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Isolation, Purification and Characterization of Large-Ring Cyclodextrins (CD_{36}∼CD_{39})

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Abstract

Large-ring cyclodextrins (LR-CDs) composed of more than 9 D-glucose units are not well studied. In this study, LR-CDs composed of 36, 37, 38 and 39 D-glucose units (CD_{36}∼CD_{39}) were isolated and purified from a LR-CD mixture, and their physicochemical properties including aqueous solubility, surface tension, specific rotation and acid-catalyzed hydrolysis rate were elucidated. The aqueous solubilities of CD_{36}∼CD_{39} were greater than those of α-, β-, γ-CD, CD_9, CD_{10}, CD_{14} and CD_{26}. CD_{36}∼CD_{39} did not show any surface activity. The acid-catalyzed hydrolysis of CD_{36}∼CD_{39} was a little faster than that of other LR-CDs (CD_9∼CD_{35}). There was no marked difference in specific rotation or the acid-catalyzed hydrolysis rate among CD_{36}∼CD_{39}. Furthermore, we compared these findings with the physicochemical properties of α-, β-, γ-CD and other LR-CDs (CD_9∼CD_{35}).

Introduction

LR-CDs are the cyclic α-1, 4-glucans composed of more than 9 D-glucose units. Until today, LR-CDs composed of several hundred D-glucose units have been reported. Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19), which produces mainly α-, β-, γ-CD, is widely used as a 4-α-glucanotransferase to form cyclic compounds. Disproportionating enzyme (D-enzyme, EC 2.4.1.25) from potato, heat resistance amylomaltase (EC 2.4.1.25) cloned from the thermophilic bacterium *Thermus aquaticus*, and glycogen debranching enzyme (GDE, EC 2.4.1.25/EC 3.2.1.33) from yeast (*Saccharomyces cerevisiae*) and so on are among the enzymes used for LR-CD production [1–4]. Furthermore, it was reported that LR-CDs were produced with the initial action of CGTase [5]. It is interesting that LR-CDs have different degrees of polymerization (DP) depending on the enzyme: the minimum DP of the LR-CD produced by amylomaltase, D-enzyme, GDE and CGTase was 22, 17, 11 and 9, respectively.

Recently, there has been an increase in research into the crystal structure and/or inclusion complex of LR-CDs composed of more than 9 D-glucose units [6–14]. Additionally, a LR-CD mixture has already been commercialized as an artificial chaperon to refold denatured proteins, and it is reported that the complexation of single-wall carbon nanotubes with LR-CD composed of 12 D-glucose units enables their solubilization in water [15, 16]. To develop further applications for LR-CDs, investigations of physicochemical properties would be indispensable. We have focused on LR-CDs for several years and already reported the isolation, purification, physicochemical properties and ability to form inclusion complexes of LR-CDs with 9∼35 D-glucose units [17–23]. In this study, cyclomaltohexatridecaose (CD_{36}), cyclomaltoheptatridecaose (CD_{37}), cyclomaltooctatridecaose (CD_{38}) and cyclomaltononatridecaose (CD_{39}) composed of 36, 37, 38 and 39 D-glucose units were isolated and purified from a mixture of LR-CDs (The subscript denotes the number of D-glucose units). Their physicochemical properties, such as aqueous solubility, surface tension, specific rotation and acid-catalyzed hydrolysis rate were elucidated. Furthermore, we compared these findings with the known physicochemical properties of α-, β-, γ-CD and other LR-CDs (CD_9∼CD_{35}).

Experiment

Materials

The LR-CD mixture was provided by the Biochemical Research Laboratory in Ezaki Glico Co., Ltd. (Osaka, Japan). The preparation of amylomaltase and production of the mixture were reported in a previous paper [2]. The procedure used to prepare the LR-CD mixture

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containing DP ranging from 9 to 21 was described in detail previously [24]. Other chemicals were obtained from commercial sources and were used without further purification. Milli-Q Water (Milli-Q Gradient, Millipore Co., USA) was used in all experiments as purified water.

Isolation and purification of CD_{36}~CD_{39}

The purification and testing of the purity of CD_{36}~CD_{39} was carried out with HPLC using an Octadecyl silica (ODS) column (YMC-Pack ODS-AQ, 10 ϕ×250 mm: for purification, 4.6 ϕ×250 mm: for testing purity, YMC Co. Japan) and amino (NH2) column (Asahipak NH2P-50, 10 ϕ×250 mm: for purification, 4.6 ϕ×250 mm: for testing purity, Showa Denko Co., Japan). The conditions and procedure used are shown in Figure 1.

Identification of CD_{36}~CD_{39} by mass and NMR Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF MS) were measured in the positive-ion mode with an AXIMA-CFR plus (Shimadzu Co., Japan) using 2,5-Dihydroxybenzoic acid as the matrix. The acceleration voltage was 20 kV. The external standard was insulin. ^{1}H-NMR, ^{13}C-NMR and two-dimensional ^{1}H-^{13}C correlation (^{1}H-^{13}C COSY) NMR spectra were recorded on a JNM-LA500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C, JEOL, Japan) in 99.8% Deuterium oxide with Tetramethylsilane for ^{1}H and Dioxane for ^{13}C as an external standard at 50 °C.

Physicochemical properties of CD_{36}~CD_{39}

The aqueous solubility of the CDs was determined as follows. Water was carefully added to a glass vessel containing 50 mg of each CD. The quantity of water varied progressively from 0.01 to 0.1 mL. The samples were vigorously shaken for 1 min at 10 min intervals at 25 °C, until the CD had completely dissolved. The total volume of water added was measured, and the saturated solubility was calculated. Surface tension measurements were made on a Wilhelmy surface tensiometer. The glass vessels used were treated with 20% sulfuric acid before each measurement. Optical rotation measurements were taken on a polarimeter at 25 °C. The polarimeter was calibrated with 26 w/v% sucrose solution before measurements. In the acid-catalyzed hydrolysis, samples of 30 mg of CDs were dissolved in 1.5 mL of 1 mol/L HCl, and the reaction solution was heated 50 °C in an incubator. Samples of the reaction solution were taken at appropriate intervals and neutralized by the addition of 1 mol/L NaOH. The samples were quantified by HPLC.

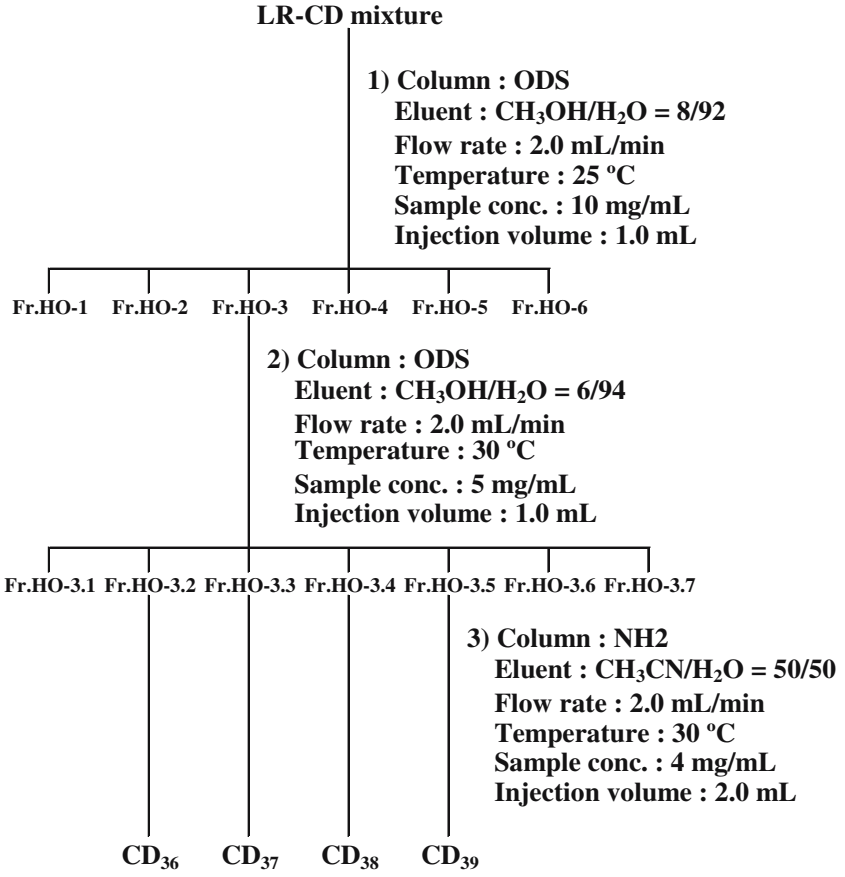

Figure 1. Summary of the isolation and purification of CD_{36}, CD_{37}, CD_{38} and CD_{39} by HPLC.

Results and discussion

Isolation and Purification of CD_{36}∼CD_{39}

HPLC with an ODS column can separate branched and non-branched CDs composed of the same number of D-glucose units. However, the use of an ODS column caused a tailing of chromatographic peaks. Accordingly, isolation and purification were carried out with HPLC using an ODS column and NH2 column. Figure 2 shows the chromatograms obtained for the LR-CD mixture (a) and Fr.HO-3 (b) using an ODS column. The purified Fr.HO-3.2∼Fr.HO-3.5 fractions exhibited a singlet peak on the chromatograms obtained using an ODS column and NH2 column, respectively (data not shown). Therefore, the purity of each of these fractions was >98%.

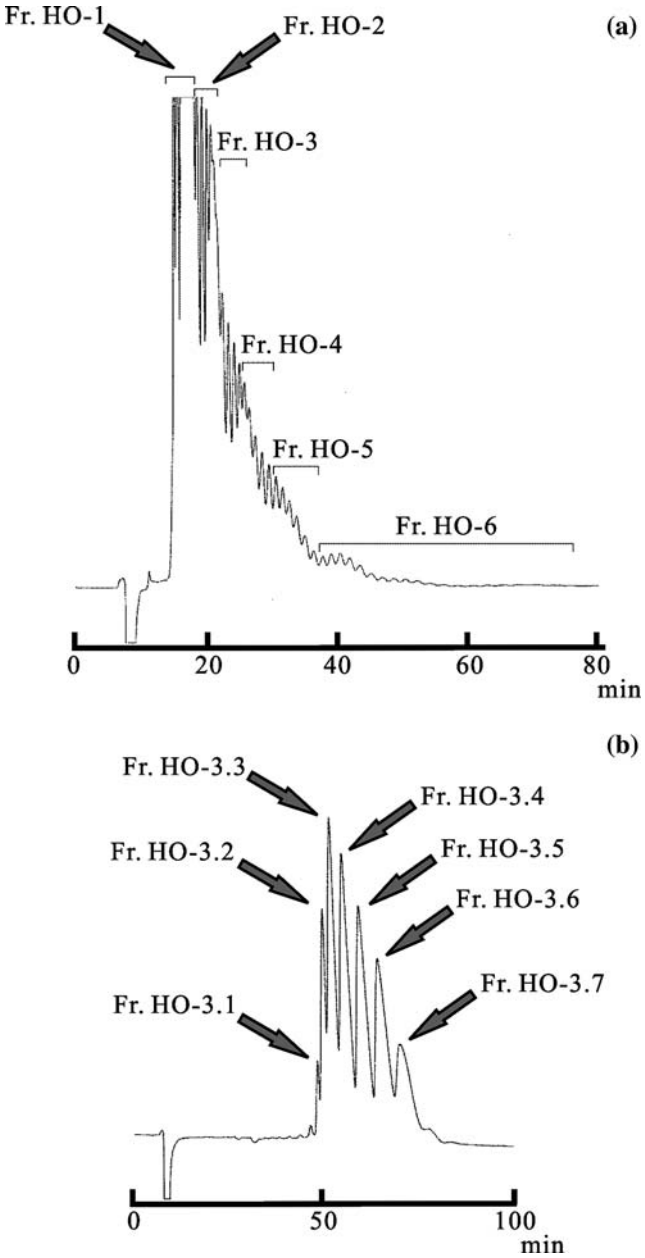

Figure 2. Chromatograms of the LR-CD mixture (a) and Fr.HO-3 (b) obtained with an ODS column.

Identification of CD_{36}∼CD_{39}

The fractions Fr.HO-3.2∼Fr.HO-3.5 were identified with NMR spectroscopy and mass spectroscopy. The ^{13}C NMR spectra of Fr.HO-3.2∼Fr.HO-3.5 indicated six clear singlet signals attributed to equivalent D-glucose units in solution. These signals were assigned from the ^{1}H-^{13}C COSY NMR spectra of Fr.HO-3.2∼Fr.HO-3.5 (Figure 3). The results showed similar spectra of other LR-CDs (CD_9∼CD_{35}). The molecular weights of Fr.HO-3.2∼Fr.HO-3.5 determined by MALDI-TOF MS agreed with the theoretical values of CD_{36}∼CD_{39} calculated from $(C_6H_{10}O_5)_n$, where n is the number of D-glucose unit (Figure 4).

Physicochemical Properties of CD_{36}∼CD_{39}

Table 1 summarizes the physicochemical properties of α-, β-, γ-CD and LR-CDs. CD_{36}∼CD_{39} were more soluble than α-, β-, γ-CD, CD_9, CD_{10}, CD_{14} and CD_{26} but similar in solubility to other LR-CDs. The low aqueous solubility of CD_9, CD_{10}, CD_{14} and CD_{26} was caused by high crystallinity [6–10]. α-, β-, γ-CD and CD_9∼CD_{39} did not show any surface activity. This result was consistent with the general behavior of sugars. There was no marked difference in specific rotation among CD_{36}∼CD_{39}. In homologous compounds with different molecular weights, the evaluation of molecular rotation is based on rotation power.

Molecular rotation ($[\phi]_\lambda^t$) is expressed by:

$$[\phi]_\lambda^t = \frac{M}{100}[\alpha]_\lambda^t$$

where M, $[\alpha]$, t and λ are the molecular weight, the specific rotation, the temperature and the wavelength, respectively. Figure 5 shows the calculated molecular rotation of CDs (CD_6∼CD_{39}). The line was classified into three-sections using Akaike's information criteria (AIC). AIC is widely used for the selection of models in various fields [25]. If the CDs have no structural difference, molecular rotation must increase linearly with the number of D-glucose units. This result showed the possibility of structural differences (CD_6∼CD_{39}). The crystal structures of α-, β-, γ-CD, CD_9, CD_{10}, CD_{14}, and CD_{26} have already been reported: α-, β-, γ-CD and CD_9 have the familiar perforated bucket structure (The overall shape of CD_9 is elliptic), CD_{10} and CD_{14} have two band flips and a distorted structure, and CD_{26} has two band flips and a helical structure: a band flip is a 180° inverted glycoside linkage [6–10, 26–30]. Therefore, it is presumed that the three different straight lines of α-, β-, γ-CD and CD_9, CD_{10}∼CD_{20} and CD_{21}∼CD_{39} have an influence on these structural differences, respectively.

The half-lives of the ring openings of CD_{36}∼CD_{39} were a little shorter than those of other LR-CDs (CD_9∼CD_{35}). Those of CD_9∼CD_{30} showed a pattern of

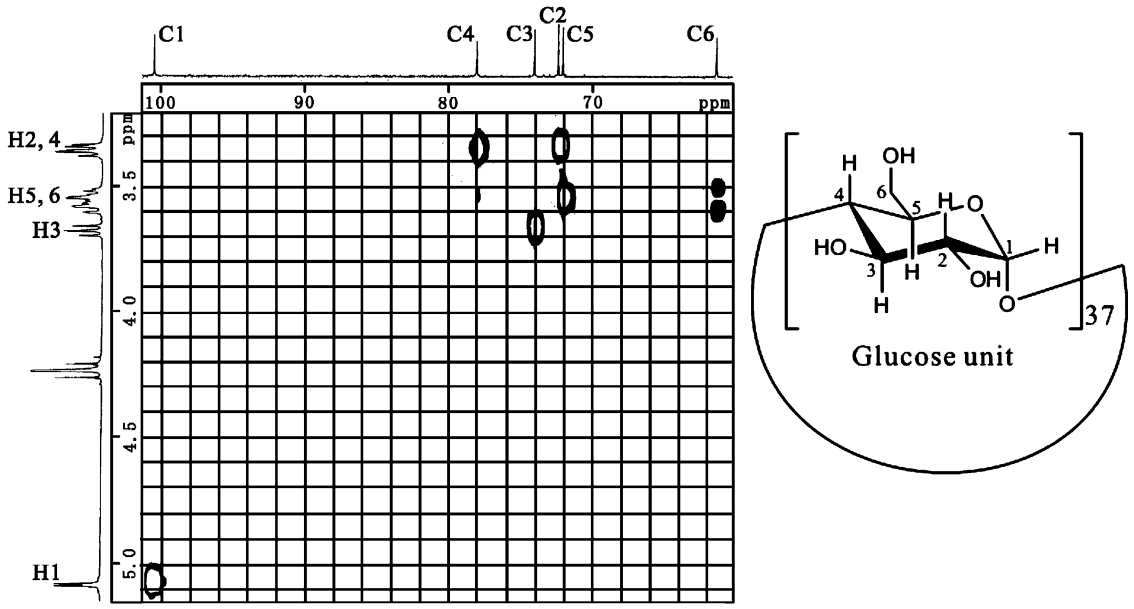

Figure 3. ^{1}H-^{13}C COSY NMR Spectrum of Fr.HO-3.3 (CD_{37}). Solvent: Deuterium oxide, Temperature: 50 °C.

one every 6 or 7 D-glucose units. Furthermore, the ^{13}C NMR chemical shifts of C1 and C4 used for binding to two D-glucose units showed a similar pattern, and a strong correlation was observed between the half-lives of ring openings and ^{13}C NMR chemical shifts as shown in Figure 6. Therefore, we suspected that there is a relationship between the stability and structure of LR-CDs. Six or seven D-glucose units compose a relatively stable helix, so LR-CDs do have a stable structure periodically. However, since the glycoside linkage is readily attacked by acid, the half-life decreased as the number of glycoside linkages increased. In addition, periodical change of half-lives and ^{13}C NMR chemical shifts of LR-CDs is not clear as the D-glucose unit in the range from CD_{31} to CD_{39}. It was considered that the changes of these half-lives indicate the relaxation of a distortion of the ring structure in LR-CD as glycoside linkage increases. These results are probably related to the structures of LR-CDs. However, we have great difficulty in explaining their true cause. A more detailed investigation is required to obtain a clear conclusion.

There are two problems that remain to be solved: first, mass production is still difficult, and second, the isolation of LR-CDs is very expensive and troublesome.

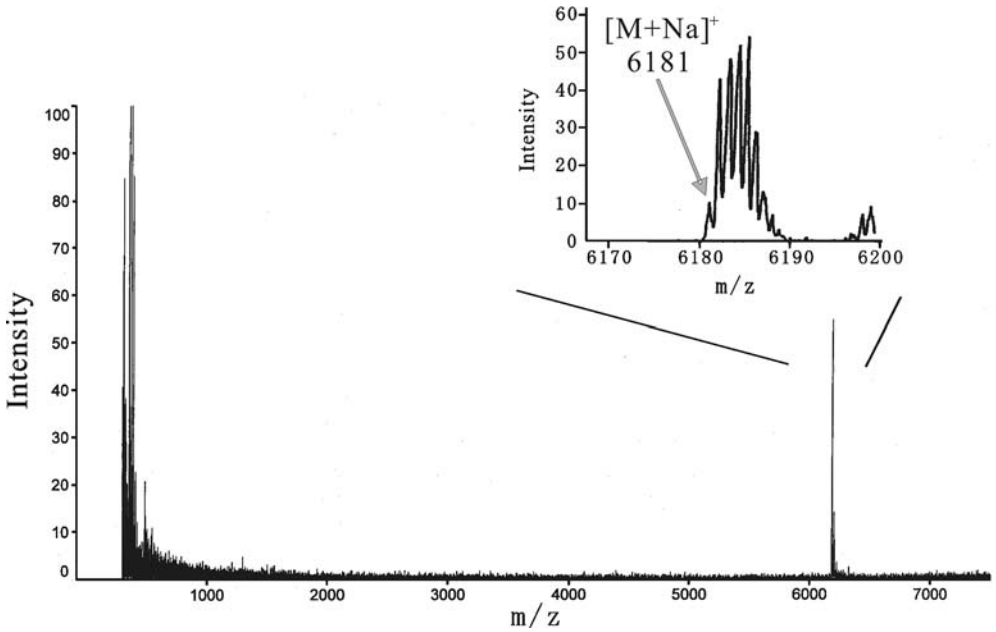

Figure 4. MALDI-TOF MS of Fr.HO-3.5 (CD_{38}). Matrix: 2,5-Dihydroxybenzoic acid, Acceleration voltage: 20 kV.

Table 1. Physicochemical properties of CDs

	Number of D-glucose unit	Molecular wight [a]		Aqueous solubility (g/100 mL) [b]	Surface tension (mN/m) [b]	Specific rotation $[\alpha]_D^{25}$	Half-life of ring opening (h)[c]
		Theoretical	Experimental				
α-CD	6	973	973	14.5	72	+147.8	33
β-CD	7	1135	1135	1.85	73	+161.1	29
γ-CD	8	1297	1297	23.2	73	+175.9	15
CD_9	9	1459	1459	8.19	72	+187.5	4.2
CD_{10}	10	1621	1621	2.82	72	+204.9	3.2
CD_{11}	11	1784	1783	>150	72	+200.8	3.4
CD_{12}	12	1946	1946	>150	72	+197.3	3.7
CD_{13}	13	2108	2107	>150	72	+198.1	3.7
CD_{14}	14	2270	2270	2.30	73	+199.7	3.6
CD_{15}	15	2432	2432	>120	73	+203.9	2.9
CD_{16}	16	2594	2594	>120	73	+204.2	2.5
CD_{17}	17	2756	2756	>120	72	+201.0	2.5
CD_{18}	18	2919	2919	>100	73	+204.0	3.0
CD_{19}	19	3081	3081	>100	73	+201.0	3.4
CD_{20}	20	3243	3243	>100	73	+199.7	3.4
CD_{21}	21	3405	3405	>100	73	+205.3	3.2
CD_{22}	22	3567	3567	>100	73	+197.7	2.6
CD_{23}	23	3729	3729	>100	73	+196.6	2.7
CD_{24}	24	3891	3891	>100	73	+196.0	2.6
CD_{25}	25	4054	4053	>100	73	+190.8	2.8
CD_{26}	26	4216	4215	22.4	73	+201.4	2.9
CD_{27}	27	4378	4375	>125	72	+189.4	2.8
CD_{28}	28	4540	4537	>125	72	+191.2	2.6
CD_{29}	29	4702	4699	>125	72	+190.2	2.5
CD_{30}	30	4864	4860	>125	72	+189.1	2.3
CD_{31}	31	5026	5023	>125	71	+189.0	2.4
CD_{32}	32	5188	5185	>125	71	+192.7	2.4
CD_{33}	33	5351	5349	>125	71	+192.1	2.2
CD_{34}	34	5513	5510	>125	72	+189.6	2.2
CD_{35}	35	5675	5671	>125	71	+193.7	2.1
CD_{36}	*36*	*5837*	*5835*	*>100*	*71*	*+190.6*	*1.9*
CD_{37}	*37*	*5999*	*5995*	*>100*	*71*	*+189.9*	*1.8*
CD_{38}	*38*	*6161*	*6158*	*>100*	*71*	*+190.1*	*1.9*
CD_{39}	*39*	*6323*	*6321*	*>100*	*70*	*+188.1*	*1.8*

[a] Theoretical masses were calculated as 162.1406×*n*, where *n* is the number of glucose unit. Experimental masses of α-CD∼CD_{26} and CD_{27}∼CD_{39} were determined as the average mass and the monoisotopic mass, respectively.
[b] Observed at 25 °C.
[c] In 1 mol/L HCl at 50 °C.

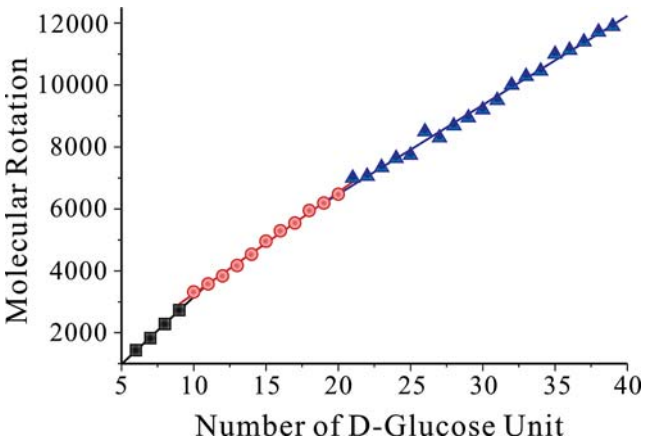

Figure 5. Dependence of molecular rotation on number of D-glucose unit.

Overcoming these problems will lead to the further development of LR-CDs.

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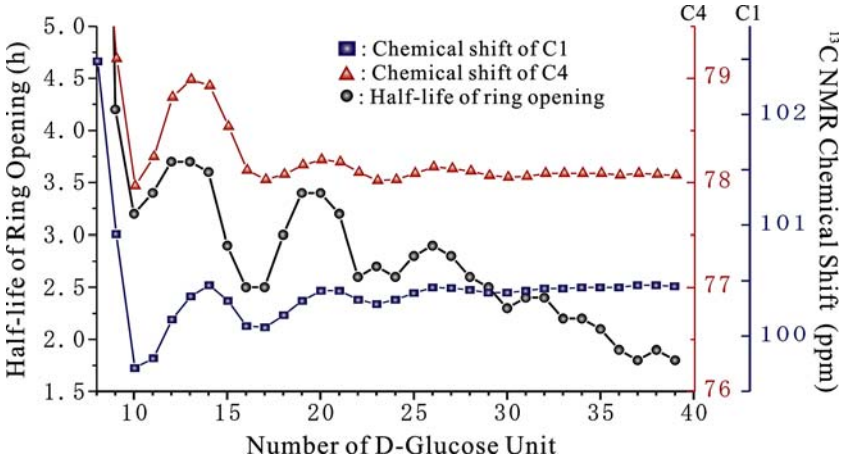

Figure 6. Dependence of half-life of ring opening and ^{13}C NMR chemical shifts on number of D-glucose unit.

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