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ORIGINAL ARTICLE

Inclusion complexation of naproxen with cyclosophoraoses and succinylated cyclosophoraoses in different pH environments

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Abstract Cyclosophoraoses [cyclic β-(1,2)-glucan, Cys] isolated from *Rhizobium leguminosarum biovar trifolii* TA-1 have unique structures and high solubility, which make it a potent solubilizer for host–guest inclusion complexation. Succinylated cyclosophorasoses (S-Cys) were also synthesized by chemically modifying isolated cyclosophoraoses. In ultraviolet-visible studies using naproxen (NAP), Cys was shown to form the most stable complexes with NAP ($K_{1:1} = 2457.9\ M^{-1}$), which was followed by the negatively charged S-Cys ($K_{1:1} = 357.1\ M^{-1}$) at pH 3.4. A further strong reduction in the complex stability constant was observed at pH 7.5. When the reduction in the stability constant was compared with other cyclic oligosaccharides (Cys; 119.2 M^{-1}, CD; 14.48 M^{-1} and HP-CD; 6.75 M^{-1}), S-Cys ($K_{1:1} = 5.6\ M^{-1}$) was shown to have the highest decrease in stability constant. These results suggest that the S-Cys could regulate the efficiency of inclusion complexation at external pH values. NMR studies of complex formation between NAP and Cys also showed a different correlation pattern at pH 3.4 and 7.5. This difference in correlation demonstrates that the inclusion complexes between Cys and NAP formed as a result of the differential charge distribution of the carboxyl groups of NAP. The pH-dependent inclusion behavior of Cys for NAP was also evaluated using molecular docking simulations.

Keywords Cyclosophoraose · Naproxen · Succinylated cyclosophoraose · Inclusion complexation · Stability constant

Abbreviations

Cys	Cyclosophoraoses
S-Cys	Succinlyated cyclsophoraoses
NAP	Naproxen
CD	β-Cyclodextrin
HP-CD	Hydroxypropyl-β-cyclodextrin
MALDI-TOF	Matrix assisted laser desorption/ ionization-time of flight
NMR	Nuclear magnetic resonance
DSC	Differential scanning calorimetry
ROESY	Rotating frame nuclear overhauser effect spectroscopy
DP	Degree of polymerization
TLC	Thin layer chromatography
DS	Degree of substitution
Deuterium oxide	D_2O
Deuterium methanol	CD_3OD
DEAE	Diethylaminoethyl
FID	Free induction decay

Introduction

Cyclosophoraoses (Cys) are a class of unbranched cyclic oligosaccharides composed of β-(1,2)-D-glucans and vary in size from 17 to 40 in a neutral or anionic form [1–3].

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Cyclosophoraoses are the major cell envelope constituents of all members of the Rhizobiaceae family, and these molecules have been shown to functions in both living bacteria and during the process of plant infection [4, 5]. Cyclosophoraoses are synthesized in the cytosol and transported to the periplasmic space where they play an important role in regulating osmolarity in response to external osmotic shock [6–8]. Recent reports have shown that cyclosophoraoses form inclusion complexes with a variety of hydrophobic guest molecules such as amphotericin B, fluorescein, flurbipropen, indomethacin, paclitaxel and vitamins [9–15]. Much attention has been focused on using these compounds to form inclusion complexes with other lipophilic molecules as well as evaluating their biological functions. Cyclosophoraoses have also been modified with various functional groups such as carboxymethyl [16–18] sulfonyl [19] and succinyl [20] groups to further their potential applications. In a recent report, inclusion complex formation between succinylated cyclosophoraoses (S-Cys) and 4′-hydroxy flavanone was shown to be pH dependent [20–23].

Naproxen (NAP; D-2-(6-methoxy-2-naphthyl)-propionic acid) (Fig. 1a) is a non-steroidal anti-inflammatory drug commonly used because of its anti-inflammatory, analgesic and antipyretic properties and has been used to treat rheumatic diseases and analgesic [21–23]. However, NAP has a very low solubility in water and when administered orally, it causes gastrointestinal side-effects, drowsiness and dizziness [24]. Therefore, the aim of the present study was to increase the solubility of NAP through inclusion complex formation with cyclosophoraoses.

In this study, we investigated the pH-dependent inclusion complex formation of NAP with Cys and S-Cys. The phase solubility studies of NAP with Cys and S-Cys were performed using ultraviolet–visible (UV–vis) spectroscopy as described by Higuchi and Connors [25] and the results were compared with commercial solubilizers such as β-cyclodextrin (CD) and HP-β-cyclodextrin (HP-CD). Nuclear magnetic resonance (NMR) spectroscopy and molecular docking simulation studies of the complexation of NAP with Cys were conducted to better understand the interactions between the benzene ring of NAP and Cys during complex formation.

Materials and methods

Materials

Naproxen (NAP; D-2-(6-methoxy-2-naphthyl)-propionic acid), β-cyclodextrin, Hp-β-cyclodextrin (2-hydroxypropyl-β-cyclodextrin) were purchased from Sigma-Aldrich Chemials Co. (St, Louis, MO, USA). D_2O (99.9 at% D) and CD_3OD (40 wt% D) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

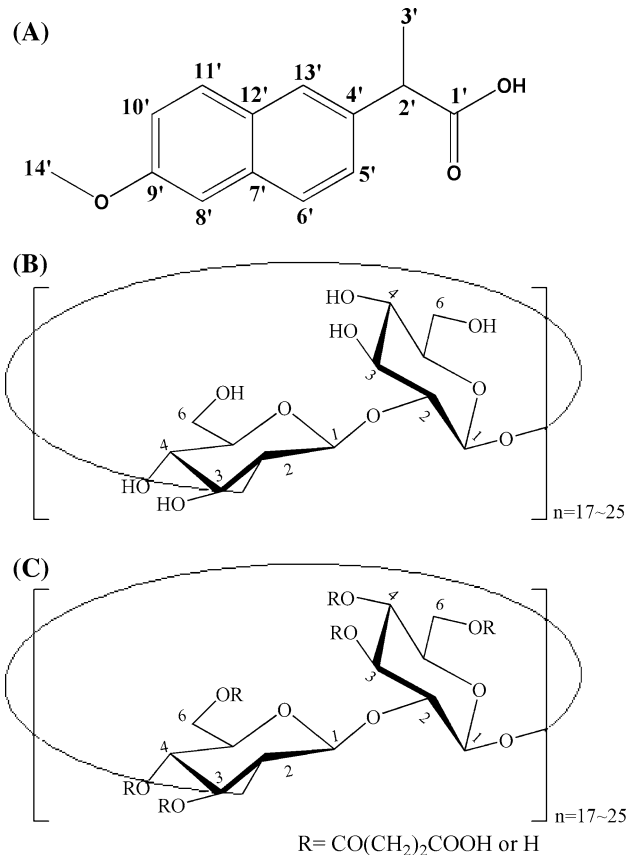

Fig. 1 Chemical structures of naproxen (NAP) (**a**), cyclosophoraoses (Cys) (**b**), and succinylated cyclosophoraoses (S-Cys) (**c**)

Isolation and purification of cyclosophoraoses (Cys)

The *R. trifolii* TA-1 was grown in 500 mL of GMS medium at 30 °C for 12 days [18]. Cells were harvested by centrifugation (8,000 rpm for 10 min), and then culture supernatants were concentrated by rotary evaporation. Next, to remove high-molecular-weight (HMW) glycans, the concentrated sample was precipitated by adding ethanol. The HMW compounds were then removed from the concentrated sample by centrifugation (12,000 rpm for 10 min). The supernatant was then concentrated by rotary evaporation, and the low-molecular-weight (LMW) glycans were collected by adding ethanol. After centrifugation, the precipitates were chromatographed on a Bio-Gel P-6. The fractions containing Cys were pooled, concentrated, and desalted on a Bio-Gel P-4. The desalted Cys was evaluated by NMR spectroscopy and matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

Cytotoxicity assay of cyclosophoraoses (Cys)

The Human Embryonic Kidney 293 (HEK293) cell lines were purchased from Korean Cell Line Bank (Seoul, Korea). Cells were maintained in Minimum Essential Medium Eagle (MEM, WelGENE Inc., Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, WelGENE Inc., Daegu, Korea), 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a humidified incubator containing 5% CO_2.

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) method was used to determine the viability of HEK293 cells [37]. HEK293 cells were seeded at a final density of 5×10^3 cells/well in 96-well microtiter plates (Costar, Cambridge, MA, USA) and incubated at 37 °C in a humidified incubator containing 5% CO_2 overnight. After the incubation cells were treated with different concentrations (0, 5, 10, 50, 100, 500, and 1,000 μM) of Cys and incubated at 37 °C in a humidified incubator containing 5% CO_2 for 24 h. After 24 h incubation cells were washed with PBS and the 20 μL MTS (CellTiter 96® AQueous One Solution; Promega, Madison, WI, USA) solution was added to each well incubate the plate at 37 °C for 4 h in a humidified, 5% CO_2 atmosphere. After 4 h of incubation, record the absorbance at 490 nm using a 96-well plate reader (Zenyth 1100; ANTHOS, Austria). Cell viability was expressed as the percentage of the untreated control. Experiments were carried out in triplicate.

Preparation of succinlyated cyclsophoraoses (S-Cys) from cyclosophoraoses (Cys)

The S-Cys were prepared as reported elsewhere [20]. First, 500 mg of neutral Cyclosophoraoses (0.16 mmol; 3119) was dissolved in 5 mL of anhydrous pyridine. One gram of succinic anhydride (SA) (10 mmol) was then solubilized in 3 mL of pyridine. The solutions were mixed, the mixture was heated at 100 °C and 5 mg of DMAP [4-(dimethylamino)pyridine] (0.041 mmol) were added. The reaction solution was incubated for 24 h. The mixture was evaporated to remove solvent and 10 mL of water was added. The S-Cys were then precipitated by adding 50 mL of isopropyl alcohol. The precipitate was washed three times with 10 mL of isopropyl alcohol and finally dried to remove acetone. The S-Cys were finally purified over a DEAE Sephadex and Bio-gel P2 to remove any remaining chemicals. The S-Cys was evaluated by MALDI-TOF mass spectrometry and NMR spectroscopy.

Phase solubility studies

The phase solubility studies of the NAP with Cys and S-Cys were performed using ultraviolet–visible (UV–vis) spectroscopy as described by Higuchi and Connors [25] and the results were compared with commercial solubilizers such as β-cyclodextrin (CD) and HP-β-cyclodextrin (HP-CD). Excess amounts of NAP were weighed and placed in 20 mL tubes. 10 mL of aqueous solutions containing various concentrations of Cys, S-Cys, β-CD, and HP-CD (0–10 mM, respectively) were then added and the samples were shaken at 25 ± 0.5 °C in different pH buffer solutions. After 1 day, an aliquot was filtered through a Whatman 0.2 μm filter. A portion of the sample was adequately diluted and analyzed by spectrophotometry. The experiment was carried out in triplicate. Complex formation between NAP and cyclic oligosaccharides was also examined using the spectral shift method [29]. The change in absorbance of the substrate (NAP) after the addition of various concentrations of ligand (cyclic oligosaccharides) was measured at 301 nm. The apparent formation constant, $K_{1:1}$ was calculated from the straight line in the phase solubility diagram according to the following equation [25]:

$$K_{1:1} = \frac{slope}{S_0(1 - slope)}$$

where, S_0 is the solubility of the drug in each examined buffer solution and in the absence of ligand.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was conducted using a TA Instruments DSC Q2000 equipped with a low temperature cell and nitrogen as the purging gas. The DSC calibrated with indium (3.21 mg, 99.99% pure, melting point 156.6 °C) and each sample was analyzed at a scanning speed of 20 °C/min from 0 to 275 °C. The thermal behavior was carried out by heating 1–5 mg of samples in aluminium crimped pans under nitrogen gas flow over the temperature range 30–250 °C. Measurements were made in duplicate. The physical mixture of NAP and Cys in 1:1 molar ratio was prepared by mixing individual components that had previously been pulverized in a ceramic mortar and sieved through mesh number 60. The complex formation between NAP and Cys was obtained after the samples of equal molar ratios (1:1) were equilibrated at 25 ± 0.5 °C in buffer solutions (pH = 3.4) for 1 day. The solution was then filtered through a Whatman 0.2 μm filter and lyophilized.

NMR spectroscopic analysis

All NMR measurements were performed with 0.7 mL samples in 5 mm NMR tubes. Tetramethylsilane (TMS, Me_4Si) was used as an external reference, and chemical shifts were calibrated with an accuracy of 0.05 ppm.

Rotating frame nuclear overhauser effect spectroscopy (ROESY) techniques were also performed in phase-sensitive mode with 32 scans per FID, using a pulse train to achieve a spin-lock field with a mixing time (τ_{mix}) of 400 ms on a Bruker spectrometer at 500 MHz. ROESY experiments were carried out using NAP and Cys samples mixed at a molar ratio of 1:1 in different pH buffers (pH = 3.4 and 7.5).

Computational methods

Molecular docking simulations were performed using the Glide module [30] in Maestro 9.2 software (Schrodinger Inc.,). All compounds were docked to receptors in the flexible mode. The three-dimensional coordinates for the β-CD were taken from the crystal structure. Since the crystal structures of the cyclosophorase were not yet available, a theoretically modeled structure was used in this study. A previously calculated conformation of the cyclosophoroheptadecaose (DP = 17) was used as the model structure of cyclosophoraose [31]. The starting configurations of NAP were prepared using the Builder module of the Maestro software. To mimic each acidic and neutral pH condition, the protonated and deprotonated form of the carboxyl group was introduced on the naproxen. The prepared molecular structures were further relaxed using the energy-minimization method. A molecular grid was defined for each β-CD and Cys using the Receptor Grid Generation tool in Glide with a 20 Å cubic box. Molecular docking was conducted under extra precision *XP* mode using the Glide XP 5.0 scoring function to obtain accurate binding mode and affinity data. In this docking mode, an energy window of 2.5 kcal/mol and distance-dependent dielectric constant ($\varepsilon = 1$) was used in the pose sampling process. The highest-scoring docked pose for each NAP model with β-CD or Cys was ranked according to the Glide Score.

Results and discussion

Identification of cyclosophoraoses (Cys)

The structure of the purified native cyclosophoraoses was evaluated by NMR spectroscopy as described elsewhere [11]. In addition, the ring sizes of the Cys (Fig. 1b), which ranged from 17 to 25, were confirmed through MALDI-TOF mass spectrometry.

Cytotoxicity assay of cyclosophoraoses (Cys)

Cytotoxicity of the Cys to the HEK 293 cell is determined by MTS assay. The HEK293 is commonly used in drug toxicity studies. HEK293 is used to assess the effect of the drug candidate on the renal system [38]. Figure 2 shows cytotoxicity curves for Cys. The percentages of surviving HEK293 cells are plotted against the Cys concentrations. MTS assay for HEK293 cells indicated that Cys produced only a slight decrease in cell viability. These results suggest Cys has good biocompatibility.

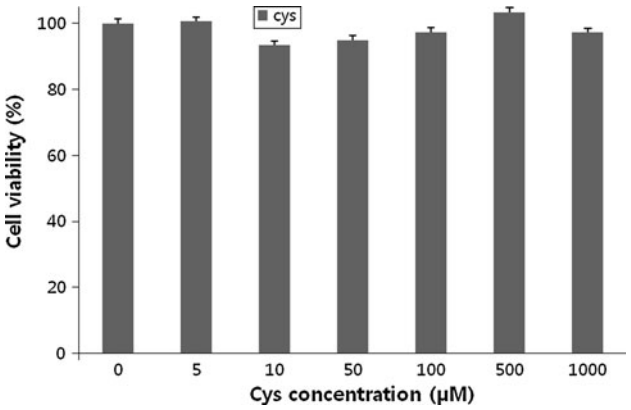

Fig. 2 Cytotoxicity of Cys at the concentration of 0, 5, 10, 50, 100, 500, 1000 μM against HEK293 cells ($n = 3$)

Synthesis and structural analyses of succinylated cyclosophoraoses (S-Cys)

The succinylated cyclosophoraoses (S-Cys) were synthesized through one-step reaction [20, 26]. The reaction was monitored on thin layer chromatography (TLC) and the products were purified by ion-exchange and size-exclusion gel chromatographic methods. The S-Cys were synthesized in a one-step process with a yield of 90% and the reaction was monitored by TLC. ^{1}H NMR analysis also confirmed that the S-Cys contained a high level of succinate with the presence of two prominent signals between 2.6 and 2.8 ppm. We confirmed that the S-Cys (Fig. 1c) had degree of substitution (DS) values ranging from 2.16 to 2.84 through MALDI-TOF mass spectrometry (data not shown) [20]. Using NMR spectroscopic analysis, we also found that the neutral cyclosophoraoses were highly substituted with the succinate groups at the 3, 4-OH and 6-OH positions (Fig. 1c) [20].

Study of the interactions between NAP and cyclic oligosaccharides

The phase solubility experiments for NAP with with α-, β-, and γ-cyclodextrin were reported [33, 34]. Recently, phase-solubility analysis at various temperatures was carried out in order to evaluate the solubilizing power of acetylated β-cyclodextrin and γ-cyclodextrin toward NAP [35]. In this study, the phase solubility interaction between

NAP and cyclic olilgosaccharides (Cys and S-Cys) at differnt pH values was investigated to determine the apparent stability constants of the equimolar complexes.

Figure 3 shows the solubility of NAP in the presence of various concentrations of cyclic oligosaccharides under different pH conditions. According to a conventional host–guest complexation theory, dissolved host molecules only interact with drugs through formation of inclusion complexes whose guest(G)-host(H) (G_mH_n) stoichiometry (m:n) is most frequently 1:1, 1:2 and 2:1. Five types of phase-solubility relationship were suggested by Higuchi and Connors [25]. AL-type has a linear relationship between guest solubility and host concentration and it is interpreted as formation of a complex with m:1 stoichiometry;. AP-type represents positive deviation from linearity and which is usually interpreted as m:n complex. AN-type represents negative deviation from linearity with no reasonable explanation. BS- and BI-types correspond to complexes possessing limited solubility [36]. NAP solubility increased linearly with an increase in concentration of host cyclooligosaccharide as AL-type profiles in all cases. This AL-type profile indicates that a 1:1 complexation occurred at different pH values. The apparent stability constant values of the various complexes were calculated from the phase-solubility diagrams in different pH buffer solutions as described by Higuchi–Connors [25]. The effect of drug ionization on the stability of its complexes with the different cyclic oligosaccharides is shown in Table 1. Since NAP (pKa = 4.2) is in the unionized form at pH 3.4, the complex formed between the cyclic oligosaccharides and NAP was the most stable stability. At pH 3.4, when the drug is in the uncharged state, it has a higher affinity for the hydrophobic cavity of the cyclic oligosaccharides. The Cys formed the most stable complex ($K_{1:1} = 2457.9\ M^{-1}$), followed by the negatively charged S-Cys ($K_{1:1} = 357.1\ M^{-1}$). The succinate moeities of S-Cys may affect the spatial arrangement of the cyclic oligosaccharide and provide steric repulsion during complexation with NAP, which would decrease the stability of the NAP complex. Their apparent stabilities were also compared with commercial solubilizers such as CD ($K_{1:1} = 364.1\ M^{-1}$) and HP-CD ($K_{1:1} = 348.6\ M^{-1}$) (Table 1). The complex stability rapidly decreased with increasing pH, which occurred because of a lower affinity for inclusion in the cyclic oligosaccharide cavity. A large reduction in the complex stability constant was observed at pH 7.5, where the drug was almost completely (99.5%) in the ionized form. At this pH value, the stability constant of the complex with Cys ($K_{1:1} = 119.2\ M^{-1}$) was higher than that with the β-CD ($14.48\ M^{-1}$) and other negatively modified cyclic oligosaccharides (S-Cys; $K_{1:1} = 5.6\ M^{-1}$ and HP-CD; $K_{1:1} = 6.75\ M^{-1}$). These results indicate that the degree of ionization had a significant effect on the stability constant, and hence on the solubility of NAP at different pH values. Both protonated and neutral NAP can form complexes with the cyclic oligosaccharide. However, at acidic pH, a major portion of NAP exists in the unionized form, which is hydrophobic; hence it can easily become entrapped in the hydrophobic cavity of the cyclic oligosaccharides. However, complex formation between NAP and cyclic oligosaccharides rapidly decreased with increasing pH. Since the drug remained in its ionized form, the ionized molecules may disrupt the complex formation with the cyclic oligosaccharides at pH 7.5.

Determination of complexes stoichiometry between NAP and Cys

The continuous variation method can be used to determine the complex stoichiometric ratios of inclusion phenomena [27], and this method is based on the difference in a physical parameter, for example, absorbance ΔA ($\Delta A = A_0 - A$) of NAP in the presence of Cys. ΔA values could be calculated by measuring the absorbance of NAP solutions in the absence (A_0) and presence (A) of the corresponding concentration of the Cys. The continuous variation plots demonstrated that the complex had a 1:1 stoichiometry, since the ratio, r, had a maximum value of 0.5 at pH 3.4, 5.6 and 7.5, respectively (Fig. 4) [32].

Differential scanning calorimetry (DSC)

DSC provided information on the solid-state interactions between NAP and Cys. The inclusion complex formation between NAP and Cys was examined by differential scanning calorimetry (DSC) and compared with the corresponding physical mixture in the same molar ratio (Fig. 5). In DSC analysis, the melting peak corresponding to NAP was observed at 157.2 °C; however, this peak was observed at 157.0 °C in the physical mixture, which indicated that there was no interaction in the physical mixture. The disappearance of an endothermic peak (Fig. 5d) may be attributed to the interaction and inclusion complexation of NAP with Cys, as would be expected [29].

ROESY NMR spectra of the complexes

Nuclear overhauser effect (NOE) can be used to provide information on the geometry of the Cys complexes and this method is based on the spatial distance between the interacting protons. ROESY experiments have frequently been used to elucidate the intermolecular interaction of the inclusion complex [28]. The ROESY spectra of the complexes between NAP and Cys at pH 3.4 and 7.5 are shown in Fig. 6a and b, respectively. In the two-dimensional ROESY experiments, the NOEs between the Cys and NAP

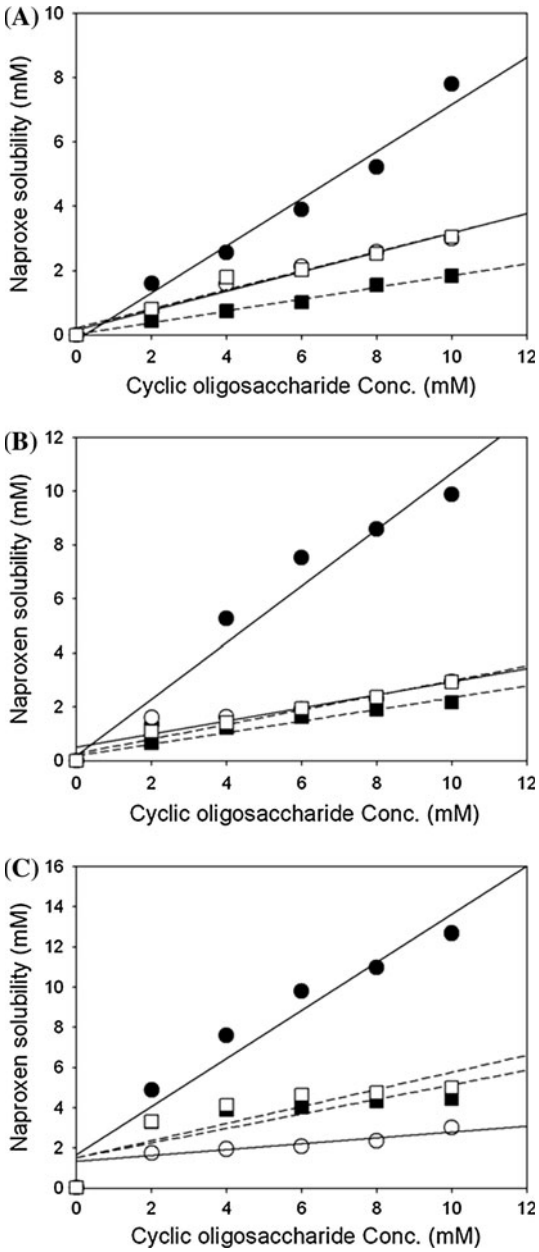

Fig. 3 Solubility of naproxen as a function of cyclic oligosaccharide concentration in different pH buffers; **a** pH = 3.4, **b** pH = 5.6, and **c** pH = 7.5. Symbols; Cys (*filled circle*), S-Cys (*open circle*), CD (*filled square*), and HP-CD (*open square*)

Table 1 Apparent stability constants for the interaction between naproxen and various cyclic oligosaccharides

pH	Apparent stability constant, $K_{1:1}$ (M^{-1})			
	Cys	S-Cys	CD	HP-CD
3.4	2457.9	357.1	364.1	348.6
5.6	1204.6	116.9	113.4	161.6
7.5	119.2	5.6	14.48	6.75

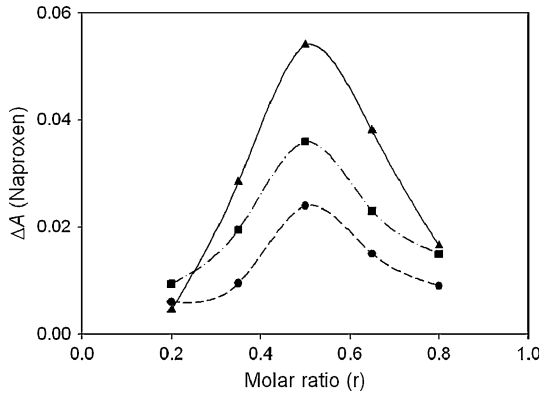

Fig. 4 Continuous variation plot of the naproxen/cyclosophoraoses complexes at pHs 3.4 (*filled triangle*), pHs 5.6 (*filled square*) and pHs 7.5 (*filled circle*)

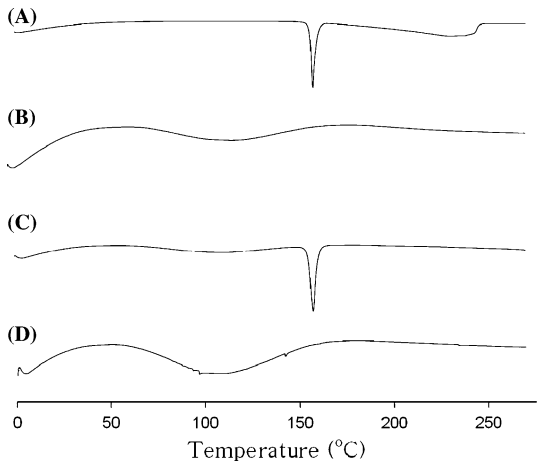

Fig. 5 Differential scanning calorimetry curves of the NAP/Cys complex **a** intact NAP, **b** Cys alone, **c** physical mixture of NAP/Cys, **d** NAP/Cys complex

protons were different between pH 3.4 and 7.5 (Fig. 6). Intermolecular cross-peaks appeared between protons (H3 and H6b) of the Cys and protons (H5′ and H13′) of NAP at pH 3.4, but the cross-peaks disappeared and were weak at pH 7.5 (Fig. 6). This different correlation pattern at pH 3.4 and 7.5 demonstrates that the inclusion complexes between the Cys and NAP formed as a result of the different molecular interaction, which was due to the ionization changes of NAP at the different pH values. Therefore, these results suggest that the protonated carboxyl group of NAP may effectively interact with the proton of the Cys and complex formation occurs through two hydrogen bonds between the carboxyl group of NAP and the hydroxyl groups of the Cys. However, the deprotonated NAP at pH 7.5, where NAP is almost completely in the ionized form, has a low affinity for Cys due to the loss of its hydrogen bond.

Conformation analysis of Cys and CD

To investigate the binding mode of NAP with Cys and CD, docking simulations were carried out using the Glide docking suite [30]. The effect of acidic or neutral pH during the docking job was simply modeled using the protonated or deprotonated state of NAP in molecular detail. Table 2 summarizes the final Glide score of each docking complex between the NAP molecules and Cys or CD. The docking scores suggest that Cys is a more suitable solubilizing agent for NAP at all pH values. The docking scores for NAP with Cys or CD were correlated with the apparent stability constants. The binding mode of each complex from the docking results indicated that Cys forms a complex with NAP through a different mechanism than CD (Fig. 7). Cys are in the similitude of the distorted doughnut form. The NAP molecule squeezes in through the narrow crack made by the critical arrangement of the glycosyl residues. Cys tightly enclosed the NAP with the sugar rings. The interaction between the NAP and Cys were mainly mediated by sugar-aromatic ring contacts without a void space. The protonated NAP was further stabilized by two hydrogen bonds between the carboxyl group and the hydroxyl groups of the Cys (Fig. 7a, c). This is comparable with the deprotonated NAP/Cys complex, which has only one hydrogen bond (Fig. 7b, d). The additional hydrogen bond mediated by the carboxyl group of protonated NAP seems to be responsible for the higher binding constant of the NAP/Cys complex at acidic pH. The CD formed a typical inclusion complex with the NAP molecule (Fig. 7). The NAP was inserted into the central cavity of the circular CD structure. The orientation of NAP in the cavity was different between the protonated and deprotonated form. However, these interactions were not tight when compared to the NAP/Cys complex. The carboxyl group of NAP strayed out of its space around the inner cavity of CD due to its molecular size. It was concluded that the planar shape of the NAP resulted in the formation of a stable complex with the narrow crack in the distorted Cys conformation as compared with the circular cavity structure of CD.

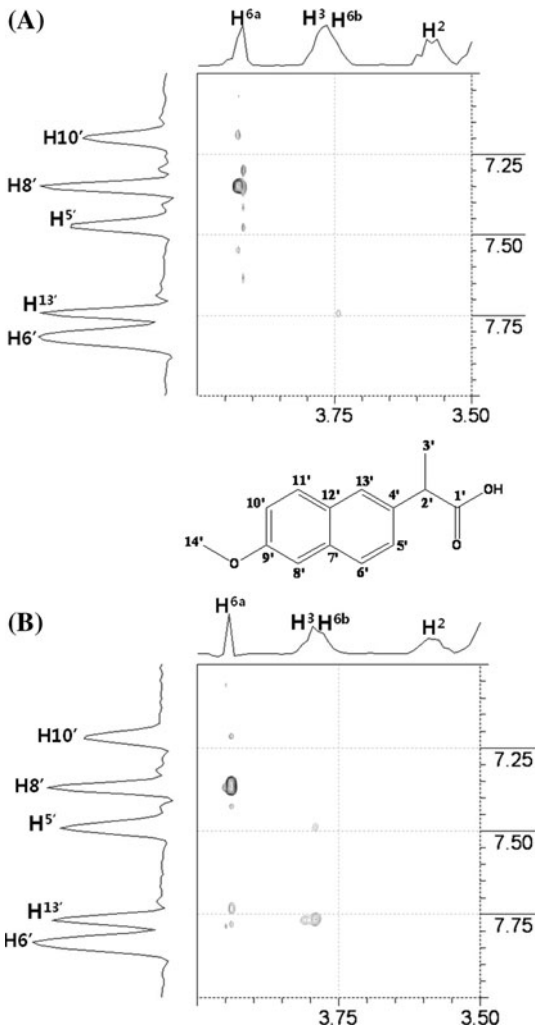

Fig. 6 Expansion of ROESY spectra of the naproxen/cyclosophoraoses complexes at pH 3.4 (**a**) and 7.5 (**b**)

Table 2 Glide docking scores for each protonated and deprotonated naproxen with Cys or CD

	Cys	*β*-CD
Protonated-naproxen	−7.986	−6.890
Deprotonated-naproxen	−7.118	−5.444

Conclusion

In this study, we evaluated pH-dependent inclusion complex formation between NAP and Cys. The results of UV–vis and NMR spectroscopic studies showed that Cys formed complexes with NAP in a molar ratio of 1:1 at different pH values (pH = 3.4, 5.6, and 7.5). The Cys could form inclusion complexes with NAP and the binding strength was dependent on pH. The Cys produced the most stable complex formation with NAP ($K_{1:1}$ = 2457.9 M^{-1}), followed by the negatively charged S-Cys ($K_{1:1}$ = 357.1 M^{-1}) at pH 3.4. A strong reduction in the complex stability constant was observed at pH 7.5. When the reduction in the stability constant was compared with other cyclic oligosaccharides (Cys; 119.2 M^{-1}, CD; 14.48 M^{-1} and HP-CD; 6.75 M^{-1}), S-Cys ($K_{1:1}$ = 5.6 M^{-1}) was shown to have the highest reduction in stability constant. These results suggest that S-Cys could regulate the efficiency of inclusion complex formation at external pH values. The ROESY experiments also showed that the intermolecular cross-peaks between Cys and NAP were different at pH 3.4 and 7.5. In the

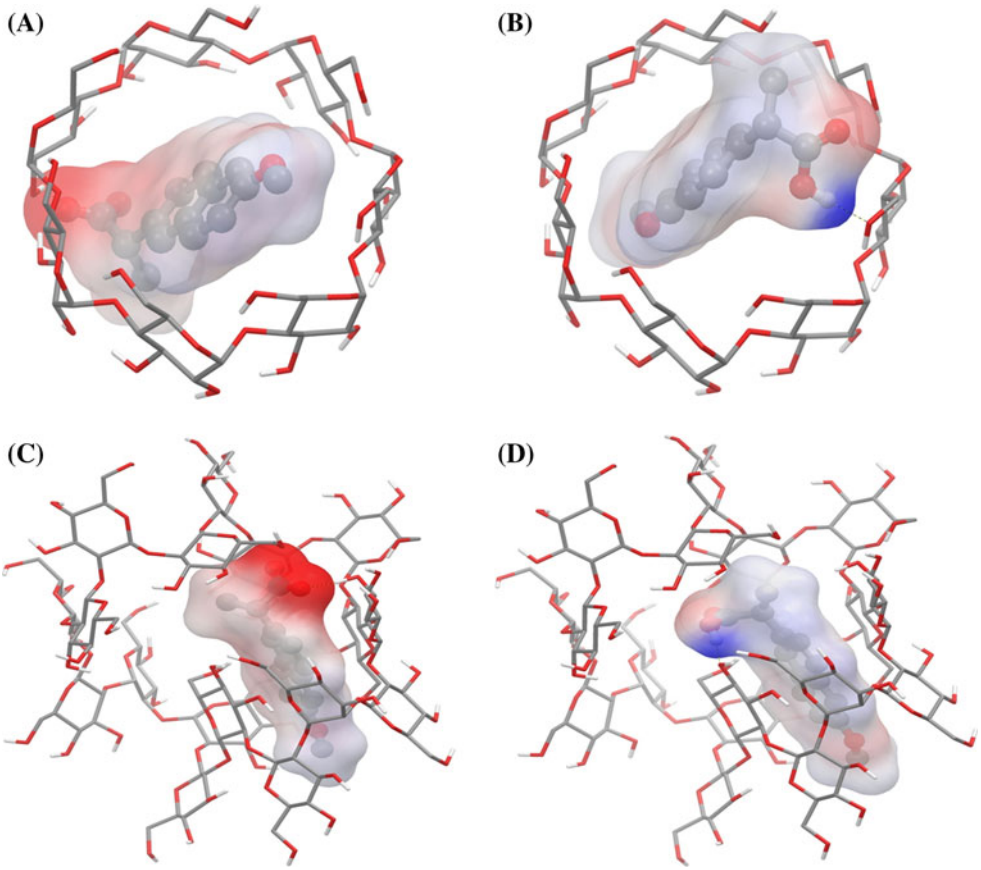

Fig. 7 Top-ranked docked poses for the protonated (**a, c**) and deprotonated (**b, d**) form of naproxen with the CD or Cys. Naproxen molecules were represented as a ball and stick model

molecular docking simulations, the carboxyl groups of NAP was shown to have a different orientation and position in its space around the inner cavity of CD and Cys, and this effect was dependent on the ionization changes of the NAP at different pH values (pH = 3.4 and 7.5). These results showed the pH-dependent inclusion behavior of Cys was due to the differential charge distribution of the carboxyl groups of NAP.

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