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Effects of folic acid esterification on the hierarchical structure of amylopectin corn starch

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Abstract

There are burgeoning research interests in designing biocompatible colloidal delivery systems for treating as well as delaying the recurrence of chronic diseases, including various forms of cancers. In this respect, folic acid (FA) esters and starch are particularly interesting owing to (i) the molecular recognition of FA by folate receptors and (ii) the biocompatibility of starch based delivery systems. In this study, the effects of esterification of amylopectin corn starch (ACS) with FA using an n, n'-dicyclohexylcarbodiimide/4-dimethylaminopyridine (DCC/DMAP) mediated esterification reaction were investigated at multiple length scales. Scattering (light, X-ray), spectroscopy (FTIR), electrophoretic mobility (ζ-potential) and confocal laser scanning microscopy (CLSM) confirmed that structural rearrangements (short- and long-range) occurred in the starch-folic acid ester (SF) derivatives with increased FA content (degree of substitution, 0.01-0.05). The SF ranged in size from 200 to 600 nm and were negatively charged (ca. -24 mV, SF20). FTIR revealed a loss of double-helical structure on FA substitution. Notably, CLSM and small angle X-ray scattering (SAXS) both showing an FA-assisted self-assembly and crosslinking of SF, later confirming columnar assemblies with unit cell parameter of 4.5 nm. The wide-angle X-ray scattering (WAXS) and X-ray diffraction (XRD) pattern (2θ = 6.1º, 7.7º , 13°, 17°, 20°, 22°, and 25°) in SF further gave evidence for the formation of hybrid B and V-type polymorphs, where SF may accommodate FA within a larger hybrid hexagonal lattice. This study provides structural insights for developing tunable starch-folic acid derivatives for potential applications as delivery vehicles for pharmaceuticals and nutraceuticals targeting folate receptors.

Keywords: Starch, folic acid, multiscale structural analysis, self-assembly, SAXS.

1. Introduction

There is a continuing scientific and industrial interest in designing biocompatible colloidal delivery systems for delaying the onset as well as treatment of chronic diseases, including various forms of cancers. Starch, which is the second most abundant hydrocolloid, has been recently explored for the preparation of relatively inexpensive biocompatible delivery vehicles applying physical and chemical treatments (Ahmad, Akhter, Anwar, & Ahmad, 2012; Kim, Seo, & Lim, 2013; Li, Shin, Lee, Chen, & Park, 2016; Shalviri, et al., 2012). These nano- or sub-micron-sized modified starch-based delivery systems are promising for nutraceutical and pharmaceutical applications owing to their large surface area-to-volume ratio, but generally suffer from lack of cellular specificity and molecular recognition. The molecular recognition of these nanoparticles can be greatly improved by attachment of high-affinity targeting ligand molecules. Folic acid (an oxidized form of folate), a naturally water-soluble vitamin, is such a widely explored targeting ligand molecule. Due to its high binding affinity ($K_d \sim 10^{-10}$ M) along with its specific binding properties to folate receptors in the human cells, it improves the targeting properties to the cancer cells of breast, lung, kidney, colon and brain, that are known to overexpress folate receptors by 100-300 times as compared to that of non-cancerous cells (Antony, 1996; Kamen & Capdevila, 1986). Thus, there has been significant research efforts to esterify folic acid to modify starch via a wide variety of chemical synthesis routes.

Folate esterified to polyethylene glycol (PEG) using n, n'-dicyclohexylcarbodiimide (DCC) and n-hydroxysuccinimide (NHS)-mediated esterification was conjugated to the surface of modified starch nanoparticles, latter designed via a water-in-oil microemulsion templating by Xiao, et al. (2006). An increase in particle size of the starch nanoparticles was specifically observed upon folic

acid esterification (from 50 to ~130 nm) with a folic acid content of 0.8 μg/mg of PEG-Starch nanoparticle. In another instance, folic acid was esterified to hydrophobized pullulan, an exopolysaccharide derived from starch, using DCC and 4-dimethylaminopyridine (DMAP) mediated chemistry to produce nanoparticles (Zhang, et al., 2010). Folic acid esterification resulted in increasing the hydrodynamic diameter of the pullulan acetate nanoparticles from ca. 185 nm to 261 nm. Such increase was attributed to the enhanced swelling of the folate-pullulan esters in aqueous dispersion, which was driven by the hydrophilic nature of the folic acid. Folic acid conjugated to hydroxyethyl starch nanocapsules via n-(3-dimethylaminopropyl)-n′-ethylcarbodiimide hydrochloride (EDC)-mediated esterification (Baier, et al., 2012) also showed a similar behaviour of increasing the particle size of starch from 275 nm to 307 nm.

On the other hand, no significant changes in the hydrodynamic diameter of aminated starch was observed by Saikia, Das, Ramteke, and Maji (2017), when folic acid was esterified to aminated starch/ZnO coated iron oxide nanoparticles using an NHS/EDC mediated esterification reaction. It appears from the aforementioned studies that esterification of folic acid resulted in modification of starch nanoparticles at colloidal scale; however, rare attention has been given in literature to understand mechanistically, if such esterification has resulted in any structural rearrangements in starches.

Starch consists of two polymeric units, namely linear amylose composed entirely of D-glucose units joined by α-1,4-glycosidic linkages, and, extensively branched amylopectin composed of glucose units linked primarily by α-1,4-glycosidic bonds with occasional α-1,6-linkages forming the branching points (Zobel, 1988). The amylopectin and amylose polymers (glucose extension ~0.1 nm) are arranged as

alternating lamellae (~10 nm) of rigid mesogen units (liquid crystalline) and flexible spacer units (amorphous). The crystalline regions consist of double helices of amylopectin in ordered arrays. Additionally, single, left-handed helix are also observed. These types may (Tan, Flanagan, Halley, Whittaker, & Gidley, 2007) or may not (Borah, Deka, & Duary, 2017) include copolymers within the helical channel.

On the other hand, folic acid although hydrophilic in nature, has a tendency to self-assemble into tetramer structures even at concentrations as low as 0.1% (w/w) via hydrogen bonding and stacking interactions, which further arrange into ordered mesophases (Bonazzi, DeMorais, Gottarelli, Mariani, & Spada, 1993; Ciuchi, et al., 1994). Additionally, Kamikawa, Nishii, and Kato (2004) reported the formation of non-symmetric supramolecular assemblies in folic acid derivatives, which were synthesized using EDC/DMAP mediated esterification. The self-assembled columns of the folic acid derivatives were thought to be formed via the secondary cooperative interactions, involving hydrogen bonding, ion dipolar interactions, stacking interactions, and segregation into nanophases of molecular block structures. Hence, it is plausible that during esterification with folic acid, starch may undergo a folic acid assisted structural reorientation.

Since such structural rearrangements might result in changes of the properties of the delivery system and its release kinetics, it is vital to gain fundamental understanding of the multiscale structure of starch on esterification with folic acid, which has not been reported in literature until now. Such crucial insights will enable the optimisation of future design and fabrication of folic-acid-functionalized, colloidal starch delivery vehicles tailored for targeted drug and nutraceutical delivery applications.

In this study, we have designed different starch-folic acid esters focusing mainly on the structural rearrangements of the amylopectin corn starches mediated by esterification with folic acid. Amylopectin corn starch was utilized as the starch model, since it is devoid of amylose and thus was expected to provide distinct peaks for the lamellar phases in X-ray scattering studies. We hypothesize that controlling the degree of folic acid esterification will profoundly alter the hierarchical structure of starch particles, including colloidal properties (size, charge) and its molecular properties (lamellar structure and crystalline structure). A combination of complementary techniques of dynamic light scattering (DLS), small-angle and wide-angle X-ray scattering (SWAXS), X-ray diffraction (XRD), Fourier transform infrared (FT-IR) spectroscopy, electrophoretic mobility and confocal laser scanning microscopy (CLSM) were assessed to understand the effect of folic acid esterification on the structure of starch. To the best of our knowledge, this is the first study that systematically characterizes the structural rearrangements of starch on multiple length scale. This is the first in a series of papers by the present authors on the structure-function relationship of folic acid-starch esters and its overall implications towards designing biocompatible colloidal vehicles for delivery of pharmaceuticals and nutraceuticals targeted at cancer cells.

2. Materials and methods

2.1. Materials

Amylopectin corn starch (ACS) was obtained from Sigma-Aldrich Company Ltd., Dorset, UK. The ACS contained no amylose as assessed using colorimetric procedure (Morrison & Laignelet, 1983), which was in agreement with the supplier's specification. Folic acid (FA), n, n'-dicyclohexylcarbodiimide (DCC), 4-

dimethylaminopyridine (DMAP), dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich Company Ltd., Dorset, UK. Milli-Q water purified using a Milli-Q apparatus (Millipore Corp., Bedford, MA, USA) was used throughout the experiments. All other chemicals were of analytical grade unless otherwise stated.

2.2. Preparation of starch-folic acid esters

Starch folic-acid (SF) ester derivatives were synthesized using an esterification reaction between the carboxyl group of folic acid (FA) and the hydroxyl group of starch (ACS) as described previously for synthesis of stearate-grafted dextran (Du, Weng, Yuan, & Hu, 2010), with some modification. The "zero length" crosslinker, n, n'-dicyclohexylcarbodiimide (DCC) served as the coupling reagent, and, 4-dimethylaminopyridine (DMAP) was the reaction catalyzer. The SF esters with the different degree of substitutions of FA were synthesized by controlling the feed ratios of FA to starch.

Briefly, 1g FA was dissolved in 30 mL anhydrous DMSO, and, DCC, DMAP were added in the FA:DCC:DMAP molar ratio of 1:1:0.3. Activation of the FA carboxylic groups was achieved by stirring the solution for 30 min at 30 ºC while maintaining dark conditions. Following this, starch was added in various concentrations to the FA solution (5-30 wt% of FA to starch dry weight) and was reacted in the dark at 30 ºC for the next 24 h. The DMAP was removed by washing the reaction products first with 1N HCl and then with Milli-Q water using a Whatman No. 4 filter paper. The exposure time to 1N HCl was < 5 min to avoid any degradation of the starch polymer. The reaction product was then dialyzed (3.5 kDa MWCO) against 10 mM phosphate buffer at pH 7.4 containing 0.10 M NaCl for 24 hours, and, then with water for another 24 h to remove any unbounded FA and DCC. The samples were then lyophilized for 48

h, ground to a fine powder using mortar and pestle, and the SF ester derivatives (SF5, SF10, SF20, and SF30) were obtained. Control samples included the native amylopectin corn starch, ACS; ACS treated with DMSO, S/DMSO; ACS reacted with DCC and DMAP in DMSO but without FA substitution, S/DCC.

2.3. Characterization of the Starch-Folic acid (SF) ester derivatives

2.3.1. Degree of substitution and folic acid content

The amount of FA esterified to ACS was determined spectrophotometrically. Briefly, SF (4–10 mg) were dissolved in 0.5 mL DMSO. 0.5 mL of acetic acid solution (60 vol%) and 9.0 mL of water/sulfuric acid (1.3:1, vol%) was added to the solution. The sample solution was stirred, heated at 70 ºC for 30 min, and then cooled to room temperature. The UV absorbance of the solution was measured at 380 nm against a blank. Absorbance calibration curves were plotted against known FA concentrations. The degree of substitution was defined as the number of FA per glucose residues of starch and calculated as,

$$Degree\ of\ substitution\ = [\frac{c/M_{FA}}{(m-c)/M_{starch}}] \quad \text{Eq. (1)}$$

where c is the content of the FA determined from the corresponding calibration curve, m is the amount of the starch used in the experiment; M_{FA} is the molecular weight of the FA; M_{starch} is the molecular weight of anhydrous glucose unit of starch.

2.3.2. Mean hydrodynamic diameter and ζ-potential

The mean hydrodynamic diameter (D_h) and ζ-potential of the samples were measured on a Zetasizer (Nano ZS series, Malvern Instruments, Worcestershire, UK) equipped with a 4-mW helium/neon laser at a wavelength output of 633 nm. 0.1 mg

199 mL^{-1} of the sample in DMSO:water (1:10, vol %) was prepared, and all measurements
200 were made at 25 °C.
201
202 **2.3.3. Fourier transform infrared spectroscopy**
203 The FTIR spectra (4000 to 400 cm^{-1}; 64 scans were averaged with a resolution
204 of 2 cm^{-1}) of samples were collected using a Bruker ATR-FTIR Spectrometer (Bruker
205 Optics GmbH, Ettlingen, Germany). Spectra (1200–800 cm^{-1}) were baseline corrected
206 using anchor points at 1200 and 800 cm^{-1}, and then interpolated. The peaks at 947, 995,
207 1022 and 1047 cm^{-1} were selected and Lorentz peak fitting was performed using
208 OriginPro 8.0 (OriginLab Corp, Northampton, USA).
209
210 **2.3.4. Confocal laser scanning microscopy**
211 Confocal images were obtained on a Zeiss inverted LSM880 confocal
212 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) using an argon laser at
213 an excitation wavelength of 688 nm and 40×/1.25 oil objective. Approximately, 2 mg
214 of sample was dispersed in Milli-Q water before imaging. Anionic FA groups were
215 labeled using methylene blue dye (Zhang, et al., 2011).
216
217 **2.3.5. Small and wide-angle X-ray scattering, and, X-ray diffraction**
218 The small and wide-angle X-ray scattering (SWAXS) beamline (SAXSpace,
219 Anton Paar, Austria) setup used in this study has been described elsewhere (Adal, et
220 al., 2017; Patil-Sen, Sadeghpour, Rappolt, & Kulkarni, 2016). Samples were loaded
221 onto a 1.5 mm quartz capillary, hydrated with water, sealed using paraffin wax, and
222 then placed in a vacuum stage at 25 ± 0.1 °C for measurements. Silver behenate with a
223 known lattice spacing of 5.84 nm was used to calibrate the scattering vector q as,

$$q = \frac{4\pi}{\lambda} sin\theta \quad \text{Eq. (2)}$$

where λ = 0.154 nm and 2θ is the scattering angle. The scattering background from the capillaries (with and without water) was subtracted after normalizing for sample transmission, and then deconvoluted (slit length de-smearing). The parameters of the lamellar structure from SAXS (0.1 $nm^{-1} < q < 2.5\ nm^{-1}$) were obtained by least square fitting employing the Levenberg-Marquardt optimisation algorithm to a Cauchy-Lorentz-Power Law equation (Yuryev, et al., 2004) as,

$$I(q) = I_{max}\left[1 + \left(\frac{2(q-q_{\max})}{\Delta q}\right)^2\right]^{-1} + Aq^{-\delta} \quad \text{Eq. (3)}$$

where I_{max}, q_{max}, Δq (FWHM), A and δ are positive adjustable parameters.

The half width at half maximum (HWHM), $\Delta q/2$, in reciprocal space, was converted to real space to calculate the average lamellar thickness variations as,

$$HWHM\ (real\ space) = \frac{\pi \Delta q}{{q_{max}}^2 - (\Delta q/2)^2} \quad \text{Eq. (4)}$$

The scattering in the wide-angle X-ray (WAXS) data ($2.5\ nm^{-1} < q < 14.5\ nm^{1}$) was background subtracted and then smoothed applying a spline function which minimized,

$$p \sum_i w_i (y_i - s(x_i))^2 + (1-p) \int \left(\frac{d^2 s}{dx^2}\right)^2 dx \quad \text{Eq. (5)}$$

where a smoothing parameter p was applied on all the scattering patterns. Note, for the WAXS regime no desmearing procedure was applied, since its effect is small at wide angles and only amplifies the signal's noise.

Powder X-ray diffraction (XRD) data (2θ = 10–30°) of the samples ACS and SF20 were recorded at room temperature (ca. 25 °C) on a D8 Focus X-ray

diffractometer (Bruker AXS, Germany) using Cu Kα (λ = 0.154 nm) as the incident X-ray source. In one instance, SF20 was heated to 130 °C at the rate of 2 °C/ min, and then cooled to room temperature at the same rate (sample was re-indexed as $SF20_{heated}$) before recording the XRD as described by Vermeylen, et al. (2006), with some modification. Spectra are reported as $q = (4\pi/\lambda) \cdot sin\ (2\theta/2)$ to complement SWAXS. The spectra were baseline corrected for representation using OriginPro 8.0 (OriginLab Corp, Northampton, USA).

2.4. Statistical analysis

The SWAXS fitting was performed in MATLAB R2016a (version 9.0, The MathWorks, Inc., Natick, MA, USA). Analysis of variance (ANOVA) and Tukey's HSD post hoc analyses were conducted using SPSS 8.0 (SPSS, Inc., Chicago, IL, USA). Mean values were considered significantly different at $p<0.05$.

3. Results and discussion

Fig. 1 shows the degree of substitution of ACS using FA. The ratio of FA to ACS from 5 to 30 wt% markedly increased the degree of substitution of ACS from 0.01 to 0.05, with FA content ranging from 3.84 ± 1.65 % to 12.45 ± 3.42 % ($p<0.05$), respectively. It is noteworthy that the increase in the degree of substitution was observed to slow down beyond SF20, where it seems that the steric zone formed by the addition of excessive levels of FA deterred further conjugation. In the next section, we have focused on the effects of different FA/starch ratios on the multiscale structure of SF using a range of complementary techniques.

3.1. Effect of FA esterification on mean hydrodynamic diameter and ζ-potential

The mean hydrodynamic diameter (D_h) of the samples is shown in Fig. 2. The D_h of the ACS granules (5488.16 ± 2198.47 nm) should be considered with precaution as the polydispersity index was ≥ 0.4. In comparison, the D_h of the control samples, i.e. S/DMSO (600.44 ± 135.98 nm) and S/DCC (999.15 ± 160 nm) were significantly lower *($p<0.05$)* (Fig. 2). It appears that the treatment of ACS with DMSO resulted in the dissolution of the supramolecular ACS, thereby dramatically reducing the size by almost an order of magnitude. It is quite tempting to state that blocklets were generated after disruption of the lamellar arrangement in ACS granules, as D_h of S/DMSO was in close agreement with the size ranges of the spheroid type blocklets (20-500 nm) reported previously in literature (Pérez & Bertoft, 2010; Tang, Mitsunaga, & Kawamura, 2006). However, an event of the disruption of ACS granules into the single blocklets is highly unlikely during dissolution in DMSO. The DMSO is a hydrogen bond acceptor and results in the complete disruption of intra- and inter-molecular hydrogen bonding in starch, which might lead to lamellar melting. As such, the remnants of granule disruption were possibly clusters of amylopectin.

The FA substitution was seen to systematically reduce the mean hydrodynamic diameter (D_h) from 596.01 ± 112.17 nm (SF5) to 204.23 ± 3.16 nm (SF30) (Fig. 2). This was also reflected in the corresponding ζ-potential values of the samples (Fig. 2). The ζ-potential value of ACS (-3.95 ± 0.32 mV), significantly *($p<0.05$)* decreased upon esterification with FA reaching -24.50 ± 6.41 mV for SF20. Hence, it appears that the gradual binding of anionic FA molecules, to ACS, was responsible for the net negative charge acquisition in the SF samples (SF5-SF30) at pH 7.4 (Fig. 2). It is worth noting that despite such high degree of substitution, starch esters did not achieve the magnitude of the negative charge of native FA molecules (ζ-potential value of FA molecule = -

295 34.36 ± 1.75 mV, data not shown). This suggests that, despite the binding of
296 considerable quantities of FA molecules to starch during formation of SF, the FA-
297 induced coverage of SF might not have been complete.
298
299 **3.2. Microstructural analysis**
300 Fig. 3 shows the CLSM images of SF samples with different degree of
301 substitution. The ACS and SF5 did not show any fluorescence (S/DMSO, S/DCC
302 provided in Fig. S1 in Supplementary Information). However, clear methylene blue-
303 induced fluorescence was observed in the SF samples (SF10-SF30), once the FA
304 content was increased. Methylene blue is a cationic dye and has a higher affinity
305 towards anionic molecules (Zhang, et al., 2011). In our case, the dye was thus well
306 adapted to interact with the anionic FA-bound domains of SF. In addition, FA groups
307 appeared to be distributed throughout the SF samples. Since DMSO allowed for the
308 dissolution of the supramolecular starch (as discussed in section 3.1), FA molecules
309 could react with the entire sub-structural moieties of starch. It was interesting to observe
310 certain small patches of fluorescence-dense regions in the SF samples (SF10-SF30,
311 indicated by arrow) (Fig. 3). These fluorescence-dense regions might be the typical
312 signature of columnar assemblies, latter formed via hydrogen bonding in intra-folic acid
313 derivatives (Bonazzi, et al., 1993). The appearance of these fluorescence-dense regions
314 are further discussed in section 3.4 dealing with X-scattering.
315
316 **3.3. FTIR and Short range molecular order**
317 Fig. 4 shows the FTIR spectra of FA, ACS, and SF20. The characteristic peaks
318 around 3322, 2927, and 2849 cm^{-1} can be attributed to the hydroxyl (O-H) stretching
319 vibrations of the glutamic acid moiety and NH–group of pterin ring, respectively. An

increase was observed in the peak at 860 cm^{-1} representing the C−H, CH_2 deformation, the carbonyl group (C=O stretching) at 1695 cm^{-1}, and C-O stretching at 1149 cm^{-1}. These indicate increased vibrations in the esters group, suggestive of esterification via the glutamate moiety of FA. The glutamate moiety of FA houses two carboxylic acid groups that in theory should be able to esterify, yielding the α- or γ- activated derivative (α- and γ- carboxyl groups of FA are shown in Fig. S2 in Supplementary Information). It has been reported that the γ-activated carboxyl group is more accessible in FA (Eisele, et al., 2010; Singh, Gupta, Asthana, & Jain, 2008), therefore, the esterification reaction might have occurred between the γ-carboxyl group of FA and the hydroxyl group of starch.

The FTIR peaks in the range of 1200-800 cm^{-1} are considered as the fingerprint region for polymer conformations and hydration of starches (van Soest, Tournois, de Wit, & Vliegenthart, 1995). The peaks at 1022 cm^{-1} and 1047 cm^{-1} represent the amorphous and the ordered structures (crystallinity) of starch, whereas, the peak at 995 cm^{-1} is related to the hydrated crystalline samples (Bello-Pérez, Ottenhof, Agama-Acevedo, & Farhat, 2005; Htoon, et al., 2009; van Soest, et al., 1995). Therefore, the ratios of absorbance of 1047/1022 cm^{-1} and 1022/995 cm^{-1} were calculated (peak fitting is shown in Fig. S3 in Supplementary Information) and the former seemed to decrease and the later seemed to increase (Fig. 5), as the degree of substitution increased. This was suggestive of a loss in crystallinity and double helical molecular order. S/DMSO, S/DCC provided in Fig. S4 in Supplementary Information. Additionally, the peak at 947 cm^{-1} was more pronounced in S/DMSO, S/DCC and SF esters as compared to that in ACS (Fig. S5 in Supplementary Information). This peak represents V-type helices. As it might be expected, the introduction of FA made the peak more distinct. Such V-type polymorphism relates to the left-handed single helix formation in the presence of

low molecular weight molecules and even solvents (DMSO in our case). The V-type helices were seen to increase with an increase in the degree of substitution of FA.

3.4. SAXS analysis on the mesoscopic structure of starch

To gain further insights into the starch structure on the nanometre scale, SAXS analyses were carried out to investigate the lamellar structure and the corresponding quasi-long-range order within the samples. We concentrated on ACS, S/DMSO, S/DCC and SF20 esters as representatives. Fig. 6 shows the solution scattering behaviour of the samples, and evidence the presence of a characteristic peak positioned at ca. 0.7 nm^{-1} in ACS (Fig. 6a), widely accepted to originate from the stacking order in the semi-crystalline regions of starch granules, which is given by a regular lamellar repetition of crystalline and amorphous regions in the radial direction of the granules. All data have been fitted by applying Eq. 3 with q_{max} determining this lamellar repeat distance d = 9.10 nm, for ACS. For S/DMSO (Fig. 6b), the stacking distance increased slightly (d = 9.66 nm; Table 1), and, the exponentially decaying diffuse scattering contribution (second term in Eq. 3) was strongly increased (note, identical exposure times were applied). On the other hand, the scattering peak arising from the semi-crystalline regions was drastically reduced in its intensity (about four-fold). This means we can estimate that roughly ¾ of the semi-crystalline volume has been impaired by DMSO. It is noteworthy to mention that the remaining intact semi-crystalline regions displayed a 6% looser stacking density (cp. d-spacings; Table 1), but no significant loss in local stacking disorder was observed (cp. HWHM; Table 1).

It is worth noting though, that the crystalline region was not eliminated by DMSO, but only after esterification with DCC. Transformation of the hydroxyl groups of amylopectin by DCC could have resulted in the depreciation of the hydrogen bonds

amidst starch resulting in total structural disassembly. This was observed in the total absence of the characteristic ca. 9 nm peak in S/DCC and SF20 ester (Fig. 6c,d), indicating that the lamellar stacking was destroyed during the esterification process with the corresponding decrease in the long-range order of amylopectin.

For SF20 (Fig. 6d), a new peak at ca. 1.60 nm^{-1} was observed, which corresponds to a characteristic repeat distance of 3.92 nm. While one diffraction peak alone is not sufficient to identify any lattice type, in any case, its appearance indicates a novel process of reassembly. Based on previous literature, it is tempting to assume that this new molecular organization is mainly caused by the self-assembly of FA tetramers via hydrogen bonds stacking into rod-like piles, which in turn fill the space in a closed packed hexagonal fashion (Bonazzi, et al., 1993). These self-organisations have been reported to be concentration dependent and the distance between such tetramer helices ranged from 3.6 to 4.9 nm, which corresponds to the d_{10}-spacing ranging from 3.1 to 4.2 nm (note, this is the strongest reflection of this columnar phase with the Miller indices h = 1 and k = 0). The apparent peak at q =1.60 nm^{-1} in our data (Fig. 6d) agrees with such an interpretation.

Analysis of the Porod's law deviations (Fig. S6 in Supplementary Information) represented by $ln\ (I \cdot q^4) \sim q^2$ revealed almost no deviations of scattering at higher q regimes for ACS, indicating a two-phase system with a relatively smooth electron density interface. Positive deviations at higher q regimes indicating a quasi-two-phase system with electron density fluctuations were observed in S/DMSO and S/DCC. This observation was interesting and could be an indication of mixing up of the repeated lamellar structures (crystalline and amorphous) leading to the loss of lamellar structure with smooth boundaries. The SF20 ester demonstrated a slight negative deviation, indicating a reduction of scattering at higher q regimes. Such reduced scattering is

usually thought to occur due to a transition zone. Least square fitting using Levenberg-Marquardt optimisation algorithm was employed, and $\ln(I(q) \cdot q^4) = \ln(K) - \sigma^2 q^2$ (Li, 2013) was fitted to the negative scattering deviations ($1.2 < q < 4.0$ nm^{-2}) from the Porod's plot (Fig. S7 in Supplementary Information). Here, K is the Porod's constant and σ is related to the thickness of the transition zone. The average thickness, σ, of the transition zone (E) was found to be 1.3 nm, calculated as, $E = (2\pi)^{\frac{1}{2}} \sigma$. Such negative deviations could be resultant from microstructural reorientation and reassembly caused by FA esterification, as observed above in section 3.3.

Upon substitution with FA, a hydroxyl group in starch forms one ester bond with FA, thus leaving possibilities for a glucose sub-unit in starch to form other ester bonds with FA as well. This leaves the pterin tails free to form tetramer complexes, presenting a scenario where starch polymers can be crosslinked. Such crosslinking alongside helix formation (as observed in section 3.3) can also induce curling in the SF chains, thereby shrinking them. On one hand, it explains the gradual reduction in D_h of SF with increasing degree of substitution (Fig. 2). On the other hand, FA has been reported to self-assemble into a nematic, columnar phase at lower concentrations (c < ca. 45 wt%) (Bonazzi, et al., 1993). Kamikawa, et al. (2004) has also reported the formation of non-symmetric supramolecular structures in FA derivatives (hexagonal columnar phase; lattice parameter = ca. 4.7 nm). We explicate the fluorescence dense regions observed in the CLSM micrographs (Fig. 3) to be columnar assemblies, latter formed via hydrogen bonding between intra-SF derivatives with a first order lattice spacing of 3.92 nm (note, the according unit cell parameter $a = 2/\sqrt{3} \cdot d_{10} = 4.5$ nm).

3.5. WAXS and XRD investigation on the molecular packing

Fig. 7 shows the WAXS patterns of the samples under study. Major peaks with scattering vector q were recorded for the ACS sample at 10.6, 12.0, and 12.6 nm^{-1}, corresponding to 2θ angles of (λ = 0.154 nm^{-1}) 15º, 17º, and 18º (specific for the A-type monoclinic crystals), respectively. Such Bragg peaks are known to be characteristic of A-type starch (Tawil, Viksø-Nielsen, Rolland-Sabaté, Colonna, & Buléon, 2011). Thus, ACS sample was identified (Fig. 7a) as an A-type starch, which agrees well with literature (Haaj, Thielemans, Magnin, & Boufi, 2016).

When ACS was treated with DMSO (S/DMSO) and DCC (S/DCC), only very weak and broad reflections were observed with WAXS (Fig. 7b, c), which demonstrates that also on a molecular scale, the crystalline order was destroyed, i.e. both, stacking (SAXS, Fig. 6) and packing order (WAXS, Fig. 7) were broadly impaired under the influence of DMSO and DCC. It is well evidenced that 1, 6-α linkages in A-type starches are highly scattered and are present in both amorphous and crystalline domains. These branching regions contained in the crystalline regions result in the generation of weakened points that make A-type structures more prone to dissolution (Zhang, et al., 2014).

Esterification with FA resulted in a new structural arrangement in the starch (Fig. 7d). This observation is also consistent with our SAXS findings. The SF20 can be characterized by major peaks with q at 4.4, 5.5, and 12.3 nm^{-1} corresponding to 2θ angles of 6.1º, 7.7º, and 17.4º, respectively. It is noteworthy that the 6.1º peak is classified as the characteristic $(100)_H$ reflection of the B-type hexagonal crystals (Huang, et al., 2014). Furthermore, the 17.4º is a characteristic B-type starch peak (Tawil, et al., 2011). Additionally, the strong Bragg peaks at 7.7° is characteristic of V-type starch polymorph (Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2010). Thus,

444 a gradual transformation is observed from an A-type to a hybrid B and V-type
445 polymorph. This interpretation is supported by our FTIR observations (section 3.3) that
446 show an increase of V-type polymorphs in SF.

447 Since it was noted that the WAXS regime was noisy, XRD was used to
448 complement the information on the lattice arrangements of ACS and SF20. Use of XRD
449 complemented the WAXS data. The ACS was a typical A-type pattern with peaks at 2θ
450 = 15°, 17°, 18° and 23° (Tawil, et al., 2011). The SF20 was a hybrid B-type with peaks
451 at 2θ = 17°, 22°, and 25° (Tawil, et al., 2011; Zhang, et al., 2014), and, V-type with
452 peaks at 2θ = 13° and 20° (Zabar, et al., 2010) (Fig. S8 in Supplementary information).

453 In summary, we have observed that that semi-crystalline lamellar structure of
454 the A-type starches was largely disintegrated during the DMSO treatment. This might
455 have led to a change from the smectic state of the A-type starch to a more nematic state,
456 illustrated more conveniently by the arrangement of double helices along the ac-plane
457 in Fig. 7e (left). When FA was introduced into the system, FA was esterified to the
458 starch backbone. This led to an isotropic gel-like arrangement. When the SF ester
459 derivative was lyophilized, FA molecules might have acted as nuclei for crystallization,
460 and the structure reoriented into a B-type polymorph, illustrated more conveniently by
461 the arrangement of the double helices along the ab-plane in Fig. 7e (right). Note, that
462 the lattice spacing of this B-type arrangement was much larger than the ones in A-type
463 starches and common pure B-type starches. The FA attached to the starch backbone has
464 a bulky, overhanging pterin tail. Thus, during crystallization, it seems plausible for SF
465 esters to exert a space filling effect. Existence of 36 molecules of water in the crystal
466 structure of B-type starches has been suggested in literature (Imberty & Perez, 1988).
467 The water molecules are positioned between the wide channels of the double helices
468 and occupy greater than a quarter of the central cavity of the unit cell. The B-type

polymorphs having such positioning of water in the channels does cast doubt on our hypothesis. Other arguments outlined by Vermeylen, et al. (2006) suggested that the B-type crystals get irreversibly converted into A-type after heating for longer periods of time. Accordingly, Fig S8b, c in the Supplementary information presents the XRD diffractograms of the samples SF20 and $SF20_{heated}$. Our experiments did not appear to corroborate the noticeable B- to A-type polymorphic phase transitions as observed by Vermeylen, et al. (2006), suggesting that the B-polymorph cell (SF20) were possibly constructed by FA occupying the central cavity of the unit cell unlike the case of common B-type starches. However, further study is required to fully understand the role of water in these hybrid B- and V-type polymorphs of SF.

4. Conclusion

Using an optimal combination of complementary scattering (light, X-ray), spectroscopic (FTIR), electrophoretic (ζ-potential), and imaging (CLSM) techniques, we observed that FA esterification greatly influences the colloidal properties as well as the short and long range molecular structure of SF ester derivatives. SF acquired a net negative charge when crosslinked with FA. Such crosslinking also led to the systematic reduction in the mean hydrodynamic diameter with the increase in the degree of substitution. Further, the starch structure was strongly compromised, i.e., the common lamellar stacking arrangement was lost, in which FA assisted in the altered self-assembly of SF. Observations of the packing order revealed a reorientation of ACS from the typical A-type arrangement to hybrid B and V-type polymorphs. This study has demonstrated for the first time, the multiscale structural alterations that occurred in amylopectin corn starch mediated by esterification with folic acid. Although preliminary, the present results may provide new design strategies for developing

494 functional starch-folic acid esters with tunable size and charge, capable of
495 supramolecular association and molecular recognition. Further work is in progress
496 where the structural aspects are further evaluated using electron microscopy and nuclear
497 magnetic resonance (NMR) techniques and functional properties of these starch-folate
498 esters are assessed in various *in vitro* physiological conditions, in order to design smart,
499 targeted delivery systems for carrying pharmaceuticals and nutraceuticals.
500

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