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Biomimetic Glycosylated Polythreonines by *N*-Carboxyanhydride Polymerization

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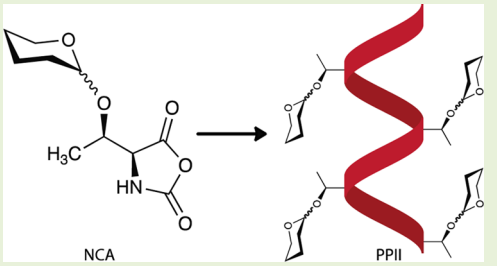
ABSTRACT: Glycosylated threonine (Thr) is a structural motif found in seemingly disparate natural proteins from deep-sea collagen to mucins. Synthetic mimics of these important proteins are of great interest in biomedicine. Such materials also provide ready access to probe the contributions of individual amino acids to protein structure in a controlled and tunable manner. *N*-Carboxyanhydride (NCA) polymerization is one major route to such biomimetic polypeptides. However, challenges in the preparation and polymerization of Thr NCAs have impeded obtaining such structures. Here, we present optimized routes to several glycosylated and acetylated Thr NCAs of high analytical purity. Transition metal catalysis produced tunable homo-, statistical, and block-polypeptides with predictable chain lengths and low dispersities. We conducted structural work to examine their aqueous conformations and found that a high content of free OH Thr induces the formation of water-insoluble β-sheets. However, glycosylation appears to induce a polyproline II-type helical conformation, which sheds light on the role of glyco-Thr in rigid proteins such as mucins and collagen.



INTRODUCTION

Polymerization of α-amino acid *N*-carboxyanhydrides (NCAs) is a technique widely used to obtain polypeptides and polypeptide hybrid materials. NCA-derived polypeptides are widely used as commodity chemicals[1] and as biomaterials for applications from tissue engineering and drug delivery to biomineralization and nanoelectronics.[2] In our own work, NCA chemistry has been used to prepare synthetic mimics of the natural glycoproteins mucins.[3−5] Mucins are the primary protein component of mucus and are involved in epithelial hydration, lubrication, and defense.[6]

All mucins are characterized by high-molecular-weight regions rich in proline (Pro) and *O*-glycosylated threonine (Thr) and serine (Ser).[7] Glycosylation initiates with α-linked *N*-acetylgalactosamine (αGalNAc). Mucin peptide backbones are thought to be highly rigid and extended due to the Pro content, which induces a polyPro (PP) II helical structure that is proposed to be further stabilized by a peptide-αGalNAcSer/Thr H-bond.[4,8−10] β-linked glycans are not observed on Ser/Thr in native mucins, and peptides with this glycan linkage take on an entirely different conformation.[11] Similarly, galactose (Gal)-Thr replaces Pro residues in the collagen of deep-sea hydrothermal vent worms and stabilizes the essential PPII helical structure.[12−14] In these studies, the anomeric linkage was not determined. Despite these interesting structural properties and biological significance, glycosylated-Thr-containing polypeptides by NCA polymerization are relatively unexplored. This is partly due to general challenges in synthesis, purification, and polymerization of Thr NCAs. Here, we report efficient methods for synthesis and polymerization of several glycosylated and acetylated (Ac) Thr NCAs and structural characterization of the resulting polypeptides.

Thr NCA was presumably first prepared in 1963 as its OAc derivative.[15] Treatment of the amino acid with phosgene in dioxane was reported to generate the NCA; however, the product was only characterized by elemental analysis and was not polymerized. Similarly, in 1964, Saito reported that free β-OH Thr NCA could be formed but polymerization attempts led to rearrangement to the 2-oxazolidinone derivative rather than the polypeptide.[16] Recent work on free β-OH Thr NCA has also shown that epimerization of the α-carbon leads to diastereomers.[17] Therefore, NCA studies have used various β-OH-protected Thrs including trimethylsilyl (TMS),[18,19] *tert*-butyl,[20] benzyl (Bn),[21] and diphenylphosphate[22] as protecting or functional groups.

Early work attempting NCA preparation under standard conditions of phosgene in dioxane or ethyl acetate was met with limited success. Thr monomers were typically only incorporated into polypeptide copolymers at low monomer feed ratios (i.e., <10%).[21] NCA yields were often not reported, and characterization was limited to elemental analysis, Fourier transform infrared spectroscopy (FTIR), melting/decomposition point, or CO_2 evolution.[19−21] One study noted that the

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NCA gave only 49% of the expected CO_2 evolution upon H_2SO_4 treatment.[15] In another study, it was observed that even at an NCA feed ratio of 50%, amino acid analysis indicated that Thr composed only 13% of the resulting polypeptide.[20] Such reports imply that NCA was of low purity.

In 1992, cyclization of *N-tert*-butoxycarbonyl (Boc) BnO-Thr to the NCA was reported in 66% yield by treatment with triphosgene followed by triethylamine (TEA), as evidenced by NMR.[23] It was noted that even after crystallization, traces of TEA hydrochloride could be observed by ^{1}H nuclear magnetic resonance (NMR). Attempts to purify Thr NCAs include passage through celite[24] and *N*-methyl morpholine as an acid scavenger in combination with complex microflow devices for washing at various pHs.[25] In these cases, no polymerization data were reported and therefore quality of the monomer cannot be judged.

The triphosgene/TEA method was later used by Gibson and Cameron to prepare BnO-Thr NCA. They added cold aqueous workup and crystallizations to further purify the NCA which was obtained in 68% yield.[26,27] Related work by Heise et al. reported similar synthesis of the NCA from BnO-Thr via treatment with phosgene and acid scavenger α-pinene followed by recrystallization to give the NCA in 60% yield.[28] In these cases, polymerization was initiated with primary amines and chain lengths of up to ca. 150 residues were achieved in tetrahydrofuran (THF). Chain extension experiments failed in this solvent but could be accomplished in dimethylacetamide. Side reactions were identified and included the formation of cyclic compounds, water- and dimethylamine-initiated polymers, and formamide-terminated chains. Protecting groups were never removed in these materials and they were noted to gel in dichloromethane. Follow-up work by Jan and co-workers utilized deprotected Thr-containing hydrogels but noted that the benzyl-protecting groups could not be fully removed as evidenced by NMR.[29−32]

Preparation of glycosylated Thr NCAs has been reported in one prior publication.[33] Peracetylated GalOAc$_4$Thr and glucose-OAc$_4$Thr NCA were prepared from the Boc AAs in ca. 50% yield. These monomers could not be polymerized due to insufficient monomer purity. Presumably, the material was also a mixture of α- and β-anomers. GalOAc$_4$Thr NCA was also mentioned in a conference proceeding but this lacked complete characterization data or polymerization data.[34] Our data reported here represent the first polymerization of glycosylated Thr (glycoThr) NCAs to give well-defined homo- and block-co-polypeptides.

EXPERIMENTAL SECTION

Materials and Instrumentation. Reactions were conducted under an inert atmosphere of N_2 and with oven-dried glassware and anhydrous solvents unless otherwise stated. Deionized water (18 MΩ cm) was obtained by passing in-house deionized water through a Thermo Scientific MicroPure UV/UF purification unit. Infrared spectra were recorded on a Bruker Alpha attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectrophotometer. Tandem size exclusion chromatography/refractive index (SEC/MALS/RI) was performed on an Agilent 1260 Infinity liquid chromatograph pump equipped with Wyatt DAWN HELEOS-II light scattering (LS) and Wyatt Optilab T-rEX refractive index (RI) detectors. Separations were achieved using 10^5, 10^4, and 10^3 Å Phenomenex Phenogel 5 μm columns using 0.10 M LiBr in dimethylformamide (DMF) as the eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 3 mg/mL. d*n*/d*c* values were calculated by batch injection of a series of polymer concentrations or determining the degree of polymerization by ^{1}H NMR of polyethyleneglycol endcapped polymers and fitting to the observed RI data. The applied d*n*/d*c* for Pα/βGalOAc$_4$Thr was 0.0295 and that for PαGalNAcOAc$_3$Thr was 0.042. d*n*/d*c* values for copolymers were calculated using the weighted average of the homopolymers with the d*n*/d*c* for PZLys being 0.123 and the d*n*/d*c* for PBnGlu being 0.104. CD measurements of the polypeptide solutions were recorded in quartz cells with a path length of 0.1 cm, on a JASCO J-1500 CD spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury spectrometer (400 MHz) or an Agilent DirectDrive spectrometer (500 MHz) and are reported relative to deuterated solvent.

O-Acetyl-L-threonine NCA, AcOThr NCA. H-AcOThr-OH (1.287 g, 1 equiv) was suspended in dry THF (79.8 mL, 0.1 M) under N_2. Phosgene (15 wt % in toluene, 11.39 mL, 2 equiv) was added to the suspension. The reaction mixture was cooled to 0 °C and distilled TEA (1.22 mL, 1.1 equiv) was added dropwise. The reaction mixture was stirred for 3 h and reaction progress was monitored by ATR-FTIR. After 3 h, the reaction mixture was filtered through cotton to remove TEA−HCl salts. The reaction solution was evaporated under reduced pressure. The evaporate was sequestered in a tandem solvent trap system cooled by liquid N_2. The traps were immediately quenched with ammonium hydroxide. The crude product (yellow oil) was purified using anhydrous silica chromatography[35] with 5% THF in DCM. The collected fractions were analyzed by ATR-FTIR. NCA-containing fractions were combined resulting in 1.05 g of white solid (70.5% yield).

***O*-((2,3,4,6-Tetra-*O*-acetyl)-β-D-galactopyranose)-L-threonine-*N*-carboxyanhydride, βGalOAc$_4$Thr NCA.** The Boc-βGalOAc$_4$Thr (0.1555 g, 1 equiv) was dissolved in anhydrous THF (2.916 mL, 0.1 M). Triphosgene (0.034 g, 0.4 equiv) was added and the solution was cooled to 0 °C. Distilled TEA (0.0445 mL, 1.1 equiv) was added dropwise. The reaction mixture was stirred for 2.5 h and reaction progress was monitored by ATR-FTIR. After 2.5 h, the reaction mixture was filtered through cotton to remove TEA−HCl salts. The reaction solution was evaporated under reduced pressure. The evaporate was sequestered in a tandem solvent trap system cooled by liquid N_2. The traps were immediately quenched with ammonium hydroxide. The crude product was purified using anhydrous silica chromatography[35] with 5 to 10 to 15% THF in DCM. The collected fractions were analyzed by ATR-FTIR. NCA-containing fractions were combined resulting in 0.0992 g of white solid (74% yield).

***O*-((2,3,4,6-Tetra-*O*-acetyl)-α-D-galactopyranose)-L-threonine-*N*-carboxyanhydride, αGalOAc$_4$Thr NCA.** The NCA was synthesized and purified following the same conditions as described for βGalOAc$_4$Thr NCA,

***O*-(2-Acetamido-2-deoxy-3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonine-*N*-carboxyanhydride, αGalNAc(OAc)$_3$Thr NCA.** The NCA was synthesized and purified following the same conditions as described for βGalOAc$_4$Thr NCA. The crude product was purified using anhydrous silica chromatography[35] with 10 to 30% THF in DCM.

General Method for Polymerization of NCAs. All polymerizations were prepared in a N_2-filled glovebox. NCAs were dissolved in anhydrous THF at 50 mg/mL in a vial or bomb tube. To the NCA solution, a 30 mg/mL solution of $(PMe_3)_4Co$ in THF was added and the tube was sealed. The NCA/$(PMe_3)_4Co$ ratio ranged from 10:1 to 50:1, yielding different length polypeptides. The vials were left in the glovebox at RT, and the bomb tubes were removed from the glovebox and heated at 50 °C for 5−72 h. The reaction progress was monitored by ATR-FTIR. Upon completion, the polypeptides were analyzed with SEC/MALS/RI.

General Method for Polymerization of Statistical Copolymers. Copolymers were prepared in a N_2-filled glovebox in a manner similar to homopolymers. The NCAs were dissolved in THF at 50 mg/mL and mixed at a variety of NCA molar ratios. The $(PMe_3)_4Co$ catalyst in THF (30 mg/mL) was added to the combined NCA solutions and the reaction progressed at RT and was monitored by ATR-FTIR. Polypeptides that remained soluble were analyzed using SEC/MALS/RI.

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Table 1. Representative Polymerization Data for Tunable polyThrs

		block 1			block 2		
entry	name[a]	[M]/[I][b]	M_n[c]	$Đ$[d]	[M]/[I][b]	M_n[d]	$Đ$[d]
1	PβGalOAc$_4$Thr$_{47}$	10	20 060	1.46			
2	PβGalOAc$_4$Thr$_{80}$	25	34 390	1.13			
3	PβGalOAc$_4$Thr$_{154}$	50	66 430	1.17			
4	PAcOThr$_{150}$	50	21 450	n.d.[g]			
5	(PAcOThr$_{0.75}$-*s*-PZlys$_{0.25}$)$_{150}$	50	25 912	n.d.[g]			
6	(PAcOThr$_{0.5}$-*s*-PZLys$_{0.5}$)$_{208}$[e]	50	42 150	1.37			
7	(PAcOThr$_{0.25}$-*s*-PZLys$_{0.75}$)$_{173}$[e]	50	40 190	1.44			
8	(PβGalOAc$_4$Thr$_{0.5}$-*s*-PZlys$_{0.5}$)$_{103}$[e]	50	35 570	1.19			
9	(entry 8)-*b*-PZLys$_{622}$[f]				90	198 700	1.20
10	PβGalOAc$_4$Thr$_{80}$-*b*-PBnGlu$_{237}$[f]	25	34 390	1.13	50	96 990	1.51

[a]Sample name and observed polypeptide DP. [b]Molar ratio of NCA to $(PMe_3)_4Co$ in THF. [c]M_n as determined by GPC/MALS/RI and ^{1}H NMR end-group analysis. [d]$Đ$ as determined by GPC/MALS/RI. [e]The two NCA monomers were mixed before polymerization. [f]NCA 2 was added sequentially after consumption of NCA 1. [g]Sample could not be analyzed by GPC/MALS/RI or ^{1}H NMR due to poor solubility in organic and aqueous solvents, M_n is theoretical based on the M/I ratio.

General Method for Polymerization of Block Copolymers. A total of 3.8 mg of βGalOAc$_4$Thr NCA was dissolved in anhydrous THF inside a N_2-filled glovebox to form a 50 mg/mL solution. A total of 3.87 μL of 30 mg/mL $(PMe_3)_4Co$ in anhydrous THF was added to the NCA solution resulting in a 25:1 NCA/catalyst solution. The polymerization reaction was monitored by ATR-FTIR. After the reaction was complete (24 h), 4.21 mg of BnGlu NCA in THF (50 mg/mL) was added to the polypeptide solution. The polymerization progress was monitored by ATR-FTIR. SEC/MALS/RI analysis was conducted for the PβGalOAc$_4$Thr block 1 and the diblock polypeptide. A similar method was utilized to prepare the statistical block 1 polymer shown in Table 1 entry 8, 9 except that for block 1 βGalOAc$_4$Thr NCA and Z-Lys NCA were mixed prior in an equimolar ratio prior to the addition of the catalyst.

General Method for Deacetylation of PAcOThr, AcO-Containing Copolymers, and PglycoThrs. The polymer was suspended in 1 M K_2CO_3 in 1:1 MeOH/H_2O for AcOThr or 0.25 M K_2CO_3 in 1:1 MeOH/H_2O for PglycoThrs and stirred overnight. The solution was transferred to a 1 kDa spin filter and concentrated at 4000*g* for 20 min. The concentrate was diluted three times with Milli-Q and spin-filtered at 4000*g* for 20 min each time. The concentrate was recovered, frozen, and lyophilized. Samples can also be dialyzed against Milli-Q water in 2000 MWCO dialysis tubing.

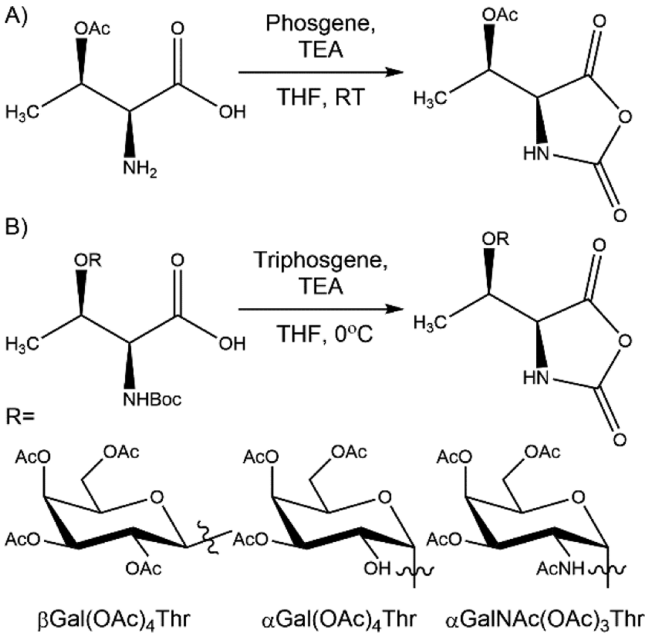
RESULTS AND DISCUSSION

We synthesized several glycoThrs as shown in Scheme 1. We selected αGalOAc$_4$, βGalOAc$_4$, and αGalNAc(OAc)$_3$ Thr as these are the structures found in natural mucins and collagen and are of interest in biomimetic materials. α/βGalOAc$_4$Thr was formed in 63% yield by coupling GalOAc$_4$-SPh with *N*-carbobenzyloxy (Z) ThrOBn using *N*-iodosuccinimide and TMS triflate (see the Supporting Information).[36] The observed α/β ratio was 1:4.7 and these could be separated by chromatography. Absolute stereochemistry at the anomeric center was determined by 2-D NMR experiments (see the Supporting Information). An alternative coupling method using Gal(OAc)$_5$, Z-Thr-OBn, and BF_3-EtO_2 was also explored but resulted in a lower yield (42%). αGalNAc-(OAc)$_3$Thr was formed via coupling of the azido phenyl-selenide sugar with Z-Thr-OBn using published procedures (see the Supporting Information).[36] Protecting groups were readily removed by hydrogenolysis on palladium over carbon.

We first explored cyclization of AcOThr, which is commercially available. We selected the Ac group since it is readily removed by treatment with a mild base, as compared to the previously used benzyl group which required harsh TMS iodide and did not result in quantitative removal. Typical NCA formation conditions are treatment of the amino acid with phosgene in THF for ca. 3 h. These conditions resulted in little formation of AcOThr NCA. We used the ratio of the ATR-FTIR AcO carbonyl stretch at 1741 cm^{-1} versus the NCA carbonyl stretch at 1787 cm^{-1} to judge reaction progress in this case and all others (see Figure S1). However, we found that the NCA was readily formed by treatment with 2 equiv phosgene and 1.1 equiv TEA in THF (Scheme 1A). The crude product was purified by anhydrous silica chromatography[35] to give the NCA as an analytically pure white solid in 71% yield. Treatment with triphosgene and TEA resulted in the formation of the NCA with a 37% yield. We also compared cyclization of Boc-AcOThr using triphosgene and TEA but found that the yield was reduced to 36%.

Next, we attempted cyclization of βGal(OAc)$_4$Thr using the same phosgene/TEA method that had resulted in good yield for AcOThr. However, ATR-FTIR did not show NCA formation after extended reaction times. Next, we attempted

Scheme 1. Synthesis of Acetylated and Glycosylated Thr NCAs



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cyclization of Boc-βGal(OAc)$_4$Thr using the previously reported method with triphosgene/TEA in THF (Scheme 1B). NCA formation was observed within 3 h via ATR-FTIR. However, the Boc group was still visible by in the FTIR spectra (see Figure S2). Neither extended reaction times nor additional triphosgene/TEA drove the reaction further. After filtration of the TEA salts and anhydrous flash column chromatography, the NCA was isolated in 74% yield and with high analytical purity. αGal(OAc)$_4$Thr could be prepared by the same method.

Kramer et al. previously reported the synthesis of αGalNAc-(OAc)$_3$Ser NCA in 73% yield by treatment of the free amino acid with phosgene and acid scavenger α-pinene.[11] We attempted cyclization of αGalNAc(OAc)$_3$Thr under these same conditions. However, after chromatographic purification, the yield was only 21%. We presume that this is due to steric hindrance by the Thr methyl group as compared to the Ser proton. Next, we Boc-protected the amino acid and used identical conditions as those for βGal(OAc)$_4$Thr. Gratifyingly, αGalNAc(OAc)$_3$Thr NCA formed in 70% yield and was obtained in high purity after chromatographic purification.

In related work in our lab cyclizing sterically hindered AAs to NCAs, we found that crude reaction materials contain significant contamination by unreacted amino acid salts, uncyclized *N*-carbamoyl chloride, and TEA salts.[37] Precipitation and repeated crystallization did not remove these materials in our hands. Other reported NCA impurities are diketopiperidine, isocyanate, and HCl.[38,39] Additional impurities can result from the reaction of THF and HCl.[40] All of these impurities are known inhibitors of polymerization or could lead to competing mechanisms.

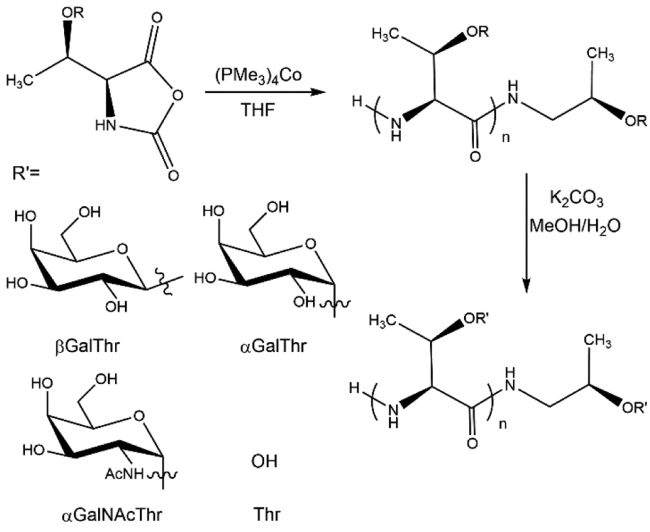
We postulate that in prior studies, such impurities were the main culprit of lower-than-expected Thr content in the polypeptides and the inability to polymerize the NCAs. Key to our work was the application of the anhydrous chromatography method which effectively removes the scope of impurities present in the NCAs.[35] The method utilizes heat-treated silica and anhydrous solvents and can be performed in the fume hood analogously to traditional chromatography.

With highly pure NCAs in hand, we explored polymerization using transition metal catalysts reported by Deming (Scheme 2).[41] Previous polymerizations of Thr NCAs were all conducted with amine or alkoxide initiators which are known to possess competing mechanistic pathways that result in poorly controlled polymerizations, low chain lengths, high dispersities, and the inability to perform chain extensions to prepare diblock polymers.[2] Transition metal catalysts typically result in controlled and living NCA polymerization and have been previously applied to a wide variety of glycosylated NCAs.[42−45]

We were pleased to observe that treatment of all the Thr NCAs with $(PMe_3)_4Co$ in THF at 25 or 50 °C (heat was applied for reactions of [M]/[I] > 50:1) resulted in complete conversion to poly(glycoThr)s (PglycoThrs) within 2−48 h, depending on monomer to initiator ratio ([M]/[I]). Complete monomer consumption was evidenced by ATR-FTIR via disappearance of the NCA carbonyl stretches at ca. 1850 and 1790 cm^{-1} and appearance of peptide carbonyl stretch at ca. 1660 cm^{-1} (Figure 1A).

We performed polymerization experiments to check for chain-breaking side reactions and to verify living chain ends. Control over PglycoThr molecular weight was demonstrated by variation of [M]/[I] ratio and subsequent analysis of the

Scheme 2. Transition Metal-Catalyzed Polymerization of Glycosylated and Acetylated Thr NCAs and Deprotection of Acetate Groups to Give Biomimetic Polypeptides



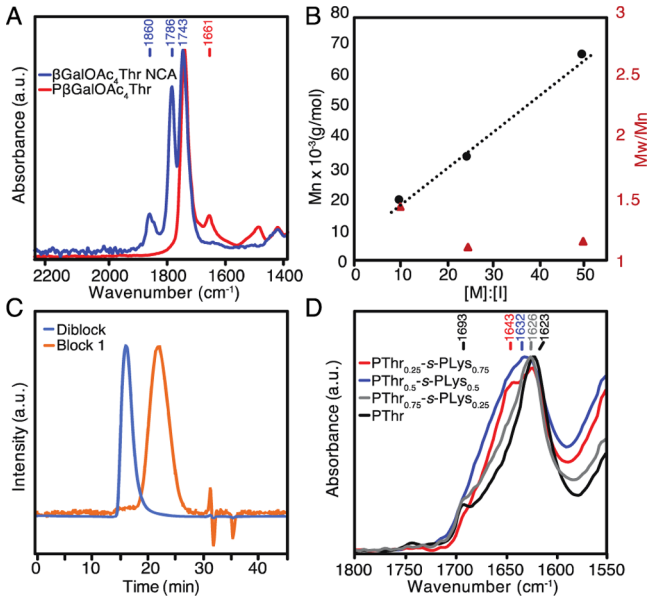


Figure 1. Polymerization data for functionalized THR NCAs. (A) ATR-FTIR spectra indicating the formation of PβGal(OAc)$_4$Thr from βGal(OAc)$_4$Thr NCA; (B) polymerization data for PβGal(OAc)$_4$Thr indicating controlled polymerization and predictable M_n, R^2 = 0.9946; (C) GPC/MALS traces indicating complete conversion from (PβGalOAc$_4$Thr$_{0.5}$-*s*-PZLys$_{0.5}$)$_{103}$ copolymer to (PβGalOAc$_4$Thr$_{0.5}$-*s*-PZLys$_{0.5}$)$_{103}$-*b*-PZLys$_{622}$ diblock copolymer, peaks at ca. 30 and 35 min are solvents; and (D) ATR-FTIR spectra of PThr homo- and co-polypeptides indicating varied sheet-forming propensity that increases with Thr content.

chain lengths by a combination of ^{1}H NMR end-group analysis and gel permeation chromatography coupled with tandem 18-angle LS and RI analysis (GPC/MALS/RI). Increasing [M]/[I] ratios gave rise to PglycoThrs whose lengths increased linearly with stoichiometry. Data for polyβGal(OAc)$_4$Thr (PβGal(OAc)$_4$Thr) are shown in Figure 1B and Table 1 entries 1−3. Similar results were obtained for the other glycosylated monomers (see the Supporting Information). Chain lengths up to ca. 150 residues were observed and they

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possessed narrow distributions (M_w/M_n). We observed a higher M_w/M_n for the lowest M_n sample and for an AcOThr copolymer (*vide infra*, Table 1 entries 1 and 7) likely due to broad elution as the GPC columns are not as efficient at separating low-molecular-weight materials and the copolymer has limited solubility in the GPC solvent. As expected, polymers had higher than theoretical molecular weights due to the known efficiency of $(PMe_3)_4Co$ initiation in THF.[46]

To check that the polymerization is indeed living, we performed chain extension experiments. βGal$(OAc)_4$Thr and *N*-ε-Z-L-Lys (ZLys) NCAs were mixed at a 1:1 ratio and treated with $(PMe_3)_4Co$ at an [M]/[I] of 50:1. After complete consumption of the NCAs by ATR-FTIR, a sample was removed for analysis and a 1.9 molar equivalent solution of ZLys NCA was added to the remainder. Again, all monomer was consumed. GPC/MALS/RI analysis of the $(P\beta GalOAc_4Thr_{0.5}$-*s*-$PZLys_{0.5})_{103}$ block 1 versus the diblock copolypeptide showed a complete shift with no evidence of unreacted block 1 (Figure 1C, Table 1 entries 8 and 9). We also conducted a chain extension experiment in the same manner with the homopolymer $P\beta GalOAc_4Thr_{80}$ and the addition of γ-benzyl-L-glutamate (BnGlu) NCA to form the second block. Again, the monomer was completely consumed and an M_n shift was observed by GPC/MALS/RI (Table 1 entry 10, Figure S7).

We also explored polymerization of AcOThr NCA. Similar to the glycoThr NCAs, AcOThr NCA was readily polymerized with $(PMe_3)_4Co$ in THF at 25 °C as evidenced by ATR-IR (Table 1 entry 4, see the Supporting Information). However, upon treatment with the initiator, AcOThr NCA solutions immediately become opaque and the poly(AcOThr) (PAcOThr) eventually precipitated (Figure S1). The homopolypeptide is not soluble in common organic solvents, including our GPC solvent of 0.1 M LiBr in DMF. This is due to the formation of β-sheet structures (*vida infra*). Therefore, we explored statistical copolymers comprising AcOThr and ZLys.

AcOThr NCA was copolymerized with ZLys NCA at 25, 50, and 75% ratios (Table 1 entries 5−7). Statistical copolymers with 25 and 50% AcOThr were fully soluble in THF and DMF. However, polypeptides with 75% AcOThr were poorly soluble (see Figure S3). The 25 and 50% AcOThr copolymers were analyzed by GPC/MALS/RI and M_n values were as expected (Table 1 entries 4 and 5). Dispersities were slightly higher than those of the PglycoThrs, which we presume is due to aggregation of minor equilibrium structures with sheet-like conformations (*vide infra*). Table 1 entries 4 and 5 are expected molecular weights as predicted by the M/I ratios used in their preparation since the sample could not be dissolved for GPC or NMR analysis.

$PAcOThr_{150}$ was deacetylated by treatment with K_2CO_3 in methanol/water overnight at room temperature. Complete removal of the protecting groups was evidenced by ATR-FTIR (see the Supporting Information). Co-polypeptides $(PThr_{0.75}$-*s*-$PZLys_{0.25})_{150}$, $(PThr_{0.5}$-*s*-$PZLys_{0.5})_{208}$, and $(PAcOThr_{0.25}$-*s*-$PZLys_{0.75})_{173}$ were first Z-deprotected by treatment with HBr in acetic acid and then subjected to the same K_2CO_3 and methanol/water conditions.[47] All of the resulting polypeptides were insoluble in water and organic solvents. In an effort to rationalize the poor solubility of polypeptides that would seemingly be water-soluble, we used ATR-FTIR to analyze their secondary structures. Peptide amides absorb infrared light via strong C═O stretching (amide I) and more minor N−H bending (amide II). Hydrogen bonds involved in the formation of secondary structures via these chemical motifs will affect the wavelength of light absorbed by these bonds. Prior work indicated that peptide bonds in β-sheet conformations have strong amide I absorbances from 1623 to 1641 and 1674 to 1695 cm^{-1}, while peptide bonds in disordered structures have shifted absorbances from 1642 to 1657 cm^{-1}.[48,49]

Analysis of the polypeptide carbonyl absorbances in the ATR-FTIR spectra revealed that the co-polypeptide secondary structures take on increasing β-sheet content with increasing Thr ratios, which explains the poor solubility. PThr had a strong absorbance at 1623 cm^{-1} and a minor peak at 1693 cm^{-1}, indicating very high β-sheet content (Figure 1D). Spacing of the Thr residues with 25% Lys only slightly shifted these absorbances (strong 1626 and 1693 cm^{-1} shoulder). PLys is completely disordered when charged but can take on helix and sheet conformations with changes in pH and temperature.[50,51] Increasing the Lys content to 50% shifted the amide I to a broader absorbance at 1632 cm^{-1}. This indicates a mixture of sheet and disordered conformations, but sheets still dominate. Finally, increasing the Lys content to 75% revealed a structure with amide I absorbances at both 1643 cm^{-1}, characteristic of disordered peptides, and 1625 cm^{-1}, characteristic of sheets, indicating that both structures are present. These data indicate that Thr is a remarkably strong sheet-inducing amino acid.

PglycoThrs were quantitatively deprotected by treatment with K_2CO_3 in methanol/water and then were purified by dialysis or spin-filtering (Scheme 2). All of the PglycoThrs were fully water-soluble. We used ATR-FTIR and circular dichroism (CD) to investigate their aqueous conformations. All ATR-FTIR spectra revealed amide I absorbances from 1648 to 1658 cm^{-1} (see the Supporting Information). α helices and PPII helices absorb at longer wavelengths from 1648 to 1657 cm^{-1} as compared to sheets and disordered structures.[48,49] Absorbances in this range could indicate ordered, helical structures. Clearly, the presence of the sugars disrupts the sheet formation that was so strongly induced by free-OH Thr.

CD spectroscopy was utilized to further characterize PglycoThr conformations. We observed interesting non-canonical spectra for all three glyco-polypeptide variants (Figure 2A). PβGalThr had a minimum at 198 nm and a positive maximum at 218 nm. There was a very slight shift in the observed dichroisms for PαGalThr with a minimum at 196 nm and a maximum at 217 nm. PαGalNAcThr had a strong positive maximum at 192 and a positive maximum at 213 nm. We attribute the shift from 217 to 218 nm for PGalThrs to 213 for PαGalNAcThr to be due to the αGalNAc amides, which are thought to be involved in hydrogen-bonding to the peptide backbone.[8] Such a hydrogen bond could affect the energetics of the $\eta \rightarrow \pi^*$ transition. We attributed the differing absorption in the 190−200 nm range to be due to absorption by the GalNAc amide itself. We obtained a CD spectrum of free GalNAc monosaccharide for comparison and observed a strong positive absorption between 190 and 200, similar to that of the PαGalNAcThr spectra (see Figure S8). Details of CD sample preparation and concentrations can be found in the Supporting Information and Table S2. All data are reported in molar ellipticity, which normalizes for samples of differing concentrations.

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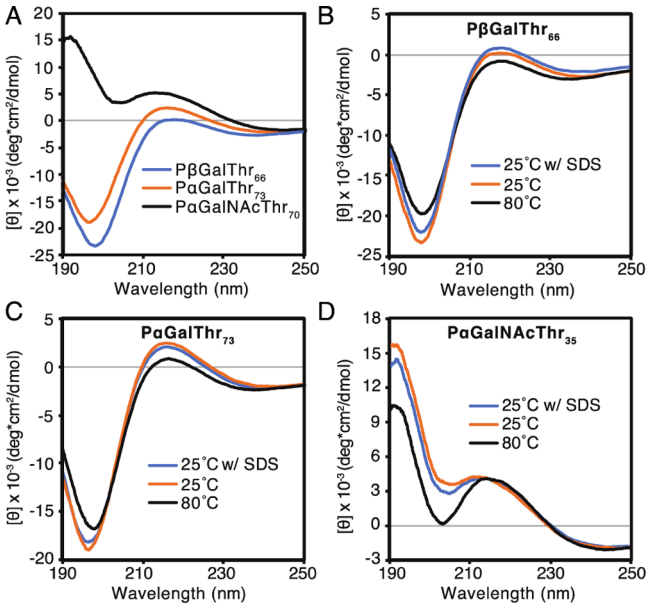

Figure 2. Conformational analysis of PglycoThrs by CD spectroscopy in water and under denaturing conditions. (A) Overlaid spectra of PβGalThr$_{66}$, PαGalThr$_{73}$, and PαGalNAcThr$_{70}$ at 25 °C in Milli-Q water; spectra of (B) PβGalThr$_{66}$, (C) PαGalThr$_{73}$, and (D) PαGalNAcThr$_{35}$ in Milli-Q water at 25, 80 °C, or in SDS solution. All spectra are reported in molar ellipticity.

β-sheets typically have negative bands at 218 nm and positive bands at 195 nm, while disordered proteins have a very minor, negative ellipticity above 210 nm and a negative band around 195 nm.[52] α helices have negative minima at 208 and 222 nm and a positive maximum near 195 nm. PglycoThrs do not fit these patterns. Considering the high content of glycosylated Thr in mucins and specialized forms of collagen that take on PPII conformations, we wondered about the contributions of Thr to this structure and if those conformations might be present in these structures. Distinct CD signatures have been shown for PPII vs unordered secondary structures. Collagen is a particularly useful example. Ordered PPII collagen has a large negative minimum at 197 and a small positive maximum at ~220.[53] However, denatured collagen has a negative minimum at 200 nm and no positive maxima fitting the canonical disordered pattern. The positive maxima between 213 and 218 nm of the glycoThrs fit most closely with PPII-type structures. It is worth noting that despite equivalent phi and psi angles, the absorbances of pure polyPro in the PPII form are red-shifted 8−10 nm as compared to ordered collagen since Pro imino groups alter the energetics of the $\eta \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions.[54]

To further probe the notion of order vs disorder in these structures, we investigated their CD conformations under denaturing conditions. Ellipticities that increase or decrease in magnitude with changes in temperature or denaturant are uncharacteristic of disordered peptides. We examined the CD of the PglycoThrs with high heat and sodium dodecyl sulfate (SDS). For PαGalThr, PβGalThr, and PαGalNAcThr, the dichroism strength was reduced in terms of the positive maxima between 213 and 218 nm and the absorbance at 192 and 198 nm (Figure 2C,D). This was true for both heat and SDS, indicating that the peptide backbone is ordered and that the order can be disrupted, likely due to breaking hydrogen bonds. We noted that after heating to 80 °C, the original conformation was restored upon return to 25 °C (see Figure S9).

Considering the dominating, sheet-inducing effects of free-OH Thr that we observed with copolymers containing only 25% PThr, we wondered about the induction of PPII-type structures by glycosylated Thr. To explore this, we prepared a panel of statistical copolymers composed of glutamic acid (Glu) and αGalNAcThr, with αGalNAcThr content ranging from 10 to 80%. PolyGlu is a disordered coil when charged and α helical when neutral.[55] CD of these co-polypeptides revealed that the αGalNAcThr-induced PPII-like structure dominates the conformation but that spacing of these residues relaxes the structure (Figure 3D). The $\eta \rightarrow \pi^*$ transition for 100%

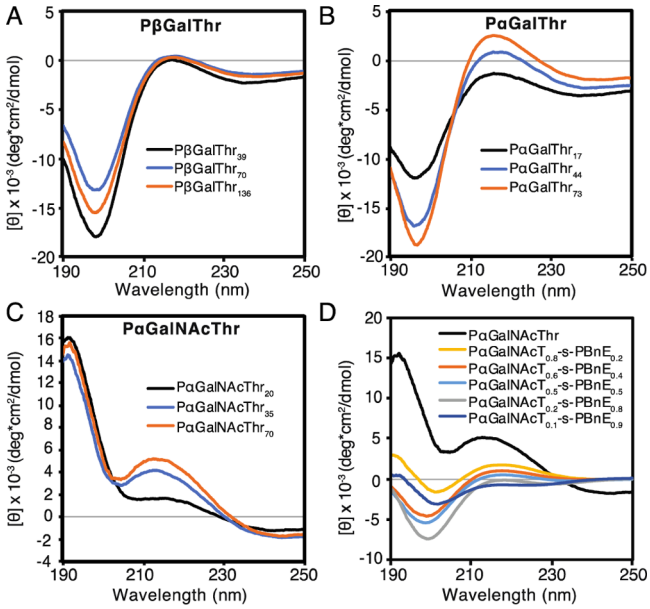

Figure 3. Conformational analysis of glyco-PThr homo- and co-polypeptides by CD spectroscopy in water. Varied molecular weights of (A) PβGalThr; (B) PαGalThr; (C) PαGalNAcThr; (D) PαGalNAcThr-*s*-PGlu co-polypeptides. All spectra were obtained at 25 °C in Milli-Q water and reported in molar ellipticity.

αGalNAcThr occurred at 213 nm and was of higher magnitude than that of 80% αGalNAcThr, whose transition shifted to 217 nm. Further spacing of the residues in 50 and 60% αGalNAcThr continued this trend. Polypeptides with 20% αGalNAcThr have an $\eta \rightarrow \pi^*$ at 218 but the ellipticity remained negative. Finally, at only 10% αGalNAcThr and 90% Glu, a disordered structure was observed. Similar to free-OH Thr, αGalNAcThr is a strong secondary structure inducer.

Prior experimental and theoretical work has shown that the dichroism per residue the greater, the longer the structure and that the absorbances of rigid molecules are typically more intense than those of flexible molecules.[56,57] This rationalizes the decreasing intensity of the 213−218 nm absorbance in the Glu/αGalNAcThr co-polypeptides. To further probe this effect, we examined PglycoThrs of varied molecular weights. This study revealed that absorbance strength increased with increasing chain length but the magnitude of the effect varied depending on anomeric linkage (Figure 3A−C). Interestingly, structures in α-glycan-linked PThrs appear to be more strongly affected by chain length as compared to the β-linked. It is interesting to speculate upon the undetermined anomeric linkage in thermally stable deep-sea collagen. We hypothesize

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that the α-linkage may be predominant. In sum, our data indicate that glycosylated Thrs may play a role in stabilizing the biologically essential PPII-type structure found in mucins, collagen, and other structural proteins.

CONCLUSIONS

We have demonstrated optimized routes for preparation and polymerization of acetylated and glycosylated Thr NCAs. Glycosylated Thr is a conserved structure found in mucins and in heat-stable collagen. We prepared glycoconjugate amino acids featuring these native linkages. We explored various NCA cyclization substrates and conditions, which yielded NCAs of high analytical purity. Polymerization using transition metal catalysts produced tunable homo-, statistical, and block-polypeptides with predictable chain lengths and low dispersities. We found that polypeptides with high content of free-OH Thr form water-insoluble β-sheets. Glycosylated PThrs, however, are water-soluble and appear to take on a PPII-type conformation, providing insights into the evolutionary advantage of these molecular motifs in rigid proteins such as mucins and collagen.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.2c00020.

Detailed information about experimental procedures, NMR, FTIR, CD, and related data (PDF)

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ABBREVIATIONS

THF, tetrahydrofuran; ATR-FTIR, attenuated total reflectance-Fourier transform infrared; GPC MALS/RI, gel permeation chromatography, 18-angle light scattering/refractive index; NMR, nuclear magnetic resonance; CD, circular dichroism; SDS, sodium dodecyl sulfate

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