MODIFICATION OF β-CYCLODEXTRIN THROUGH SOLUTION RING-OPENING OLIGOMERIZATION OF β-BUTYROLACTONE

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β-cyclodextrin (CD) derivatives were prepared through ring-opening of β-butyrolactone (BL) in DMSO solution initiated by CD at relatively low temperature in the presence of (-)-sparteine (SP) as nucleophilic activator. The reaction was proved to yield a mixture of polyhydroxybutyrate (PHB) and 3-OH butyrated cyclodextrin derivatives (CD-HB). Following separation, the resulted products were thoroughly characterized through NMR spectroscopy and electrospray ionization mass spectrometry (ESI MS) coupled with liquid chromatography (LC). The CD-HB products are described in view of their substitution degree, substitution pattern at glycoside ring level and length of the oligomer chains attached to the CD, taking into account both NMR and ESI MS characterization methods. The kinetics of β-butyrolactone ring-opening in the presence of CD and SP is evaluated through ^{1}H NMR spectroscopy showing that secondary processes are contributing to the monomer conversion.

Keywords: β-cyclodextrin, β-butyrolactone, electrospray mass spectrometry, sparteine, polyester, liquid chromatography

INTRODUCTION

The development of new cyclodextrin derivatives has become increasingly important in the past years due to the expanding needs in pharmaceutics for molecules with the capacity to host various bioactive molecules.[1] Among the possible functionalization pathways, the esterification of CD's hydroxyl groups can lead to compounds with interesting properties. This may be achieved via classical esterification reactions with a carboxylic acid of choice. Cyclodextrins can be also functionalized through ring-opening of cyclic esters solely in the presence of CDs, the reaction being initiated by the hydroxyl groups. Harada and co-workers were the first to prove the ring-opening polymerization of various cyclic esters, like β-butyrolactone, ε-caprolactone and δ-valerolactone initiated by CD.[2] The reaction was conducted in bulk at a relatively high temperature (100 °C) and the products were described structurally through NMR and MALDI MS as polyester tethered cyclodextrins. However, the possible formation of side products was not discussed. Later, the ring-opening oligomerization (ROO) of lactide monomers initiated by CD in dimethylformamide solution, at a lower temperature of 80 °C was demonstrated.[3] Another strategy employed for the synthesis of cyclodextrins tethered with oligoester chains is represented by the use of organocatalysts. An example consists in the use of sparteine (SP) in the bulk polymerization of β-butyrolactone at a relatively low temperature (50 °C).[4] The lactide was also polymerized in bulk at 120 °C in the presence of 4-dimethylaminopyridine or in CH_2Cl_2 at 35 °C using perbenzylated β-cyclodextrin diol as initiator.[5] A system composed of the amido zinc catalyst and β-CD-$(OBn)_{19}(OH)_2$ acting both as a diol co-initiator

and a chain transfer agent has been reported for ring-opening polymerization (ROP) of lactides (LA), β-butyrolactone (BL), and trimethylene carbonate (TMC).[6] A deeper review of the recent publications in the field of polyester functionalized cyclodextrins has also been published.[7]

On the other hand, the reactions involving the cyclodextrin as initiator have raised some questions concerning the nature of the products and their structural assignment and in particular the fact that more than one OH group from the same CD molecule can initiate polymerization.[8,9] In one of our papers, we have shown beyond any doubt that cyclodextrin is capable of initiating more than one polymerization reaction per a single molecule.[4] The reaction has been performed in bulk and the CD transformation was not fully quantitative due to the inhomogeneous reaction environment. Therefore, in this paper, we investigate the solution polymerization of BL in the presence of CD/SP system, using DMSO as solvent. We investigate the reaction kinetics in order to explain the formation of various products in the final reaction mixture. The characterization techniques used for the structural elucidation of cyclodextrins and their derivatives often employ mass spectrometry methods like MALDI and ESI MS, but in general without prior chromatographic separation.[10-18] Herein we demonstrate the usefulness of such a procedure by using MS coupled with liquid chromatography and NMR correlations.

EXPERIMENTAL

β-Butyrolactone, BL, (98%, from Aldrich) was dried by distillation over calcium hydride under reduced pressure and kept under argon. β-Cyclodextrin, CD, (98%, from Sigma) was dried at 80 °C for 24 h in a vacuum oven and kept under argon. (-)-Sparteine, SP, (99%, from Aldrich) was used as received. Dry DMSO was purchased from Aldrich, kept on molecular sieves and further used without any supplementary drying.

Solution polymerization of BL

The polyhydroxybutyrate-cyclodextrin conjugates (CD-HB) were prepared by solution ring-opening of β-BL monomer at 50 °C in the presence of β-CD as initiator and SP as nucleophile activator. In a typical procedure, 1.02 g (0.898 mmol) of CD powder, 1.5 mL (17.973 mmol) BL and 0.2 mL (0.898 mmol) SP were introduced into a dried flask under argon together with DMSO (total concentration, 20% w/w) and the flask was sealed with a septum. The reaction mixture was heated at 50 °C under magnetic stirring in a thermostated oil bath for 12 h. Then, the reaction mixture was transferred into a crystallizer, frozen by adding liquid nitrogen and lyophilized for 24 h (freeze-drying conditions: pressure 0.100 mbar, temperature -42 °C) to remove DMSO. The lyophilized sample was then suspended in CH_2Cl_2, stirred overnight and centrifuged (for 30 minutes with 3000 rpm). The procedure was repeated two more times to ensure the full separation of the pure PHB (soluble in CH_2Cl_2) from the CD-HB white precipitate. A polyhydroxybutyrate (PHB) homopolymer (fraction F1) was obtained after evaporation of CH_2Cl_2. The CD-HB precipitate (fraction F2) was dried overnight in a vacuum oven at 40 °C and kept into a desiccator at room temperature.

Characterization

NMR – H, C, HSQC

The NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer equipped with a 5 mm multinuclear, inverse detection z-gradient probehead. DMSO-d6 was used as solvent. Polymerization kinetics was performed directly into the NMR tube at 50 °C, by inserting the appropriate amounts of reactants in deuterated DMSO.

LC-ESI MS

LC-MS experiments were conducted using the AGILENT 6520 LC QTOF mass spectrometer equipped with a dual ESI source. The data were analyzed using the Mass Hunter software. The concentration of each solution was of 0.5 g/L for chromatographic analyses and 0.1 g/L (water/acetonitrile 1:1 v/v mixture) for mass spectrometric analysis performed via direct infusion of the sample. The ESI MS parameters were set as follows: Vcap = 4000 V, fragmentor voltage = 200 V, drying gas temperature = 325 °C, drying gas flow = 10 L/min and nebulizer pressure = 35 psig. Nitrogen was used as spraying gas.

Direct injection ESI MS was performed by sample infusion via an external syringe pump (KDS Scientific) with a flow of 0.05 mL/min. The drying gas flow was set at 3.5 L/min and nebulizer pressure at 5 psig.

LC separations were performed by using a C18 column – Agilent ZORBAX 300SB-C18 4.6 x 150 mm, 5 µm. The samples were separated by gradient elution using water–acetonitrile solvent mixture at 26 °C constant temperature in the column compartment. The samples were solved in a 1:1 (v/v) water/acetonitrile mixture and 10 µL were injected. The used eluents were: A – 2 mM formic acid and B – acetonitrile. The gradient was established as follows: 0-3 min, 20% B; 15 min, 100% B; 18 min, 100% B; 20 min, 20% B. For the LC-MS analysis of PHB secondary products, the samples were solved in pure acetonitrile and the mobile phase was delivered at 1

mL/min in the linear gradient mode: 0-3 min, 20% B; 25 min, 100% B; 30 min, 100% B; 32 min, 20% B.

RESULTS AND DISCUSSION

CD-HB products were prepared via ring-opening of BL in the presence of CD and SP, in DMSO solution. After 24 hours of reaction at 50 °C, the reaction mixture was cooled at room temperature and the cyclodextrin derivatives were purified according to the procedure described in the experimental part. The products resulted from the polymerization process were characterized by NMR spectroscopy and mass spectrometry with prior chromatographic separation (LC MS).

Electrospray ionization mass spectrometry (ESI MS)[3] or MALDI MS[2] have been employed to get a clear picture of the structure of cyclodextrins tethered with oligoester chains. These techniques generally provide high accuracy results on pure organic compounds, but when dealing with polymer samples their characteristic molecular weight distribution imposes the correlation of mass spectrometry with liquid chromatography (GPC or HPLC).[19] Moreover, when dealing with positional isomers, knowing the exact mass of the molecule, as a result of a direct MALDI or ESI mass spectrometry analysis (no chromatography), does not solve the problem. In these cases, the correlation of MS with other characterization techniques is a must. An example is given in our previous study on PHB tethered CD, where we clearly showed through NMR and MS that the CD molecule was esterified with more than a single chain of PHB.[4]

First, the PHB fraction was screened through direct injection MS followed by LC MS, and the obtained chromatogram and the associated MS spectrum extracted from the entire chromatogram are presented in Figure 1.

The LC MS procedure allowed a better quantification of the PHB secondary products having the structure depicted in the insert from Figure 1, noted as PHB-A and PHB-B, respectively. The MW ranges from 300 to 2000. The major peak series, PHB-A, with 86 Th increment is characteristic of PHB chains having OH and COOH end groups, as demonstrated by the mass values corresponding to the Na adducts calculated by the formula *m/z = nx86 + 18 + 23*. PHB-A population has the average M_n value of approx. 900 Da. The less representative peaks correspond to crotonic acid ended PHB (PHB-B) sodium adducts (m/z = nx86 + 23). Similar PHB-A and -B species as proton charged ionic species, adducts with NH_4 and doubly charged species were also observed. The formation of PHB-A and PHB-B structures during the ROP of BL initiated by CD will be later explained.

The second fraction obtained from separation, containing CD derivatives, presents a certain structural heterogeneity (Scheme 1), making its description more difficult and challenging. Structural assignment of the synthesized esterified cyclodextrins should reveal specific characteristics, like general structure, substitution degree (SD), length of the attached oligomer chains and, not least important, the substitution pattern of the glycoside ring forming the CD molecule and the distribution of the BL monomer units among different glycoside rings.

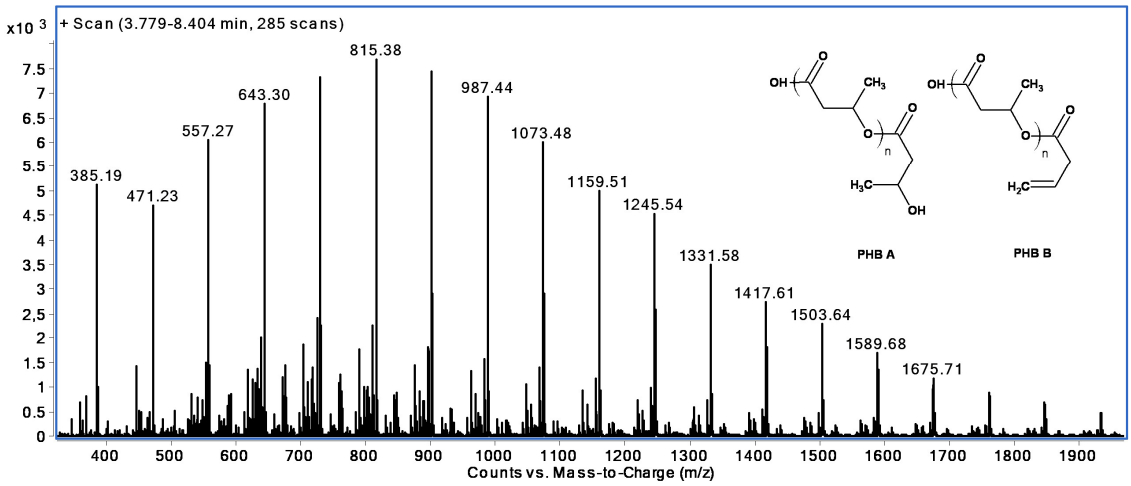
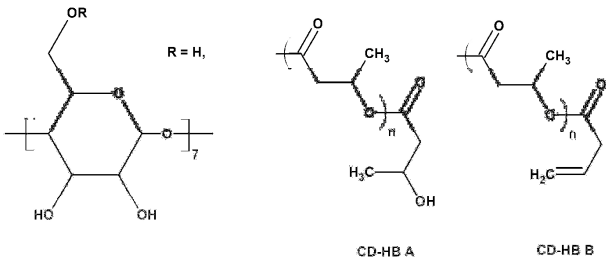


Figure 1: LC MS Spectrum obtained for PHB oligomers (fraction F1)



Scheme 1: General structure of CD derivatives

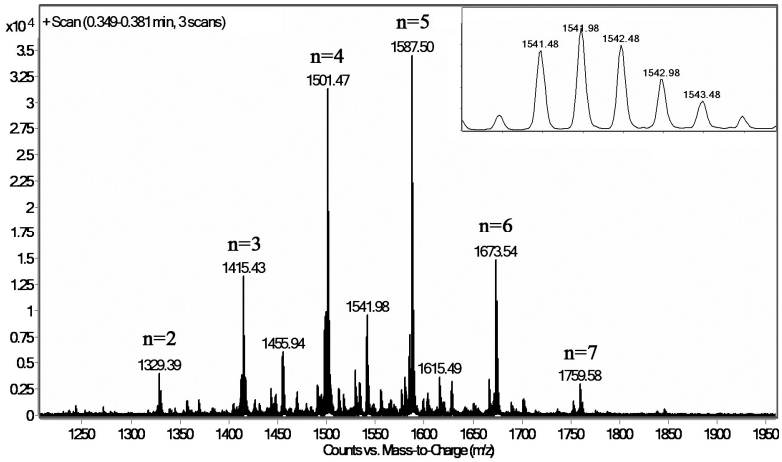


Figure 2: Direct injection ESI MS spectrum of CD-HB sample (fraction F2)

As a general manner, the cyclodextrins bearing different aliphatic oligoester chains have been described by using mass spectrometry methods like MALDI or ESI MS, without any prior chromatographic separation. However, given the complexity of these samples generated by the MW variation in the analysed mixture, we showed that the LC MS method can give a more accurate structural description.[4] The use of separation before introducing the sample into the mass spectrometer has two reasons: to enhance and to clearly isolate the signals associated with different species, which otherwise may overlap and increase the incertitude of the ESI MS measurement.

First, we used direct injection MS and the resulting spectrum is presented in Figure 2. Two main series of peaks can be seen in this spectrum. The first series, noted with CD-HB A and having 6 members shows an 86 Th difference between consecutive members and may be associated to CD esterified with PHB oligomers, as presented in Scheme 1.

The ionic species corresponding to this series are Na adducts of the CD-HB A species noted as $[CD\text{-}HB_n+Na]^+$, where n represents the number of monomer units corresponding to a specific peak. Their m/z value may be calculated using the following formula: $m/z = 1134 + nx86 + 23$, where *1134* is the mass of CD, *86* is the mass of the β-butyrolactone monomer unit, *n* is the number of monomer units and 23 represents the mass of the Na cation. For example, the base peak found at 1587 corresponds to Na adduct of CD-HB A pentamer. The average degree of substitution can be inferred from the calculation: $N_1 = \sum I_i n_i / \sum I_i$, where I_i represents the relative intensity of a specific MS peak and n_i is the number of monomer units associated with that peak. Thus, according to the direct MS measurement, the N_1 has a calculated value of 4.5 monomer units per CD molecule.

Besides the main peak series, there may be observed several peaks with lower intensities, the most notable ones being found at m/z = 1541.5 and 1455.5 (on the spectrum there may be observed the base peak, which comes the second in the isotopic distribution – see the insert in Figure 2). These peaks correspond to doubly charged species according to their isotopical profile (0.5 Th difference in isotopical distribution). The ionic species associated with

these peaks can be described as complexes of CD-HB A formed during the electrospray process as follows: $[CD\text{-}HB_n + CD\text{-}HB_m + Na + NH_4]^+$. For example, the peak found at m/z = 1541.5 can be calculated as [1478 (CD-HB_4) + 1564 (CD-HB_5) + 23 (Na) + 18 (NH_4)]/2. These species are formed during the electrospray process, as it has been described in literature for different CD derivatives.[20]

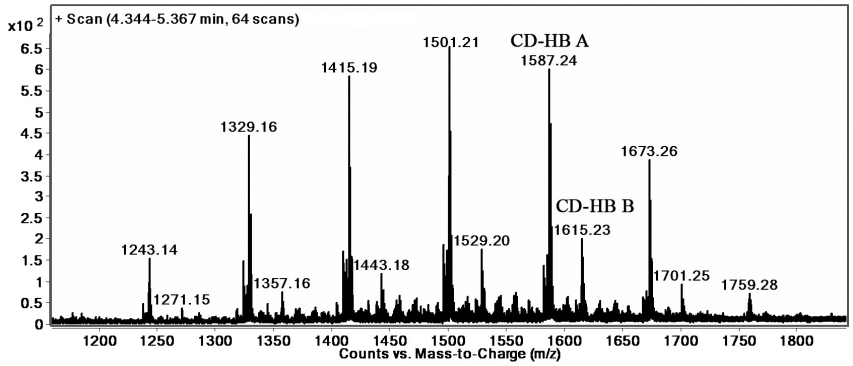


Figure 3: LC-ESI MS spectrum of CD-HB sample

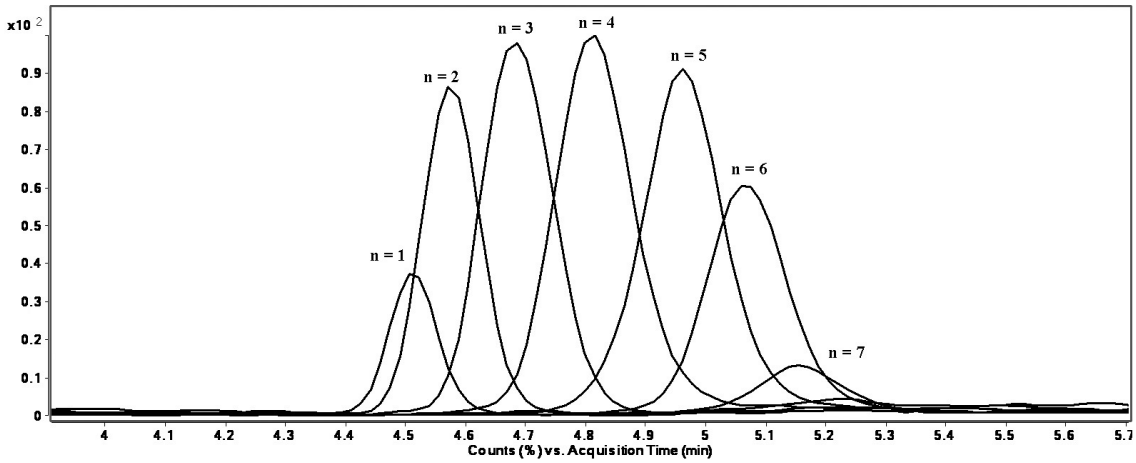


Figure 4: Extracted ion chromatograms of CD-HB A species

The CD-HB sample has been further analysed through LC MS and the extracted spectrum over the entire chromatogram is presented in Figure 3.

The mass spectrum denotes the presence of several types of peak series corresponding to the molecular species present in the mixture. The most prominent peak series has a peak-to-peak succession of 86 Th, the mass of BL monomer units, being qualitatively similar to the main series observed using direct injection MS (CD-HB A). However, from the quantitative point of view, the peak distribution in terms of relative intensities is different. In order to quantify the average substitution degree for the CD-HB A, the extracted ion chromatograms for the members of this series were plotted (Figure 4).

Assuming that peak area is proportional to the concentration of CD-HB A species in the injected solution, we may calculate the average number of monomer units (N) connected to a CD molecule by using the formula: $N_2 = \sum A_i n_i / \sum A_i$, where A_i represents the area corresponding to a specific chromatographic peak and n_i is the number of monomer units associated with that peak. Thus, 3.9 monomer units were found to be connected to a single CD molecule, which gives a difference of 0.6 as compared to the previous calculation for N_1. The LC MS method is more accurate in these calculations due to the fact that each compound from the mixture is sprayed at a different retention time.

Thus, in order to increase the accuracy of measurement when dealing with molecular weight distribution measurements, a prior separation step before MS analysis is required for this type of compounds.

In the mass spectrum presented in Figure 3, one may observe a second series of peaks up shifted with 28 Th compared with the main series, noted with CD-HB B (see the structure in Scheme 1). These peaks can be assigned to protonated ion species of CD esterified with two molecules of crotonic acid. The calculation formula for the members of this series, e.g. the peak found at *m/z* = 1529, is *m/z = 1134 (CD) + 2*68 (crotonate) + 3*86(BL) + 1 (H).*

The proportion of CD-HB-B peaks in the analyzed mixture is rather low, based on the relative ratio between the main series (CD-HB A) and the crotonate series (CD-HB B) observed in the mass spectrum. However, an exact quantification cannot be performed due to the poor separation, different cationization and consequently different ionization efficiencies characteristic of the analysed species. The appearance of the crotonated CD-HB species can be explained by the degradation processes, which will be further discussed. As peaks for pure CD were not detected in the LC MS analysis, one may suppose that CD was totally reacted when the reaction was conducted in solution, contrary to the results obtained for the process performed under bulk conditions.[4]

Usually, the direct observation of a mass spectrum may provide enough data to describe a pure and structurally homogenous sample. However, when complex isomeric mixtures are analysed, further investigation at a molecular level are required. The CD-HB sample contains positional isomers due to the multiple OH sites on the same CD molecule, which are able to initiate the opening of the BL cycle. In other words, the sum of the BL monomer units attached to the CD can be part of a single chain or can be randomly distributed among both the primary and secondary OH groups of CD structural units. Previous publications[2,3] consider the possibility that monomer ester units are attached to the CD in a single chain, but not enough data have been provided to support this hypothesis. However, we have already shown that, when the reaction is conducted in the presence of amines under bulk conditions, a single CD molecule is esterified with more than one short oligoester chain.[4]

Thus, to clearly identify the structure of CD HB product, the sample was also analysed using NMR spectroscopy. The spectrum presented in Figure 5 indicates that the CD-HB fraction contains CD molecules esterified with short chains of oligo(3-OH butyrate). Traces of unreacted β-butyrolactone, the peak annotated as BL in the ^{1}H NMR spectrum corresponding to –CH_3 protons, were also identified in the spectrum of F2 fraction. The length of oligoester chains can be calculated from the ratio of integrated signals given by the protons belonging to the "in chain" (5.07 ppm) and "end chain" (4.05 ppm) monomer units. However, these signals overlap with other signals characteristic of the modified cyclodextrin.

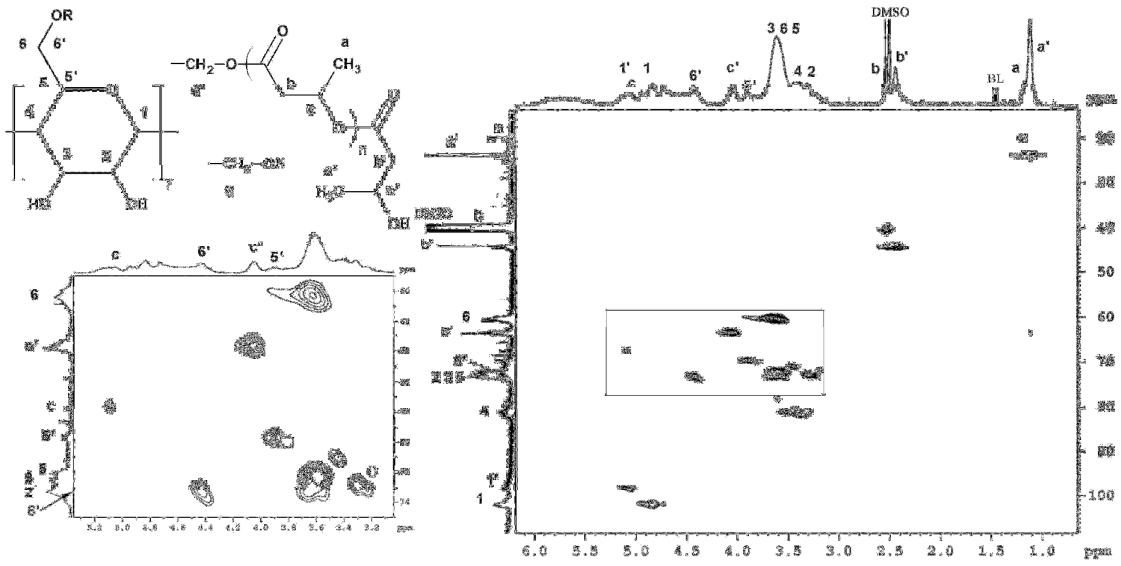


Figure 5: H–C inverse correlation NMR spectrum of CD-HB and structural assignments

Thus, this ratio was approximated using CH_3 corresponding signals at 1.12 ppm (a' – end chain) and 1.2 ppm (a – in chain) and the obtained value (a'/a = 6/1) shows that most of the monomer units are attached to the CD as 3-OH butyric acid and only small amounts can be found as oligomers (dimers, trimers, etc). This information is important as it confirms the fact that almost all monomer units observed in MS analysis are esterified on different OH groups of the CD and not in a single oligomer chain. This finding clearly supports the assumption that CD may initiate a ring-opening with more than one OH group.

The modification of CD molecules is also denoted by the broad signals in the NMR spectra. The most notable modifications that could be assigned are the shifts of the protons from 5 to 5' and from 6 to 6' positions. The assignments were supported by the HSQC H-C inverse correlations detailed in Figure 5. According to the literature reference in the case of caprolactone modified CD, the H signal at 3.9 ppm can be correlated with the C signal found at 69 ppm, demonstrating that CD are esterified at C6.[21] However, this assignment has some flaws, since in our opinion the substituent change (from OH to ester) of CH_2 from 6 positions cannot be justified by a shift of only 0.2 ppm. In fact, the peak present at 3.9 ppm may be associated to the shift of the proton belonging to C5, noted with 5', while the signal belonging to the protons of C6 may be found at 4.5 ppm, noted with 6'. Our assignment comes in agreement with the one made in the case of the lactide polymerization in the presence of cyclodextrins.[5] Thus, the HSQC correlation spectra revealed the corresponding ^{13}C NMR peaks at 69 ppm, C5', and 75 ppm, C6', respectively. This assignment is supported by the COSY correlation spectrum, in which a clear correlation between the 5' and 6' protons may be observed (Figure 6).

So far, the NMR analysis confirmed that CD is esterified mostly with 3-OH butyric acid, which may be the consequence of the fact that the reaction is carried in DMSO solution. Under bulk conditions, the CD were esterified with short chains having an average of three monomer units, while in the present case the average number of BL units per chain is close to one. Also, the total number of BL units per CD drops from an average of 12 for the bulk polymerized sample[4] to about 4 for the solution obtained product. This can be justified by the termination processes occurring during the ROP process, as depicted in Scheme 2. The degradation of the growing chains can be slowed down by the conditions of bulk polymerization, when the increase in viscosity can lead to the confinement and protection of the growing chains. The processes responsible for PHB degradation can be α-deprotonation[22] under the basic conditions created by the presence of SP or even by E1cb mechanism proposed by some of us.[23]

The resulting species proposed in Scheme 2 were actually identified in the MS analysis of the reaction mixture when both, PHB with OH and COOH or crotonate end groups, on the one hand, and crotonated CD, on the other hand, were found. In fact, once the PHB homopolymer is formed, it can continue the ROP process as COOH groups are known to be good initiators for the ROP of BL. Thus, most of the conversion of the BL monomer is related to the carboxylate and not to alcoholate propagating centres. This fact was also confirmed by a kinetic study (Figure 7) where, for two different CD/BL ratios (1/20 and 1/30), the slope which signifies the rate of conversion of BL monomer is similar with a small offset due to different CD initial concentration and suggests that the polymerization process occurs through a carboxylate propagation mechanism and not through an alcoholate one in which a CD initiator is involved.

The polymerization of cyclic esters in the presence of CDs was proposed to undergo through a ring-opening process initiated by CD OH groups, followed by an insertion of the monomer between the CD and the growing chain.[2] The addition of amines to the reaction activates the OH groups and the polymerization temperature may be decreased from 100 °C to 50 °C.[4] However, the amine addition has secondary effects like polymer degradation due to increased basicity of the system. Thus, in the case of CD-HB derivatives, the polymerization process is overlapping with the degradation of the newly formed CD-HB chains. BL consumption at relatively high conversion doesn't proceed through cyclodextrin initiation followed by propagation but through a different process, probably through an anionic mechanism initiated by carboxylic groups. The origin of the carboxylic groups may be explained by the occurrence of degradation of PHB chains grafted onto CD, as described in Scheme 2.

By comparing the results presented in this paper with the previous results obtained in bulk polymerization of BL in the presence of CD,[4] one may conclude that a homogenous reaction environment (DMSO) leads to an augmentation of the degradation processes.

The monomer conversion proceeds slowly initially and as conversion increases the polymerization process is speeded up probably due to a concurrent process. Thus it may be suspected that in the first step the polymerization proceeds via OH group initiation and propagation, while in the second step the COOH groups become responsible for the monomer conversion, as previously described.[24]

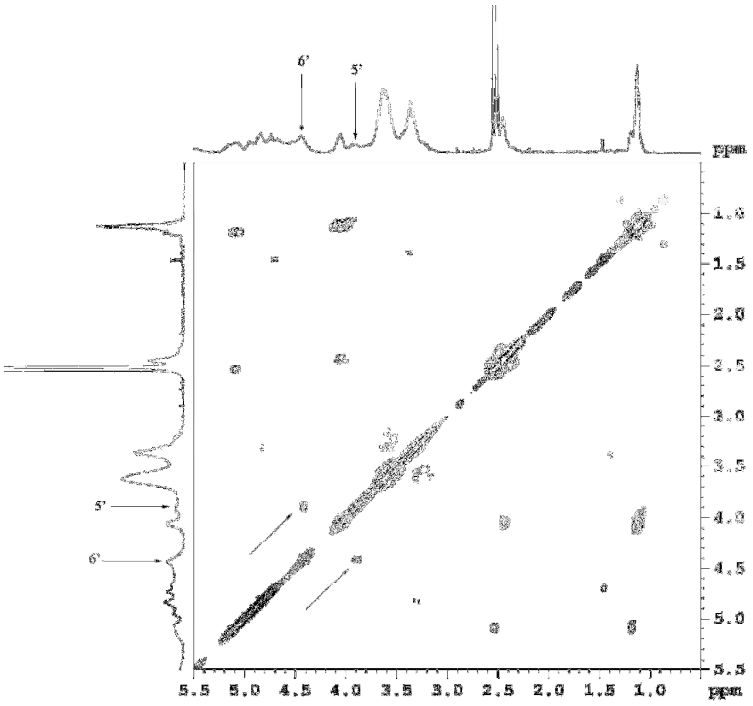
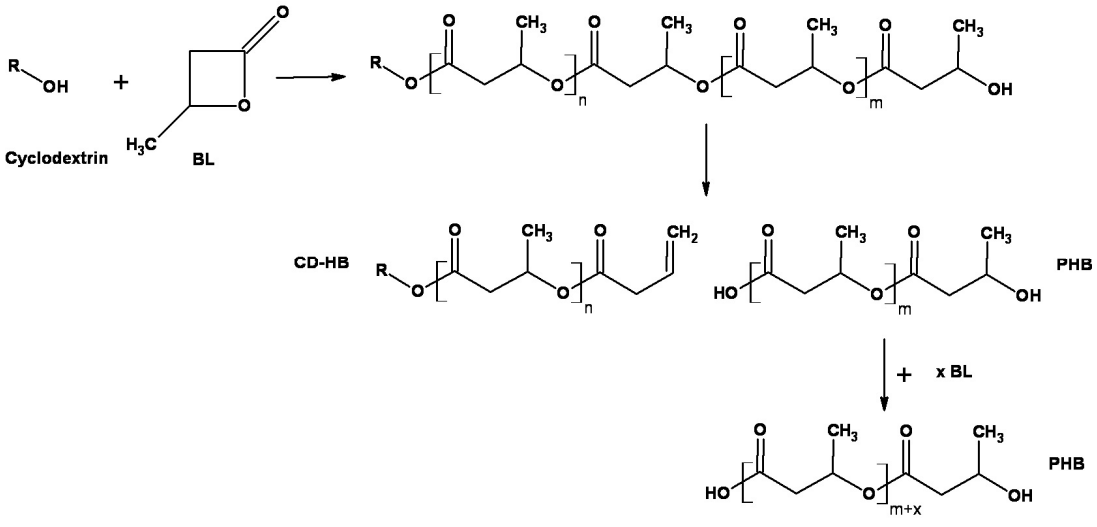


Figure 6: COSY H-H correlation NMR spectrum of CD-HB products



Scheme 2: Synthesis and degradation of PHB in presence of cyclodextrin

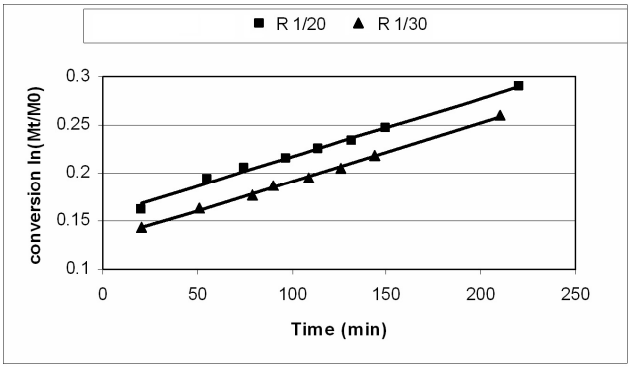


Figure 7: Variation of monomer conversion in time as a function of CD/BL molar ratio

CONCLUSION

The current study showed that 3-OH butyrate tethered β-cyclodextrins (CDHB) with an average number of 4 monomer units, esterified mainly at primary OH (C6), can be obtained through a relatively simple procedure in a DMSO solution, in a CD/SP catalyzed system. The reaction proceeds at a relatively low temperature, 50 °C, when non-negligible amounts of PHB oligomers with OH and COOH end groups are also obtained. The molecular structure has been demonstrated thoroughly using mass spectrometry coupled with liquid chromatography and supported by NMR COSY and HSQC correlations. Thus, this study showed that CD is fully reacted, the average substitution degree in CD-HB product is 4 and the BL units are distributed as 3-OH butyric acid among different glycoside units. Also, NMR studies showed that the initiation sites are located mainly at C6 position. The presence of crotonic acid functionalized CD suggests the cleavage of growing CD-HB chains. Most of the monomer conversion occurs through the carboxylate propagation mechanism, as shown by the kinetic study, resulting in PHB with OH and COOH end groups.

Overall, the presented experiments support the possibility to prepare 3-OH butyrate cyclodextrin derivatives through a relatively simple procedure.

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