Electronic Supporting Information for:

"Carboxylated Glucuronic Poly-amido-saccharides as Protein Stabilizing Agents"

Sarah E. Stidham, Stacy L. Chin, Eric L. Dane, and Mark W. Grinstaff

Departments of Chemistry and Biomedical Engineering, Boston University

Contents

General Methods and Instrumentation

All solvents were dried and freshly distilled prior to use or were purchased dry from Sigma-Aldrich. All reagents were obtained from Sigma-Aldrich, Acros, or Carbo-Synth and used without further purification. The EnzChek® lysozyme assay kit was purchased from Invitrogen and used as per manufacturer instructions. ^{1}H NMR spectra were recorded on a Varian INOVA 400MHz spectrometer or a Varian INOVA 500MHz spectrometer. Broad or overlapping peaks, often noted in the spectra of polymers, are denoted "br." 2D NMR spectra were recorded on a Varian 500MHz VNMRS spectrometer. Infrared spectroscopy was performed on a Nicolet FT-IR with ATR on powder samples. Gel permeation chromatography (GPC) in THF or aqueous buffer (0.1M $NaNO_3$, 0.01M Na_2HPO_4, 0.02% NaN_3, pH 7.6) was performed using a Waters HR-5 organic column with a flow rate of 1 mL/min or a PL aquagel-OH 60 column with a flow rate of 0.5 mL/min. The system was calibrated against polystyrene or dextran standards. Thermogravimetric analysis (TGA) was performed on a TA instruments TGA Q50. Circular dichroism (CD) experiments were performed on an Applied Photophysics CS/2 Chirascan spectrometer with a standard Mercury lamp in 7mM phosphate buffer or ethanol. Lyophilization was performed using a Virtis Benchtop 4K freeze dryer Model 4BT4K2L-105 at -40^{o}C.

Monomer and Initiator Synthesis

(1S,3R,4S,5R,6R)-4,5-bis(benzyloxy)-3-((benzyloxy)methyl)-2-oxa-8-azabicyclo [4.2.0] octan-7-one. The procedure was previously reported[1]. ^{1}H-NMR matched that reported in the literature.

Perfluorophenyl 2-((*tert*-butoxycarbonyl)amino)acetate. Boc-protected glycine (2.00g, 11.4mmol) and pentafluorophenol (2.31g, 12.6mmol) were dissolved in 30mL dichloromethane. *N,N'*-Dicyclohexylcarbodiimide (2.59g, 12.6mmol) in 20mL dichloromethane was added to the amino acid solution slowly. The reaction was stirred at room temperature under N_2 for three hours, then filtered through Celite to remove the resulting dicyclohexylurea side-product. The product was precipitated from hexanes as a white, fluffy solid (2.83g, 73% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.05 (s, 1H, NH), 4.30 (s, 2H, NH-*CH*$_2$-C=O), 1.47 (s, 9H, *tert*-butyl).

Polymerization Procedure

20mer Polymerization. Lactam monomer (1.5g, 3.26mmol) and glycine initiator (56mg, 0.163mmol) were dissolved in 15mL freshly distilled dried tetrahydrofuran. The reaction was cooled to 0^{o}C under N_2. 0.6 M lithium hexamethyldisilazide (0.7mL, 0.408mmol) in tetrahydrofuran was added and the reaction was stirred for one hour. After checking for completion by thin layer chromatography, the reaction was quenched with one drop of saturated ammonium chloride solution. The THF was removed, and the resulting solid was redissolved in dichloromethane. The organic layer was washed with 1M HCl, $NaHCO_3$, and brine, then dried over Na_2SO_4, filtered, and concentrated. The solid was redissolved in a minimum amount of dichloromethane and precipitated into stirred cold

pentane, to isolate a white solid (930mg, 60% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.86 (br, 1H), 7.2-6.9 (br, 15H), 5.76 (br, 1H), 4.70-4.25 (br, 5H), 4.04 (br, 2H), 3.75-3.4 (br, 4H), 2.80 (br, 1H), 1.37 (s, Boc-group).

Debenzylation of Polymers

20mer Debenzylation. Polymer (930mg, 2.02mmol) dissolved in 8mL freshly distilled tetrahydrofuran. 0.6M lithium hexamethyldisilazide (4.4mL, 2.63mmol) added. 100mL of ammonia condensed at -78^{o}C under N_2. Sodium metal washed in toluene/hexanes added to ammonia until dark blue color persists. Polymer solution added to ammonia solution, and reaction stirred at -78^{o}C under N_2 for one hour. Reaction quenched with saturated ammonium chloride, then warmed to room temperature to evaporate ammonia. Reaction mixture was washed with diethyl ether, and the aqueous layer was dialyzed for 24 hours against 100MW cutoff, then lyophilized to collect a fluffy, white powder (310mg, 81% yield). ^{1}H-NMR (400 MHz, D_2O): δ 5.705 (d, *J*=4.0 Hz, 1H, H_1), 4.07 (pseudo t, *J*=10.0 Hz, 1H, H_3), 3.69 (br s, 2H, H_6), 3.40 (m, 2H, H_4 and H_5), 2.99 (dd, *J*=11.0, 4.0 Hz, 1H, H_2), 1.39 (s, 0.5H, Boc-group); IR (ATR): 3349.62, 1672.03, 1503.95 cm^{-1}.

TEMPO Oxidation of Polymers

20mer Full Oxidation. Polymer (150mg, 0.793mmol), (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl (TEMPO, 1.25mg, 0.008mmol), sodium bromide (16.5mg, 0.16mmol), and sodium hypochlorite (14% solution, 1mL, 1.9mmol) dissolved in 8mL distilled water. Reaction stirred at room temperature while pH maintained at 10.5 by addition of 0.5M

NaOH by pH-stat. Reaction complete when pH remains stable, usually within five minutes. Reaction quenched with ethanol, then dialyzed for 24 hours against 500MW cutoff, then lyophilized to collect a fluffy, white powder (150mg, 83% yield). ^{1}H-NMR (400 MHz, D_2O): δ 5.80 (t, *J*=26 Hz, 1H, H_1), 4.14 (pseudo t, *J*=14 Hz, 1H, H_3), 3.75 (d, *J*=8 Hz, 1H, H_5), 3.46 (pseudo t, *J*=12 Hz, 1H, H_4), 3.025 (dd, *J*=8, 4 Hz, 1H, H_2), 1.40 (s, 0.8H, Boc-group). ^{13}C-NMR (126 MHz, D_2O): δ 176.20, 169.78, 74.26, 73.07, 72.24, 68.13, 51.22, 27.65. IR (ATR): 3340.69, 1675.43, 1607.05, 1524.38, 1414.36 cm^{-1}.

20mer Half-oxidation. Polymer (160mg, 0.846mmol), (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl (TEMPO, 1.25mg, 0.008mmol), sodium bromide (17.5mg, 0.17mmol), and sodium hypochlorite (14% solution, 0.45mL, 0.846mmol) dissolved in 8mL distilled water. Reaction stirred at room temperature while pH maintained at 10.5 by addition of 0.5M NaOH by pH-stat. Reaction complete when pH remains stable, usually within five minutes. Reaction quenched with ethanol, then dialyzed for 24 hours against 500MW cutoff, then lyophilized to collect a fluffy, white powder (110mg, 70% yield). ^{1}H-NMR (400 MHz, D_2O): δ 5.77 (br d, *J*=16 Hz, 1H, H_1), 4.13 (br, 1H, H_3), 3.73 (br, 1.8H, ox-H_5, H_6), 3.46 (br, 1.6H, ox-H_4, H_4, H_5), 3.05 (br, 1H, H_2), 1.43 (s, Boc-group). IR (ATR): 3340.74, 1674.04, 1599.89, 1524.75, 1410.25 cm^{-1}.

Periodate Oxidation of Polymer

Oxidation of 20mer. Polymer (50mg, 0.26mmol) and sodium periodate (68mg, 0.32mmol) dissolved in 10mL distilled water. Reaction stirred at room temperature in the dark overnight. Quenched with ethylene glycol and stirred for a further 1 hour.

Dialyzed for 24 hours against 500MW cutoff, then lyophilized to collect a white solid (40mg, 80% yield). ^{1}H-NMR (500 MHz, d6-DMSO): δ 13.50 (br, enol H), 9.42 (br, aldehyde), 8.24 (br, aldehyde), 6.71 (br, amide), 5.86 (br), 5.36 (br), 3.95-4.55 (br m), 3.45-3.65 (br m).

Lysozyme Assay

Aliquots of lysozyme alone or with 100-fold by mass trehalose, free-OH polymer (50mer), sodium alginate (MW = 80k-120k), sodium hyaluronate (MW = 10k-30k), or carboxylate polymer (50mer) in PBS were frozen before solvent removal by lyophilization. Lysozyme concentration was approximately 10uM, while lyoprotectant concentration was less than 100mM. The samples were redissolved in deionized water, and the process was repeated for a total of ten lyophilization cycles. The EnzChek® lysozyme assay kit was used to measure lysozyme activity of the stressed samples relative to unstressed lysozyme. Diluted sample was incubated with *Micrococcus luteus* labeled with FITC (1mg/mL) at 37^{o}C for 1 hour. Fluorescence of lysed cell membrane (lysozyme activity) was measured as FITC fluorescence (abs 480 nm/ em 530 nm) and quantified relative to known standards. The results are provided for 5 repeats. Statistics to determine significance were calculated using the Students t test.

Polyacrylamide Gel Electrophoresis

A 10% polyacrylamide gel was loaded with a Thermo Scientific PageRuler Low Range Unstained Protein Ladder and hen egg-white lysozyme at 1 mg/mL concentration with or without DP10 PAS, DP20 oxPAS, or trehalose at 100x concentration. The gel was run at

100V for 1 hour under native conditions. The gel was stained with Coomassie blue and imaged.

Gel Permeation Chromatography Results

Protected polymers were eluted in THF on a Waters HR-5 organic column with a flow rate of 1 mL/min, calibrated against polystyrene standards. Free-OH and oxidized polymers were eluted in aqueous buffer (0.1M $NaNO_3$, 0.01M Na_2HPO_4, 0.02% NaN_3, pH 7.6) on a PL aquagel-OH 60 column with a flow rate of 0.5 mL/min, calibrated against dextran standards. PDI is calculated as Mn/Mw.

DP	MW (calc)	Prot. MW	PDI	Free-OH MW	PDI	Ox. MW	PDI
20	9.2k	6.4k	1.1	3.9k	1.1	3.9k	1.2
30	13.8k	13.0k	1.2	5.2k	1.2	5.5k 5.3k (half)	1.2 1.2
50	23k	20.0k	1.1	8.7k	1.2	8.9k	1.3

Bn-PAS Circular Dichroism Spectrum

Figure S1. CD spectrum of benzyl-protected PAS acquired in ethanol.

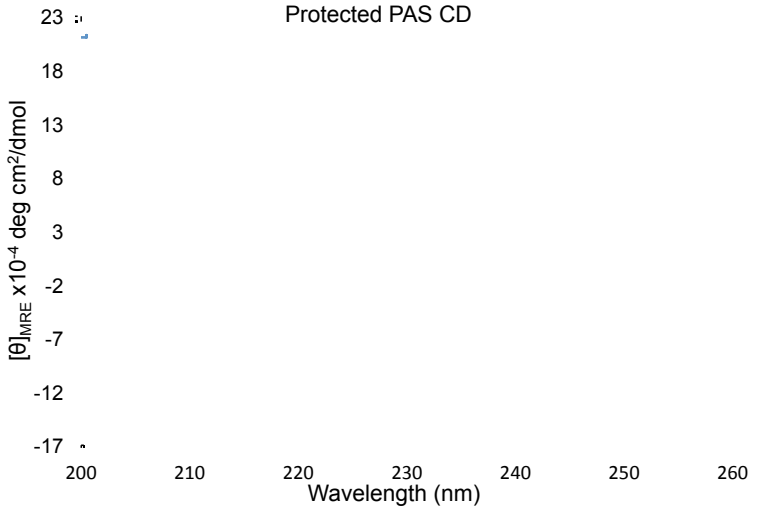


Figure S2. PAS ^{1}H-NMR

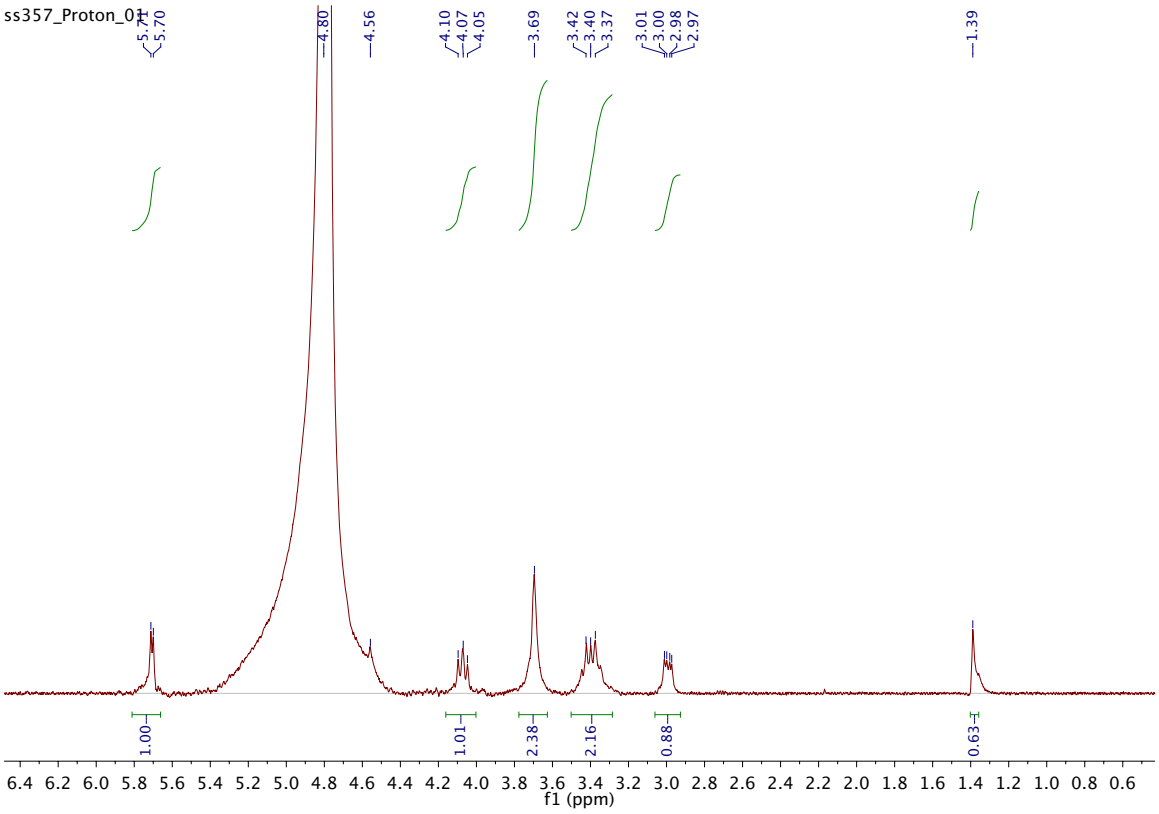


Figure S3. oxPAS ^{1}H-NMR

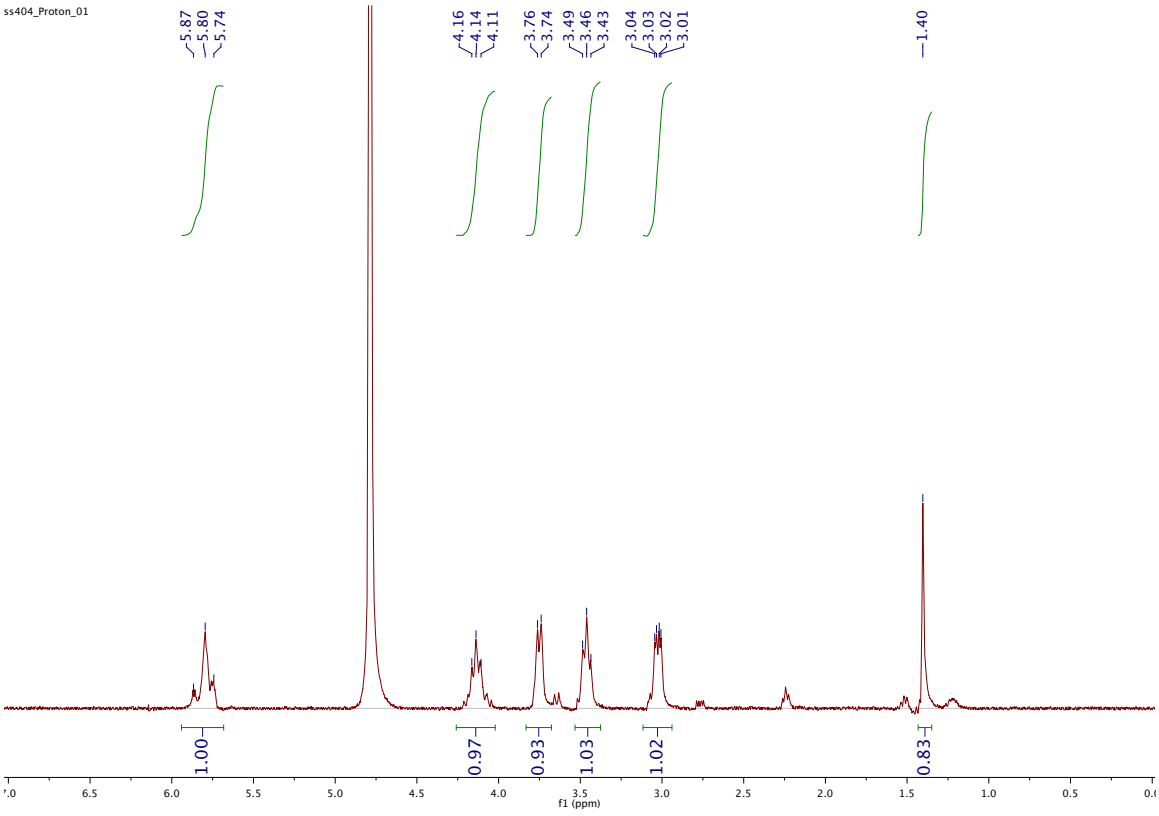


Figure S4. oxPAS ^{13}C-NMR

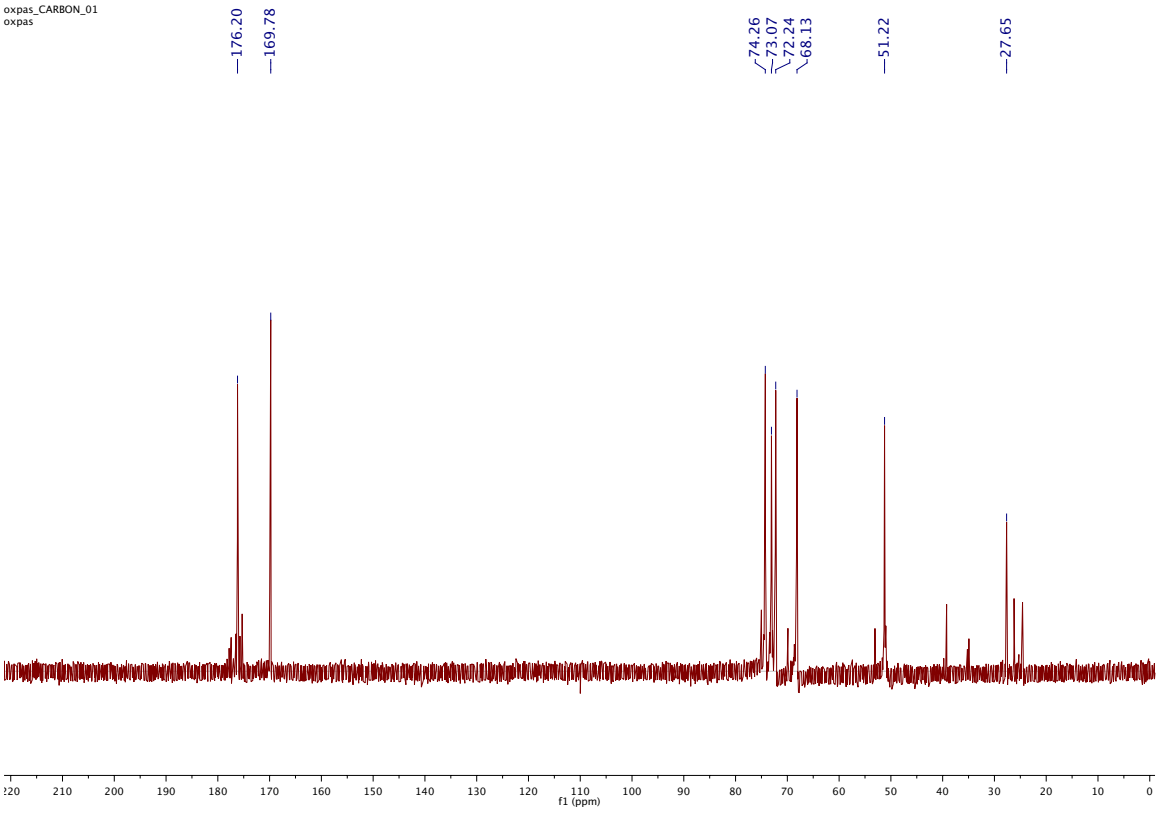


Figure S5. Half-oxPAS ^{1}H-NMR

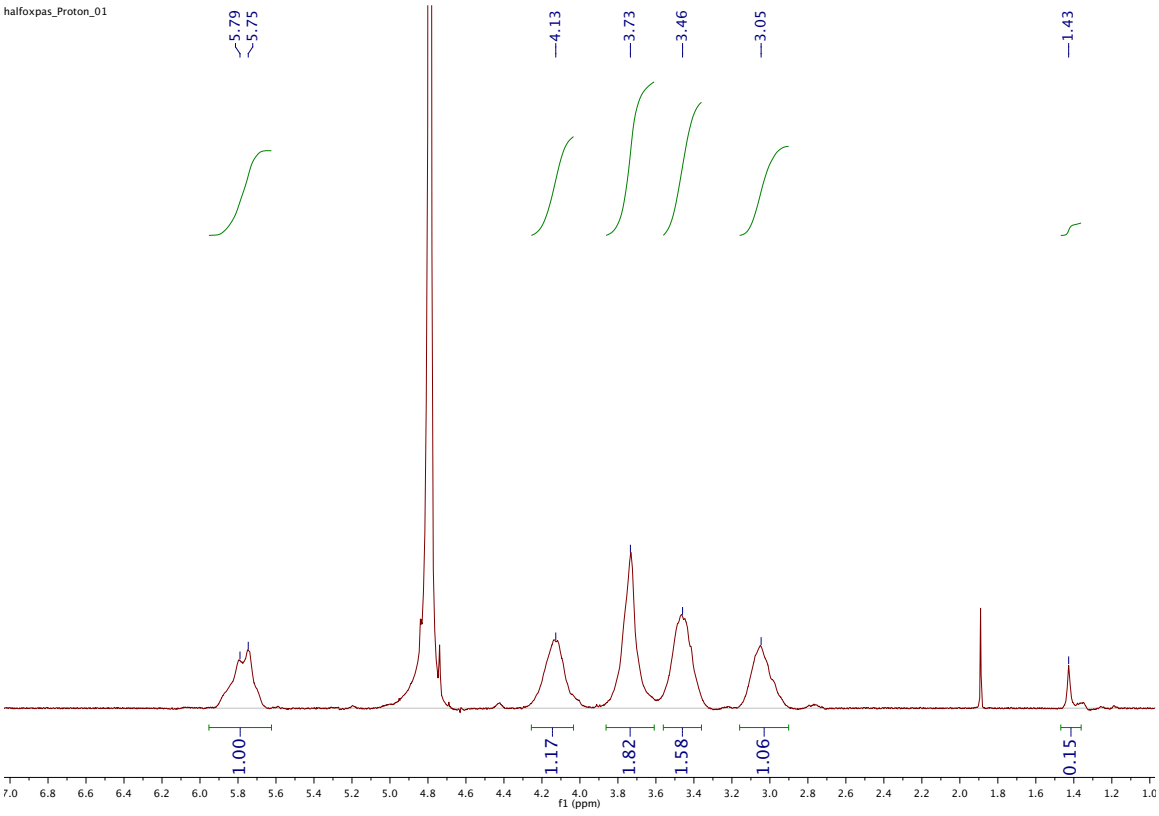
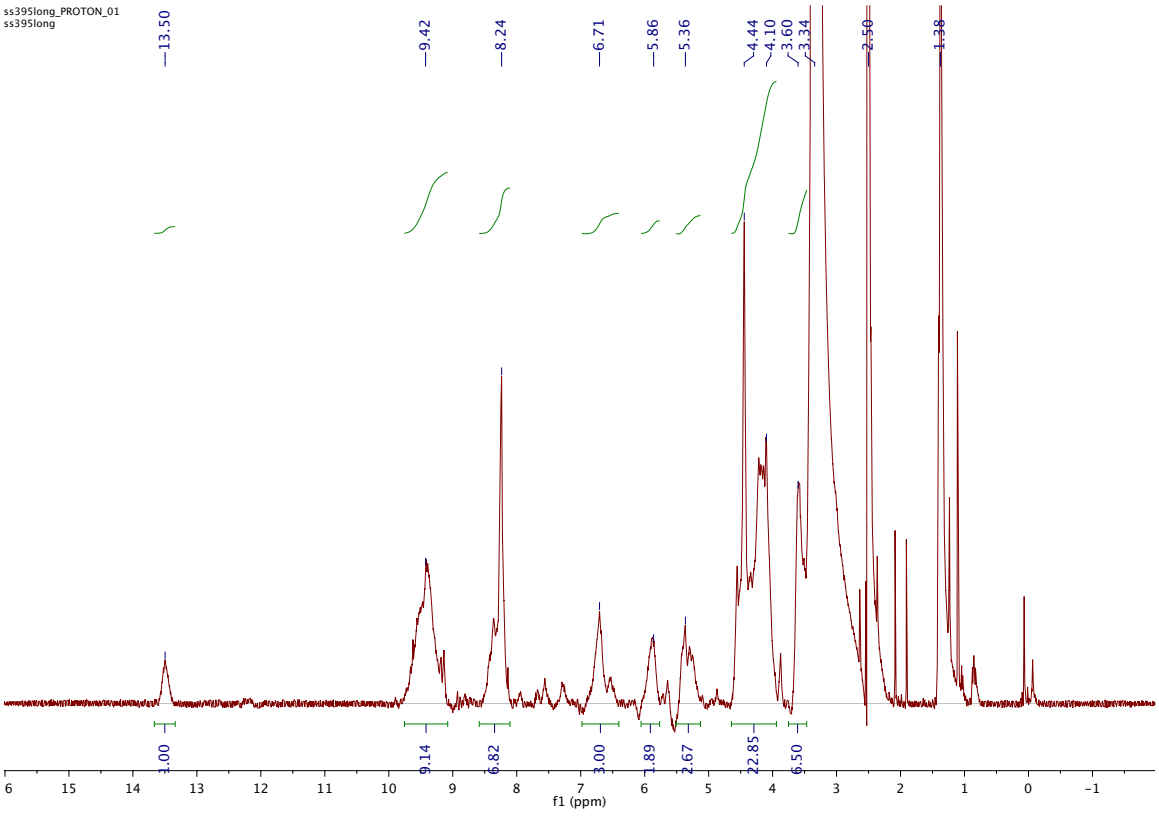
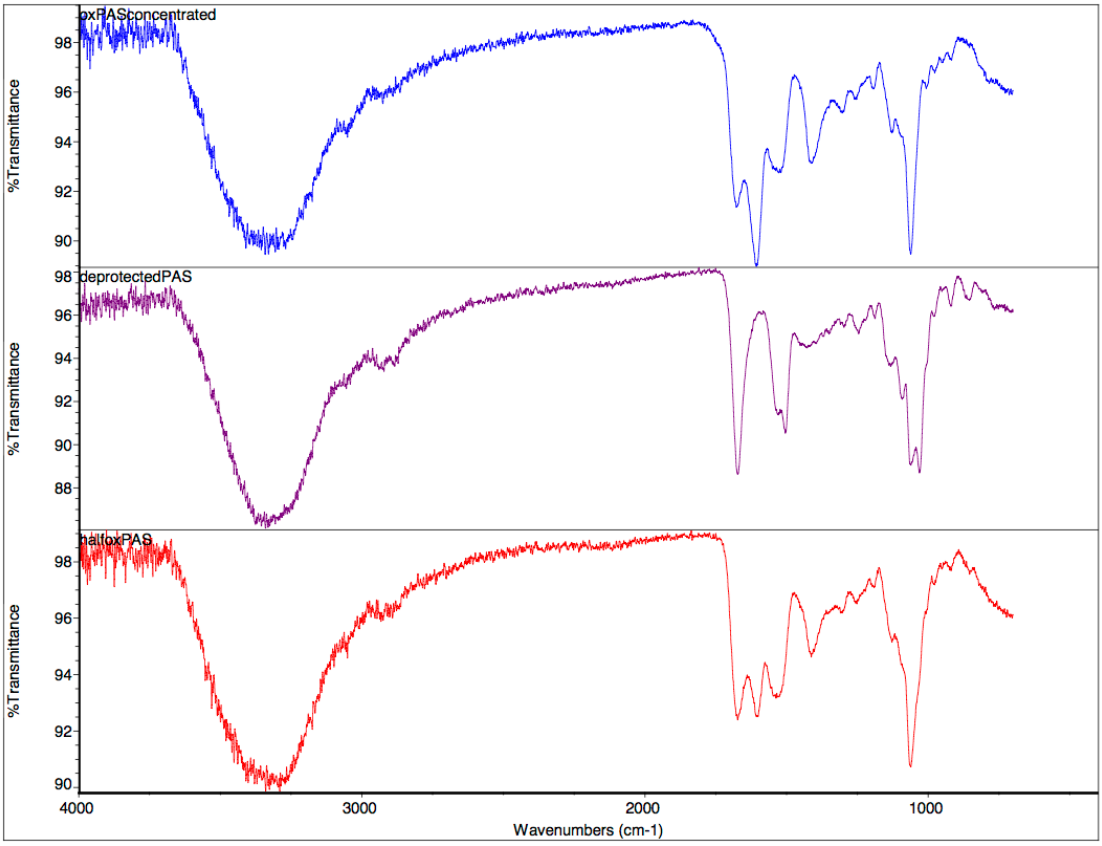


Figure S6. openPAS ^{1}H-NMR



*openPAS molecular weight did not decrease as seen by ^{1}H-NMR and GPC in aqueous buffer

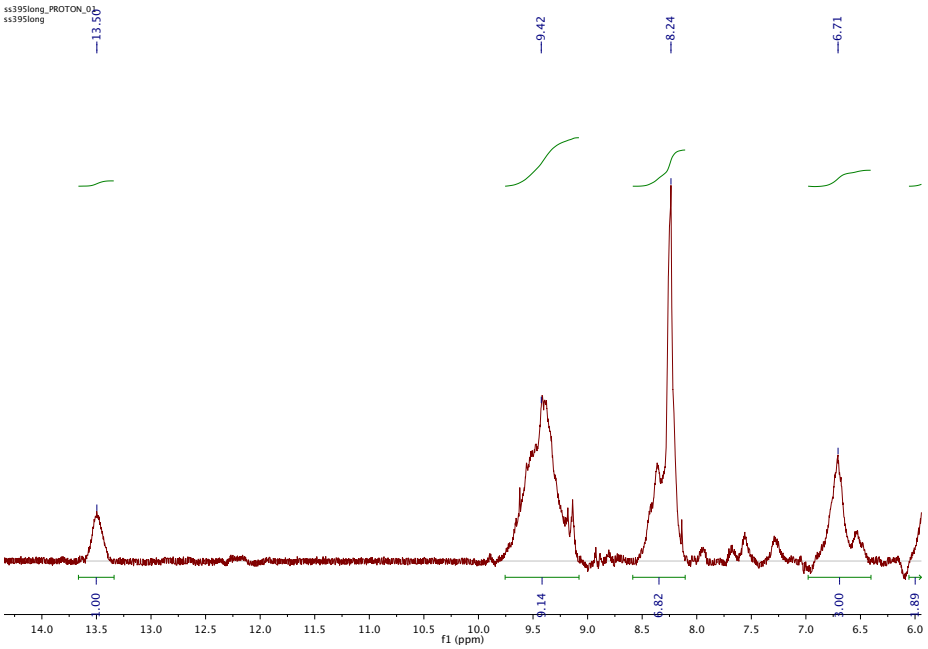
Figure S7. IR Spectra



Top: oxPAS 20mer. Middle: PAS 20mer. Bottom: halfoxPAS 20mer.

Figure S8. NH/ND Exchange

OpenPAS ^{1}H-NMR in d6-DMSO



OpenPAS ^{1}H-NMR in d6-DMSO + 10% D_2O

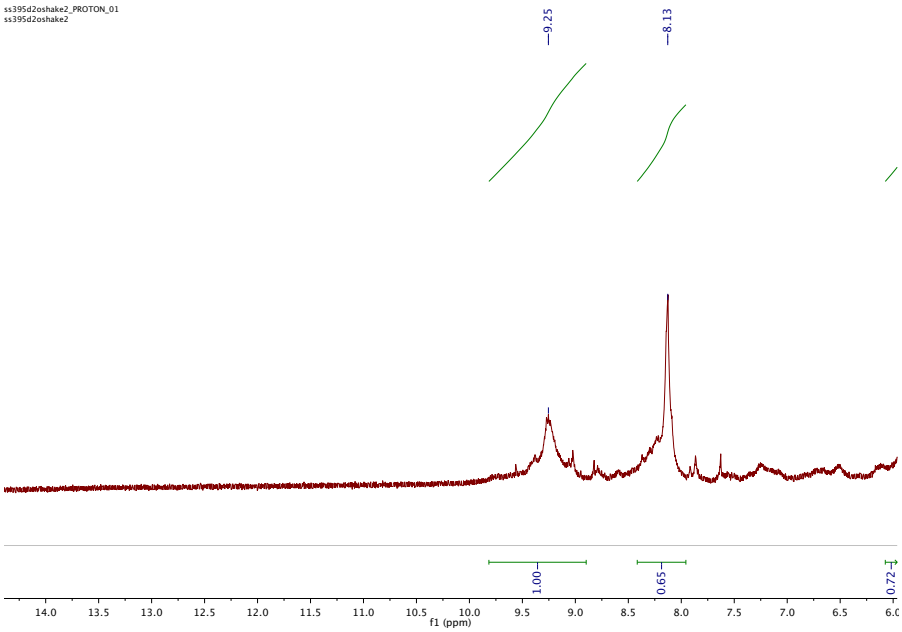
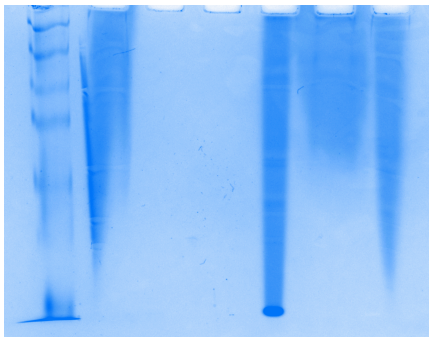


Figure S9. Lysozyme and Lyoprotectant TGA Results

Material	% Water Cycle 1	% Water Cycle 10
Lysozyme	21.33	17.79
oxPAS	12.78	8.22
Lysozyme + oxPAS	17.06	12.10
Lysozyme + sodium alginate	14.77	4.06
Lysozyme + sodium hyaluronate	4.82	1.60

Figure S10. PAGE Results

1 2 3 4 5 6 7



1. Ladder
2. Lysozyme
3. PAS
4. oxPAS
5. PAS + Lysozyme
6. oxPAS + Lysozyme
7. Trehalose + Lysozyme

References

1. E. Dane and M. Grinstaff, *J. Am. Chem. Soc.*, 2012, **134**, 16255.