

# Designing synergistic crystallization inhibitors: bile salt derivatives of cellulose with enhanced hydrophilicity

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## Abstract

Crystallization inhibitors in amorphous solid dispersions (ASD) enable metastable supersaturated drug solutions that persist for a physiologically relevant time. Olefin cross-metathesis (CM) has successfully provided multifunctional cellulose-based derivatives as candidate ASD matrix polymers. In proof of concept studies, we prepared hydrophobic bile salt/cellulose adducts by CM with naturally occurring bile salts. We hypothesized that increased hydrophilicity would enhance the ability of these conjugates to maximize bioactive supersaturation. Their selective preparation presents a significant synthetic challenge, given polysaccharide reactivity and polysaccharide and bile salt complexity. We prepared such derivatives using a more hydrophilic hydroxypropyl cellulose (HPC) backbone, employing a pent-4-enyl tether (Pen) for appending bile acids. We probed structure-property relationships by varying the nature and degree of substitution of the bile acid substituent (lithocholic or deoxycholic acid). These conjugates are indeed synergistic

inhibitors, as demonstrated with the fast-crystallizing prostate cancer drug, enzalutamide. The lithocholic acid methyl ester derivative, AcrMLC-PenHHPCPen (0.64), increased induction time 68 fold vs. drug alone.

Keywords: olefin cross-metathesis, bile-salts, cellulose, amorphous solid dispersion, enzalutamide, chemoselectivity

## **1. Introduction**

Bile acids are complex, interfacially active, amphiphilic compounds. They promote the emulsification of fats and solubilize poorly aqueous-soluble drugs through micelle formation (Mukhopadhyay & Maitra, 2004). These properties inspire use of bile acids and derivatives in biomedical applications, including to solubilize hydrophobic drugs and aid permeation (Pavlović et al., 2018). Bile acids can enhance supersaturated systems by promoting liquid-liquid or glass-liquid phase separation (LLPS or GLPS), stabilizing the sub-micron diameter droplets of highly concentrated drug (Jackson et al., 2016; Trasi & Taylor, 2015). Recently, bile salts have been shown to inhibit crystallization of structurally diverse drugs (Chen, Mosquera-Giraldo, Ormes, Higgins, & Taylor, 2015; Li et al., 2016; Lu, Ormes, Lowinger, Mann, Xu, Litster, et al., 2017; Lu, Ormes, Lowinger, Mann, Xu, Patel, et al., 2017). Molecular dynamic simulations indicate that van der Waals and hydrogen bonding interactions strongly influence crystallization inhibition (Li et al., 2016), while the degree of aggregation was found to influence effectiveness in delaying crystallization, as monomeric bile salts presented superior inhibition properties (comparable to HPMCAS-MF) for telaprevir (Lu, Ormes, Lowinger, Mann, Xu, Litster, et al., 2017). Poorly water soluble drugs currently pose challenges for oral therapy. ASDs are molecular dispersions of drug in polymeric matrices, from which the drug rapidly dissolves

in the gastrointestinal (GI) tract to form a supersaturated solution. ASD polymers play multiple roles, including providing adequately high formulation glass transition temperature ( $T_g$ ) even when challenged by high humidity or drugs which are plasticizers, to prevent drug aggregation and crystallization. The polymer also must dissolve in the aqueous GI milieu at a rate similar to that of the drug (Craig, 2002; Taylor & Zhang, 2016). Sufficient dissolved polymer thereby can associate with the drug and prevent it from nucleating from the supersaturated solution, or if crystal seeds are present, from undergoing crystal growth via polymer adsorption to kinks or steps, therefore blocking molecular incorporation (Hasegawa et al., 1988). Some critical ASD challenges are still unmet by current polymers, including formulations that must contain a high bioactive concentration, and/or where the drug is a fast crystallizer (Baird et al., 2010; Van Eerdenbrugh et al., 2010, 2014). Thus synthesis of high-performance ASD polymers is crucial, informed by elucidation of the complex and interwoven structure-property relationships that are key to meeting all performance criteria (Arca et al., 2018; Frank & J. Matzger, 2018; Ricarte et al., 2019; Liu et al., 2014, 2015; Mosquera-Giraldo et al., 2016; Mosquera-Giraldo, H. Borca, et al., 2018; Pereira et al., 2013; Wilson et al., 2020).

Recently we reported synthesis of the first model bile salts and steroids substituted with Grubbs type II olefins (Chatterjee et al., 2003; Dong, Matson, et al., 2017) as substrates for conjugation with cellulose ether derivatives, e.g. ethyl 5-pent-1-enyl cellulose (EC2.30C5) (Dong et al., 2019). The objective was to form a construct between these two known classes of crystallization inhibitors that would exhibit synergistic performance. While the CM approach was successful, these bile ester derivatives of polysaccharides did not exhibit the expected synergy. In fact they *reduced* the time to

crystallization of the poorly soluble drug, telaprevir, vs. drug alone. We hypothesize that the hydrophobicity of both the ethyl cellulose backbone, (DS(Et) = 2.3, DS(Pen) = 0.7), and the bile acids caused the observed poor ASD performance, creating a mismatch between polymer and drug dissolution rates. We now hypothesize that bile salt/cellulosic polymer conjugates based on benign, water-soluble cellulose derivatives will have improved dissolution rates, therefore showing crystallization inhibition synergy.

We report attempts to design and prepare a series of conjugates with enhanced hydrophilicity, based on water-soluble HPC. Building on our previous work (Dong et al., 2019), we sought to etherify HPC with a Type I olefin-terminated side chain for olefin CM (PenHPC) (Dong et al. 2016). We broadened the series of bile acid derivatives by probing two attachment sites (A-ring OH vs. D-ring COOH) to determine which bile salt most influences crystal growth inhibition. Bile salt carboxyl groups were previously protected as methyl esters (Dong et al., 2019), while in this study we examine both ester and carboxylate-terminated conjugates. Carboxyl substituents are frequently elements of effective ASD polymers (Liu et al., 2014; Mosquera-Giraldo et al., 2016; Mosquera-Giraldo, H. Borca, et al., 2018). We investigate the influence of these polymers upon nucleation induction times of enzalutamide, an important, fast-crystallizing, hydrophobic drug for prostate cancer.

## **2. Experimental**

### *2.1. Materials and methods*

Hydroxypropyl cellulose (HPC,  $M_w = 100 \text{ kg mol}^{-1}$ , DP = 100, DS(HP) 2.2, MS(HP) 4.4) (Dong, Mosquera-Giraldo, Troutman, et al., 2016)), sodium hydride (95%), anhydrous tetrahydrofuran (THF), 5-bromo-pent-1-ene, Hoveyda-Grubbs' 2<sup>nd</sup> generation

catalyst, 3,5-di-*tert*-butylhydroxytoluene (BHT), triethylamine (TEA), lithocholic acid, deoxycholic acid, ethylene glycol, *para*-toluenesulfonyl hydrazide (*p*TSH), and potassium bromide (KBr) were from Sigma-Aldrich (Saint Louis, MO, USA). *N,N*-Dimethylacetamide (DMAc), *N,N*-dimethylformamide (DMF), lithium chloride (LiCl), dichloromethane (DCM), methanol, ethanol, and dialysis tubing (MWCO 3.5k Da) were from Fisher Scientific (Fair Lawn, NJ, USA). Enzalutamide was obtained from ChemShuttle (Hayward, California).

<sup>1</sup>H and <sup>13</sup>C NMR spectra were acquired on a Bruker Avance II spectrometer operating at 500 MHz except as indicated. Polymer (ca. 10 mg for <sup>1</sup>H NMR, 50 mg for <sup>13</sup>C NMR) was dissolved in ca. 1 mL CDCl<sub>3</sub> or dimethylsulfoxide (DMSO-*d*<sub>6</sub>); CF<sub>3</sub>CO<sub>2</sub>H (three drops) was added to shift the water peak downfield. Fourier transform-infrared (FT-IR) spectra were recorded in transmission mode with a Thermo Nicolet 8700 instrument (Madison, WI, USA); samples prepared as KBr pellets (1 mg polymer / 99 mg KBr mixed by mortar and pestle). Glass transition temperatures (*T*<sub>g</sub>) were measured by a TA Instruments Q2000 using modulated differential scanning calorimetry (MDSC), with N<sub>2</sub> as purge gas. Each polymer sample (~0.3-6 mg in a Tzero aluminum pan) was first equilibrated at -50 °C and then heated as high as 270°C at ramp rate 3°C/min, with modulation amplitude of 1 °C and oscillation period of 60s (results from first heating cycle). Molecular weights (*M*<sub>w</sub>) were measured by size exclusion chromatography (SEC) applying a Wyatt Technologies TRIOS II light scattering and Optilab T-REX refractive index (RI) detectors, using two Agilent Technologies PLgel 10 μm mixed-bed columns, DMAc/LiCl solvent, and a Shimadzu LC-20AD at 50 °C at a flow rate of 1 mL min<sup>-1</sup>.

## 2.2. Synthetic methods

### 2.2.1. Synthesis of hydroxypropyl 1-pent-4-enyl cellulose (PenHPC DS 0.60 and 1.00)

PenHPC (DS = 0.6, 1) was prepared according to Dong et al., (2016) (NMR (Fig. 2a, 3a), FTIR (Fig. S1a-d, top) and DS calculations (Fig. S7) in ESI (S1.1).

### 2.2.2. Syntheses of lithocholic acid (LCA) and deoxycholic acid (DCA) methyl esters

Syntheses carried out as adapted from Dong, et al., 2019; characterization information in ESI (S1.2).

### 2.2.3. Acrylation of A-ring bile acids and their methyl esters (exemplary procedure for LCA provided)

LCA (0.500 g, 1.32 mmol) was dissolved in anhydrous THF (5 mL, 0 °C), and triethylamine (0.134 g, 1 eq), then acrylic anhydride (Acr<sub>2</sub>O, 0.201 g, 1.2 eq) was added gradually. The solution was stirred at RT for 20 h, then concentrated under rotary evaporation. The crude lithocholate 3-O-acrylate (AcrLC) product thus obtained was recrystallized from water. The product was isolated by filtration, then dried under vacuum at 80 °C. Methyl lithocholate acrylate (AcrMLC) and methyl deoxycholate acrylate (MDCAc) were prepared according to Dong et al., (2019) (NMR data in ESI).

**Lithocholate acrylate (AcrLC).** Yield: 0.37 g, 64.5 %. <sup>1</sup>H NMR (selected signals, CDCl<sub>3</sub>): 0.63 (s, CH<sub>3</sub>), 0.91 (s, CH<sub>3</sub>), 0.93 (s, CH<sub>3</sub>), 4.80 (m, 1H, C3 CH<sub>2</sub>=CHCOOCH<sub>2</sub>), 5.78 (dd, COOCH=CH<sub>2</sub>, *trans*), 6.06 (dd, COOCH=CH<sub>2</sub>), 6.36 (dd, COOCH=CH<sub>2</sub>, *cis*). <sup>13</sup>C NMR δ 12.1, 18.2, 20.8, 23.3, 24.2, 26.3, 26.6, 27.0, 28.2, 30.75, 31.0, 32.2, 34.6, 35.0, 35.3, 35.8, 40.1, 40.4, 41.9, 42.7, 55.9, 56.5, 74.6 (C3 CH<sub>2</sub>=CHCOOCH<sub>2</sub>), 129.1 (COOCH=CH<sub>2</sub>), 130.2 (COOCH=CH<sub>2</sub>), 165.8 (CH<sub>2</sub>=CHCOO), 180.3 (C=OOH).

Deoxycholate acrylate (AcrDC) was prepared similarly by dissolving DCA (3.714 g, 9.57 mmol) in 20 mL anhydrous DCM containing 1 eq trimethylamine and 1 eq Acr<sub>2</sub>O

for 12 h. The solution was then concentrated under rotary evaporation, then the material was recrystallized from acetone, and collected as a pellet via centrifugation (0.782 g, 18 % DCA). The liquid filtrate was collected, concentrated, and dried under vacuum at 80 °C. The resulting crude oil was then redissolved in DCM, and purified by column chromatography (silica gel, 15% EtOAc in DCM) to isolate the A-ring 3-*O*-acrylate, AcrDC (15% EtOAc/DCM, TLC Rf = 0.35), DCA (15% EtOAc/DCM, TLC Rf = 0), and D-ring 12-*O*-acrylate (15% EtOAc/DCM, TLC Rf = 0.65).

***Deoxycholate acrylate (C3-O-AcrDC)***. Yield: 1.17 g, 27%. <sup>1</sup>H NMR (selected signals, CDCl<sub>3</sub>): δ 0.69, 0.93, 4.00 (t, 1H, C12 HOCH), 4.80 (m, 1H, C3 CH<sub>2</sub>=CHCOOCH), 5.78, 6.05, 6.35. <sup>13</sup>C NMR (DMSO) δ 12.87, 17.41, 23.25, 23.72, 26.13, 26.61, 27.07, 27.56, 28.80, 30.78, 31.13, 32.26, 33.78, 34.27, 34.98, 35.18, 36.09, 41.97, 46.60, 47.43, 48.39, 73.36 (C3 CH<sub>2</sub>=CHCOOCH), 74.58 (C12 HO-CH), 129.19 (COOCH=CH<sub>2</sub>), 130.42 (COOCH=CH<sub>2</sub>), 165.98 (COOCH=CH<sub>2</sub>), 179.98 (24C C=OOH).

***Deoxycholate diacrylate (C3 & C12-O-AcrDC)***. Yield: 2 g, 46.1%. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.86, 0.90, 0.92, 1.25, 1.48, 1.51, 1.62, 1.63, 1.69, 1.71, 1.80, 1.85, 2.24, 2.27, 2.33, 2.35, 2.37, 4.77, 5.17, 5.77, 5.80, 5.85, 5.88, 6.06, 6.08, 6.12, 6.16, 6.18, 6.20, 6.23, 6.34, 6.39, 6.42, 6.46. <sup>13</sup>C NMR δ 12.45, 14.14, 17.40, 22.70, 23.09, 23.45, 24.69, 25.69, 25.91, 26.50, 26.88, 27.34, 29.07, 29.25, 29.37, 29.44, 29.60, 29.65, 29.67, 29.71, 30.56, 30.85, 31.93, 32.24, 33.97, 34.48, 34.66, 34.72, 35.69, 41.81, 45.23, 47.59, 49.64, 74.37, 76.00, 128.91, 129.03, 130.28, 130.61, 165.62, 179.60, 179.88.

#### 2.2.4. General procedure for olefin CM of PenHPC (DS (Pen) = 1.00)

**Preparation of AcrLC-PenHPC(1.00)** exemplifies the process: PenHPC(1.00) (190 mg, 0.22 mmol C=C), AcrLC (500 mg, 1.2 mmol, 2.9 equiv), and BHT (25 mg) were

dissolved in ethyl acetate (EtOAc, 12 mL) under N<sub>2</sub>. HG II catalyst (39 mg, 12 mol%) was dissolved in anhydrous EtOAc (1 mL) and gradually added to the solution, which was stirred at 50 °C for 3 h before adding three drops of ethyl vinyl ether to terminate the reaction. The solution was concentrated under vacuum and added to hexanes to precipitate the product, which was redissolved in THF and reprecipitated into water, then dried overnight under vacuum at 40 °C. Additional NMR data in ESI, **S1.3**.

*AcrLC-PenHPC(1.00)*. Yield: 350 mg, 87.5%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.64 (s, CH<sub>3</sub>), 0.91 (s, CH<sub>3</sub>), 0.92 (s, CH<sub>3</sub>), 1.00-2.57 (m, steroid ring protons, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH=CHCOO-LCAc), 2.90-4.68 (m, cellulose backbone, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH=CHCOO-LCAc, 4.77 (m, LCAc C3 CH), 5.77 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH=CHCOO-LCAc), 6.94 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH=CHCOO-LCAc). <sup>13</sup>C NMR (126 MHz, DMSO) δ 12.32, 17.78, 18.59, 20.71, 23.50, 24.28, 24.90, 25.71, 26.44, 26.76, 28.19, 28.89, 29.52, 29.81, 31.16, 32.38, 34.62, 35.27, 35.76, 41.64, 42.72, 56.05, 56.40, 65.73, 67.97, 68.52, 72.98 - 79.16 (cellulose C2, C3, C5 and bile acid C3 CH<sub>2</sub>=CHCOOCH), 83.10 (cellulose C4), 101.72 (cellulose C1), 121.78 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH=CHCOO-LCAc), 149.38 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH=CHCOO-LCAc), 172.65 (C=OOAcrLC), 175.25 (24C C=OOH).

#### 2.2.5. General procedure for hydrogenation of PenHPC-Bile ester conjugates

Preparation of AcrDC-PenHHPC(1.00) was adapted from Dong et al., 2019; all details found in ESI (**S1.4**), with remaining hydrogenated product characterizations on (**Fig. S4 and S6**).

*AcrDC-PenHHPC(1.00)*. Yield: 160 mg, 71%. <sup>1</sup>H NMR (selected, DMSO) δ 0.58 (s, CH<sub>3</sub>) 0.86 (s, CH<sub>3</sub>), 1.01-2.21 (m, steroid ring protons, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>COO-

DCAcAn), 2.76-4.54 (m, cellulose backbone, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>COO-DCAcAn, C12 HO-CH), 4.60 (DCAcAn C3 -OCH). <sup>13</sup>C NMR δ 12.44, 16.94, 17.34, 19.12, 20.29, 22.83, 23.49, 24.45, 25.25, 25.92, 26.14, 26.67, 27.18, 28.53, 29.37, 30.77, 30.88, 31.89, 32.83, 33.79, 34.55, 34.97, 35.55, 41.18, 46.03, 46.24, 47.42, 64.98, 65.29, 68.14, 71.06 - 79.28 (cellulose C2, C3, C5, and bile ester C3 CH<sub>2</sub>CH<sub>2</sub>COOCH and C12 HO-CH), 82.52 (cellulose C4), 101.37 (cellulose C1), 172.28 (C=OODCAc), 174.97 (C=OODCAc).

### 2.2.6. Selective hydrolysis of D-ring methyl ester, AcrMLC-PenHHPC

AcrMLC-PenHHPC(1.00) (250 mg, 0.29 mmol -OMe) dissolved in 5 mL THF was heated to reflux (~ 59 °C) with stirring, followed by dropwise addition of 2M NaOH (5 mL). The solution was stirred 1h, then cooled to RT, followed by partitioning between EtOAc/water; water extraction was repeated 3X. Aqueous layer was dialyzed against methanol (1 day) and water (2 days), then lyophilized. Yield: 18 %, <sup>1</sup>H and <sup>13</sup>C NMR signals agreed with those of AcrLC-PenHHPC obtained via direct acrylation and CM of the bile acid (section 2.2.5).

### 2.3. Measurement of nucleation induction times

Polymer was dissolved in DMSO (20 mg/mL) by sonication (60 min, 40 °C). Next, small aliquots (125 μL) of this solution were added, using constant agitation, to 50 mL of pH 6.8 100 mM buffer at 37 °C to obtain 50 μg/mL polymer solutions (<1% DMSO). Supersaturated enzalutamide solutions (35 μg/mL) were prepared by adding 175 μL of the enzalutamide methanolic stock solution (10 mg/mL) to 50 mL phosphate buffer (0.100M) with polymer concentration 50 μg/mL, maintained at 37 °C and magnetically stirred (300 rpm). Crystallization induction time from unseeded samples was measured using an SI

Photonics UV/vis spectrometer (Tucson, AZ) coupled to a fiber optic probe (path length 5 mm). Wavelength scans (200-450 nm) were performed at 60s time intervals. Crystal formation onset time was determined from the drop in drug concentration (absorbance at 237 nm) in the absence and presence of pre-dissolved polymer at 37 °C, designated as the nucleation induction time; measurement repeated 3X.

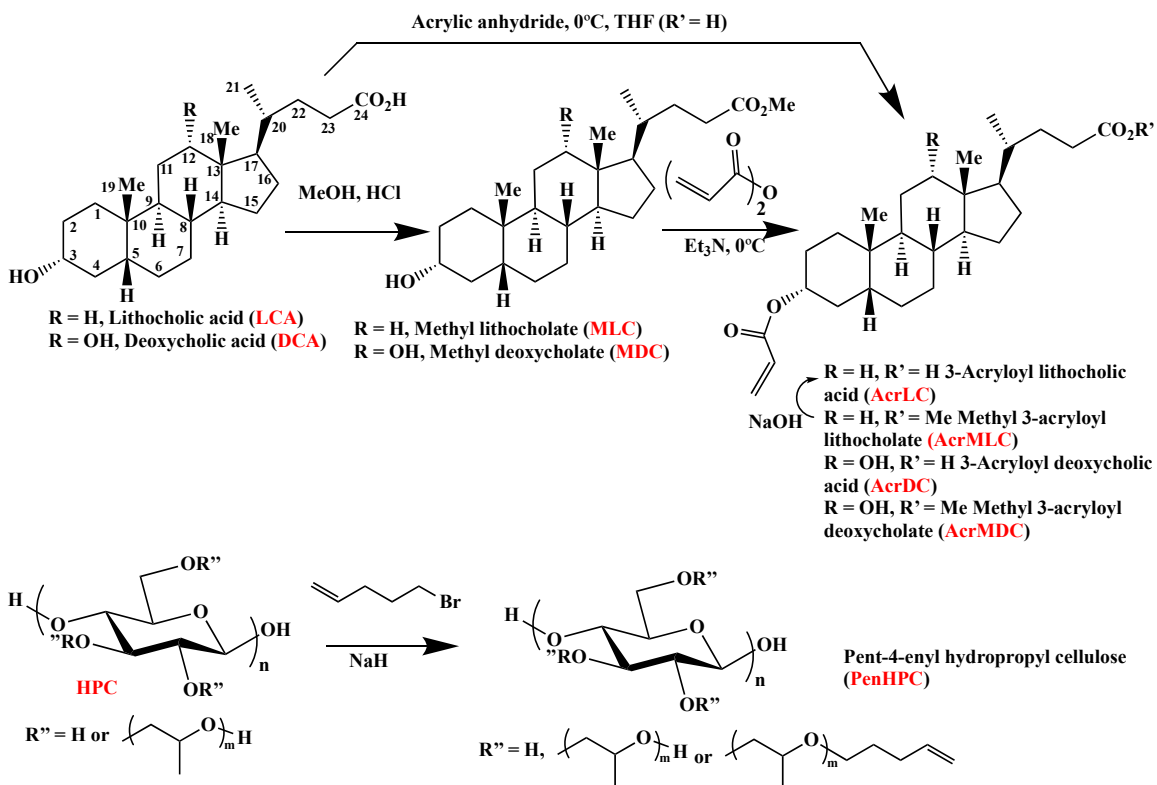
#### *2.4. Solubility parameter (SP) calculation*

Cellulose derivatives and enzalutamide SP were determined using Fedor's method (Fedors, 1974). Procedural details in ESI of Dong, Mosquera-Giraldo, Taylor, et al., 2016.

### **3. Results and discussion**

Our strategy exploited the 4-pent-1-enyl ether of HPC (PenHPC, Scheme 1) as the Grubbs Type I olefin in CM (Dong, Mosquera-Giraldo, Troutman, et al., 2016; Grubbs, 2004) with an acrylated bile salt derivative (Type II olefin). We faced chemoselectivity

challenges, including selectivity between the neutral A-ring hydroxyl and the anionic D-ring carboxylate nucleophiles of LCA.



Scheme 1. CM partner syntheses. Structures do not imply regioselectivity; particular positions of substitution in all schemes only for convenience of depiction and clarity.

The DCA A-ring OH-nucleophile competes with both the D-ring carboxylate and the C-ring hydroxyl. Cellulose derivatives are relatively unreactive since they diffuse slowly, have relatively high Mw, and hydroxyls have relatively narrow approach angles; all non-conductive to regioselectivity (Fox et al., 2011). Fortunately, PenHPC has a flexible oligo(HP) tether and broader approach angles.

The key issue was selective bile salt acrylation. We began by making bile acid methyl esters to eliminate carboxylate/hydroxyl competition (Scheme 1). LCA (methyl lithocholate, MLC) and deoxycholic acid (methyl deoxycholate, MDC) esters were

prepared by Fischer esterification (Fischer & Speier, 1895) as reported previously (Dong et al., 2019). Acrylation of bile salt methyl esters was by Dong's procedures, catalyzed by triethylamine at 0°C using  $\text{Ac}_2\text{O}$ , with excellent yield from MLC, and moderate, somewhat selective acrylation yield from difunctional MDC.  $^1\text{H}$  NMR spectra for bile acid and acrylates are shown on Fig. 1;  $^{13}\text{C}$  NMR spectra are in **Fig. S2**. As exemplified for LCA reactions, successful methyl esterification was confirmed by the new  $^1\text{H}$  NMR singlet at 3.66 ppm and the methyl ester  $^{13}\text{C}$  NMR resonance (C25) at 51.39 ppm. Subsequent A-ring hydroxyl acrylation was confirmed by downfield shift of the C3-OCH methine multiplet (3.62 to 4.66 ppm) due to presence of 3-*O*-acrylate (Fig. 1b). Product acrylate olefin protons were observed as doublets (5.79, 6.10, 6.39 ppm) (Fig. 1b), with corresponding  $^{13}\text{C}$  NMR quaternary resonances (129.15, 130.2 ppm) (**Fig. S2b**).

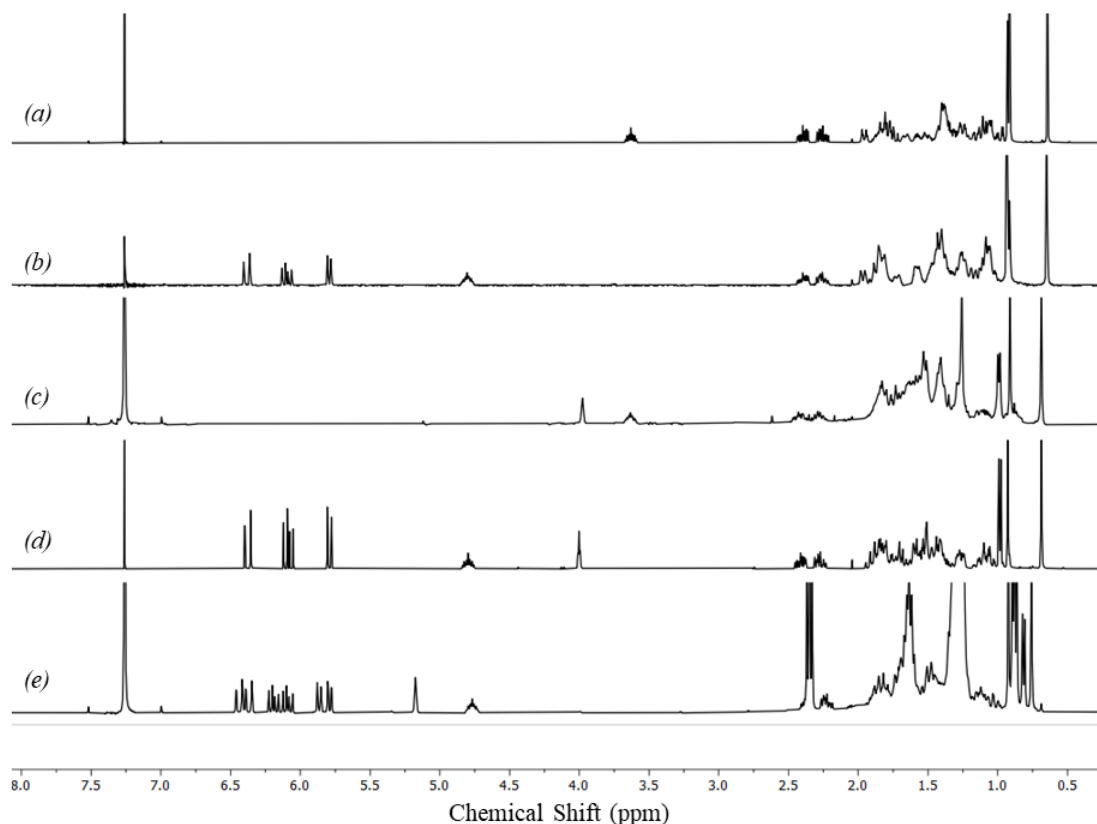


Figure 1: Proton NMR spectra of a) LCA, b) acrylated LCA (AcrLC), c) DCA, d) monoacrylated C3-OAc DCA (AcrDC), and e) diacrylated DCA (AcrDC).

Direct bile salt acrylation would afford a carboxyl-functionalized adduct, which we hypothesized would be an effective crystallization inhibitor based on earlier structure-property studies (Mosquera-Giraldo et al., 2016; Mosquera-Giraldo, Li, et al., 2018). We chose Acr<sub>2</sub>O to effect selective acrylation of the less hindered LCA A-ring hydroxyl (Dong et al., 2019; Hu et al., 2005; Li & Ray Dias, 1997; Zhu & Nichifor, 2002); Table 1. C3-OH selectivity was maximized by limiting the Acr<sub>2</sub>O /OH ratio. One equivalent each of Acr<sub>2</sub>O and triethylamine worked best; after 20h, we were gratified to observe complete C3-OH chemoselectivity. Purity and identity of the 3-*O*-acrylated AcrLC product were confirmed by FTIR and NMR spectroscopy. Downfield shift of the 3-CH-OH proton (3.63 to 4.66 ppm) upon acrylation was diagnostic; (Figs. 2a, 2b), supported by the downfield <sup>13</sup>C NMR

shift of C3-OAc (71.39 to 74.6 ppm). Additional strong evidence was provided by the acrylate proton resonances (5.79, 6.05, 6.36 ppm), acrylate carbons (129.1, 130.2), and the new ester carbonyl (165.8 ppm) (Fig. 2b, Fig. S3c). FT-IR spectroscopy was also supportive, with new olefinic stretches at 1621 cm<sup>-1</sup> (Fig. S1).

With its additional C-ring hydroxyl (C12-OH), regioselective DCA acrylation is more challenging (Hu et al., 2005). We sought selectivity by brief esterification with Acr<sub>2</sub>O at 0 °C. Selectivity was modest, affording 57% 3-*O*-Ac, 20% 12-*O*-Ac, and 42% unreacted DCA by <sup>1</sup>H NMR (emergence of three acrylate olefin protons, and characteristic downfield shifts protons alpha to acrylation sites). Disappearance of the methine (alpha to methoxyl) resonance at 3.62 ppm was accompanied by emergence of the 3-*O*-Ac C3-OCH monoacrylate at 4.44 ppm, as well as new olefinic protons (5.78, 6.05, 6.35 ppm) (Fig. 1d); analogously, the C12-OCH methine proton (4.0 ppm) disappeared, replaced by the deshielded monoacrylate 12-*O*-Ac-OCH (5.17 ppm), whereas new olefinic acrylate protons emerged (5.84, 6.16, 6.40 ppm) in the diacrylate mixture (Fig. 1e). Longer reaction times, more acrylate, and use of more reactive acryloyl chloride all harmed regioselectivity (Table 1). Despite modest selectivity, we were able to isolate pure deoxycholic acid 3-acrylate by flash column chromatography.

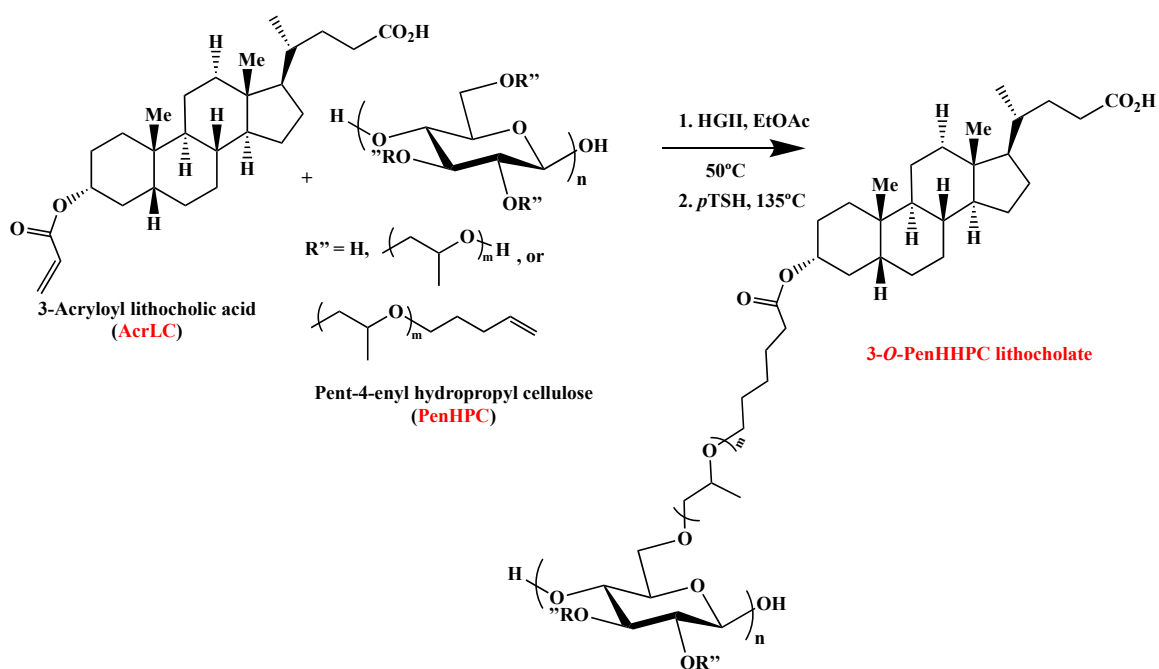
Table 1. Selectivity vs. conditions for esterification of A-ring hydroxyl (C3-OH)

Substrate	Acrylate/TEA molar ratio	Time (h)	Starting material (%)	3- <i>O</i> -Acrylate (%)	12- <i>O</i> -Acrylate (%)
LCA	1.2 : 1	12	0	100	n/a
		16			
16					
	1.2 : 3	12	56.7	43.2	n/a

	1.2 : 5	12	31	69	n/a
DCA	1.2 : 1	12	42	57	20
		16	63	36	29
		16	40*	50*	30*
	1.2 : 3	16	60*	41*	32*

\* = 16 h using acryloyl chloride

Our previous successful CM with ethyl pent-4-enyl cellulose (EC2.30C5; Dong et al., 2019) inspired the methodology herein, reacting PenHPC (Grubbs type I olefin) with acrylated bile acids and esters (Grubbs Type II olefins) (Chatterjee et al., 2003; Dong, Matson, et al., 2017). Exemplary reaction of 3-*O*-acryloyl-lithocholic acid (AcrLCA) with pent-4-enyl HPC is shown (Scheme 2).



Scheme 2. Bile salt CM chemistry.

We explored effects of solvent (EtOAc, THF, DCM) and temperature (37, 50°C) on CM reaction time and conversion (Table 2). Earlier bile ester CM conjugation with ethyl cellulose derivatives reached completion within 24 h (37 °C) (Dong et al., 2019), but here

we made the useful observation that EtOAc accelerates CM, affording higher efficiency (<equiv (2.9) of precious acrylate required, vs. 5 equiv in THF or CH<sub>2</sub>Cl<sub>2</sub> (Dong et al., 2019)) in reaching 100% conjugation. Even with this smaller excess, kinetics in EtOAc were significantly faster; 100% conversion within 5h at 37 °C. At 50 °C, achievable because of the higher EtOAc boiling point, 100% completion was reached within 3h even at only 2.9:1 type II:type I olefin.

Table 2. Reaction times, conversion achieved (DS<sub>bile acid conjugate</sub>) vs. acrylated bile acid, solvent, and reaction temperature\*.

PenHPC DS(pent-4-enyl)	Solvent, (Temp. (°C))	Conversion (reaction time, h)			
		AcrMLC	AcrLC	AcrMDC	AcrDC
<b>1.00</b>	THF (37°C)	100 (36)	--	--	--
	DCM (37°C)	100 (14.5)	50 (12)	--	--
	EtOAc (50°C)	100 (3)	100 (3)	100 (3)	100 (3)
<b>0.60</b>	THF (37°C)	50 (39.5)	50 (39.5)	--	--
	DCM (37°C)	80 (24)	100 (54)	--	--
		100 (36)	--	--	--
	EtOAc (50°C)	100 (3)	100 (3)	100 (3)	100 (3)

Catalyst HGII, 10 mol%.

Conjugate formation was monitored by disappearance of terminal olefin proton resonances (4.96, 5.82 ppm) from PenHPC and 5.78-6.36 ppm from the acrylates (e.g. Figs. 2a, 2b) along with emergence of new, distinct conjugated olefin proton resonances (5.82, 6.89 ppm). Products also showed expected steroid nucleus proton resonances, between 2.4-

0.6 ppm (Fig. 2c). Adduct structures were further supported by new olefinic carbon resonances (Figs. 3b, S3), which shifted as expected upon conjugation (PenHPC from 114.5, 138.4; acrylate from 129.1, 130.2 ppm) to 121 and 149.4 ppm, respectively (Fig. 3a,b). After CM completion, we demonstrated the possibility of recovering valuable, unreacted bile salt acrylate, by concentrating the hexanes filtrate after precipitation, and redissolving the resulting crude solid in minimal solvent for subsequent injection into a silica plug for further isolation from residual catalyst or polymer. The more polar PenHPC or PenHPC-bile ester conjugate residue was retained by the silica, while the less polar acrylate and potentially some HG II catalyst coeluted with the mobile hexanes, from which it was readily recovered by evaporation.

We have previously shown that  $\alpha$ ,  $\beta$ -unsaturated CM products are prone to radical-initiated  $\gamma$ -H-atom abstraction (Meng & Edgar, 2015), followed by undesired radical reactions. We eliminated this risk in our CM adducts by final transfer hydrogenation of the conjugated olefins (Dong et al., 2017; Dong & Edgar, 2015; Meng et al., 2014; Meng & Edgar, 2015), refluxing the conjugate at 135°C in the presence of *p*-toluene sulfonyl hydrazide (*p*TSH). Complete hydrogenation was clearly indicated by the absence of vinyl proton or carbon resonances in product  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (**Fig. S4**). Further, expected FT-IR shifts and absorbance reductions were observed for conjugates and their corresponding hydrogenated products (**Fig. S1a-d, bottom**). We refer to the hydrogenated conjugate of PenHPC and AcrLC as 3-*O*-PenHHPC lithocholate (Scheme 2; where the “H” that precedes “HPC” refers to “hydrogenated”); in this case DS(pent-4-enyl) was 1.0 (exemplary  $^1\text{H}$  NMR spectra Fig. 2, 3).

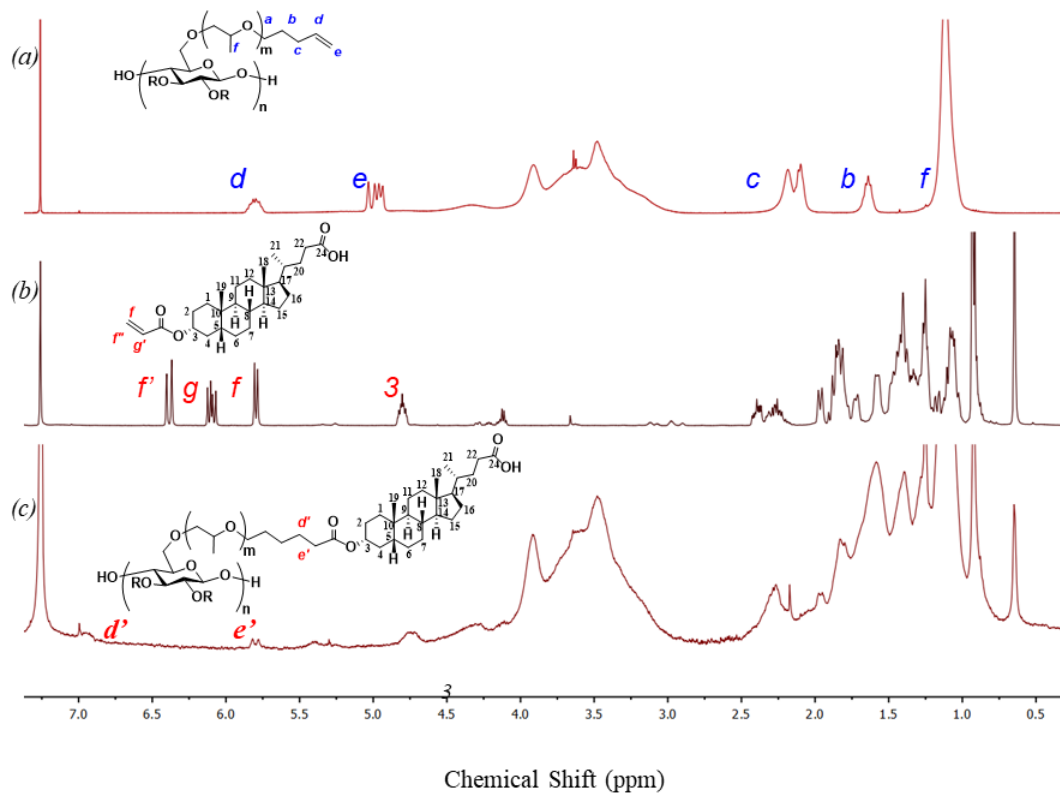


Figure 2. Proton NMR spectra of a) PenHPC b) AcrLC , c) CM conjugate, 3-*O*-PenHHPC lithocholate (1.00).

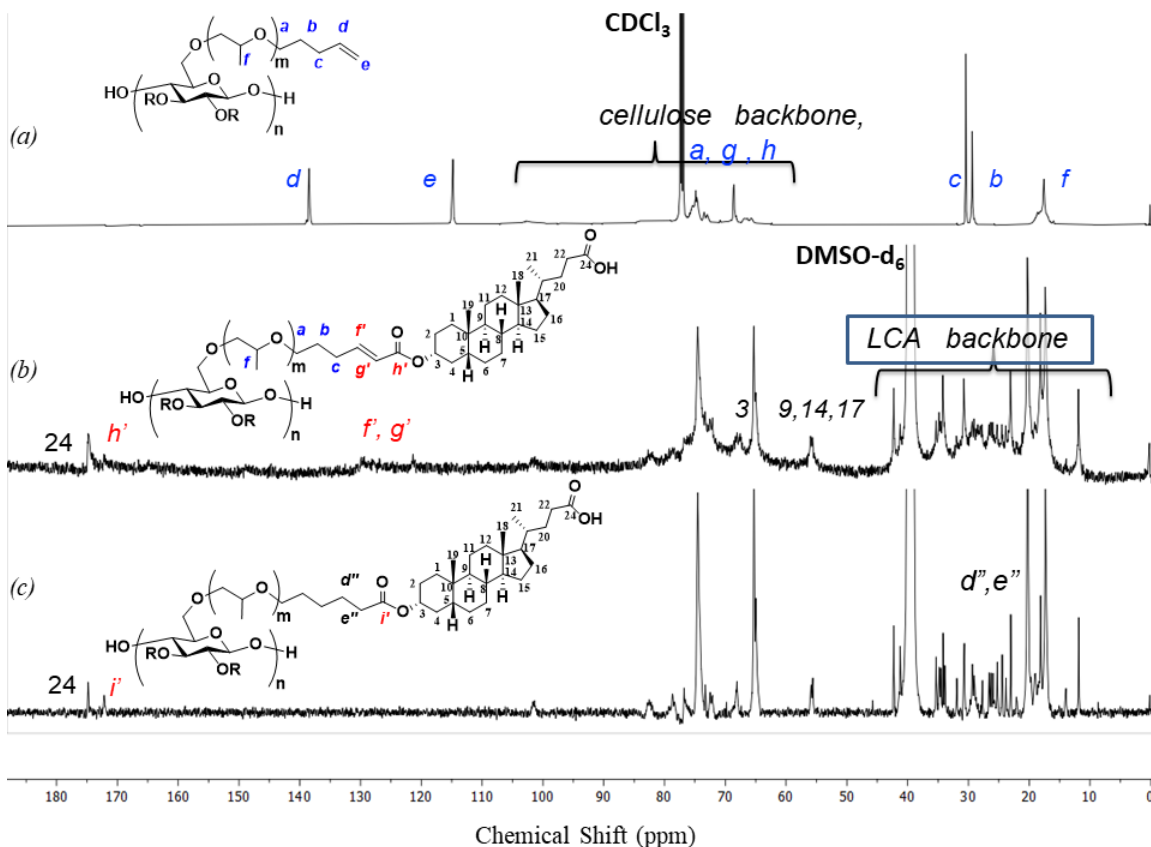


Figure 3.  $^{13}\text{C}$  NMR spectra of a) PenHPC b) AcrLC , c) CM conjugate, 3-*O*-PenHHPC lithocholate (1.00).

We demonstrated selective hydrolysis of 3-*O*-PenHHPC lithocholate D-ring methyl ester. Hydrolysis was performed after hydrogenation, via brief reflux (2M NaOH, 1h). This afforded a ready, straightforward approach to obtain two of our target compounds (methyl ester- or carboxylic acid-bearing), avoiding potential cleavage of acrylate ester. In spite of this success, purification afforded a modest yield (~18%). It is possible that, despite the brief heating, sufficient depolymerization may have occurred to cause polymer loss during dialysis (MWCO 3.5kDa).

Polymers with sufficiently high  $T_g$  (40-50°C above ambient temperature) are desired to combat effects of humidity and possible drug plasticization upon the

formulation. Many cellulose derivatives have high  $T_g$  values (Dong et al., 2019), but it was important to investigate possible internal plasticization by bile acid substituents. PenHPC (DS (Pen) = 1.00)  $T_g$  is 91 °C, (Dong, Mosquera-Giraldo, Taylor, et al., 2016). Each hydrogenated conjugate displayed  $T_g$  well above ambient temperature, from 80.71 - 145 °C, though some were quite weak. The higher  $T_g$  derivatives have significant potential for sustaining ASD formulations in the glassy state (Fig. S9-16, Table S1).

We tested performance of these complex bile acid- and bile ester-tethered polysaccharides *in vitro* as drug crystallization inhibitors. The time at which drug crystallization became detectable due to drop in drug solution content (inflection point in  $A_{237\text{ nm}}$ ) was designated as the nucleation induction time, measured in the absence and presence of each polymer. Enzalutamide is a hydrophobic, fast crystallizing, poorly water-soluble (crystalline solubility = 2.9 µg/mL) prostate cancer therapeutic (Wilson et al., 2018).

We had hypothesized that more hydrophilic polymers would result in effective crystallization inhibition. In the absence of polymer (Fig. 4), the drug crystallized quickly, within 7 min.

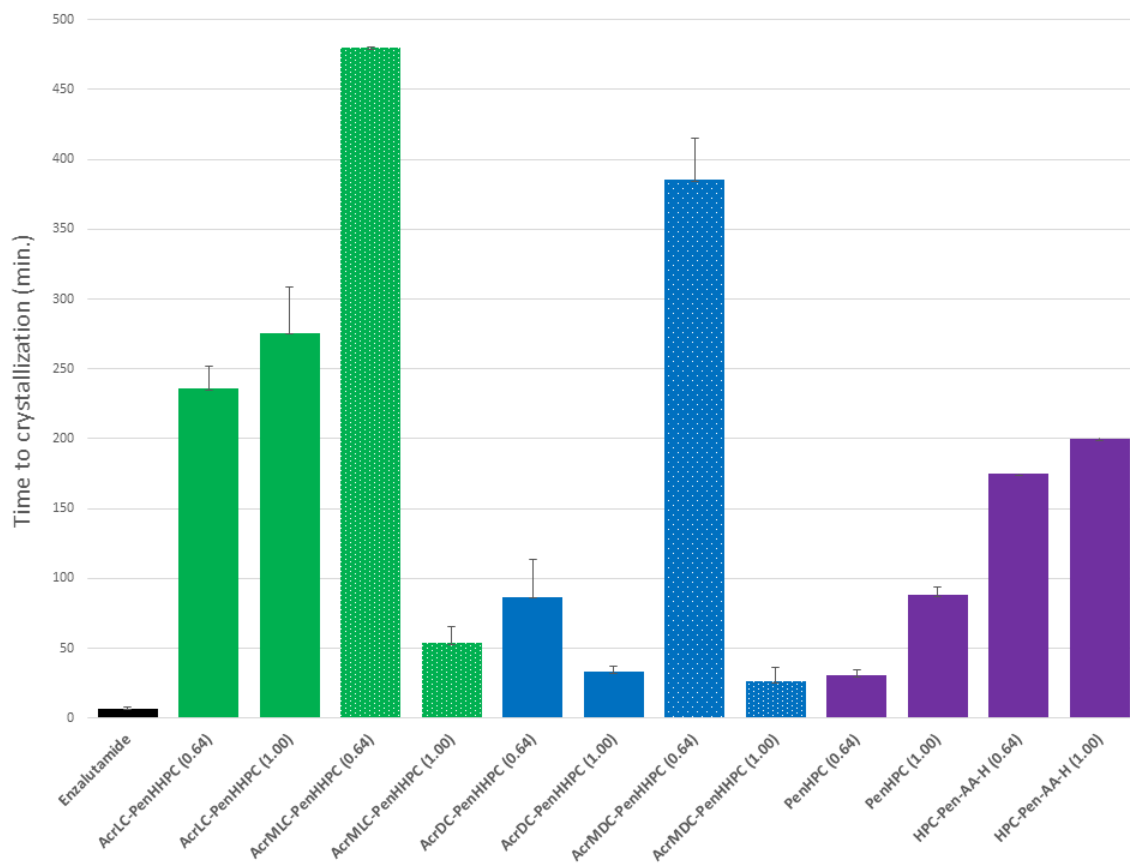


Figure 4: Enzalutamide nucleation induction times in absence and presence of pre-dissolved polymers (n = 3; polymer abbreviations and structures see Table S2).

Previous studies showed the importance of carboxylic acids in effective crystallization inhibitors (Mosquera-Giraldo et al., 2016), but also showed that higher DS(CO<sub>2</sub>H) at some point does not enhance polymer effectiveness (Mosquera-Giraldo, Borca, et al., 2018). Using computer simulations, we have shown that drug molecules tended to interact with the hydrophobic portion of the polymer rather than forming specific interactions with the carboxylic acids. This suggested that carboxylic acids may provide amphiphilicity, but do not necessarily form specific interactions with drug molecules. Herein, we found that the best performing ASD polymers with regard to inhibition of enzalutamide crystallization were methyl esterified, in particular those with lower DS(bile

salt ester). 3-*O*-PenHHPC methyl lithocholate (DS(bile ester) 0.64) can effectively maintain supersaturation for ca. 8h (when experiment was terminated). Meanwhile, 3-*O*-PenHHPC methyl deoxycholate (DS bile ester 0.64) afforded induction time until crystallization of ca. 385 min. It appears that for enzalutamide, hydrophobic interactions between bile salt ester and drug were more important than amphiphilicity provided by the carboxylic acid substituents, which seemed to be of importance for many polysaccharide derivatives (Arca et al., 2018; Dong, Mosquera-Giraldo, et al., 2017; Dong, Mosquera-Giraldo, Troutman, et al., 2016; Ilevbare et al., 2013b; Liu et al., 2014; Tanno et al., 2004). Alternatively, the combination of bile ester and hydrophilic cellulose ether backbone generates a degree of amphiphilicity that enables the appropriate balance between polymer-water interactions and polymer-drug interactions, as suggested in previous studies (Mosquera-Giraldo, Borca, et al., 2018). All of our bile acid/ester conjugates with HPC pentyl ether prolonged enzalutamide crystallization time. We were pleased to observe that LCA derivatives (simpler to make) consistently out-performed DCA derivatives.

We compared performance of these novel ASD polymer candidates to their cellulose ether precursors (pent-4-enyl HPC, DS(pent-4-enyl) 0.64, 1.0), which were not conjugated to bile salts and thus could serve as negative controls. As positive controls, we used CM adducts of pentenyl HPC with acrylic acid (after hydrogenation), HPC-Pen-AA-H (0.64) and HPC-Pen-AA-H (1.00), since we previously shown that they are effective crystallization inhibitors (Wilson et al., 2020). Enzalutamide crystallizes in < 150 min using similar conditions (50 µg/mL polymer, 50 mM phosphate buffer) in the presence of 5-carboxypentyl HPC with DS(carboxypentyl) 1.0 (Wilson et al., 2020), supporting the

concept that sufficiently hydrophilic bile salt-appended cellulose ethers are synergistic and effective crystallization inhibitors.

Fedor SPs of our derivatives were calculated (Table S3). Effective crystallization inhibitors have been associated with moderate hydrophobicity, ranging from SP 20-23 MPa<sup>1/2</sup> for promising cellulose esters (Ilevbare et al., 2013a, 2013b) and ethers (Dong, Mosquera-Giraldo, Taylor, et al., 2016). The pentenyl HPC derivatives are more hydrophilic, with SP values 22.8 and 21.8 MPa<sup>1/2</sup> for DS (Pen) = 0.64 and 1, respectively; vs. the more hydrophobic pentenyl substituted EC2.30C5 DS(Pen) = 0.69 (SP values 18.0 to 19.9 MPa<sup>1/2</sup>). Thus SP values for HPC-bile salt adducts prepared herein, falling within 21.0-23.2 MPa<sup>1/2</sup> indicate improved hydrophilicity, which contributes to longer nucleation induction times in most cases.

## Conclusions

We confirmed our original hypothesis by demonstrating synergistic, enhanced ASD performance of conjugates of bile acids with HPC derivatives, with their improved hydrophilicity. Grubbs Type II olefin acrylates of bile salts or their methyl esters were prepared chemo- and regioselectively by acrylation of bile salts and esters, followed by efficient, mild CM, in order to probe both A- and D-ring structure-property relationships regarding crystal growth inhibition. Careful selection of acrylating agent, minimizing its excess, and optimizing base stoichiometry allowed us to achieve chemoselective acrylation of the LCA A-ring hydroxyl. By so doing we eliminate the extra methyl esterification step as well as the sensitive saponification of the methyl ester in the presence of the acrylate ester. These methods also provided a degree of regioselectivity in acrylation of the equatorial 3-OH of DCA in the A-ring vs. to the C-ring 12-OH. We significantly improved

both CM kinetics and efficiency of preparing these complex structures by optimizing conditions. EtOAc provides much faster CM conversion. Rates of initiation in olefin metathesis are known to increase with solvent polarity (Sanford et al., 2001), and DCM (20.2) has a higher Hildebrand SP than EtOAc (18.2), so the faster rates in EtOAc are an important and pleasant surprise. The higher EtOAc boiling point must certainly be influential on kinetics; for example, AcrMLC CM completion in EtOAc taking 3h at 50 °C vs. 14.5 h in DCM at 37°C (**Table 2**). Although their polarities are similar and THF boiling point is slightly higher (Hildebrand SP 18.3, BP 66°C (Sanford et al., 2001)), CM was faster in EtOAc than in THF, 100% conversion of AcrMLC in the latter solvent for example taking 36h. The better ability of THF to coordinate metals may be a factor, perhaps by competing for Ru coordination sites. Superior CM performance in EtOAc promises greater efficiency than previously reported bile salt CM chemistry (Dong et al., 2019), reducing usage of expensive bile acid starting materials, and allowing recycle of unreacted bile salt acrylate; virtually 100% efficiency with regard to bile salt acrylate may be possible.

These results illuminate structure-property relationships of bile acid vs. bile ester decorated polysaccharides for oral drug delivery, simultaneously highlighting the remarkable potential of mild, flexible, and efficient CM chemistry for synthesis of complex polysaccharide derivatives. It is worth emphasizing that even with large, sterically demanding, slow diffusing molecules like these cellulose derivatives, and even when the CM type II olefin partner is a relatively bulky small molecule like a bile salt derivative, relatively rapid and fully complete, selective CM conversions were achieved, enabling preparation of these challenging structures in a few synthetic steps.

The improved hydrophilicity of each ASD polymer vs. the corresponding ethyl cellulose-bile salt adduct led to substantially increased nucleation-induction times (e.g., slower crystallization) even with the fast-crystallizing prostate cancer drug, enzulatamide. In some cases, bile salt adduct methyl esters performed better than the corresponding acids. This was a surprising result, given our earlier studies showing the value of carboxylic acid substituents in prolonging nucleation times. While carboxyl-containing polysaccharides have often performed well in crystallization inhibition (e.g., hydroxypropyl methyl cellulose acetate succinate is a leading commercial ASD polymer (Curatolo et al., 2009)) and in structure-property studies (Liu et al., 2014), it is also true that neutral cellulose ethers like HPC and hydroxypropyl methyl cellulose are effective ASD polymers for certain drugs (Arca et al., 2018), and neither contains carboxyl groups. This study highlights the importance of amphiphilic character of the polymer to ASD performance, indicating that, in this family of polymers at least, the absence of a pendent carboxylic acid to act as a pH trigger and provide amphiphilicity can be overcome by interactions between the hydrophobic bile salt methyl ester substituent and the drug. It also illustrates the complex nature of these structure property relationships, illustrated in Fig. S17 by comparing crystallization induction time of these polymers to solubility parameter, bile salt type and DS, and whether the terminus of the bile salt substituent was a carboxylic acid or a methyl ester. It is clear from the relationship that LCA derivatives performed better, as did those with higher DS(bile salt). While the methyl esterified LCA adduct gave the best performance, there was not a clear overall trend to better performance for the methyl esters. The best performers had solubility parameters between 21.5-22.5, but there were also several poor performers in that SP range.

In the best case, the methyl ester of LCA and pentenyl HPC adduct, *in vitro* enzalutamide crystallization time was nearly 8 h, considerably exceeding normal small intestine residence time. It was also gratifying and useful that the LCA derivatives performed best, since they are simpler to synthesize and pose less complex regioselectivity problems. It will be of great interest to probe further the structure property relationships of these cellulose ether–bile salt adducts, further illuminating features needed for truly superior crystallization inhibition even in difficult cases like enzalutamide, and providing further insight into the fundamental nature of and structural requirements for crystallization inhibition.

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### **Appendix A. Supplementary data**

The supplementary information for this article can be found at:

### **Notes**

The authors declare no competing financial interest.

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