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Drug carrier systems based on water-soluble cationic β-cyclodextrin polymers

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Abstract

This study was designed to synthesize, characterize and investigate the drug inclusion property of a series of novel cationic β-cyclodextrin polymers (CPβCDs). Proposed water-soluble polymers were synthesized from β-cyclodextrin (β-CD), epichlorohydrin (EP) and choline chloride (CC) through a one-step polymerization procedure by varying molar ratio of EP and CC to β-CD. Physicochemical properties of the polymers were characterized with colloidal titration, nuclear magnetic resonance spectroscopy (NMR), gel permeation chromatography (GPC) and aqueous solubility determination. The formation of naproxen/CPβCDs inclusion complexes was confirmed by NMR and fourier transform infrared spectroscopy (FT-IR). Cationic β-CD polymers showed better hemolytic activities than parent β-CD and neutral β-CD polymer in hemolysis test. The morphological study of erythrocytes revealed a cell membrane invagination induced by the cationic groups. The effects of molecular weight and charge density of the polymers on their inclusion and release performance of naproxen were also investigated through phase-solubility and dissolution studies. It was found that the cationic β-CD polymers with high molecular weight or low charge density exhibited better drug inclusion and dissolution abilities.
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Keywords: Cationic β-cyclodextrin polymer; Physicochemical analysis; Inclusion complexes; Human erythrocytes; Hemolysis; Morphological changes

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six to eight glucose unites linked by α-1,4-glucosidic bonds. The internal hydrophobic cavities in the CDs facilitate the inclusion of a number of guest molecules (Szejtli, 1998). During the past decades, CDs have been used successfully as drug carriers to improve drug solubility, chemical stability, dissolution and bioavailability or decrease unfavourable side-effects (Hedges, 1998). However, the low water solubility and toxicity of parent CDs limit their further application in pharmaceutical formulations (Irie and Uekama, 1997). The in vivo cytotoxicity of CDs (and β-cyclodextrin in particular) appears to involve binding to and extraction of membrane cholesterol, mainly through inclusion complexation, altering the permeability properties of the membrane and eventually leading to hemolysis (Ohtani et al., 1989). In some in vivo studies involving parenteral administration of CDs, it was found that renal cell damage might be a consequence of the

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selective extraction of cholesterol from kidney tubular membranes by CDs (Rajewski et al., 1995). Therefore, both cytotoxicity and nephrotoxicity of parent CDs arise from the extraction of cholesterol from cell membrane.

To overcome these serious drawbacks, various CDs derivatives have been developed. Among them, the methylated CDs, hydroxyalkylated CDs and ionic CDs are typical ones aiming at improving water solubility via disrupting the intermolecular hydrogen bond between the secondary hydroxyl groups of parent CDs. Such hydrogen bonds are mainly responsible for their low aqueous solubility (Szejtli, 1998). However, the increase of water solubility does not necessarily result in lowering toxicity. The methylated CDs showed high toxicity in spite of the improved water solubility (Uekama and Irie, 1987), whereas sulfate and sulfobutyl ether of β-CD exhibited much better hemolytic activity than parent β-CD when its hydroxyl groups are substituted by anionic groups (Macarak et al., 1991; Shiotani et al., 1995). Recent results indicated that the β-CDs modified with quaternary ammonium group were ineffective in hosting cholesterol (Zhong et al., 2001). This implies that such modified CDs might exhibit potential better hemocompatibility. On the other hand, cyclodextrin-based polymers are of interest due to their merits compared to parent CDs, such as high solubility in water and capability to solubilize a number of drugs, those with large molecular structures in particular. CD polymers can also increase the drug bioavailability (Szeman et al., 1987; Fenyvesi, 1988; Mura et al., 2002). The work presented in this paper was based on these previous findings, with the hypothesis that the β-cyclodextrin polymers modified with quaternary ammonium groups possess the advantages of CD polymers addressed above and have less tendency to bind cholesterol. Thus, we utilized choline chloride to introduce quaternary ammonium groups onto the molecular structure of CD polymers in order to improve the hemolytic activity.

Naproxen ((*S*)-(+)-6-methoxy-α-methyl-2 naphthaleneacetic acid, NAP) is a non-steroidal anti-inflammatory drug often used to relieve fever and to reduce inflammation and pain associated with arthritis. Naproxen works by inhibiting cyclooxygenase, an enzyme responsible for synthesizing prostaglandins, which are believed to cause the perception of pain and the production of fever and inflammation. However, naproxen has an extremely low aqueous solubility (approximately 27 mg/l at 25 °C). As a result, its bioavailability appears to be limited. It has been found that the low water solubility of naproxen can be improved by complexation with both native and chemically modified cyclodextrins. Among the three types of parent cyclodextrins, β-CD is the best partner of naproxen (Bettinetti et al., 1989; Mura et al., 1995, 2002).

In this work, a range of novel cationic β-cyclodextrin polymers containing quaternary ammonium groups was synthesized. The physicochemical properties of the modified CD polymers were characterized using various techniques including colloidal titration, ^{13}C NMR, GPC and aqueous solubility determination. The formation of naproxen/CPβCDs inclusion complexes was confirmed by ^{1}H NMR and FT-IR. The effects of the degree of polymerization and charge density of CPβCDs on their drug inclusion performance as well as hemolytic activity were investigated through phase-solubility, dissolution, hemolysis and morphological studies.

2. Materials and methods

2.1. Materials

β-Cyclodextrin (β-CD), epichlorohydrin (EP), choline chloride (CC) and naproxen (NAP) were purchased from Sigma Chemical Company, used as received. Standard anionic polyelectrolyte (potassium polyvinyl sulphate (PVSK) solution), standard cationic polyelectrolyte (poly(diallyldimethylammonium chloride) (poly-DADMAC) (concentration = 1 mM) and hydroxypropyl-β-CD ($M_w = 1402$) were obtained from Sigma Chemical Company. Other materials and solvent, such as standard Pulluan samples from P-5 to P-50 (P-82 from Shodex), were of analytical reagent grade. Double distilled-deionized water was used throughout.

2.2. Synthesis of β-CD based polymers

CPβCDs with various degrees of polymerization as well as cationic charge densities were synthesized by a one-step condensation polymerization. A typical synthesis procedure for a molar ratio β-CD/EP/CC

= 1/15/1 is described below: 1 g of NaOH was dissolved in 20 ml of water, and then 5.675 g of β-CD were dissolved in the sodium hydroxide solution. The solution was electromagnetically stirred at 25 °C for 24 h in a water bath. 0.698 g of CC was then fed into the solution rapidly and 6.940 g of EP were added at a flow rate of about 0.1 ml/min. After the completion of EP feeding, the mixture was heated to 60 °C. During the whole polymerization procedure, the temperature was kept at 60 °C and stirring was kept at 600 rpm. After 2 h, the polymerization was stopped by neutralized with an aqueous hydrochloride acid solution (3N). The solution obtained was dialyzed for 24 h with a dialysis membrane of molecular weight cut-off 1000. Residual unmodified β-CD and merely substituted β-CD monomers were not separated intentionally in order to investigate the properties of the proposed polymers as a whole instead of the high molecular weight fraction only. The solution obtained was evaporated and the solid was pulverized to powder. By following the similar procedure above, a series of CPβCDs with different β-CD/EP/CC ratio were synthesized. The polymerization recipes are presented in Table 1. As for the sample name, the first number after CPβCD means the ratio of EP/β-CD, and the second number refers to the ratio of CC/β-CD.

2.3. Determination of charge density

Charge density of the cationic β-CD polymers was determined via colloidal titration using a Particle Charge Detector MÜtek PCD 03 (Herrsching, Germany). 2.5 ml of 0.1% β-CD polymers solution were added into the measure cell, with 7.5 ml water. The solution was titrated with either standard anionic polyelectrolyte (PVSK solution) or standard cationic polyelectrolyte (poly (diallyldimethylammonium chloride) (poly-DADMAC) (concentration = 1 mM). Three repeats were conducted to get an average value for each sample.

2.4. Analysis of molecular weight (M_w) by GPC

Gel permeation chromatography (pump: Waters 600E System Controller; detector: Waters 410 Differential Refractometer) was carried out with Ultrahydrogel 120 and Ultrahydrogel 250 columns at 40 °C and the flow rate 0.7 ml/min. Water was used as an eluent. Calibration was made using standard Pulluan samples from P-5 to P-50 (P-82 from Shodex), hydroxypropyl-βCD (M_w = 1402) and β-CD (M_w = 1135). Samples were used with concentration of 0.2–0.4% (w/v) and filtrated with a 0.45 μm Nylon Cameo filter-syringe prior to the use.

2.5. NMR studies

Nuclear magnetic resonance (NMR) spectra were conducted in D_2O using a Varian Unity 400 spectrometer operated at 400 MHz for ^1H NMR and at 100 MHz for ^{13}C NMR.

2.6. FT-IR

Fourier transform infrared spectroscopy was performed on Nexus 470 FT-IR (Thermo Nicolet

Table 1
Polymerization recipe and molecular weight of obtained CPβCDs

Sample	β-CD (mol)	EP (mol)	CC (mol)	NaOH (mol)	H_2O (g)	M_n
PβCD5	0.005	0.025	0	0.025	20	2638
CPβCD5_0.5	0.005	0.025	0.0025	0.025	20	2578
CPβCD5_1	0.005	0.025	0.005	0.025	20	2383
CPβCD5_1.5	0.005	0.025	0.0075	0.025	20	2320
PβCD10	0.005	0.05	0	0.025	20	4936
CPβCD10_0.5	0.005	0.05	0.0025	0.025	20	4957
CPβCD10_1	0.005	0.05	0.005	0.025	20	4448
CPβCD10_1.5	0.005	0.05	0.0075	0.025	20	4468
PβCD15	0.005	0.075	0	0.025	20	5382
CPβCD15_0.5	0.005	0.075	0.0025	0.025	20	5103
CPβCD15_1	0.005	0.075	0.005	0.025	20	4864
CPβCD15_1.5	0.005	0.075	0.0075	0.025	20	4822

Company), running from 4000 to 650 cm^{-1} using zinc selenide as a sample holder. The blend of NAP with CPBCD15_1.5 at 1:1 (mol/mol) ratio was manually ground using a mortar with a pestle for 10 min (Arias et al., 1997; Mura et al., 2002). The amount of CPBCD15_1.5 was determined by taking the β-CD repeat unit as its molecular weight.

2.7. Aqueous solubilities of CD-polymers

0.3 g of polymer samples was added to 0.5 ml of water to ensure the solution reaching saturation. The solution was mechanically shaken for 4 h and then incubated overnight at room temperature. The solution was then filtered through a 0.22 μm Nylon Cameo filter-syringe. The filtrate was dried in an oven for sufficient period until a constant weight being reached. The solubility was estimated in terms of the weight of samples in the saturated solution and solution volume. Two repeats were conducted.

2.8. Solubility of drug complexes

Solubility measurement of NAP were carried out by adding 10 mg of drug to 1 ml of aqueous solution of CPβCDs (concentration ranging 1–7%, w/v) to a sealed glass container which was mechanically shaken at 25 °C until equilibrium was achieved (approx. 72 h). After the equilibration, an aliquot was withdrawn and filtered (pore size 0.22 μm). The NAP concentration was determined by measuring the ultraviolet absorbance of the saturated solutions at 274 nm wavelength and compared with the calibration curve (Bettinetti et al., 1989). Each experiment was performed in duplicate. Changes in NAP solubility (S) were plotted as a function of cyclodectrin (CD) concentration. In the case of the formation of a 1:1 inclusion complex, the stability constant $K_{1:1}$ can be obtained from the slope S_1 and intercept S_0 of the initial straight line portion of the diagram in terms of the equation described by Higuchi and Connors (1965):

$$K_{1:1} = \frac{S_t - S_0}{S_0\{[\mathrm{CD}]_t - (S_t - S_0)\}} = \frac{\mathrm{slope}_1}{S_0(1 - \mathrm{slope}_1)}$$

When $K_{1:1}$ was applied to evaluate the phase-solubility of CD polymers using the equation above, the molar concentration of CD polymers was calculated by taking the β-CD repeat unit as its molecular weight to convert the units of CD polymer concentration from % (w/v) to mol/l. In other words, the CD repeat unit was treated as the "host" instead of the entire polymer chain. Thus, the $K_{1:1}$ of different cationic CD polymers with different degrees of polymerization can be compared (Mura et al., 2002).

It should be addressed that the potential effect of the varying amount of EP and CC residues on the $K_{1:1}$ calculation was assumed to be negligible due to the much lower molecular weights of EP and CC compared with CD unit. Moreover, the ratios of EP and CC residues to CD in various polymers should be relatively constant due to the similar reactivity of EP towards CD in the range of molecular weights being studied, which further minimizes the impact of the residues.

2.9. Dissolution of drug complexes

NAP/CPβCD complexes were prepared by adopting the procedures described by Arias et al. (1997) and Mura et al. (2002). Equimolecular of naproxen and CPβCDs were mixed together then manually ground using a mortar with a pestle for 10 min, in condition leading to the best yield and to the most stable complexes

Dissolution rates of NAP from the NAP/CPβCDs complexes in the equimolar ratio, as well as those of the drug alone were determined in water at 37 ± 0.3 °C according to a dispersed amount method (Mura et al., 2002). One hundred milligrams of NAP or NAP equivalent were added to 300 ml of water and stirred. Suitable aliquots were withdrawn with a filter-syringe (pore size: 0.45 μm) at appropriate interval times and assayed for NAP content as in the solubility studies. The same volume of fresh medium was added to the beaker and the correction for the cumulative dilution was calculated. Each test was repeated twice.

2.10. Hemolysis test

Hemolytic activities of CPβCDs were evaluated according to the method of Ohtani et al. (1989). Sodium citrate was added (0.47%) to freshly drawn human blood. The erythrocyte fraction obtained after centrifugation (1000 rpm for 5 min) was washed three times with phosphate buffer (0.154 M sodium chloride and 0.01 M phosphate, pH 7.4) and re-dispersed in a buffer solution to give a hematocrit of 5%. 0.1 ml of cell

suspension was added to 2.0 ml of the buffer solution containing cationic PβCDs at various concentrations. Each mixture was incubated for 30 min at 37 ± 0.5 °C, and then centrifuged at 1000 rpm for 5 min. The supernatant concentration was measured using a 721 UV spectrophotometer at 543 nm, which corresponded to the release of haemoglobin from the cells. The degree of hemolysis was presented as percentages of the total efflux of haemoglobin, which was obtained when water was used instead of the buffer solution containing CPβCDs.

2.11. Morphological study of erythrocytes

The erythrocyte suspension (5%, 0.1 ml) was incubated with the phosphate buffer solution (2 ml) containing test samples at 37 °C for 60 min and then fixed with 2% glutaraldehyde solution (5 ml) on ground slides covered with poly(vinyl formal) membrane. After being stood at room temperature for 1 h, the fixed cells were washed with 50–70–90–100% acetone solutions separately and then dehydrated with 50–70–90–100% isoamyl acetate solutions (in acetone) step by step. The sample were then dried in CO_2 for 16 h, and coated with gold. The prepared samples were finally observed under a scanning electron microscope (SEM) (Akashi, MSM4C, Tokyo, Japan).

3. Results and discussion

3.1. Physicochemical characterization of cationic PβCDs

CPβCD15_1.5 was chosen to investigate the structure of cationic β-CD polymers. Fig. 1 shows its ^{13}C NMR spectrum and the carbon groups to which these chemical shifts are assigned. The assignment of C-1 to C-9 is referred to the findings of Harada et al. (1981) and Renard et al. (1997). In the spectrum, “A” is the overlap of the substitution of C-3 and C-6. “B” indicates to two peaks at 65.356 and 65.801 ppm, which

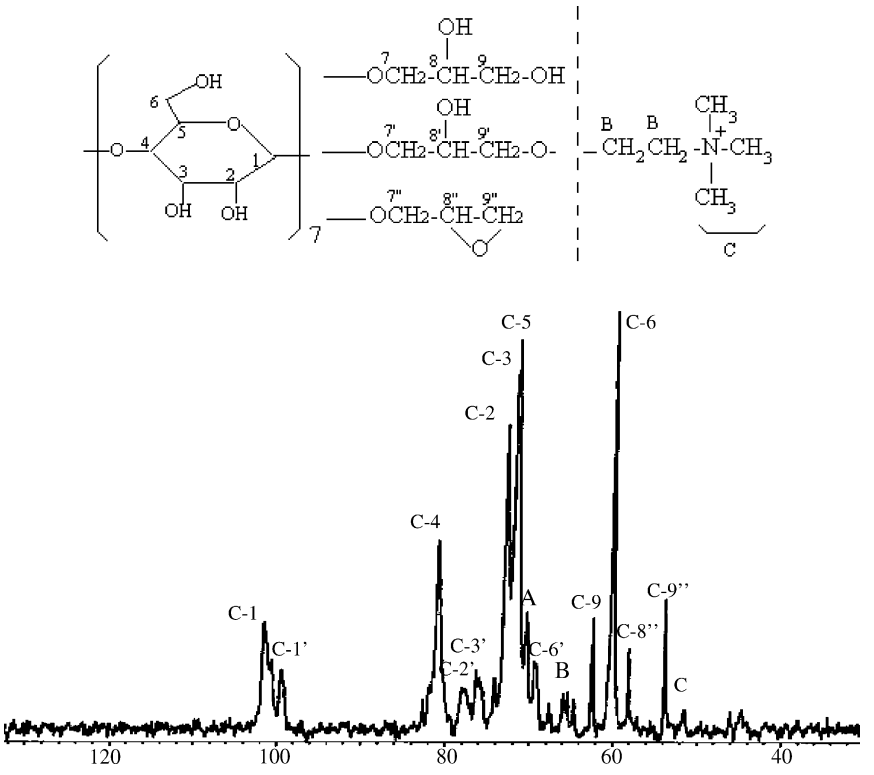


Fig. 1. ^{13}C NMR spectrum and the proposed structure of cationic CPβCD15_1.5 in D_2O.

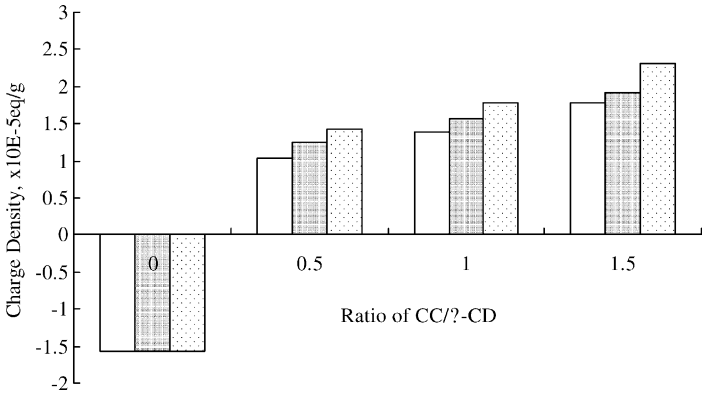


Fig. 2. Charge density of CPβCDs: (□) EP/β-CD = 5, (▨) EP/β-CD = 10, (▩) EP/β-CD = 15.

should be assigned to the two CH_2 groups introduced by the choline chloride because they are closed to each other and have the similar area. "C" indicates a signal at 51.880 ppm, which may be attributed to the resonance of the methyl choline group. These signals results are similar to those reported by Simkovic (1997). The resonance of C-2, C-3 and C-6 are all shifted and indicated as C-1′, C-2′, C-3′ and C-6′, respectively. Such information suggests that the substitution occurred on both of the primary and secondary sides of β-CD cavity of CPβCDs prepared in this paper.

Fig. 2 shows the influence of the different ratios of choline chloride/β-cyclodextrin (CC/β-CD) and epichlorohydrin/β-cyclodextrin (EP/β-CD) on the charge density of obtained CPβCDs. In the absence of choline chloride, PβCDs appeared to be anionic (charge density $= -1.56 \times 10^{-5}$ eq./g). With the increasing of the ratio of CC/β-CD, a similar increasing trend of charge densities of samples with a same EP/β-CD ratio was observed due to the cationic property of choline chloride. Charge density also increased with the enhancement of EP ratio in the polymers. This is because choline chloride was incorporates into the CPβCDs through its reaction with epichlorohydrin. Thus, the increase of the ratio of epichlorohydrin provides more opportunities for cationic groups to enter the polymers. Since there was an obvious trend in charge density of obtained CPβCDs, we only chose PβCD15, CPβCD15_1.5 and CPβCD5_1.5 to further investigate the influence of the charge density on drug solubility and dissolution.

The number average molecular weights (M_n) of various CPβCDs are presented in Table 1. The M_n increases with the increasing of the ratio of EP/β-CD, which is consistent with previous studies (Renard et al., 1997; Yudiarto et al., 2001). On the other hand, the M_n decreases slightly with the increasing of the ratio of CC/β-CD while at the same EP/β-CD ratio. This is attributed to more EP consumed as the amount of CC increases. EP is a bifunctional linking agent, which controls the degree of polymerization of PβCDs. Due to the same reason the M_n of cationic PβCDs obtained in this paper are relatively lower than those with the same ratio of EP/β-CD obtained by other authors (Renard et al., 1997; Yudiarto et al., 2001). Fig. 3 presents the effect of different ratios of EP/β-CD and CC/β-CD on M_w distribution of cationic PβCDs. The results indicated that the low ratio of EP/β-CD (i.e., 5) produced the CPβCDs with lower MW ranging from 2000 to 3000. This implies the domination of βCD ether and dimer fractions. M_w distribution of CPβCD15_1 ranges mainly between 3000 and 7000, representing that most cationic β-CD polymers contain more than three of β-CD repeat units. The molecular weight distribution of both CPβCD5_1 and CPβCD15_1 are much broader than that of PβCD5. It is also note there are small peaks at low M_w range, which indicate the existence of relatively low molecular weight fractions consisting of residual unmodified β-CD and merely substituted β-CD monomers. This fraction was not separated intentionally in order to investigate the properties of the proposed polymers as a whole instead of the

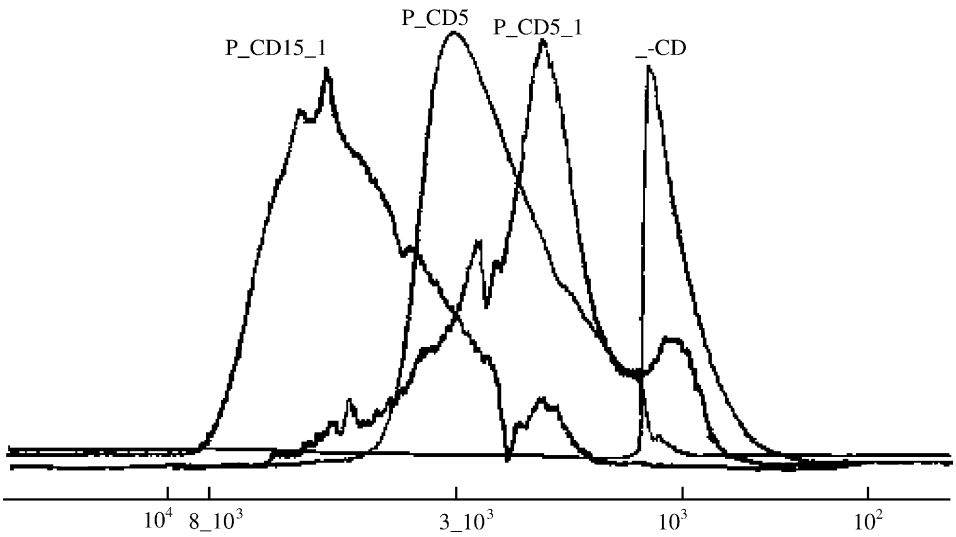


Fig. 3. Molecular distribution of several cationic PβCDs.

high molecular weight fraction only. The low MW fractions, estimated from the peak areas, ranged approximately from 3.8 to 5.8% in different samples. The number average molecular weights (M_n) of various CPβCDs are the statistic results of all fractions.

As expected, polymerization and introduction of cationic group significantly enhance the aqueous solubility of β-CD (shown in Table 2.). The solubilities of the CPβCDs are twenty times higher than that of the parent β-CD. The low aqueous solubility of parent β-CD is attributed to the intermolecular hydrogen bonding between the secondary hydroxyl groups, which are unfavourable to the interaction between cyclodextrin and surrounding water molecules (Szejtli, 1998). The introduction of cationic groups via condensation polymerization disrupts the intermolecular hydrogen bonding, thus increasing the aqueous solubility. However, the aqueous solubility of CPβCDs was insensitive to the ratio of epichlorohydrin and choline chloride in the compositions, implying that the increasing of polymerization degree or charge density does not necessarily lead to further interruption of original crystallinity of parent β-CD. This may be due to the fact that cationic group does not combine directly to the β-CD but to the epichlorohydrin chain. Consequently, the increasing of choline chloride does not further interrupt the intermolecular hydrogen bonding between the secondary hydroxyl groups of β-CD. On the other hand, since all of the CPβCDs are water-soluble, which implies the crosslinking did not occur or limited hydroxyl groups in a single β-CD molecule were connected by epichlorohydrin. The increment in the degree of polymerization resulted from the combination of more β-CD molecules, but did not necessarily lead to further interruption of intermolecular hydrogen bonding in a single β-CD molecule.

Table 2
Aqueous solubility of CPβCDs at 25 °C

Sample	Aqueous solubility (g/100 ml)	Solubility relative to β-CD
βCD	1.850[a]	1
PβCD5	40.125	21.69
CPβCD5_0.5	41.325	22.34
CPβCD5_1	40.875	22.09
CPβCD5_1.5	41.175	22.26
PβCD10	41.725	22.55
CPβCD10_0.5	39.200	21.19
CPβCD10_1	40.775	22.04
CPβCD10_1.5	39.425	21.31
PβCD15	39.600	21.41
CPβCD15_0.5	40.825	22.07
CPβCD15_1	42.275	22.85
CPβCD15_1.5	41.200	22.27

[a] Data taken from Szejtli (1998).

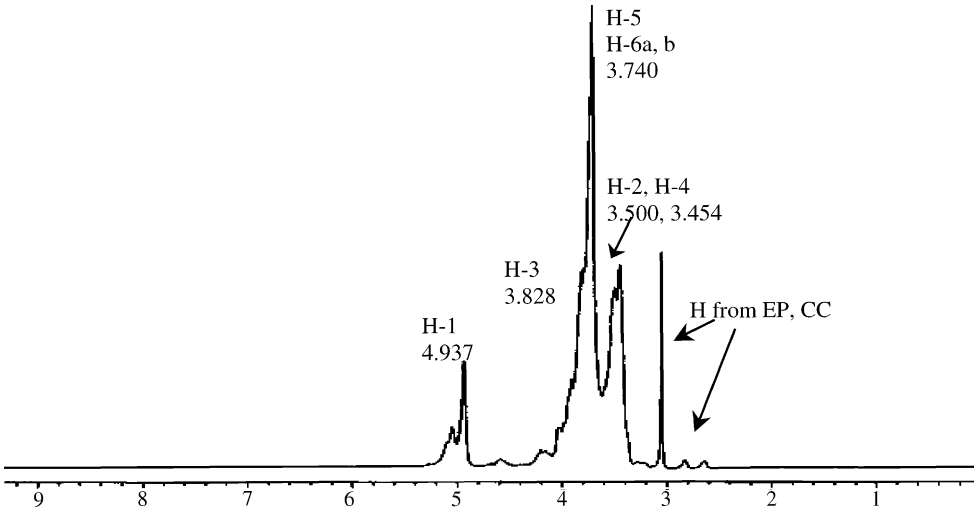


Fig. 4. ^{1}H NMR spectrum of CPβCD15_1.5 in D_2O.

3.2. NAP/CPβCDs complexes

Figs. 4 and 5 are ^{1}H NMR spectrum of CPβCD15_1.5 and NAP/CPβCD15_1.5 (co-ground in a molar ratio of 1:1), which confirm the presence of drug complexation. In the spectrum of CPβCD15_1.5, the peak near 5 ppm is assigned to the anomeric proton attached to the C-1 of the glucose unit; and two broadened peaks between 3 and 4 ppm correspond to the protons in pyranose rings. The peak assigned to the proton of 2-hydroxypropyl ether segments occurs at about 3 ppm (below the two peaks of the pyranose units), reflecting the chemical shift caused by the introducing of EP. ^{1}H NMR spectrum of NAP/CPβCDs complexes, in which both CPβCDs peaks and NAP peaks are observed, demonstrates a possible displacement of cyclodextrin protons located both inside and outside of the cavity (namely H-1 to H-6) resulting from drug

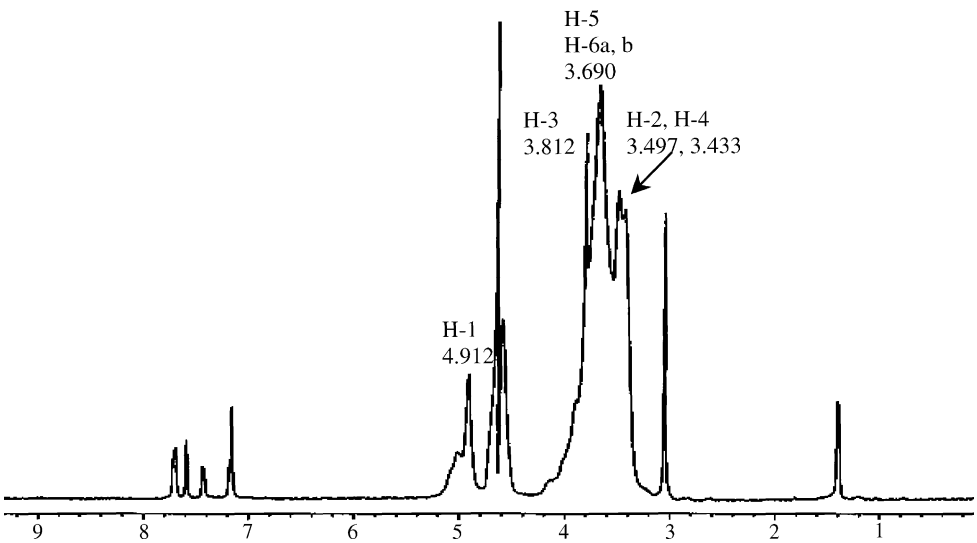


Fig. 5. ^{1}H NMR spectrum NAP/CPβCD15_1.5 complex in D_2O.

inclusion and interaction. There are slightly downfield chemical shifts of these protons after complexation of NAP. This represents the environment around these protons are changed by the guest molecules included. This may also indicate the existence of weak interaction between drug and CPBCDs and the formation of inclusion complexes in the products (Schneider et al., 1998; Hedges, 1998).

FT-IR spectrums provide further information on the formation of drug/CPβCDs inclusion complexes. Naproxen is characterized by peaks appearing between 1750 and 650 cm^{-1} (Fig. 6a), which are different from the strong peaks of CPβCD15_1.5 at about 1000 cm^{-1} (Fig. 6b). The peak at 1726 cm^{-1} of the C=O stretching of the carboxylic groups is the important characteristic band of NAP. As for CPβCD15_1.5, the bands at 1023 and 1080 cm^{-1} are due to the coupled C–C and C–O stretching vibrations; and the band at 1154 cm^{-1} is contributed by the antisymmetric stretching vibration of the C–O–C glycosidic bridge. In the complex of NAP/CPβCD15_1.5 (Fig. 6c), the spectra are the superposition of that of the two substances with attenuation of the NAP peaks. The peaks of NAP almost disappear whereas the characteristic peaks of CPβCD15_1.5 remain strong. It can be concluded that free NAP are mostly included in the cavities of CPβCD15_1.5, thus it cannot be characterized obviously in the FT-IR spectrum. The peak of the spectrum in the region at about 1000 cm^{-1} is broadened and slightly shifted due to the superposition of the band associated with the stretching of NAP. Meanwhile, a shift of the NAP characteristic peak from 1726 to 1728 cm^{-1} is observed in the complex. The results suggest the modification of electronic environment of NAP chemical groups by CPβCDs, which also confirms the formation of the NAP/CPβCD15_1.5 complex.

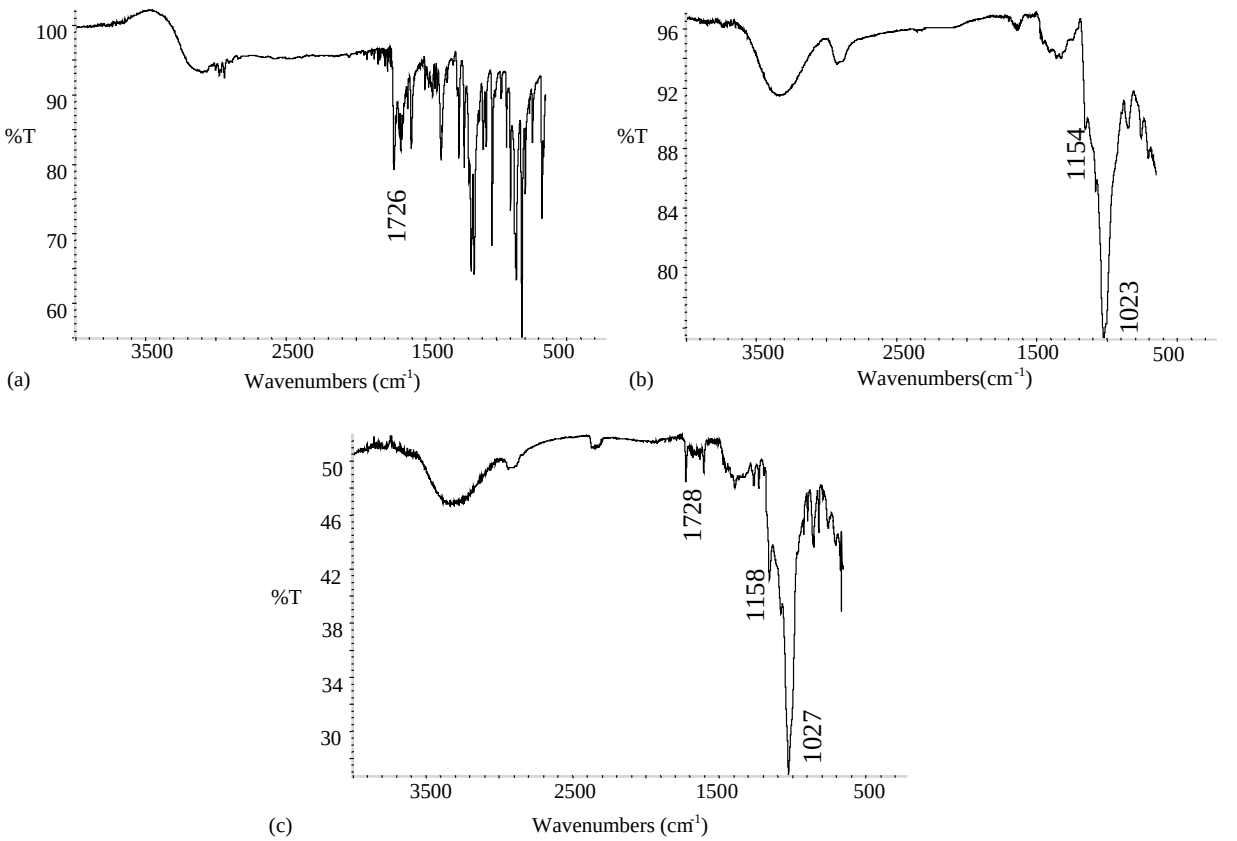


Fig. 6. FT-IR spectra of: (a) naproxen; (b) CPβCD15_1.5; (c) NAP/CPβCD15_1.5.

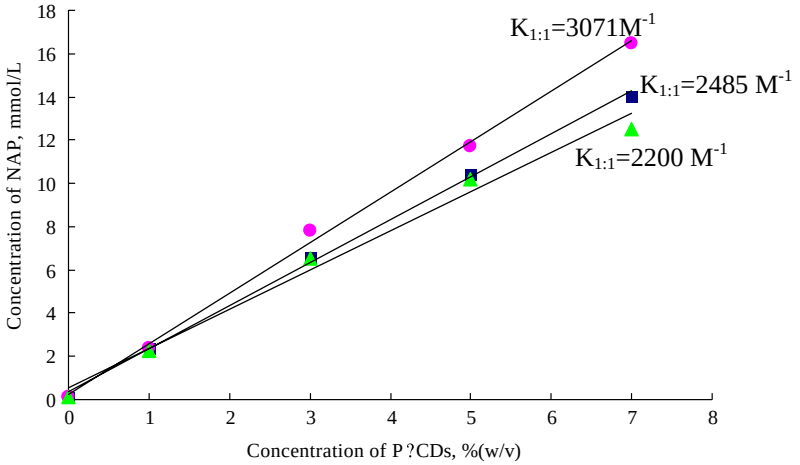


Fig. 7. Solubility diagrams of naproxen in (▲) CPβCD5_1.5; (■) CPβCD15_1.5; (●) PβCD15.

3.3. Phase-solubility and dissolution studies

The extremely low water solubility of naproxen (about 27 mg/l at 25 °C) has been significantly improved by complexation with CPβCDs (as shown in Fig. 7). For example, after included with CPβCD15_1.5 at a concentration of 7% (w/v), naproxen solubility increases up to 3.2 ± 10^3 mg/l, about 120 times higher than the original value. The high solubility may contribute to a higher drug bioavailability as reported by Uekama et al. (1985). The values of the apparent stability constants $K_{1:1}$ of the soluble complexes are 3071, 2485 and 2200 M^{-1} for PβCD15, CPβCD15_1.5 and CPβCD5_1.5, respectively (calculated according to the molecular weight of β-CD repeat unit as described previously). Cationic PβCDs with a higher polymerization degree (CPβCD15_1.5) has better complexation stability than that for CPβCDs with a lower polymerization degree (CPβCD5_1.5). Whereas both cationic PβCDs are more stable than parent βCD ($K_{1:1} = 1700\ M^{-1}$, as reported by Bettinetti et al. (1989)). This may be attributed to the cooperative action in binding between the adjacent β-CD units and polymer chains (Harada et al., 1981; Szeman et al., 1987). The adjacent units and polymer chain can act like arms of the CD cavities to facility the drug inclusion, which is especially useful when binding large molecule having two or more binding sites for inclusion. However, the apparent stability constants of CPβCDs are lower than that of neutral PβCDs (PβCD15). This suggests the potential decrease of the accessibility to the cavity of βCD units resulting from the structural restriction due to the introduction of quaternary ammonium groups.

Fig. 8 shows the dissolution profiles of the co-ground complexes of NAP with obtained CPβCDs. Compared with NAP alone, all NAP/CPβCD samples exhibit a much faster dissolution rate and much higher the maximum amount of drug dissolution. This may be mainly attributed to the high hydrophilic characteristics of CPβCDs, lowering the interfacial tension between NAP and water. The co-ground procedure of preparation of drug complexes may also have some positive impacts on this: it increases the total inter-surface between NAP and CPβCDs while decreases the crystallinity of NAP (Mura et al., 2002). Compared with the dissolution rate of the NAP/parent β-CD reported by Bettinetti et al. (1989) and Mura et al. (1995), the rate of NAP dissoluted from CPβCDs was higher due to the same cooperative action mentioned above. Moreover, the effects of the degree of polymerization on both NAP dissolution rate and NAP solubility appeared to be similar, so did polymer charge density. Therefore, inclusion stability constants might play a vital role in profiling the drug dissolution for the drug/CPβCDs complexes: it is easier for the samples with higher stability constant to accommodate more NAP thus improving the dissolution.

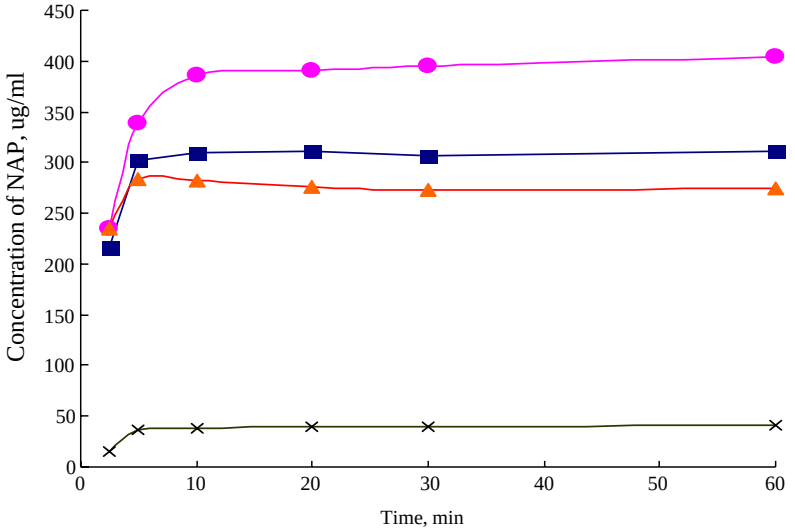


Fig. 8. Dissolution studies of naproxen alone (×) or from (▲) NAP/CPβCD5_1.5; (■) NAP/CPβCD15_1.5; (●) NAP/PβCD15.

3.4. Hemolysis and morphological studies

One of the most important requirements for drug carriers is that they have either no or acceptable low levels of intrinsic cytotoxicity. Studies with isolated erythrocytes may provide a simple and reliable measure to classify the CPβCDs according to their cytotoxicity because the interaction of CDs with plasma membranes must be the initial step of cell damage (Irie and Uekama, 1997). All tested samples induced hemolysis immediately at a low concentration and eventually caused almost total hemolysis at a high concentration (Fig. 9). The relative hemolytic activities is in the order of β-CD > PβCD15 > CPβCD5_1.5 > CPβCD15_1.5, implying that both increasing polymer chain length and increasing the amount of quaternary ammonium groups tend to enhance the structure hindrance of cationic PβCDs. Such hindrance correspondingly decreases the inclusion ability of their hydrophobic cavities to cholesterol, which acts as a key rigidifier in cell lipid bilayers. Thus, the damage of cationic PβCDs to cell membrane can be minimized.

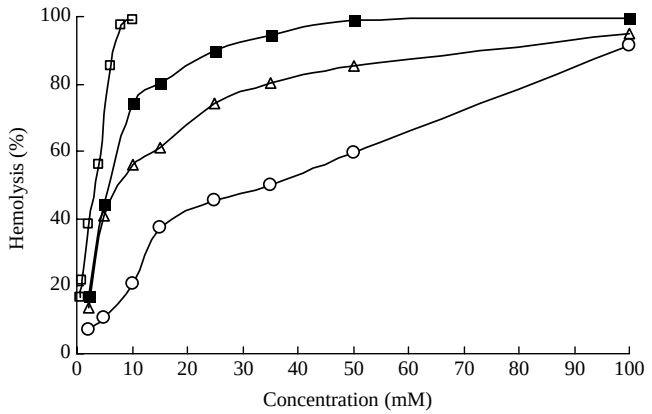
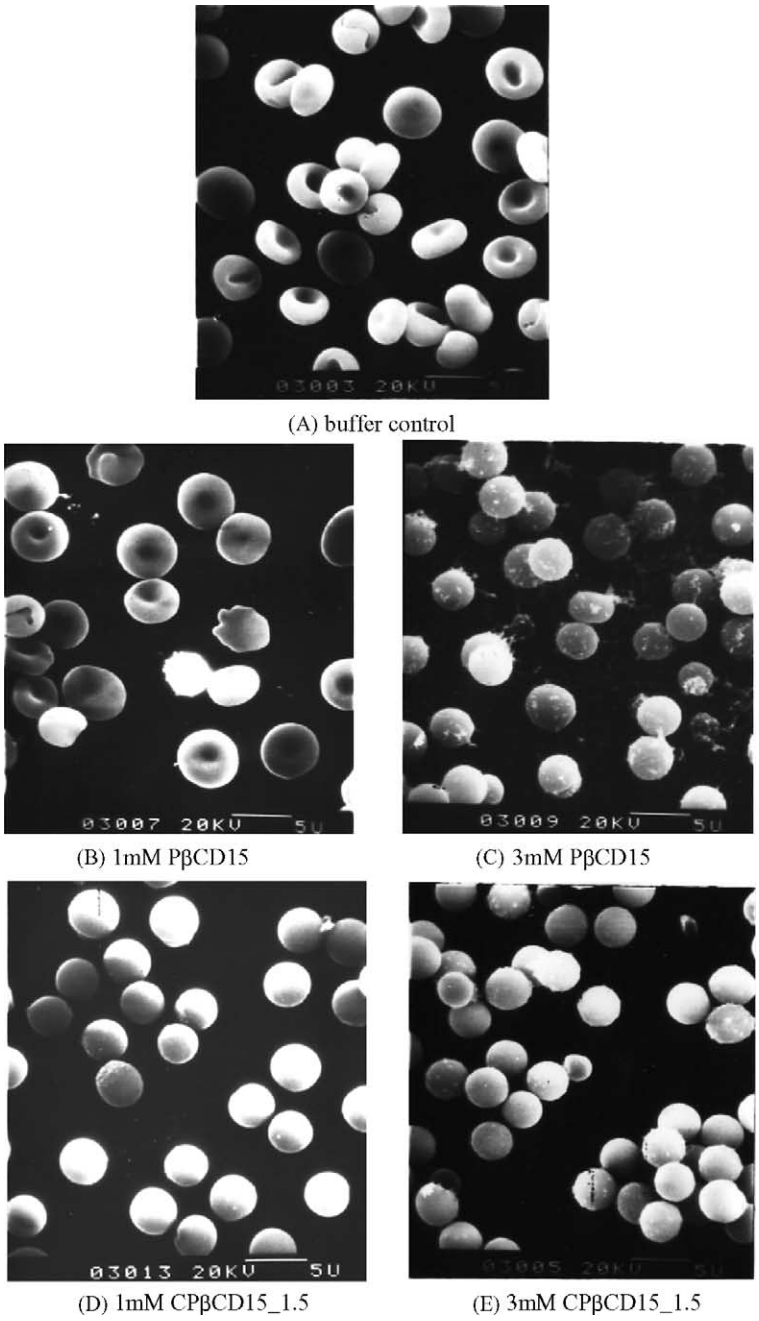


Fig. 9. Hemolytic effects of β-CD (□); PβCD15 (■); CPβCD5_1.5 (△); CPβCD15_1.5 (○) on human erythrocytes in isotonic phosphate buffer (pH 7.4).

However, at a high concentration (such as 100 mM), CPβCDs still induced total hemolysis due to the effect of increasing amount of CD cavities in conjunction with the inevitable change of osmotic pressure of the medium resulting from the addition of cationic substance (Fujii et al., 1979; Irie and Uekama, 1997).

Further information on the morphological changes of human erythrocytes with CPβCDs, obtained from



(A) buffer control

(B) 1mM PβCD15

(C) 3mM PβCD15

(D) 1mM CPβCD15_1.5

(E) 3mM CPβCD15_1.5

Fig. 10. Scanning electron micrographs of human erythrocytes treated with PβCDs and CPβCDs in phosphatebuffer (pH 7.4) at 37 °C.

SEM observation, is shown in Fig. 10. In the buffer control (Fig. 10A) erythrocytes are almost in good biconcave disc shape. At a low concentration (1 mM), PβCD15 does not induce the visible morphological changes of erythrocytes, compared with the control sample. However, at the same concentration PβCD15_1.5 turns the morphology of erythrocytes into spheres (Fig. 10D). This membrane internalization (invagination) ultimately leads to spherical cells with invaginated vesicles in the interior due to the electronic attraction force between positive charge of PβCD15_1.5 and the negative charge of the lipid bilayer of cells (Fujii et al., 1979). Even at a relatively high concentration of CPβCD15_1.5 (3 mM, Fig. 10E), the micrographs of those cells remain clear and the boundaries of cells do not show obscure hemolysis phenomena (hemolysis degree 10.5%). Meanwhile, the hemolysis degree of erythrocytes in 3 mM PβCD15 is high as shown in both morphological shape (Fig. 10C) and hemolysis test (44.4%). Overall, CPβCD15_1.5 shows lower hemolysis degree than PβCD15 does, regardless of the concentration at 1 or 3 mM. Thus, it can be concluded that the cationic charge of CPβCDs does not cause high hemolysis although it can induce an obvious change in erythrocytes shape. The hemolysis degree of cationic β-CD polymers apparently relies on their ability to attract cholesterol from the cell membrane, which is similar to those reported by Zhong et al. (2001). Consequently, the relatively low hemolysis degree of CPβCD15_1.5 might be attributed to their low inclusion towards cholesterol.

4. Conclusions

The condensation polymerization of β-CD, EP and CC generated a range of novel water-soluble CPβCDs with various characteristics, depending on the feed ratios. The characteristics of CPβCDs, such as charge density, molecular weight as well as MW distribution, are controllable during polymerization. The inclusion complexation of CPβCDs with NAP enhanced the water solubility of NAP significantly (from 27 to 3.2 ± 10^3 mg/l at 25 °C). The complex formation was confirmed by NMR and FT-IR measurements. CPβCDs also showed relatively low hemolytic activity, compared with parent β-CD. Such good physicochemical and hemolytic properties permit CPβCDs to be used as drug carriers in pharmaceutical applications.

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