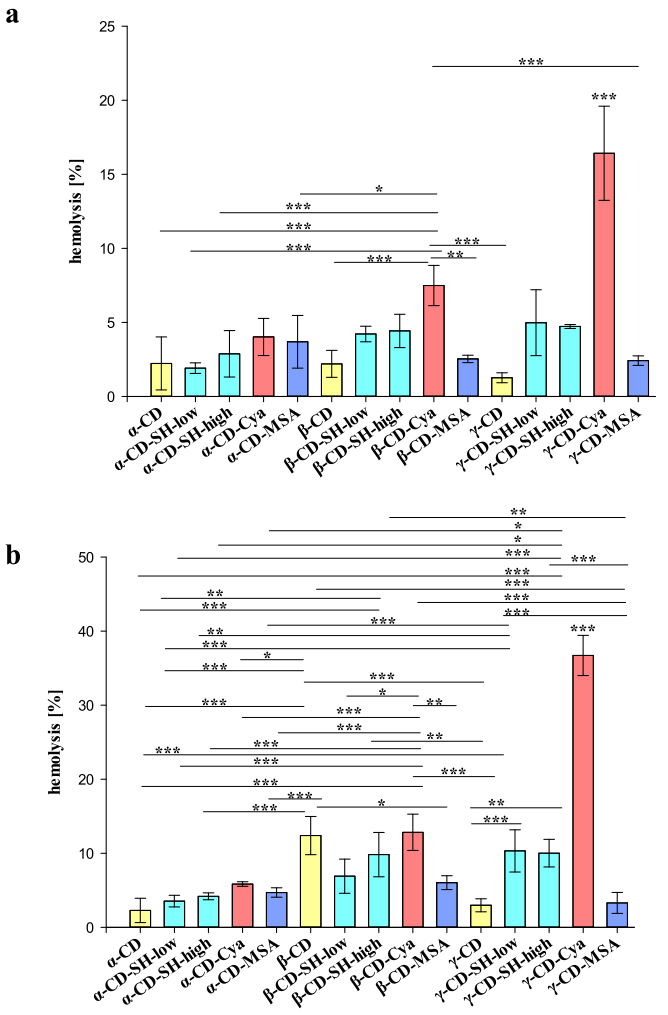

Fig. 4. Lysis of red blood cell concentrate within (a) 1 h and (b) 3 h of incubation with 0.15% (m/v) (thiolated) CDs at 37 °C. Native CDs = yellow bars, nonionic thiolated CDs = cyan bars with low degree of thiolation (CD-SH low) and high degree of thiolation (CD-SH high), cationic thiolated CDs = red bars, and anionic thiolated CDs = blue bars. Indicated values are depicted as means ± SD ($n \geq 3$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Evaluation of toxicity

3.3.1. Resazurin assay

Cellular metabolic activity was evaluated employing resazurin assay as viable cells are able to metabolize resazurin to its reduced form, resorufin. As illustrated in Fig. 3, generally similar high cell viability of all nonionic and anionic thiolated CD having been tested in a concentration of 0.15% (m/v) was found within 4 h or 24 h of incubation. Cell viability was close to or even higher than that of native CDs while incubated on a Caco-2 cell monolayer within 24 h (Fig. 3). In case of cationically charged thiolated CDs, a decrease in cell viability could be observed within 24 h of incubation. Likely because of the cationic charge affecting the cell membrane (Bernkop-Schnürch, 2018), viability of 62.77 ± 16.13, 79.95 ± 5.79, and 72.75 ± 5.87% for cysteamine modified α-CD, β-CD, and γ-CD was determined, respectively.

3.3.2. Hemolysis assay

Hemolysis assay is a valid *in vitro* test for predicting membrane damaging effects of compounds *in vivo*. This experiment can assess the safety of thiolated CDs as cell membrane rupture is associated with cytotoxicity (Leichner et al., 2019). Concerning thiolated β-CDs, a slightly decreased hemolytic effect could be observed within 3 h of incubation compared to unmodified β-CD. In contrast, for CDs with cationic character, the hemolysis was enhanced, exhibiting values up to 36.71 ± 2.71% within 3 h of incubation. A hemolytic effect of even unmodified CDs has been reported in several *in vitro* studies (Carneiro, 2019). In case of nonionic thiolated CDs, thiolation decreased hemolysis of β-CD, whereas slightly increased it of α- and γ-CD. Contrarily, the negatively charged ligand, mercaptosuccinic acid, did not affect hemolysis. Results of this study are illustrated in Fig. 4.

3.4. Rheological investigations with mucus

For all nonionic thiolated CDs an increase in dynamic viscosity (η) with mucus was shown. The higher the degree of thiolation was, the more pronounced was the increase in dynamic viscosity. Furthermore, the size of CDs also impacted the increase in dynamic viscosity. Increase in viscosity was the lowest for thiolated α-CD (3.0–5.6 fold) but the highest for thiolated γ-CD (10.7–12.3 fold) compared to the

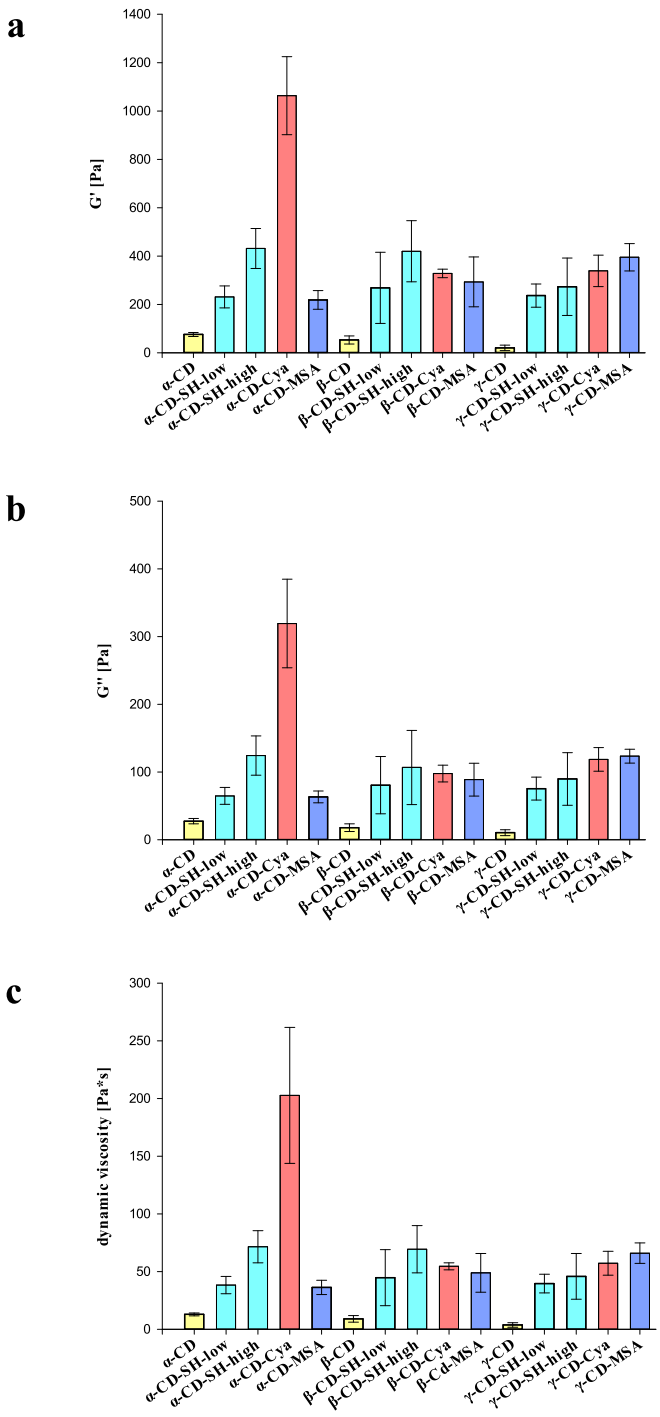

Fig. 5. Rheological behavior of cyclodextrins in porcine mucus in a ratio of 1:5 in a concentration of 0.3% (m/v) in 100 mM phosphate buffer pH 6.8 determining (a) elastic modulus (G') and (b) viscous modulus (G''), as well as (c) dynamic viscosity (η) within 6 h of incubation at 37 °C. Native CDs = yellow bars, nonionic thiolated CDs = cyan bars with low degree of thiolation (CD-SH low) and high degree of thiolation (CD-SH high), cationic thiolated CDs = red bars, and anionic thiolated CDs = blue bars. Indicated values are outlined as means ± SD (n ≥ 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

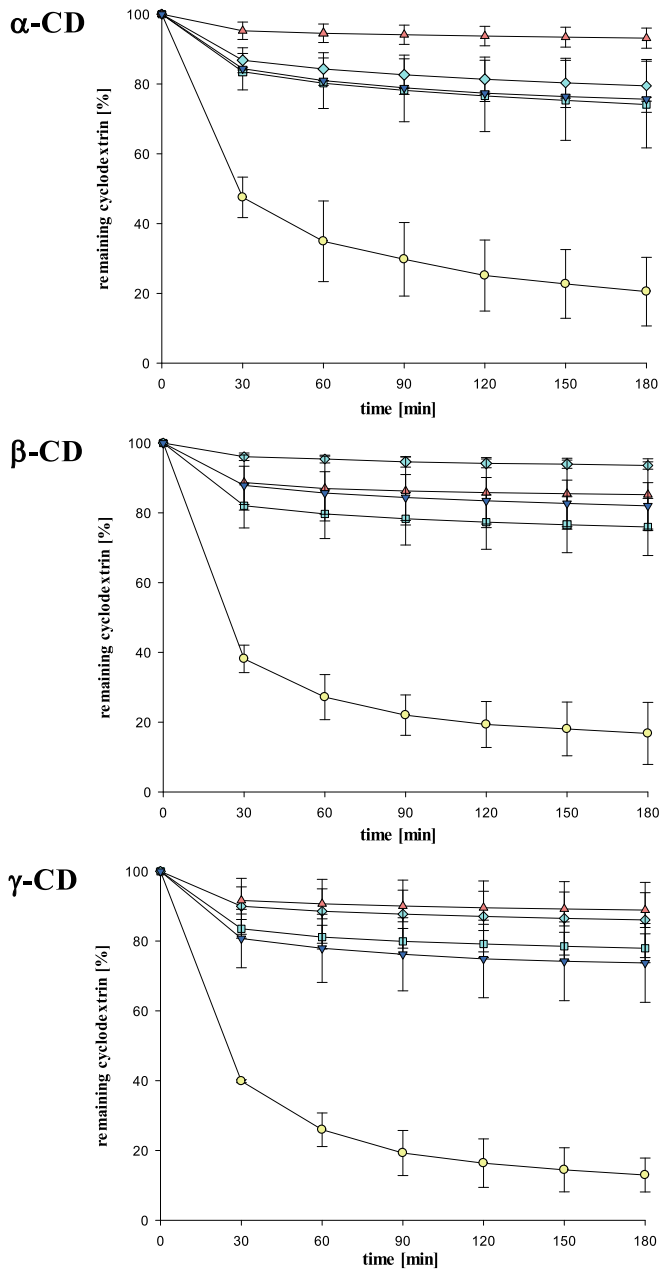

Fig. 6. Mucoadhesion of (thiolated) CD inclusion complexes with FDA (5 mg) on porcine small intestinal mucosa being rinsed with 100 mM phosphate buffer pH 6.8 at 37 °C. Native CDs = yellow circles (●), nonionic thiolated CDs = cyan squares (low degree of thiolation, CD-SH low, ■) and diamonds (high degree of thiolation, CD-SH high, ◆), cationic thiolated CDs = red triangles up (▲), and anionic thiolated CDs = blue triangles down (▼). Indicated values are outlined as means ± SD (n ≥ 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

corresponding native CDs. Referring to the highly thiolated γ-CD, the same trend was found for the elastic modulus (G') leading to a significant ($p < 0.001$) 8.6-fold improvement, as well as a 13.1-fold increase concerning viscous modulus (G''), compared to unmodified γ-CD. As the negatively charged mucus (Cone, 2009) could lead to an initially electrostatic repulsion with the anionic CDs, a decrease in η was shown compared to the nonionic and cationic thiolated CDs, except for γ-CD-MSA leading to a 17.8-fold improved dynamic viscosity. In case of cysteamine modified CDs, η increased significantly ($p < 0.001$) 15.7-, 6.1-, and 15.4-fold in comparison to unmodified α-CD, β-CD, and γ-CD, respectively. The same behavior was observed for the elastic modulus (G'). This significantly ($p < 0.001$) up to 16.3-fold higher G' compared to the corresponding unmodified CD highlighted the resistance to elastic deformation and indicated the formation of an interconnected network. Regarding the viscous modulus (G''), α-CD-Cya exhibited a significant ($p < 0.001$) 11.7-fold higher viscous behavior apart from γ-CD-Cya with

11.4-fold higher values than the unmodified CDs presented in Fig. 5.

3.5. In vitro mucoadhesion studies on porcine small intestinal mucosa

To prolong mucosal residence time, such as via the implementation of thiol groups, strong mucoadhesive features between cysteine-rich subdomains of the mucus layer and CDs can be reached via disulfide bond formation at the targeted site. The eradication of native and thiolated nonionic, cationic, and anionic CDs from porcine small intestinal mucosa via constantly rinsing with artificial instestinal fluid at pH 6.8 over 180 min was investigated. As presented in Fig. 6a, almost 70% of native α-CD was rinsed off within 180 min. Anionic α-CDs remained on the mucus layer to approximately 76%, whereas cationic thiolated α-CDs were eliminated only to a minor extent of 7% after 180 min. Native β-CDs were washed off to 83%, whereas the highly thiolated nonionic β-CD remained to almost 94% on the surface of the small intestinal mucosa with a 5.6-fold higher mucosal residence time, as shown in Fig. 6b. The lowest mucoadhesive property could be found for the native γ-CD; only 13% remained on the porcine intestinal mucosa, whereas just 11% of the cationic thiolated γ-CD was eliminated after 180 min leading to a 6.9-fold higher mucosal residence time in contrast to the unmodified γ-CD as depicted in Fig. 6c.

4. Discussion

Cyclodextrins (CDs) are cyclic oligosaccharides formed by various numbers of α-1,4-linked glucose units (Davis and Brewster, 2004). They are widely applied in food and chemical industries, cosmetics, and pharmaceuticals. The main difference between the three naturally occurring CDs, apart from the size of their central cavity, is their aqueous solubility. The various cavity sizes allow the complexation of hydrophobic drugs of different molar mass (Davis and Brewster, 2004). The solubility of CDs is also highly dependent on ligands that are covalently attached to them. Nonionic thiolated CDs used in this work showed lower solubility than the corresponding unmodified CDs, whereas ionic thiolated CDs displayed a pH-dependent increase in solubility as predicted (Könczöl and Dargó, 2018).

CD safety and toxicity usually depend on the route of administration and the type of CD being utilized. When given orally, CDs are negligibly absorbed from the gastrointestinal tract. Thus, they are almost non-toxic. According to viability studies on Caco-2 cells, nonionic and anionic thiolated CDs showed the same or even less toxicity than the unmodified ones. From a safety point of view these thiolated CDs seem to be appropriate as novel mucoadhesive excipients.

The complex structure of mucus enables a broad scope of interactions, such as non-specific Van der Waals forces or specific ionic interactions among complementary chemical structures. In case of polymers, mucoadhesion can also be achieved by an interpenetration process of the polymer chains in the mucus gel layer followed by chain entanglements. Regarding oligomers such as CDs, however, neither sufficiently strong adhesive interactions nor chain entanglements are achievable; they are not at all mucoadhesive. A way to trigger the mucoadhesion of CDs is the modification with sulfhydryl moieties, as they tend to form disulfide bonds with cysteines of mucus glycoproteins (Asim, 2020; Brannigan and Khutoryanskiy, 2019; Kali et al., 2022). Due to the formation of disulfide cross-links, the viscosity of mucus increases. In case of nonionic thiolated CDs, that were synthesized by hydroxyl-to-thiol-conversions, viscosity of the CD-mucus mixture increased more at a higher degree of thiolation, since the number of formed disulfide cross-linking points also increased. This observation confirms the crucial role of the number of thiol groups per CD ring in mucoadhesion. In case of cysteamine modified CDs mucoadhesion, as determined by the viscosity of their mixture with mucus, was similar or higher than that of the corresponding highly thiolated CD-SHs being likely attributed to the additional cationic charges. The cationic substructures are triggering ionic interactions with the negatively charged mucus contributing to increased viscosity. In case of the anionically charged ligand mercaptosuccinic acid, viscosity of the CD/mucus mixture also increased compared to the unmodified CDs due to thiol/disulfide exchange. Compared to nonionic thiolated α- and β-CDs, similar or slightly decreased mucoadhesion was found likely because of the lower degree of thiolation. The introduction of carboxylic moieties forming hydrogen bonds with mucus glycoproteins (Patel, 2003), could obviously not compensate the comparatively lower number of thiol moieties on the CD ring regarding its mucoadhesive properties. Merely γ-CD-MSA did not follow this trend. *In vitro* mucoadhesion studies on excised intestinal mucosa confirmed results obtained via rheological measurements. Generally, the formation of covalent bonds with mucus glycoproteins led to enhanced mucoadhesive features and a lower amount of CDs being washed off from the intestinal mucosa. The higher degree of thiolation resulted in a higher amount of CD remaining on the mucosa. Cysteamine modified CDs remained on the mucus layer to a greater extent than CD-MSAs or low thiolated CD-SHs, confirming that ionic interactions are highly contributing to the mucoadhesive forces. Anionic thiolated CDs showed generally a lower degree of thiolation than cationic ones, and as hydrogen bonds are weaker than ionic interactions, these CDs were washed off to a greater extent from the mucosa than nonionic and cysteamine-modified thiolated CDs.

5. Conclusion

Within the present study, nonionic thiolated α-, β- and γ-CDs with a low and high degree of thiolation were synthesized. Furthermore, via implementing an ionic character due to the ligand mercaptosuccinic acid, negatively charged thiolated CDs and via covalent attachment of cysteamine positively charged thiolated CDs were obtained. Mucoadhesive potential for all thiolated CDs highly depended on the degree of thiolation and ionic character. Generally, all thiolated CDs presented higher mucoadhesiveness than their corresponding native alternative, reaching up to 17.8-fold enhanced mucoadhesion. Although anionic thiolated CDs can form additional hydrogen bonds with mucus glycoproteins, they did not show higher mucoadhesive properties than the nonionic ones. Cationic thiolated CDs showed charge mediated ionic interactions with negatively charged substructures of mucus in addition to the formation of covalent disulfide bonds prolonging mucosal residence time. According to these results a too rapid binding of cationic thiolated CDs just to the outer loose mucus layer could be excluded. In contrast to nonionic and anionic thiolated CDs, however, cationic thiolated CDs showed raised cytotoxicity. Nonionic highly thiolated CDs are therefore the most suitable excipients in order to provide a prolonged mucosal residence time of incorporated drugs.

CRediT authorship contribution statement

Andrea Fürst: Visualization, Investigation. **Gergely Kali:** Investigation. **Nuri Ari Efiana:** Investigation. **Zeynep Burcu Akkuş-Dağdeviren:** Investigation. **Soheil Haddadzadegan:** Investigation. **Andreas Bernkop-Schnürch:** Conceptualization, Methodology, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.122719.

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