**Cellodextrins**

Chemoenzymatic Synthesis of Fluorinated Cellodextrins Identifies a New Allomorph for Cellulose-Like Materials**

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**Abstract:** Understanding the fine details of the self-assembly of building blocks into complex hierarchical structures represents a major challenge en route to the design and preparation of soft-matter materials with specific properties. Enzymatically synthesised cellodextrins are known to have limited water solubility beyond DP9, a point at which they self-assemble into particles resembling the antiparallel cellulose II crystalline packing. We have prepared and characterised a series of site-selectively fluorinated cellodextrins with different degrees of fluorination and substitution patterns by chemoenzymatic synthesis. Bearing in mind the potential disruption of the hydrogen-bond network of cellulose II, we have prepared and characterised a multiply 6-fluorinated cellodextrin. In addition, a series of single site-selectively fluorinated cellodextrins was synthesised to assess the structural impact upon the addition of one fluorine atom per chain. The structural characterisation of these materials at different length scales, combining advanced NMR spectroscopy and microscopy methods, showed that a 6-fluorinated donor substrate yielded multiply 6-fluorinated cellodextrin chains that assembled into particles presenting morphological and crystallinity features, and intermolecular interactions, that are unprecedented for cellulose-like materials.

**Introduction**

Cellulose is an abundant natural biopolymer used extensively in industry as a raw material for the production of paper, textile, food thickeners, dietary fibre, etc.[1,2] The current use of cellulose increasingly involves nanosized cellulose particles (nanocellulose), which is a promising class of renewable material due to its intrinsic characteristics and potential for a broad range of industrial applications.[3–5] The development of nanocellulose-based materials relies on assembly-driven processes, the manipulation of which can have an impact on mechanical properties or bring additional functionality to the material.[6–10] The production of cellulose nanocrystals and nanofibrillated cellulose, the main classes of nanocellulose, both rely on top-down bioprocessing methodologies, based on the isolation of nanocellulose from cellulosic biomass, which requires high energy consumption.[3] In addition, the functionalisation of nanocellulose to meet requirements for specific applications often requires harsh chemical conditions (i.e., strong acids and bases). As an alternative, enzymatic synthesis presents an at-

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Enzymatic synthesis of fluorinated cellodextrins

**Synthesis of 2-, 3- and 6-monofluorinated cellodextrins (2F-EpC, 3F-EpC, 5; and 6F-EpC, 6)**

CDP uses glucose as an acceptor substrate only poorly, compared to cellobiose and longer cello-oligosaccharides, to produce cellodextrins containing deoxy-fluoro-glucose at the reducing terminus (compounds 4–6). We therefore initially used cellobiose phosphorylase (CBP; PRO-GH94-004) to synthesise monofluorinated cellobiose analogues (1–3; Figure 1A) for use as acceptors for CDP (Figure 1B). CBP was incubated at 37 °C with Glc-1P (100 mM) and deoxy-fluoro-glucose (2F-, 3F- or 6F-Glc) (100 mM) for 16 h, at which point TLC showed approximately 80% conversion into the disaccharides 1 and 3, and ca. 60% into 2. The different conversion efficiencies may be rationalised based on a study of *Cellulomoinas uda* cellobiose phosphorylase,[41] in which $k_{\text{cat}}/K_m$ values for 2F-Glc (2.4%), 3F-Glc (0.013%) and 6F-Glc (31%) acceptors are substantially lower than that of the parent Glc substrate, but all three compounds are indeed productive substrates. CBP was removed from the reaction mixture by affinity chromatography (His6-tag nickel column purification) and the desired products were purified by gel filtration chromatography. The purification successfully removed residual deoxy-fluoro-glucose acceptors, but small amounts of cellobiose required removal by HPLC to obtain compounds 1–3 (4–9 mg) in high purity for characterisation purposes (Figures S1–S3 in the Supporting Information). It is important to highlight that the monofluorinated cellobextrins 4–6 could be obtained in one-pot reactions from the respective sugar-1P and fluorinated glucose without HPLC purification.

Once Glc-1P consumption was almost complete in the CBP reactions, more Glc-1P (4 equiv.) was added together with CDP and the reactions were incubated at 37 °C shaking for 16 h. A white precipitate was formed and isolated by centrifugation, followed by resuspension and washing with Milli-Q water to remove enzyme, salts and any soluble sugars. A further
4 equiv. of Glc-1P were added to the supernatant, and the CDP reaction was further incubated to produce more fluorinated EpCs. In this manner, monofluorinated cellodextrins were obtained (ca. 40 mg) with reasonable overall yield based on consumed fluoro-glucose (47% (2F-EpC, 4), 30% (3F-EpC, 5), and 32% (6F-EpC, 6)). MALDI-TOF mass spectrometry analysis showed these materials to have an average DP of about 9, while the unsubstituted cellodextrin (EpC, 8) produced under the same reaction conditions averaged ca. DP 8 (Figure S7). Traces of longer fluorinated cellodextrins were evident in the mass spectrometry data, which may reflect greater water solubility of the monofluorinated materials, thus resulting in further enzymatic extension. Solution-state $^{19}$F NMR analysis in 1 M NaOD (Figure S4) showed two singlets for each material, reflecting reducing terminal anomers, with peaks at $\delta$ 195.21 and 195.26 ppm (2F-EpC, 4), $\delta$ 190.86 and 197.19 ppm (3F-EpC, 5) and $\delta$ 232.55 and 234.05 ppm (6F-EpC, 6).

**Synthesis of multiply 6-fluorinated cellodextrin (multi-6F-EpC, 7)**

We also investigated CDP-mediated oligomerisation using the chemically modified glucosyl donor 6F-Glc-1P (Figures S5 and S6) and cellobiose as acceptor (Figure 1C) to achieve higher structural impact by placing multiple fluorine atoms along the cellodextrin (multiply 6-fluorinated cellodextrin, multi-6F-EpC, 7). The initial tests using 6F-Glc as an acceptor to obtain a fully 6F-substituted cellodextrin proved very slow and inefficient. Alternatively, CBP was tested with 6F-Glc as an acceptor to generate a difluorinated cellobiose, which could be a better substrate for CDP. However, only trace amounts of the product were detected, prompting us to choose the natural acceptor cellobiose. CDP was incubated at 37°C with 6F-Glc-1P (200 mM) and cellobiose (30 mM) for 72 h. The resulting white precipitate was isolated by centrifugation, followed by re-suspension and washing with Milli-Q water to give 7 with 64% yield. $^{19}$F solution state NMR analysis of 7 dissolved in 1 M NaOD (Figure S4) showed one major singlet at $\delta$ 233.25 ppm, which may correspond to fluorine from the 6F-Glc internal repeating units, and three smaller singlets at $\delta$ 233.29, $\delta$ 233.31 and $\delta$ 233.35 ppm from 6F-Glc close to the reducing terminal and the non-reducing terminal units. Analysis by MALDI-TOF mass spectrometry revealed that multi-6F-EpC (17 mg) had a higher average DP (ca. 10) than the parent EpC (ca. DP 8) and that longer chains, up to DP 15, could also be observed in the multiply 6-fluorinated material (Figure S7). These data are comparable to the monofluorinated compounds and, more importantly, the presence of multiple fluorine atoms clearly had a higher impact on the DP of the cellodextrin products. The quantities of multiply 6-fluorinated material obtained in these proof of concept studies enabled us to carry on to detailed structural characterisation at different length scales; scale up of enzymatic syntheses to provide materials for bulk physical properties assessment will be reported in due course.

**Morphological characterisation**

**Electron microscopy (EM) and atomic force microscopy (AFM)**

Transmission electron microscopy (TEM) was initially used to observe the morphological differences between EpC and fluorinated EpCs, which were prepared for analysis only by dilution of concentrated suspensions obtained after purification of pre-
cipitates formed during enzymatic synthesis. As expected, the TEM images of the monofluorinated 2F-EpC (4), 3F-EpC (5) and 6F-EpC (6; Figure S8) show a very similar morphology to EpC (8, Figure 2A, a). This crystalline sheet-like morphology is well-known for enzymatically synthesised cello-oligosaccharides, including derivatised cellulose, such as acrylated cellulose and cellulose conjugated with oligo(ethylene glycol). On the other hand, multi-6F-EpC (7) particles formed predominantly into significantly shorter platelets (<100 nm length) (Figure 2B, a). These differences were further confirmed by AFM imaging using samples prepared by depositing diluted sample suspensions on freshly cleaved mica (Figure 2B, b and c). Although a few long platelets are present in multi-6F-EpC (7), their fraction is smaller than in EpC (8). As reported in the literature, the thickness of EpC (8) platelets was found to be ca. 5 nm. Similar thicknesses were observed for long platelets of multi-6F-EpC (7, Figure 2B, c).

Long-range structural characterisation by powder X-ray diffraction (PXRD)

The PXRD patterns of the monofluorinated 2F-EpC (4), 3F-EpC (5) and 6F-EpC (6) are virtually indistinguishable from the diffraction pattern of EpC (8; Figure 3). This result indicates that the monofunctionalised cellodextrin-like molecules arrange as a cellulose type II allomorph, with three intense and sharp peaks located at 2θ = 12°, 20° and 23° (d-spacings of 0.74, 0.44 and 0.39 nm, respectively) representing (110), (110) and (020) planes. On the other hand, the experimental PXRD pattern reported for multi-6F-EpC (7) does not correspond to any allomorph previously described for cellulose. The pattern shows two well defined peaks at 2θ = 15° and 23° (d-spacings of 0.59 and 0.39 nm, respectively), as well as four different broad components at 2θ = 21°, 25°, 30° and 36° (d-spacings of 0.42, 0.36, 0.30 and 0.25 nm, respectively).

In order to verify possible similarities with previously reported cellulose structural organisations, we predicted and compared the PXRD spectra of multi-6F-EpC (7) to each known allomorph (Figure S9 and Table S1). Remarkably, the observed peak positions of multi-6F-EpC (7) are unique when compared to the diffraction patterns of the known allomorphs (Figures 3 and Figure S9, Table S1), hence demonstrating the formation of a new crystalline structure for this new cellulose-like material.
Molecular-level characterisation

Raman spectroscopy

Figure 4 shows typical Raman spectra of 2F-EpC (4), 3F-EpC (5), 6F-EpC (6), multi-6F-EpC (7) and EpC (8). The bands located at ca. 1462 (HOC and HCH stretching), 1265 (HCC and HCO stretching) and 576 cm$^{-1}$ (heavy atom stretching) and the presence of fluorinated carbon groups (CH$_2$F and CH$_3$F). Each Raman spectrum represents the average of three Lorentzian-deconvoluted spectra upon noise removal.

In contrast, the multi-6F-EpC (7) spectrum is significantly different owing to the presence of multiple fluorine atoms. Multi-6-fluorination results in new Raman bands located at 480, 496 cm$^{-1}$ to 1462 cm$^{-1}$, as expected for a single fluorine atom (at the reducing end) per oligosaccharide chain. The weak band located at 487 cm$^{-1}$ for the monofluorinated EpCs is probably an amalgamation of the 480 and 496 cm$^{-1}$ bands as a result of the single fluorine present in each chain.

Solid-state nuclear magnetic resonance (SSNMR)

Direct polarisation $^{19}$F NMR experiments (without $^1$H decoupling) were carried out at 60 kHz MAS rate for the 2F-EpC (4), 3F-EpC (5), 6F-EpC (6) and multi-6F-EpC (7) (Figure 5A). A single very broad and asymmetric peak was observed for the monofluorinated materials, centred at ca. $-190$ (2F-EpC, 4), $-197$ (3F-EpC, 5) and $-232$ ppm (6F-EpC, 6), respectively, in good agreement with the solution $^{19}$F NMR data (Figure S4). 3F-EpC (5) and 6F-EpC (6) showed broad peaks, with line widths at half height of 11.9 and 9.4 kHz, respectively (Figure 5C), while multi-6F-EpC (7) showed a sharper (3.8 kHz width at half height) Lorentzian-shaped peak (Figure 5C). $^1$H-decoupled $^{19}$F ($^{19}$F/$^1$H) NMR spectra of 3F-EpC recorded at slower MAS rate showed an even broader $^{19}$F peak (Figure S10), indicating that fast MAS is more efficient at decoupling that radiofrequency decoupling (fast MAS decouples both $^{19}$F–$^1$F homonuclear dipolar coupling as well as heteronuclear $^1$H–$^{19}$F coupling), and ii) the large $^1$F line widths of 2F-EpC (4), 3F-EpC (5), 6F-EpC (6) and multi-6F-EpC (7) in the fast MAS spectra (Figure 5A) are mostly due to the large heterogeneity of $^{19}$F chemical environments. This can be easily understood considering that these materials assemble into particles with a specific crystalline packing (cellulose type II$^{\text{a}}$ for 2F-EpC (4), 3F-EpC (5) and 6F-EpC (6)), and a new organisation for multi-6F-EpC (7); Figures 5D and 6A), and the $^{19}$F nucleus is extremely sensitive to chemical environment. Upon assembly of nanocellulose the $^{19}$F atoms of each cellulose chain can occupy any position within the nanofibril (surface, core, far from or nearby other fluorinated residues, etc.), hence presenting non-equivalent environments within the packing of EpC (Figure 5C). Assuming that $^{19}$F–$^1$H dipolar interactions are reduced considerably at fast MAS, the peak broadening reflects a multitude of orientations sampled by the C–F bonds.

To characterise the structural organisation of 2F-EpC (4), 3F-EpC (5), 6F-EpC (6) and multi-6F-EpC (7) materials at the molecular level, $^1$H$^{13}$C CP/MAS experiments were carried out. Each type of cellulose allomorph presents a characteristic $^{13}$C NMR fingerprint.$^{[53,54]}$ Monofluorinated EpCs (4, 5 and 6) 10 wt% dispersions showed the characteristic cellulose I $^1$H$^{13}$C CP fingerprint, typical of non-modified EpC (8) (Figure 5D). The only noticeable difference was the presence of a broad peak at about 61 ppm, which is characteristic of a surface/disordered population of C6 (sC6, Figure 5D).$^{[54,55]}$ Hence, the peak at 63 ppm represents the interior/ordered domains of C6 (iC6, Figure 5D). The sC6 broad peak is typically observed in bacterial cellulose (BC), which consists of cellulose particles containing both $i_C$ and $s_C$ crystalline domains and disordered regions.$^{[55]}$ Indeed, surface/disordered and interior/ordered do-
mains are typically found in nanocrystalline cellulose, bacterial cellulose and plant cell walls. We note that the presence of a fluorine atom substituting the 3-hydroxy group of glucose might affect the formation of the characteristic O3-O6 intra-chain hydrogen bond between adjacent glucose residues in cellulose II allomorph (Figure 5B). On the other hand, the formation of the O2-O2 and O6-O6 inter-sheet hydrogen bonds would be affected in 2F- and 6F-EpC, respectively (Figure 5B). Spectral deconvolution of the C6 and iC6 peaks of the 1H–13C CP/MAS NMR spectra of multi-6F-EpC (7) showed a pattern of peaks that does not correspond to either cellulose I, II or III allomorphs (Tables S4 and S5). This is evidenced by the appearance of C1 (105.9 ppm) as asinglet peak in 7, which is a singlet in cellulose type Iα and IIα and a doublet in cellulose type Iγ, IIα and IIIα (Table S4). The small shoulder observed at about 107 ppm cannot be assigned unambiguously to a specific structural feature. The different multiplicity of the peak corresponding to C1 indicates the presence of only one non-equivalent anomeric carbon per unit cell in multi-6F-EpC (Figures 6A and S12). This is different from unmodified EpC, which shows a doublet for C1 (Figure 5D) due to the presence of two non-equivalent anomeric carbons per unit cell. Also, the pattern of 13C chemical environments in multi-6F-EpC does not fully match any of the cellulose structures reported so far (Table S4). Hence, our solid-state NMR data demonstrate that the inter-chain interactions in multi-6F-

Figure 5. A) Chemical structures of unmodified EpC (8), monofluorinated 2F- (4), 3F- (5) and 6F-EpC (6), and multi-6F-EpC (7). B) 3D model of the crystalline packing of the cellulose II allomorph based on the origin-centre-origin (o-c-o) chains. The O3–O6 intra-chain (blue dashes) and O2–O2 and O6–O6 inter-sheet (yellow dashes) hydrogen bonds are shown. It should be noted that the substitution of all -OH groups at C6 with fluorine atoms precludes the formation of O6–O6 inter-sheet hydrogen bonds during self-assembly. Note: the intra-chain hydrogen bonds are only shown for the centre chain for simplicity. C) Direct detection 19F MAS NMR spectra of multi-6F-EpC (7, blue) and 2F- (4, red), 3F- (5, green) and 6F-EpC (6, orange) powders, acquired at 60 kHz MAS rate and 800 MHz 19F frequency (20 T magnetic field). D) 1H–13C CP/MAS NMR spectra of EpC powder (8, black) acquired at 10 kHz MAS rate, and 2F- (4, red), 3F- (5, green) and 6F-EpC (6, orange) 10 wt% dispersions acquired at 6 kHz MAS, and 100 MHz 13C frequency.
EpC are different from non-modified EpC or any other cellulose-like structure.

Importantly, the PXRD pattern of multi-6F-EpC (7; Figure 3) does not correspond to any cellulose allomorphs reported so far (Table S1). Hence, 7 assembles into a crystalline organisation which is unprecedented for a cellulose-type material. The formation of this novel structural motif is also supported by the new features observed in the Raman spectra, which do not correspond to either cellulose I or II (Figure 3, Table S4).

The combination of fast MAS $^{13}$C CP, low MAS $^{19}$F-decoupled $^{13}$F-$^{13}$C CP, water-polarisation transfer (WPT) solid-state NMR and $^{13}$C, COSY and HSQC solution NMR experiments enabled the assignment of the $^{13}$C spectrum of multi-6F-EpC (7; Figure 6) to be made. $^{13}$F-decoupled $^{19}$F-$^{13}$C CP experiments enabled the assignment of C6, C4 and C5 peaks of the fluorinated residues (Figure 6A). The highest intensity peak was assigned to C6 (83.8 ppm), as it is the carbon atom closest to 6F (1.3 Å). The peak at 73.1 ppm corresponds to C4 and C5 sites, based on their proximity to fluorine (Figures 6A and S12), while C2 and C3 are too far away to cross-polarise from fluorine effectively. $^{13}$C DEPT135, COSY and HSQC solution NMR experiments confirmed this assignment (Figure S13), with the methylene carbons of the fluorinated (C6) and non-fluorinated (C6*) glucose units appearing in antiphase with respect to the CH carbons (Figure 6A). Importantly, the $^{13}$C peaks at 81.9 and 73.1 ppm observed in the CP spectrum did not appear on the $^{13}$C DEPT135 or $^{13}$H-$^{13}$C HSQC solution NMR experiments carried out for a diluted dispersion of multi-6F-EpC (7). Hence, these peaks most likely correspond to the imobile interior carbons (C6* and C2,3,4,5, respectively) that are too broad to be detectable by solution NMR. The solution-NMR-observed C6 and C2,3,4,5 peaks were therefore assigned to surface/disordered domains (sC6 and sC2,3,4,5, respectively). The assignment of sC6 and iC6 was further validated by water polarisation transfer CP (WPT-CP) NMR experiments (Figure 6B).[57] The peak intensity in WPT-CP experiments depends on the distance and relative mobility of bound water at the particle surface and the number of interacting water molecules at a particular site. Hence, peaks corresponding to surface domains will show faster WPT growth at short mixing times than interior domains, as we have recently observed for BC.[55] At sufficiently long mixing times, WPT become homogeneous for both surface and interior domains due to the efficient spin diffusion. Figure 6B shows the WPT factors for a 25 wt% dispersion of multi-6F-EpC (7) at 16 ms mixing time (under our experimental conditions, homogenisation of surface-interior water polarisation transfer is achieved around 200 ms). A much higher WPT factor was observed for the sC6 (83.8 ppm) compared to the iC6 peak (81.8 ppm), confirming the assignment of sC6 and iC6 peaks to surface and interior domains, respectively (Figure 6B).

Also, sC2,3,4,5 showed higher WPT compared to iC2,3,4,5 (Figure 6B), in agreement with solution NMR data where the sC2,3,4,5 and iC2,3,4,5 peaks are visible and invisible, respectively (Figure 6A). Spectral deconvolution of sC6 and iC6 peaks of the $^{13}$H-$^{13}$C CP spectrum acquired at 60 kHz, indicated that multi-6F-EpC (7) presents an RSA of ca. 54% (Figure S11, Table S3). Although this experiment was not fully quantitative, the RSA determined for 7 is similar to what we have reported before for nanocrystalline cellulose.[58]

To summarise, we have demonstrated that the presence of multiple 6-deoxy-6-fluoro glucose residues precludes the formation of the cellulose type II crystallinity and hydrogen bond patterns that defines EpC.[59] To understand this at the molecular level, we should note that two different types of chains (centre, c, and origin, o) leading to three different types of hydrogen bond patterns (o-o-o, c-c-c and o-c-o) define the interchain interactions of the Ep cellulose II structure. In particular, the O2-H···O6, O6-H···O2, O6-H···O6 and O2-H···O2 interactions are characteristic of cellulose II packing (Figures S5 and S15).

Figure 6. A) $^{13}$C DEPT135 (blue) and $^{19}$F-decoupled $^{13}$F-$^{13}$C CP (purple) NMR spectra of multi-6F-EpC (7) powder acquired at 60 and 15 kHz MAS spinning, respectively, and 212.5 MHz $^{13}$C frequency. The $^{13}$C DEPT135 spectrum of a 1 wt% dispersion of multi-6F-EpC (7) in D$_2$O (orange) is shown for comparison. *Low-intensity peaks corresponding to the non-fluorinated glucose units 7 at the reducing terminal of each cellobextrin chain. B) Bar graph showing the number of interacting water molecules at a particular site. Hence, peaks corresponding to surface domains will show faster WPT growth at short mixing times than interior domains, as we have recently observed for BC.[55] At sufficiently long mixing times, WPT become homogeneous for both surface and interior domains due to the efficient spin diffusion. Figure 6B shows the WPT factors for a 25 wt% dispersion of multi-6F-EpC (7) at 16 ms mixing time (under our experimental conditions, homogenisation of surface-interior water polarisation transfer is achieved around 200 ms). A much higher WPT factor was observed for the sC6 (83.8 ppm) compared to the iC6 peak (81.8 ppm), confirming the assignment of sC6 and iC6 peaks to surface and interior domains, respectively (Figure 6B). Also, sC2,3,4,5 showed higher WPT compared to iC2,3,4,5 (Figure 6B), in agreement with solution NMR data where the sC2,3,4,5 and iC2,3,4,5 peaks are visible and invisible, respectively (Figure 6A). Spectral deconvolution of sC6 and iC6 peaks of the $^{13}$H-$^{13}$C CP spectrum acquired at 60 kHz, indicated that multi-6F-EpC (7) presents an RSA of ca. 54% (Figure S11, Table S3). Although this experiment was not fully quantitative, the RSA determined for 7 is similar to what we have reported before for nanocrystalline cellulose.[58]
Conclusions

In this study, we have demonstrated the enzymatic incorporation of singly and multiply fluorinated glucose residues into cellodextrin chains. The OH-to-F substitution is tolerated by the cellodextrin phosphorylase, albeit with low efficiency. Nonetheless, we were able to produce selectively fluorinated cellodextrins, averaging about DP 9 in size, that self-assemble into crystalline materials. Singly fluorinated cellodextrins display structural features reminiscent of cellulose II, as judged by solid-state NMR powder X-ray diffraction and Raman spectroscopy. In contrast, multiply 6-fluorinated cellodextrin gave rise to a new allomorph, not previously reported for either native celluloses or cellulose-like materials. Advanced solid-state NMR methods have enabled the detailed characterisation of these novel materials, deciphering the water-exposed and interior chemical environments for different carbon sites. Our findings highlight the considerable potential of chemoenzymatic synthesis for generating novel glycomaterials of controlled molecular structure and morphology.

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Conflict of Interests

The authors declare no conflict of interests.

Keywords: allomorphs • cellodextrins • chemoenzymatic synthesis • fluorine • soft-matter materials

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