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ORIGINAL ARTICLE

Syntheses and characterisation of novel cyclodextrin vinyl derivatives from cyclodextrin-nitrophenol-derivatives

Anne L. Nielsen · Kim Steffensen · Reinhard Wimmer · Martin Worm-Leonhard · Kim L. Larsen

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Abstract In this study novel reactive α-, β- and γ-cyclodextrin-esters (acrylate, pent-4-enoate and undec-10-enoate) have been synthesised and characterised. The syntheses were carried out by using nitrophenol-esters with the ability to form inclusion complexes with cyclodextrins, thereby aiming at a better control of the substitution degree and number of positional isomers of the cyclodextrin derivatives. Derivatives of α-, β- and γ-cyclodextrins modified with three different lengths of carbon-chains were successfully synthesised and characterised by MALDI-TOF MS, HPLC and LC-MS/MS, revealing some differences in LC elution patterns, substitution degrees and number of produced positional isomers. Differences were seen as an effect of changing the size of the cyclodextrin as well as the size of the side-chain being attached. The inclusion complexes between the nitrophenol esters and the different cyclodextrins were studied by ITC and selected ones by 2D ROESY NMR, showing some interesting differences in strength and structure of the complexes. These differences are speculated to be the origin of the different substitution patterns of the derivatives as observed by LC-MS/MS.

Keywords Cyclodextrin · Reactive derivative · ROESY NMR · LC-MS/MS

A. L. Nielsen
Danish Technological Institute, Kongsvang Alle 29, 8000 Århus C, Denmark

K. Steffensen
Cheminova A/S, Thyborønvej 78, 7673 Harboøre, Denmark

R. Wimmer · K. L. Larsen (✉)
Aalborg University, Sohngaardsholmsvej 57, 9000 Aalborg, Denmark
e-mail: kll@bio.aau.dk

M. Worm-Leonhard
Institute of Forensic Medicine, University of Southern Denmark, Winsløwparken 17, 5000 Odense C, Denmark

Introduction

Cyclodextrins (CDs) are cyclic oligomers consisting of six or more D-glucopyranose units linked with α(1–4) glucosidic bonds and are often shown as truncated, cone-like structures, where the exterior is polar and the interior less polar relative to water. The commercially available CDs, α-, β- and γ-CD consists of 6, 7 and 8 glucosyl units, respectively [1, 2]. Their properties are useful in a variety of applications due to their strong ability to form inclusion complexes with a large range of molecules having hydrophobic moieties corresponding in size with the cavity of the CD. The CDs can thus be used to stabilise labile molecules, to bring poorly water-soluble compounds for example drugs into solution, masking of unwanted taste and odours, modify chemical reactivity etc. [3].

The properties of the CDs can be altered in a number of ways, to e.g. increase their solubility, decrease their toxicity or alter their inclusion abilities. This can be achieved by substitution of one or more of the hydroxyl groups in the glucose units. A wide variety of derivatives of especially β-CD, but also α- and γ-CD, have been made and some are used extensively. As an example the hydroxypropyl- and sulphobutylether-derivatives of β-CD have been used extensively research on drug formulations [3]. In a number of applications it is of interest to immobilise the CDs on a surface or build them into a polymer while still maintaining their inclusion functionality. This way the CDs ability to

form complexes can be used in applications where release of free CDs is unwanted, for example in hygiene products, wound care products or removal of cholesterol with CD containing membranes [3, 4]. When incorporating CDs in polymers it is especially important to reduce the number of reactive sites per CD to a minimum. Preferably there should be only one substitution per CD molecule in order for the CDs not to act as crosslinkers in the polymer network. Small amounts of derivatives substituted on more than one hydroxyl group may however be acceptable in many applications, e.g. Janus and co-workers made water-soluble CD polymers from unpurified CD vinyl derivatives [5].

When synthesising CD derivatives it is a challenge to control the degree of substitution (DS) since the CDs have 18 (for α-CD) or more hydroxyl groups, which are chemically more or less equivalent, with the primary ones being just slightly more reactive than the primary ones [6]. It is however possible to selectively substitute only one type of hydroxyl groups to achieve a derivative with specific or regioselective substitution [7, 8]. Thereby the obtained CD is substituted on only one of the three types of hydroxyl groups on the CD surface (2-, 3-, or 6-position in the glucose ring) and this yields a better control of the DS of the produced derivative [9]. It is possible to control the reactions due to the slightly lower reactivity of the C-2 and C-3 alcohol hydroxyls compared to the C-6 alcohol hydroxyls [7, 9]. To control the DS several methods can be employed, many requiring a number of protection and deprotection steps to prevent more than one hydroxyl group from reacting and some followed by column chromatography for purification of the desired derivative [10]. Another approach to control the DS of the derivatives is to direct the reaction by using compounds with the ability to form inclusion complexes with the CDs. This was first proposed by Harada and co-workers, who used a 3-nitrophenol-acrylate ester to control the substitution of a β-CD-acrylate vinyl derivative [11]. The nitrophenol-ester is believed to form only one type of complex which will direct the reaction to the secondary alcohols of the CD (C-2 and C-3) and thereby increase the yield of mono-substituted product [11]. The yield of mono-substituted derivatives from this reaction is reported to be around 15%, with emphasis on keeping a low amount of di- and tri-substituted derivatives.

A way to determine the positional isomers of modified CDs is LC-MS, which gives a possibility to separate the different isomers on a suitable column and subsequently analyse them on MS obtaining the exact mass of each. By using collision induced dissociation (CID) MS/MS it is even possible to get a fingerprint of each, thereby revealing different regio-isomers produced. This is due to differences in collision stability during CID depending on the type of positional isomer [12, 13]. Depending on the type of derivatives and MS equipment it is possible to separate the derivatives, get a fingerprint showing how many different derivatives are formed and even specify exactly which positional isomers have been produced [13]. These types of analyses have previously been adapted to analyse derivatives of oligosaccharides or CDs, e.g. both Tüting and co-workers as well as Lesur and co-workers worked on fragmentation patterns of regioselectively *O*-methylated maltooligosaccharides using ESI-MS/MS [12, 14].

In this study syntheses of α-, β- and γ-CD vinyl derivatives are synthesised using 4-nitrophenol-esters (4-NP) as intermediates in a two-step approach, based on the work of Harada et al. [11]. The aim is a high yield of monosubstituted CD as a result of the inclusion complex formation during the reaction allowing the produced derivatives to be used for production of CD polymers via free radical polymerisation. In the present work 4-nitrophenol is used, since it forms a stronger complex with β-CD than 3-nitrophenol according to literature (K1:1 $\sim$150 and $\sim$560 M^{-1}) [4, 11]. CD monomers with different aliphatic esters (of 2, 4 and 10 carbon-chains, respectively) between the CD and the double bond are produced in this way and characterised by MALDI-TOF MS, NMR and HPLC to get a full overview of the DS and distribution of the derivatives. A CDScreen® HPLC column utilising the ability of the CDs to form inclusion complexes is applied together with MS/MS to achieve a detailed fingerprint of the derivatives. Furthermore, the binding energies of the inclusion complexes formed between the produced 4-NP-esters and α-, β- and γ-CDs are assessed by isothermal titration calorimetry (ITC) and the respective complexes formed between β-CD and NP-acrylate and NP-undec-10-enoate is studied by 2D ROESY NMR.

Materials and methods

Pharmaceutical grade α-, β- and γ-CD were purchased from Wacker Chemie, Burghausen, Germany. 4-nitrophenol (98%) (4-NP), 3-nitrophenol (98%) (3-NP), acryloyl chloride (96%, ≥200 ppm monomethyl ether hydroquinone (MEHQ) as inhibitor), pentenoyl chloride (98%) and undecenoyl chloride (>97%) were all purchased from Sigma–Aldrich, Steinheim, Germany. α-cyano-4-hydroxycinnamic acid (pro analysis) (CCA), trifluoroacetic acid (TA), nitrocellulose, acetone (99.8%) and acetonitrile (AcN) (99.9%) all of analytical grade were obtained from Sigma–Aldrich, St. Louis, Missouri, USA. Potassium hydroxide (KOH) was obtained from Sigma–Aldrich, Steinheim, Germany. Hydrochloric acid was purchased from J.T. Baker, Deventer, Holland and sodium carbonate was obtained from AppliChem, Darmstadt, Germany. Deuterium oxide (D_2O),

99.9% was obtained from Larodan Fine Chemicals, Malmö, Sweden. All chemicals were used as received without further purification. Millipore water was obtained from a Nanopure Diamond system from Barnstead International, Dubuque, Iowa USA.

Syntheses

In the first step the unsaturated acid chlorides (acryloyl chloride, pentenoyl chloride or undecenoyl chloride) were reacted with 4-NP adapting the method of Harada [11]. In brief, 1.1–2.5 g (in each case 12.1 mmoles) of the chosen acid chloride was added drop wise to a solution of 1.25 g potassium hydroxide and 1.85 g (13.2 mmoles) of 4-NP in 80 mL of water in an ice bath, keeping the temperature below 5 °C at all times. In the cases of nitrophenyl-acrylate (NP-acrylate) and -pent-4-enoate (NP-pent-4-enoate) the products were obtained as a white/yellow precipitate in 81 and 89% yields, respectively, whereas the nitrophenol-undec-10-enoate (NP-undec-10-enoate) was obtained as a brown/yellow oil (mp ~10 °C) in 87% yield. ^{1}H NMR, NP-acr: δ = d8.2, d7.05, d6.7, m6.5, d6.3 ppm; NP-und: δ = d8.2, d7.1, s1.4–1.7 ppm. For the next step 1 g of CD (α-, β-, or γ-CD) (0.77–1.03 mmoles) was dissolved in 60 mL of 0.1 M carbonate buffer, pH 10 and either of the three NP-derivatives was added in a 1:1 molar ratio to CDs. The NP-acrylate and -pent-4-enoate were dissolved in 1 mL of AcN prior to addition to the aqueous CD solution to prevent precipitation. The reaction proceeded for 15 min while stirring and was then adjusted to pH ~ 3 with diluted HCl thereby stopping the reaction. The reaction mixture was cooled to 5 °C and stored overnight before precipitates of unreacted NP and NP-derivatives were removed. The CD derivatives were collected as white powders in yields between 50 and 80% by evaporation of the solvent and purified from excess NP by recrystallisation from AcN. The derivatives are named according to the CD used, the attached chain and in HPLC and MS additionally after the number of substituents on the specific CD, for example β-CD-acr$_1$ for β-CD derivative with one acrylate chain attached.

Analyses

MALDI mass spectrometry

The CD derivatives were analysed using MALDI-TOF MS (Reflex^{TM}III from Bruker, Bremen, Germany) using a fast evaporating nitrocellulose layer and a CCA matrix. Samples were deposited using a thin layer method, with the sample being deposited on top of the applied matrix layer on the target plate. Aqueous solutions of the samples were prepared, mixed with CCA matrix and applied to the target plate. All samples were measured in reflector mode detecting positive ions. FlexAnalysis 2.4 (build 11) software (Bruker Daltonik GmbH, Bremen, Germany) was used for the analysis of the obtained data.

Nuclear magnetic resonance

^{1}H and 2D Rotational nuclear Overhauser Effect Spectroscopy (ROESY) NMR analyses were carried out on a Bruker DRX600 NMR spectrometer (Bruker, Bremen, Germany) equipped with an XYZ gradient TXI (H/C/N) probe at 298 K. All samples were prepared in D_2O with presaturation of the water signal. ROESY spectra were recorded at 300 K. Bruker TOPSPIN software version 2.0 (2006) (Bruker Biospin) was used for analysis of the data.

Isothermal titration calorimetry

ITC measurements were carried out on a VP-ITC micro calorimeter (MicroCal Inc. Company, Northampton, MA, USA) controlled by MicroCal's VP viewer software (ver. 5.0, Microcal Software Inc., Northampton, MA, USA). Titration was done with a 10 mM CD solution into 1 mM solutions of 3-NP, 4-NP and the four NP-derivatives, all in acetate buffer at pH 3.5. After thermal equilibrium of the titration cell was reached, an initial delay of 180 s followed by an initial injection of titrant of 1 μL was applied. After this injection, volumes from 10 (first 10 datapoints) to 20 (next 10 datapoints) μL and delay times between injections of 250 s were applied.

Data processing was done using Origin 7 SR2 v 7.0383 software (OriginLab Corporation, Northampton, USA) with the microcal LLC ITC add-On and manual baseline correction. The data obtained during titration were converted to heat output per injection by integration of the peaks and correction for the cell volume and sample concentration. The data were fitted by using "one set of sites" model in the Origin software based on the Wiseman Isotherm [15] as shown in Eq. 1:

$$\frac{dQ}{d[NP]_{tot}} = \Delta H^0 V_0 \left(\frac{1}{2} + \frac{1 - [NP]_{tot} - \left(\frac{1}{[CD]} K_{eq}\right)}{2\sqrt{\left(1 + [NP]_{tot} - \left(\frac{1}{[CD]} K_{eq}\right)\right)^2 - 4[NP]_{tot}}} \right) \quad (1)$$

where Q is the heat flow, $[NP]_{tot}$ the total concentration of NP or NP derivative (ligand), H^0 is the standard molar enthalpy, V_0 is the volume of the reaction cell [CD] is the concentration of CD and K_{eq} is the overall binding constant. The model assumes that only one set of binding sites,

all with same binding energy, is present in the sample. In all cases the number of binding sites (n) was fixed to one in the software as recommended by Turnbull and Daranas [16] for systems of relatively low binding constants compared to aqueous solubility (hence the Wiseman "c" parameter which is the product of the receptor concentration and the binding constant, K_a is less than approximately 10) [16].

HPLC and LC-MS/MS

CD derivatives were measured on a CDScreen® column (ChiroQuest Ltd., Budapest, Hungary) using various gradients of water and AcN (shown in the figure text of the relevant graphs) on a Dionex HPLC (Dionex Corporation, Sunnyvale, CA, USA) system, consisting of a Dionex P680 HPLC pump, a Dionex ASI-100 autosampler and an Alltech 800 (Alltech Associates Inc. Deerfield, IL, USA) ELS detector along with a UV detector (Dionex UVD 170U). For data analysis Chromeleon software (Dionex Client 6.60 SP1 Build 1447, Dionex Corporation, Sunnyvale, CA, USA) was applied. All measurements were done at ambient temperature (25°).

For LC-MS/MS analyses the abovementioned CDScreen® column was also applied together with an Agilent 1200 HPLC-system (consisting of a G1322A degasser unit, G1311A pump, G1329A autosampler and G1316A column oven) equipped with Bruker micrOTOF-Q II™ (Bruker Daltonik, Bremen, Germany) MS detector running at 4.5 kV for ionisation, 6 L/min of nitrogen as drying gas at 180 °C and evaporator of 2.5 bars. Formic acid (0.1%) and AcN were used at a flow of 0.4 mL/min under isocratic conditions, differing in composition depending on the CD derivative (α-CD-acr: isocratic conditions, 10% AcN, 90% formic acid, β-CD-acr: isocratic conditions, 13% AcN, 87% formic acid, β-CD-und: isocratic conditions, 20% AcN, 80% formic acid). The formic acid ensures a good formation of protonated sugar ions and good signal in the MS analyses. For Collision Induced Decomposition (CID) experiments, argon was used as collision gas and the collision energy was optimised by the software for each precursor ion ramping from 10 to 100 eV. Cluster ions of sodium formate were used for internal calibration.

Results and discussion

Syntheses of CD vinyl derivatives utilising the CDs ability to form inclusion complexes with NP-derivatives have been conducted in this study. The method have been developed from the work of Harada et al. [11], but expanded to produce a larger array of derivatives of CD-acrylate, CD-pent-4-enoate and CD-undec-10-enoate. In all cases the aim was to achieve a high yield of mono-substituted CDs for use in synthesis of CD-polymers where possible crosslinking arising from di-substituted products is unwanted. Controlling the reaction by formation of CD-NP-ester complexes should increase the yield of mono-substituted CDs. In this work 4-NP-esters have been used in the intermediate product, since the 4-NP is reported to be the NP having the highest stability constant in complexation with CDs [17]. The data on the obtained products of derivatives are first described and is followed by a more detailed study of the CD–NP inclusion complexes via binding energies and spatial structure predictions. Finally fingerprints of the products obtained via LC-MS/MS, giving indications on the regioselectivity of the reactions, are presented.

A sketch of the overall reaction is shown in Fig. 1 for reactions between CDs and NP-acrylate (Fig. 1a) as well as -pent-4-enoate and -undec-10-enoate (Fig. 1b). Attempts to produce α- and γ-CD derivatives with pent-4-enoate were not done, instead focus was put on syntheses of α- and γ-CD-und.

Initial characterisation of CD derivatives

The CD vinyl derivatives were produced using NP-esters as intermediate carriers of the aliphatic chains, and the obtained products were analysed by MALDI-TOF MS and HPLC to reveal the DS and the number of different derivatives. The MALDI-TOF MS data for the α-, β- and γ-CD-acr derivatives are shown in Fig. 2a and for β-CD-pent, α- and β-CD-und derivatives in Fig. 2b.

It proved possible to produce the desired acrylate-derivatives of both α-, β- and γ-CD with an acceptable yield of mono substituted product as observed in Fig. 2a where a fraction of unreacted CD is also observed. From Fig. 2b it is evident that derivatives of α- and β-CD-und as well as β-CD-pent have also been produced, whereas no product of γ-CD-und seem to be formed and only native γ-CD was observed (data not shown). The spectra show a distribution of unreacted CDs and derivatives with up to 3–4 substitutions on each CD for α- and β-CD-acr whereas γ-CD-acr seems to be of lower DS. The spectrum of β-CD-pent derivatives show small amounts of di-substituted compounds whereas the α- and β-CD-und show no trace of higher DS than mono-substituted, which may indicate differences in the reaction mechanism for these longer aliphatic chains compared to the short acrylate-chain. An explanation to this could be found in differences in the structure of the inclusion complexes between β-CD and NP-pent and NP-und respectively, this will be investigated further in the next section.

The obtained products were analysed by HPLC to obtain an overview of the relative amounts of the different DS in

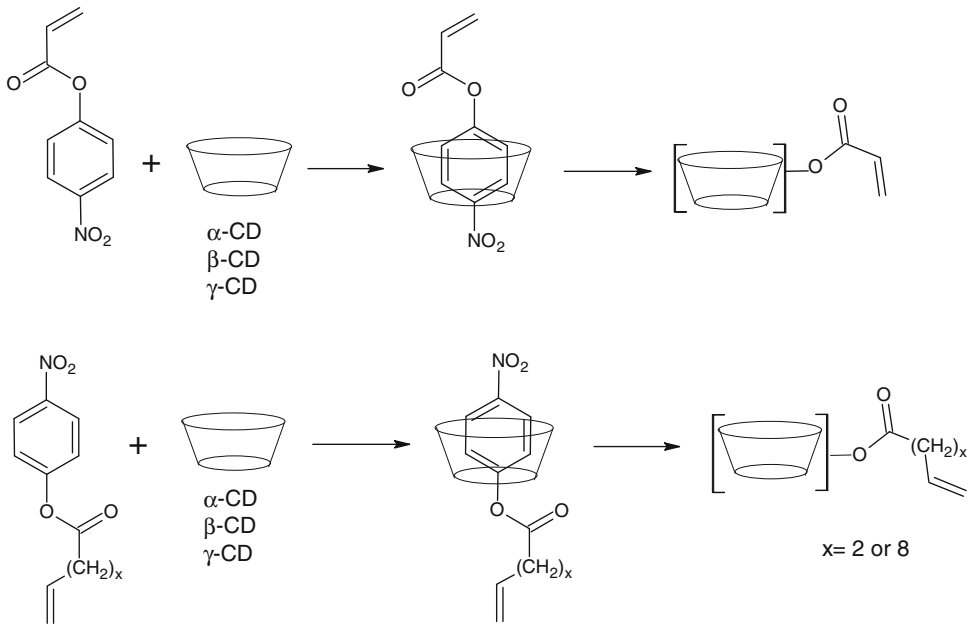


Fig. 1 Synthesis and characterization of novel reactive cyclodextrin derivatives. Proposed reaction scheme of CD derivative syntheses via inclusion complex with NP-derivative. **a** Shows the reaction with 4-NP-acrylate and **b** the reaction with 4-NP-pent-4-enoate and -undec-10-enoate. The inclusion of NP into the CD cavity will probably differ with the type of CD and this should be regarded only as a suggestion

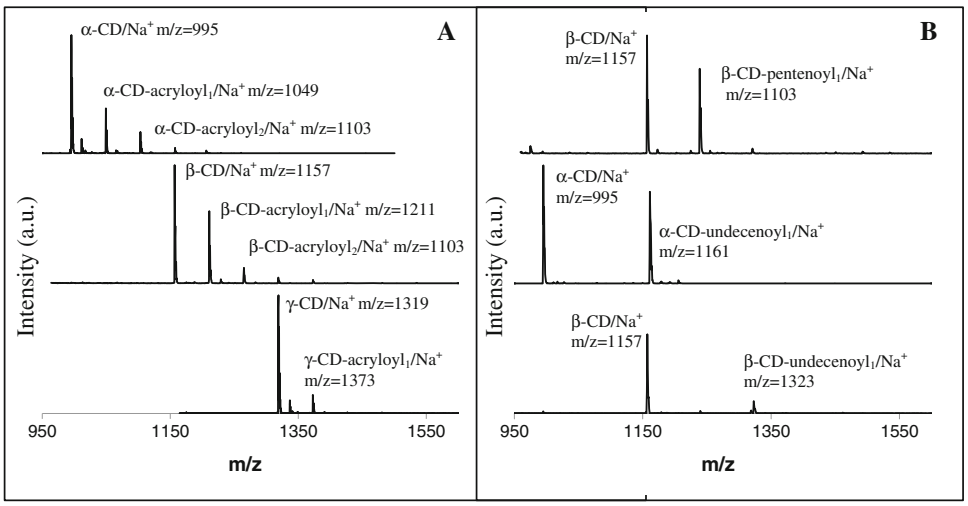
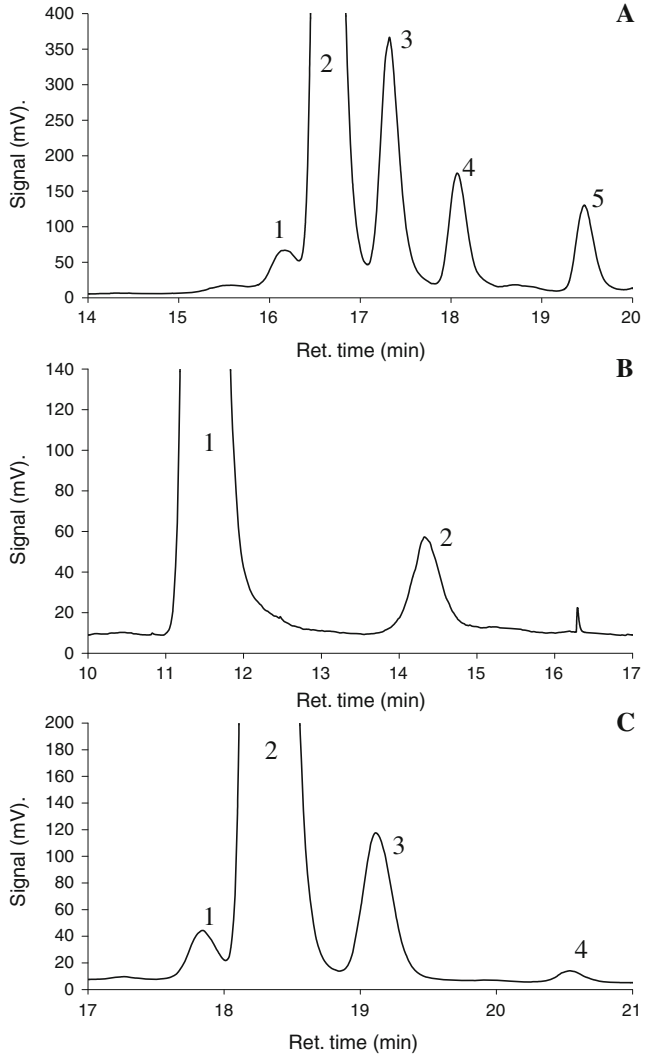


Fig. 2 **a** MALDI-TOF MS spectra of α-, *β*- and *γ*-CD-acr showing the derivatives found in the sample. **b** MALDI-TOF MS spectra of α-, *β*- and *γ*-CD-und and *β*-CD pent showing the derivatives present in the sample. All samples have been doped with small amounts of sodium chloride

the samples, since this information is not readily accessible from the MS data. For the HPLC analyses the samples were analysed on a CDScreen® column offering high selectivity since the separation is based on the ability of the CDs to form inclusion complexes with nitrophenyl groups of the stationary phase [18]. The ability of the CD derivatives to form inclusion complexes will be dependent on the DS and separation can be achieved. An evaporative light scattering detector (ELSD) was used, since it offers an excellent sensitivity towards CDs, and in the next run fractions were collected and analysed by MALDI-TOF-MS. In Fig. 3 the chromatogram of the *β*-CD-acr product is shown, along with the MALDI-TOF MS data obtained for each peak.

From the HPLC chromatograms a reasonable separation of the derivatives can be seen, even for mono-substituted product. The separation of *β*-CD from mono- and di-substituted products (*β*-CD-acr$_1$ and *β*-CD-acr$_2$) seem straightforward in Fig. 3a, whereas the higher substitution degrees doesn't seem to be well separated from native CD. The fifth peak contains mono- and di-substituted products, which where also found in peak three and four, respectively. The tri-substituted product (*β*-CD-acr$_3$) is even eluted before the unmodified *β*-CD. This is an indication that a higher number of side-chains can affect the ability of the CD to form the necessary inclusion complexes with the nitrophenyl-compound of the stationary phase in the column and affect the separation on HPLC. The chromatogram of *β*-CD-und is shown in Fig. 3b and this proves the presence of only unreacted and mono-substituted CD. This confirms the findings from the initial MALDI-TOF analyses on the crude mixture and could suggest a better control of the reaction by the NP-undec-10-enoate-ester derivative compared to the



A			
Peak #	**m/z value (Na^+)**	**Assignment**	**Percent**
1	1156 1317	β-CD, β-CD-acr_3	 2.8
2	1156.0	β-CD	77.0
3	1209.9	β-CD-acr_1	10.3
4	1263.9	β-CD-acr_2	5.9
5	1209.9 1263.9	β-CD-acr_1 β-CD-acr_2	 4.0

B			
Peak #	**m/z value (Na+)**	**Assignment**	**Percent**
1	1155.9	β-CD	96.6
2	1317.8	β-CD-und_1	3.4

C			
Peak #	**m/z value (Na+)**	**Assignment**	**Percent**
1	1317.9 1479.9	γ-CD γ-CD-acr_3	 1.1
2	1317.9	γ-CD	93.7
3	1372.0	γ-CD-acr_1	4.8
4	1372.0	γ-CD-acr_1	0.3

Fig. 3 HPLC chromatograms of CD-derivatives. Peaks are numbered and MALDI-TOF MS information for each peak is shown in the tables. **a** *β*-CD-acr; flow: 0.4 mL/min, gradient: 0–2 min: 10% AcN, 2–18 min: 10–30% AcN, 18–24 min: 30% AcN, 24–26 min: 30–10% AcN, 26–32 min: 10% AcN. **b** *β*-CD-und; flow: 0.4 mL/min, isocratic conditions 15% AcN in millipore water. **c** *γ*-CD-acr; flow: 0.4 mL/min, gradient: 0–2 min: 10% AcN, 2–18 min: 10–30% AcN, 18–24 min: 30% AcN, 24–26 min: 30–10% AcN, 26–32 min: 10% AcN

NP-acrylate-ester, but also lower yields. In the case of *γ*-CD-acr (Fig. 3c) the di-substituted product is not detected, supporting the findings from MALDI-TOF MS on the crude mixture. A possible explanation for the complex separation patterns observed on HPLC could be the ability of the column to distinguish between positional isomers of the CD derivatives, i.e. derivatives with equal number of side-chains, but attached to different hydroxyl groups on the CD. This will lead to two different positional isomers of for instance CD-acr_1 to leave the column at different retention times. This possibility of separation of the regio-isomers of the derivatives will be further tested by LC-MS/MS.

The results obtained using HPLC and MALDI-TOF MS provided the possibility to calculate the average DS as seen in Table 1. The DS are all calculated in weight-percentage relative to each CD.

Some discrepancies are observed between the numbers from different analysis methods, but overall it is possible to observe DS between 1.5 and 10 which compares well to the earlier studies with monomer-yields of around 15% [11]. In all cases the quantification method should only be considered as an overall estimate due to the nature of the methods. The MALDI-TOF method is only considered "semi-quantitative" [19], in some cases enhancing the formation of ions from one species. Interpretation of HPLC data can also be quite tricky because of the possibility of insufficient separation of the free CDs and derivatives. All in all comparing data from the methods provides some overview of the DS of the obtained derivatives.

Table 1 Overall substitution degrees obtained from MS and HPLC, respectively, given in weight percentages

	MS (%)	HPLC (%)
α-CD-acr	21.9	4.8
α-CD-und	1.3	1.5
β-CD-acr	34.2	10.3
β-CD-pent	7.2	4.4
β-CD-und	12.6	3.4
γ-CD-acr	11.7	4.8
γ-CD-und	–	–

Calculated as the average number of substituents on a CD ring

Comparing the data in Table 1 to the MS data in Fig. 2 it is evident that the synthesis of CD-acrylate-derivatives yields a number of different products appearing as quite high DS in Table 1 compared to the findings from the other syntheses. In the case of the β-CD-pent lower overall DS and yields are observed, but also higher amounts of mono-substituted CDs as shown by MALDI-TOF MS in Fig. 2. The β-CD-und synthesis seems to be relatively selective and yield only one substitution which was detected by MALDI-TOF MS, thereby leaving the rest of the CDs unreacted which is reflected by the low yield observed in Table 1. Whether the yield can be enhanced by changes in reaction time, temperature, pH or molar ratio between undec-10-enoate and CD has not been investigated, but this may be the case.

Fingerprinting of CD derivatives

A fingerprint of the CD-derivatives was obtained by LC-MS/MS in combination with the CDScreen® HPLC column. From the LC-MS/MS data extracted ion chromatograms were obtained showing the elution of an ion with a specific mass like e.g. β-CD-acr$_1$ of 1211 *m/z*. This provided a better picture of the elution pattern of each ion as the HPLC-ELSD studies showed a seemingly complex elution pattern. In addition it was possible to perform CID via argon bombardment to achieve ion fragments for selected ions. The pattern of ion fragments is specific for different positional isomers (i.e. substituted on the primary or secondary hydroxyls) on the CDs due to differences in collision stability. The obtained data are shown in Fig. 4 with total ion chromatogram, extracted ion chromatograms and fragment ions in the insert for relevant peaks.

For the β-CD-acr derivative the data shown in Fig. 4a show one peak for the ion of pure β-CD (1135.36 *m/z*) in the extracted ion chromatogram but two peaks for the β-CD-acr$_1$ ion (1189.38 *m/z*) one of them with almost the same retention time as the β-CD peak indicating an incomplete separation of these compounds. Fragmentation of β-CD-acr$_1$ ions from the two different elution times in the chromatogram (Fig. 4a, 2–3 on the right hand side) reveals some difference in the fragmentation patterns which indicates that these two peaks in fact represents two different regio-isomers of β-CD-acr$_1$. The β-CD-acr$_2$ ions are even observed to leave the column as four distinct peaks (no MS/MS data obtained). The data give strong indications of two types of regio-isomers of the CD, for example CDs being substituted on at least two different of the alcohol hydroxyl (C-2, C-3 and C-6). This can account for the different fragmentation patterns due to the differences in the spatial structure. The position of the side-chain will affect the collision stability of the modified glucose units and lead to different fragmentation patterns as observed. The same trends are observed from the data on the α-CD-acr derivative, and even though a full separation of the pure CD from the derivatives was not obtained, the data of the extracted ion chromatogram of α-CD-acr$_1$ show two semi-overlapping peaks with different fragmentation patterns as seen in Fig. 4b.

The extracted ion chromatogram of β-CD-und in Fig. 4c shows a peak of the β-CD-und$_1$ product just before the peak of the pure β-CD and another part of β-CD-und$_1$ appearing at the same retention time as the β-CD peak. It does also seem that there are some impurities being detected in this chromatogram, possibly due to the relatively low signal. No MS/MS data were obtained for this derivative, but when comparing to the data obtained for β-CD-acr it seems likely that the different peaks represent different positional isomers.

From the obtained LC-MS/MS data it has not been possible to prove the exact regiochemistry of the derivatives, since the CID did not provide sufficient amounts of different fragment ions. It is however clear that there are differences between the patterns of fragment ions arising from the same ion observed at different retention times in the chromatogram. This is an indication of different positional isomers eluting differently from the HPLC column. The separation of positional isomers has previously been shown possible with amphiphilic CDs by Kieken and co-workers, showing the same tendency but with poorer separation of the isomers due to the use of a column based on hydrophobicity instead of the CD selective one used in this study [20].

Characterisation of NP-inclusion complexes

Isothermal titration calorimetry has been applied to determine binding constants of β-CD and 4-NP, NP-acrylate, NP-pent-4-enoate and NP-undec-10-enoate, respectively. This has been done in order to reveal differences in binding with the different NP-derivatives to compare with the different substitution patterns found by MS/MS, since the

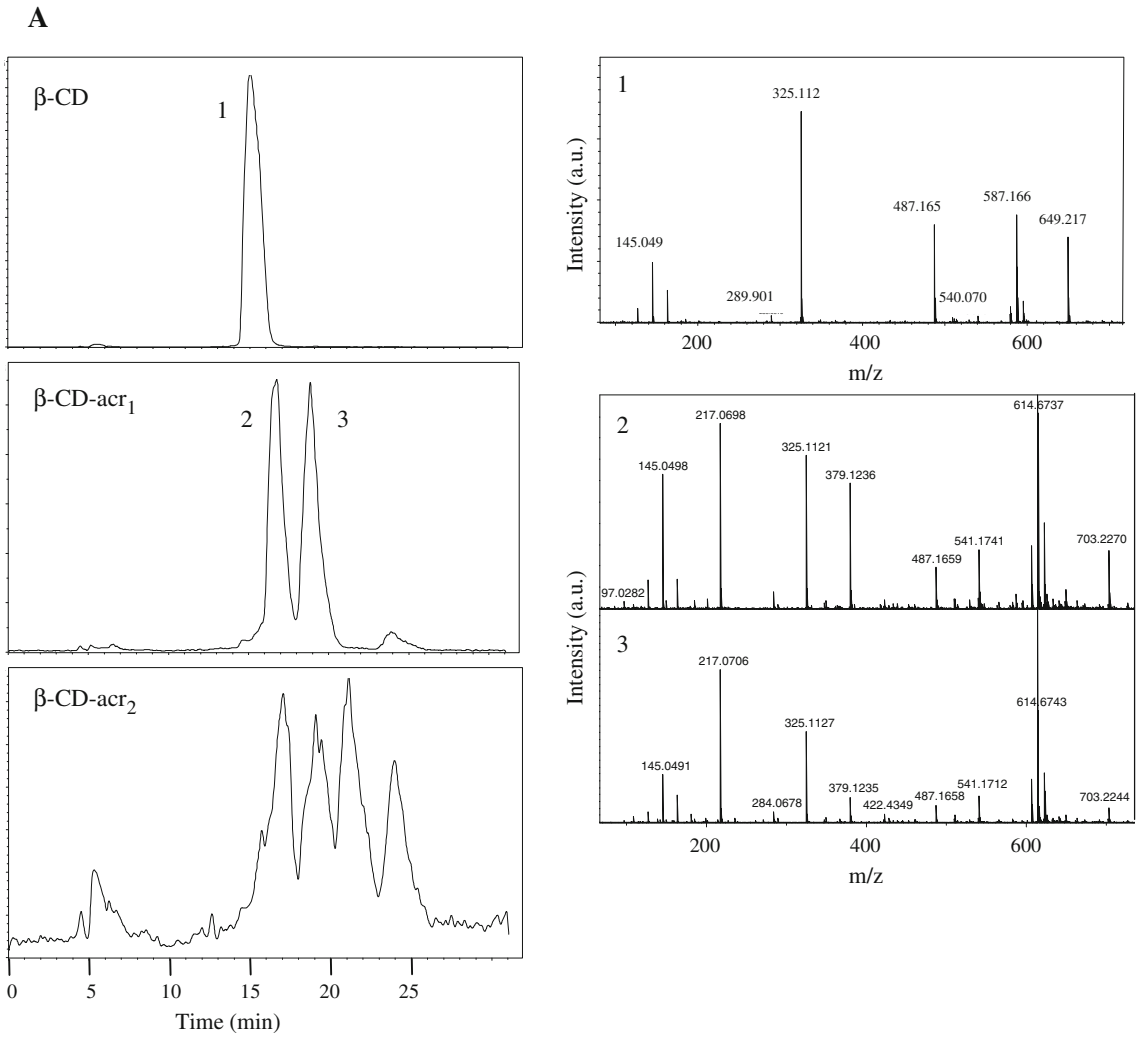


Fig. 4 Data obtained from LC-MS/MS. **a** Data from MS/MS data for *β*-CD-acr; *top left β*-CD extracted ion chromatogram, below *β*-CD-acr$_1$ ion chromatogram and *β*-CD-acr$_2$ ion chromatogram. On the *right hand side* the CID spectra are shown for each peak as marked with numbers. **b** Data from MS/MS data for α-CD-acr; *top left* α-CD extracted ion chromatogram, below α-CD-acr$_1$ ion chromatogram and α-CD-acr$_2$ ion chromatogram. On the *right hand side* the CID spectra are shown for each peak as marked. **c** Showing *top left β*-CD ion chromatogram (*top*) and *β*-CD-und$_1$ extracted ion chromatogram. No MS/MS data was obtained for this compound

strength of the complex is speculated to be the main factor of controlling the reaction. Furthermore, the inclusion between 4-NP and 3-NP with all three CDs were investigated to enable a comparison with the work of Harada where a 3-NP derivative was applied [11]. In the titrations the NP-compound was kept in the cell and CD solution was loaded in the syringe, all experiments were conducted at pH 3.5 to avoid any transesterification reaction between CD and NP-derivatives. The Wiseman Isotherm was used in fitting of the data to obtain binding constants between CD and NP-compound and these data are shown in Table 2.

For all three types of CDs it is evident that the binding constant between 3-NP and the CD is weaker than that between 4-NP and CDs, but also that all of the complexes are rather weak. The binding constant between CDs and 4-NP were in all cases found to be around twice the size of CD binding constants obtained with 3-NP. This indicates as expected that the use of 4-NP-derivatives instead of 3-NP-derivatives could possibly result in better selectivity in the reaction with CDs due to the higher strength of the complex between 4-NP and CDs.

The substitution on 4-NP with unsaturated aliphatic chains may induce a change in the binding energy with CDs and this was also tested with ITC. It is clear from the data in Table 2 that this is in fact the case. The 4-NP-acrylate and 4-NP-pent-4-enoate show a slightly lower binding constant with *β*-CD than what was observed for pure 4-NP. This could possibly be attributed to a change in the 3D structure of the complex between 4-NP and *β*-CD caused by the change of a hydroxyl group on the nitrophenol into a small aliphatic chain, which may affect the

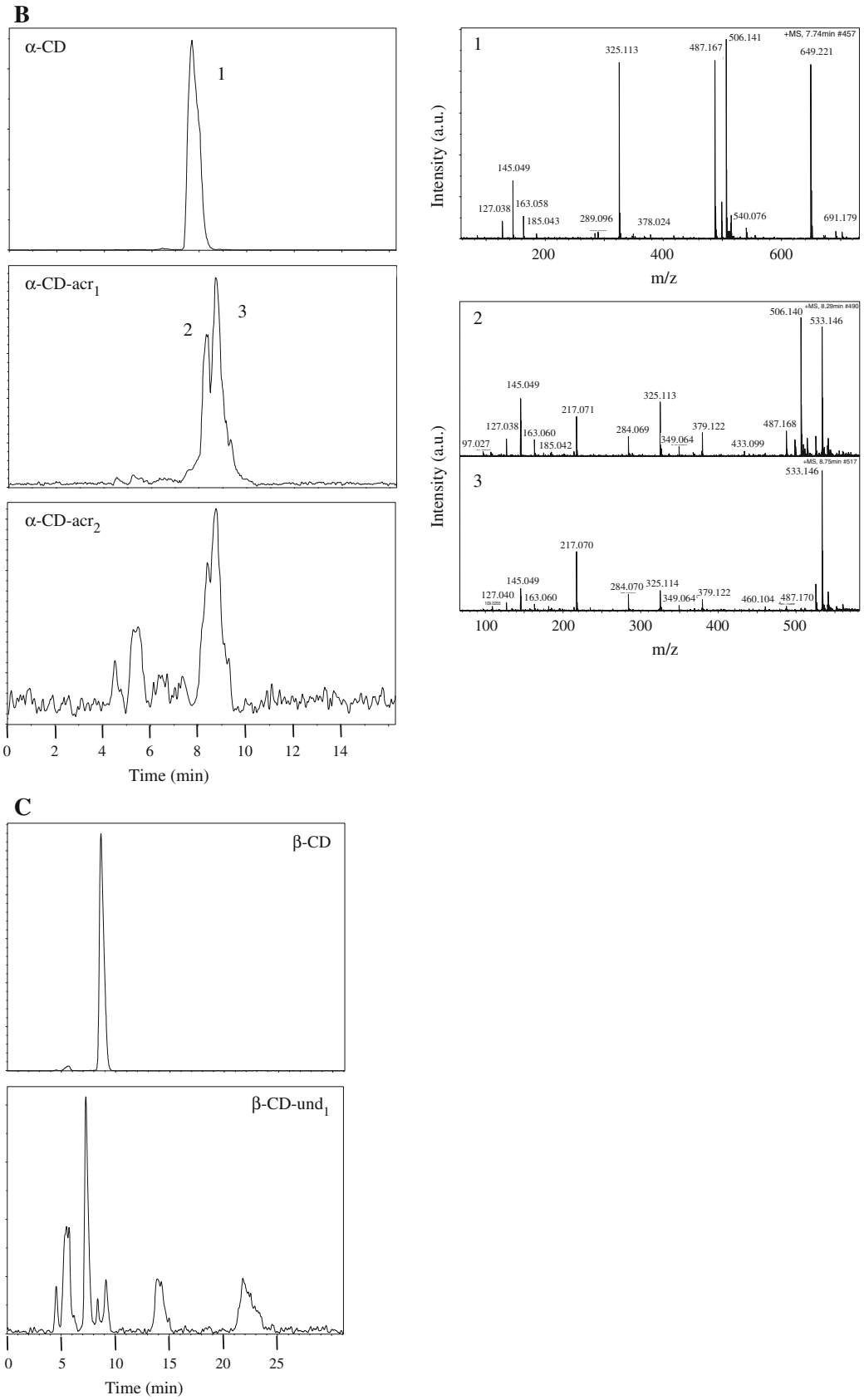


Fig. 4 continued

Table 2 Binding constants between 3-NP, 4-NP and 4-NP derivatives and α-, β- and γ-CDs given at 30 °C in M^{-1}

	3-NP	4-NP	4-NP-acr	4-NP-pent	4-NP-und
α-CD	23.3 ± 3.01	64.5 ± 3.76	–	–	–
β-CD	125 ± 3.61	240 ± 14	141 ± 7.05	111 ± 5.46	782 ± 52.7
γ-CD	145 ± 21.4	243 ± 36.7	–	–	–

Errors indicate the error of the fit of the data to the Wiseman isotherm

position of the nitrophenol inside the CD cavity. The change to a 4-NP carrying a small aliphatic chain may make the inclusion complex formation less favourable due to steric hindrance, hence affecting the stability constant negatively. The 4-NP-undec-10-enoate derivative by far has the largest binding energy with β-CD. This gives an indication of a complex formation taking place not only at the phenyl ring but also at the aliphatic undec-10-enoate chain. Binding constants of free NP and aliphatic chains of similar structure to undec-10-enoate from the literature show that the binding constant with an aliphatic chain is approximately a factor of 10 higher than that of 4-NP [17, 21], supporting the findings in this study. Another ITC study of substituted benzoic acids show data in good correlation with the ones found in this study, i.e. with a stronger binding as an effect of substitution by a longer aliphatic chain but no effect of relatively short chains [22].

Additional thermodynamic parameters were also obtained from the ITC studies as shown in Table 3. Theses thermodynamic parameters are evaluated relative to each other to give a small insight into the differences in binding for the different NPs and CDs, even though they are associated with a relatively high error in systems of low stability constants as in this case [16].

Some differences are observed between the different complex formations. α-CD seems to have the strongest binding to 3- and 4-NP seen by the large favourable enthalpy contribution, but this is counteracted by a large unfavourable entropy contribution, indicating that the fit between α-CD and NP is strong, but lead to a dramatic loss in rotational freedom. The interaction between NPs and β-CD show a weaker binding, but also a lower loss of rotational freedom observed in the entropy contribution being around zero. In the case of γ-CD, the interaction is even less as shown by smaller enthalpy change, but the entropy contribution is positive, indicating that no rotational freedom is lost.

The complex formation between NP-derivatives and β-CDs is observed to be driven by quite high enthalpy contributions, especially in case of NP-pent, but they are accomplished by a great loss of rotational freedom and negative entropy contribution. In the case of NP-und the binding is weaker (larger ΔH) but at the same time no freedom is lost. The differences in thermodynamics between the binding of the NP-derivatives indicates that the binding to NP-und may take place at another part of the molecule than in case of NP-acr and NP-pent. It could therefore be assumed that not only the NP-ring of NP-und, but also the aliphatic undec-10-enoate chain participate in inclusion complex formation.

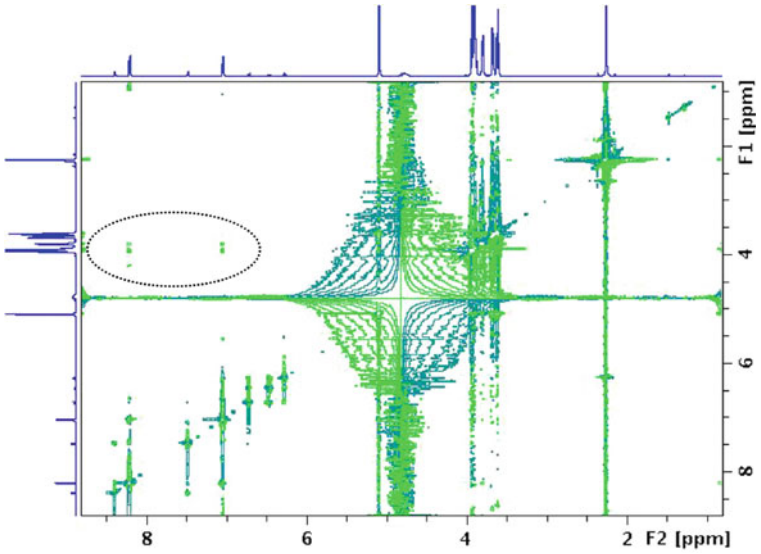
The formation of inclusion complexes between β-CD and the modified NPs was further tested by ROESY 2D NMR (Rotational Overhauser Effect SpectroscopY), a technique revealing the proximity in space of components as those found in an inclusion complex. COSY (Correlation SpectroscopY) spectra were utilised to assign the signals in the CDs. The COSY spectrum of β-CD and NP-acrylate (not shown) revealed the following shifts of the CD protons: H_1:5.2, H_2: 3.74, H_3: 3.98–4.0, H_4: 3.7–3.75, H_5: 3.86 and H_6: 3.96 ppm. In Fig. 5 a ROESY spectrum of β-CD and NP-acrylate is shown, crosspeaks in the spectrum show a spatial interaction between two molecules as indicated.

In the ROESY spectrum it is evident that the protons inside the CD cavity (protons H_3, H_5 and C_6) interact with the protons on the aromatic NP ring, corresponding to the crosspeaks at 7–8 ppm on the F2 axis and 3.8–4.2 ppm on the F1 axis. No interactions between the protons inside the

Table 3 Enthalpy and entropy of complex formation between 3-NP, 4-NP and 4-NP derivatives and α-, β- and γ-CDs all given at 30 °C in kJ/mol

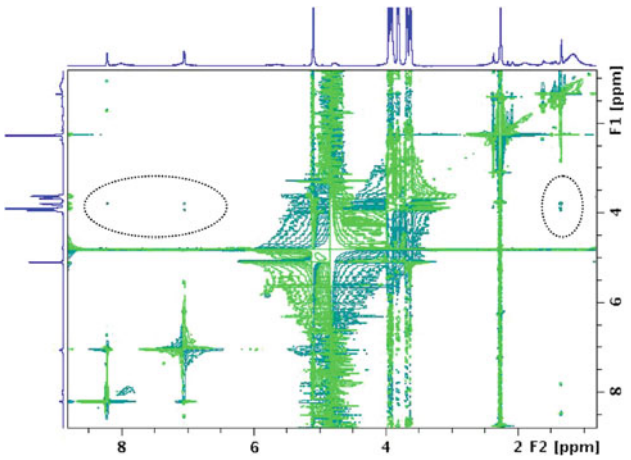
	3-NP		4-NP		4-NP-acr		4-NP-pent		4-NP-und	
	ΔH	−TΔS	ΔH	−TΔS	ΔH	TΔS	ΔH	−TΔS	ΔH	−TΔS
α-CD	−46.4 ± 5.7	38.4	−25.0 ± 1.2	14.5	–	–	–	–	–	–
β-CD	−14.4 ± 0.3	2.3	−11.3 ± 0.5	−2.5	−14.6 ± 0.6	2.1	−31.5 ± 1.3	19.7	−9.7 ± 0.3	−7.1
γ-CD	−2.9 ± 0.3	−9.6	−1.7 ± 0.2	−12.1	–	–	–	–	–	–

Errors indicate the error of the fit of the data to the Wiseman isotherm



CD-protons	H-3 (NP-acr)	H-2 (NP-acr)
H-3	x	-
H-5	xx	xx
H-6	xx	xxx

Fig. 5 2D ROESY spectrum of β-CD and NP-acrylate, showing spatial interactions between the two components. Measured in pure D_2O, pD < 6 to prevent reaction between NP-acrylate and β-CD. Crosspeaks (*away from the diagonal*) show spatial interactions. The table next to the spectrum shows the strength of the signal as marked by x for weaker interaction and xxx for stronger interaction



CD-protons	H-3 (NP-und)	H-2 (NP-und)	$(CH_2)_8$ (NP-und)
H-3	x	xx	xx
H-5	x	x	xxx
H-6	x	xx	xx

Fig. 6 ROESY spectrum of β-CD and NP-undec-10-enoate, showing spatial interactions between the two components. Measured in D_2O, pD < 6 to prevent reaction between NP-undec-10-enoate and β-CD. Crosspeaks (*away from the diagonal*) show spatial interactions. The table next to the spectrum shows the strength of the signal as marked by x for weaker interactions and xxx for stronger interactions

cavity and the acrylate-group are observed; these were expected to be visible at 2–3 ppm. Only the aromatic protons closest to the nitro group seem to interact with the protons on H_3 of the CD, at the same time the aromatic protons near the acrylate end seem to have a larger crosspeak i.e. stronger interaction with the protons on H_6 of the CD. These interactions and their strength is shown in the table and the observations indicate that the main part of the complexes have the acrylate end of the NP-ring near the narrow rim of the CD.

If the ROESY data are assumed to represent the most frequent as well as most reactive configuration of the complex, the data may suggest that the formed CD-acrylate derivatives will be mainly substituted on the H_6 (primary) of the CDs. Since more than one position of the acrylate chain on the CD was observed from LC-MS/MS experiments, it seems the conformation found from ROESY NMR is not giving all the details about the reactive conformation in solution.

The COSY spectrum of β-CD and NP-undec-10-enoate (not shown) revealed the following shifts of CD protons: H_1: 5.2, H_2: 3.74, H_3: 3.9–4.0, H_4: 3.68–3,7, H_5: 3.86 and H_6: 3.96 ppm. The ROESY spectrum of β-CD and NP-undec-10-enoate is shown in Fig. 6.

In the ROESY spectrum it is again evident that the protons inside the CD cavity (H_3, H_5 and H_6) interact with the protons on the NP ring, corresponding to the crosspeaks at 7.0–8.0 ppm on the F2 axis and 3.7–4.0 ppm on the F1 axis, as it was also observed in the case of the NP-acrylate/β-CD system. The crosspeaks at approximately 1.5 ppm on the F2 axis and 3.7–4.0 ppm on the F1 axis shows that inclusion of the aliphatic undec-10-enoate chain into the CD cavity also occurs. The intensity of the crosspeaks arising from the undec-10-enoate interaction is observed to be stronger than that of the NP interaction. The intensity of the peaks is dependent on both the amount of a given complex as well as the distance between the interacting protons in space [23], hence it is difficult to predict the ratio between CDs having included the NP and CDs having included the undec-10-enoate. It seems very unlikely that the same CD is able to include both at the same time. This is supported by the findings in ITC experiments where a reasonable fit to a 1:1 model was found. Binding constants from ITC show a stronger binding with the 4-NP-undec-10-enoate than with 4-NP, also supporting the findings that the aliphatic undec-10-enoate chain plays a role in the complex formation. The interactions between the aromatic ring and the CD cavity are observed on protons of H_3, H_5 and H_6 and it is not possible to determine which end of the ring is closest to the narrow rim of the CD. In the aliphatic region it is not possible to determine the position either, indicating that a mixture of different configurations is present in the solution. The presence of two different configurations of the NP-undec-10-enoate/β-CD complex may affect the regioselectivity of the reaction and e.g. the overall DS and distribution of derivatives. It could from these data be expected that the reaction between NP-undec-10-enoate and β-CD could follow various routes due to the many conformations found. On the contrary the analyses of the product seem to reveal a higher selectivity in this case, yielding high amounts of mono-substituted product as shown by MALDI-TOF as well as LC-MS/MS, even though more than one positional isomer was formed. One possible speculation could be a self-inclusion mechanism where the undec-10-enoate chain is included in the CD it is attached to and prevents more than one substitution to take place on each CD, but this has not been investigated further.

In this work α-, β- and γ-CD-acr derivatives have been studied along with β-CD-pent and α- and β-CD-und and the effect on the reaction of the chain length observed. From MS and HPLC data no significant differences in distribution and number of derivatives were observed from reactions with α-, β- and γ-CD, respectively, even though the ITC data reveals a rather large difference in the stability constants. The β-CD derivatives even seem to give more of the di- and tri- substituted products, in spite of the higher binding constant with 4-NP and indications of one predominant 3D conformation shown by ROESY. In addition the complexes of NPs with β- and γ-CD are around four times that of NP complexes with α-CD without any observed tendency to differences in substitution pattern. If the inclusion complex plays a key role in the reaction mechanism a higher difference in the obtained derivative distributions was expected. Since this is not the case it could be assumed that the effect of the NP-acrylate inclusion is insignificant compared to other factors affecting the DS. This is supported by the finding of relatively low stability constants between CDs and NPs. The observations of more mono-substituted product in the derivatives with longer aliphatic chains (pent-4-enoate and undec-10-enoate) indicate that the presence of an aliphatic chain on the NP-derivatives provides a better control of the DS than a small acrylate-chain, possibly by self-inclusion leading to occupation of the cavity.

Harada and co-workers reported the synthesis of β-CD-acr to be regioselective for the C-2 secondary alcohol of the CDs using a 3-NP-acrylate as intermediate [11], but in this work LC-MS/MS analyses have indicated several types of positional isomers being present in the samples.

Conclusion

Cyclodextrin monomers carrying vinyl groups have been synthesised using a method of inclusion with nitrophenyl-derivatives for controlling the substitution degrees. It proved possible to achieve CD monomers of α-, β-and γ-CDs with a chain length of 2 carbons between the CD and the double bond. Changing the NP-derivative to carry a longer carbon chain also proved possible and products of β-CD-pent-4-enoate plus α- and β-CD-undec-10-enoate were obtained. In the case of β-CD-undec-10-enoate only small amounts of substitutions higher than one was observed, whereas more different substitutions were observed with α-, β- and γ-CD-acr.

The produced products were studied in more detail by separation of the CD derivatives on a CDScreen® HPLC column and use of MS/MS. From these studies at least two different substitution patterns were distinguished in the mono-substituted CD-acr_1 product indicating low regioselectivity of the reaction. This also seemed to be the case with the β-CD-undec-10-enoate even though less of the higher substitutions were found in the analyses of the products. Studies on the complexes between NP-derivatives and CDs by ITC and 2D ROESY NMR were conducted to reveal differences between inclusion of 4-NP-acrylate and 4-NP-undec-10-enoate. From ITC it was proved that the undec-10-enoate-chain adds to the stability of the complex between β-CD and 4-NP-undec-10-enoate,

whereas the 4-NP-pent-4-enoate and 4-NP-acrylate has more or less the same stability with β-CD as the pure 4-NP. 2D ROESY NMR confirmed the presence of an inclusion complex of NP-derivatives and CDs in aqueous solution, and gave indications of the 3D structure of the complex. To sum up the results show a limited control of the substitution reaction even though the complex formation between 4-NP and CD was proved and studied by ITC. Only in the case of CD-undec-10-enoate a control of the synthesis seems to be observed; maybe due to self-inclusion leading to occupation of the cavity.

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