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Supporting Information

Carbohydrate Antigen Delivery by Water Soluble Copolymers as Potential Anti-cancer Vaccines

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1. General Experimental Procedures and Methods for Synthesis

All reactions were carried out under nitrogen with anhydrous solvents in flame-dried glassware, unless otherwise noted. Chemicals used were reagent grade as supplied except where noted. Centrifugal filter units of 3,000 molecular weight cut-off (MWCO) were purchased from EMD Millipore. Compounds were visualized by UV light (254 nm) and by staining with a yellow solution containing $Ce(NH_4)_2(NO_3)_6$ (0.5 g) and $(NH_4)_6Mo_7O_{24}4H_2O$ (24.0 g) in 6% H_2SO_4 (500mL). Flash column chromatography was performed on silica gel 60 (230-400 Mesh). NMR spectra were referenced using residual $CHCl_3$ (δ ^{1}H-NMR 7.26 ppm), D_2O (δ ^{1}H-NMR 4.79 ppm). The molecular weight and polydispersity of the block copolymer were determined by gel permeation chromatography (GPC) at 50 °C using two PLgel 10-μm mixed-B columns with DMF as the eluting solvent. The polio virus (PV) helper T cell epitope peptide (CKLFAVWKITYKDT) was synthesized by RS Synthesis (Louisville, KY).

2. Synthesis of the block copolymer 7

Monomer **6** was prepared following a literature procedure.[1] All solutions used for polymer synthesis were treated with three freeze-pump-thaw cycles and the reaction was performed under nitrogen atmosphere. A solution of 4-(4-aminophenyl) butyric acid **1** (17.9 mg, 0.1 mmol), sodium nitrite (8.3 mg, 0.12 mmol), and a 50% aqueous fluoroboric acid solution (18.7 μL, 0.15 mmol) in a 1:1 mixture of H_2O and THF (2 mL total) was cooled to 0 °C for 30 minutes. At this time, sodium cyanate (6.5 mg, 0.1 mmol), acrylamide **3** (213.2 mg, 3 mmol), and N-(2-aminoethyl)methacrylamide hydrochloride **4** (984.4 mg, 6 mmol) dissolved in water (2 mL) were added. The reaction mixture was heated to 50 °C for 40 hours. A small aliquot of the mixture was removed and polymer **5** was purified and characterized. To the remaining

mixture, monomer **6** (797 mg, 4 mmol) and acrylamide **3** (213 mg, 3 mmol) were added and reaction was allowed to proceed at 50 °C for another 40 h. The copolymer (50% yield from **1**) was obtained by dialysis against water followed by lyophilization. ^{1}H-NMR: δ 0.75-1.10 (br m, C**H**$_3$ of amine monomer), δ 1.10-2.25 (br m, aliphatic **H**, from C**H** and C**H**$_2$ of polymer backbone), δ 2.75-3.40 (br m, aliphatic **H**, from C**H**$_2$ on the amine and ester monomer), δ 3.60 (s, C**H**$_3$ of ester monomer), δ 6.75 (br s, aromatic **H**), δ 7.00 (br s, aromatic **H**).

We normalized our peak integration to the small aromatic peaks at ~6.75 ppm (2 protons) and ~7.00 ppm (2 protons) in ^{1}H-NMR spectrum (recycle delay: 1s; pulse angle: 45 degrees; number of scans: 256; acquisition time: 14 min). For copolymer **5**, the amount of amine monomer per chain was calculated by the integration of the broad peak at ~1.0 ppm, which corresponds to methyl group on the amine monomer **4**. This matches the integration of the broad peak at ~3.25 ppm and ~3.00 ppm, which correspond to CH_2 on the amine monomer **4**. The broad peak at ~1.50 ppm is responsible for CH in the polymer backbone (from acrylamide) and CH_2 in the polymer backbone (from both acrylamide and monomer **4**). Subtracting the number of CH_2 protons on amine monomer **4** from the integration of this peak and then dividing by 3 gives the number of acrylamide monomer per chain. For copolymer **7**, the number of ester monomer per chain was determined from integration of the peak at ~3.60 ppm, which corresponds to the methyl group on the ester monomer. And the number of acrylamide was calculated in the same way as mentioned above.

Due to insolubility of copolymer **7** in DMF or THF for GPC analysis, the free amines in **7** were converted to amides by reacting with a large excess of phenylacetic acid (8 eq per amine) in the presence of TSTU (16 eq per amine) and DIPEA (16 eq per amine) to obtain the derivatized polymer, which was slightly soluble in DMF for GPC analysis (**Figure S3**). The molecular weight (Mn) of polymer **7** was calculated by subtracting the molecular weight of the phenylacetic acid units from the Mn of the derivatized polymer.

3. Synthesis of peptidic glycopolymer 13

To a solution of copolymer **7** (12.6 mg) and *N*,*N*-diisopropyl ethyl amine (DIPEA) (16.6 µL, 0.095 mmol) in anhydrous DMF (1mL) was added Tn-NHS ester **8** (53 mg, 0.072 mmol)[2] at room temperature. The reaction mixture was stirred at room temperature for 2 days. The glycopolymer **9** obtained (65% yield) was dialyzed against water and lyophilized. ^{1}H-NMR: δ 0.75-1.10 (br m, C**H**$_3$ of amine monomer), δ1.50 (br s, aliphatic **H** from Tn linker), δ1.90 (s, C**H**$_3$ from Tn), δ 1.10-2.25 (br m, aliphatic **H**, from C**H** and C**H**$_2$ of polymer backbone and Tn linker), δ 2.10-2.30 (br m, aliphatic **H** from Tn linker), δ 2.75-3.40 (br m, aliphatic **H**, from C**H**$_2$ on the amine and ester monomer and Tn linker), δ3.50-3.85 (br m, **H** from Tn), δ 4.50 (s, **H** from Tn), δ 4.05 (br m, **H** from Tn), δ 4.75 (s, anomeric **H** from Tn).

To a solution of glycopolymer **9** (14.9 mg) in water (1 mL), 0.1 M LiOH solution was added until pH~11. The mixture was stirred overnight followed by neutralization with hydrochloric acid to pH ~ 7. After dialysis and lyophilization, the residue was dissolved in anhydrous DMF (1 mL), to which DIPEA (3.8 µL, 0.022 mmol), N,N,N′,N′-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU) (1 mg, 0.003 mmol) and *S*-(2-pyridylthio)cysteamine hydrochloride (1.2 mg, 0.005 mmol) were added. Stirring was continued for 2 days, and the resulting solution was dialyzed against water and lyophilized to obtain polymer **11** (62% yield). ^{1}H-NMR: δ 0.75-1.10 (br m, C**H**$_3$ of amine monomer), δ 1.50 (br s, aliphatic **H** from Tn linker), δ 1.90 (s, C**H**$_3$ from Tn), δ 1.10-2.25 (br m, aliphatic **H**, from C**H** and C**H**$_2$ of polymer backbone and Tn linker), δ 2.10-2.30 (br m, aliphatic **H** from Tn linker), δ 2.75-3.40 (br m, aliphatic **H**, from C**H**$_2$ on the amine and ester monomer and Tn linker), δ 3.50-3.85 (br m, **H** from Tn), δ 4.50 (s, **H** from Tn), δ 4.05 (br m, **H** from Tn), δ 4.75 (s, anomeric **H** from Tn), δ 7.25 (br s, aromatic **H**), δ 7.75 (br m, aromatic **H**), 8.25 (br s, aromatic **H**).

Polymer **11** (7.3 mg) and PV peptide **12** (7.4 mg, 0.004 mmol) were dissolved in water and 1M NaOH solution was added until pH~9. After incubation for 2 days, the solution was dialyzed against water and lyophilized to obtain peptidic glycopolymer **13** in 79% yield. In order to quantify the average amount of PV peptide per polymer, a calibration curve was constructed using known amounts of PV peptide by integrating the respective UV traces on reverse phase HPLC chromatograms. The peptidic glycopolymer **13** was treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to cleave the PV peptide from **13**. After centrifuge filtration (3,000 Mw cutoff) to remove TCEP, the 10,000 Mw cutoff centrifugal filter was used to collect the solution containing the PV peptide. The amount of PV peptide coupled to peptidic glycopolymer **13** was determined through HPLC analysis by comparison with the calibration curve.

4. Synthesis of the control polymer 14

DIPEA (38.4 μL, 0.22 mmol), TSTU (33 mg, 0.11 mmol) and methoxyacetic acid (8.5 μL, 0.11 mmol) were added to copolymer **7** (14.6 mg) in anhydrous DMF (1 mL) solution. Further modification and PV peptide conjugation were performed following similar procedures as synthesis of polymer **13**.

5. Mouse immunization

Pathogen-free female mice age 6−10 weeks were obtained from Charles River and maintained in the University Laboratory Animal Resources facility of Michigan State University. All animal care procedures and experimental protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University. Groups of five mice were injected subcutaneously on day 0 with 0.1 mL of the polymer as emulsions in complete Freund's adjuvant (Fisher), according to the manufacturer's instructions. Boosters were given subcutaneously on days 14 and 28 with the

glycopolymer as emulsions in incomplete Freund's adjuvant (0.1 mL). Serum samples were collected on days 0 (before immunization), 35, and 89.

6. ELISA assays

A 96-well microtiter plate was coated with a solution of bovine serum albumin-Tn conjugate (BSA−Tn)[2] in PBS buffer (10 μg mL^{-1}) and then incubated at 4 °C overnight. The plate was washed four times with PBS/0.5% Tween-20 (PBST), followed by addition of 1% (w/v) BSA in PBS to each well and incubation at RT for one hour. The plate was washed again with PBST and mice sera were added in 0.1% (w/v) BSA/PBS. The plate was incubated for two hours at 37 °C and washed. A 1:2000 dilution of horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratory) in 0.1% BSA/PBS was added to each well. The plate was incubated for one hour at 37 °C, washed and a solution of 3,3,5′,5′-tetramethylbenzidine (TMB) was added. Color was allowed to develop for 15 min, and then a solution of 0.5 M H_2SO_4 was added to quench the reaction. The optical density was then measured at 450 nm. The titer was determined by regression analysis with $\log_{10}$ dilution plotted with optical density. The titer was calculated as the highest dilution that gave three times the absorbance of normal mouse sera diluted at 1:1600 (about 0.1 for all sera). The antibody titers against the polymer backbone were determined by ELISA against polymer **7** immobilized on ELISA plates.

7. FACS of Jurkat cells

Human lymphoma Jurkat cells (kindly provided by Profs. Barbara Kaplan and Norbert Kaminski, Michigan State University) were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, minimal essential medium nonessential amino acid, 100 units/mL each of penicillin G and streptomycin (all from Invitrogen). Mouse

sera collected on day 89 were diluted 2-fold and incubated with 10^5 Jurkat cells for 30 minutes at 4 °C. The cells were washed twice with FACS buffer (1% BSA + 0.1% NaN_3/PBS) and incubated with a 1:100 diluted goat anti-mouse IgG labeled with FITC (Jackson ImmunoResearch Laboratory, catalog #115-095-164) for 30 min at 4 °C. The cells were washed again twice with FACS buffer and re-suspended in FACS buffer. Data analysis was done with LSR II (BD Biosciences).

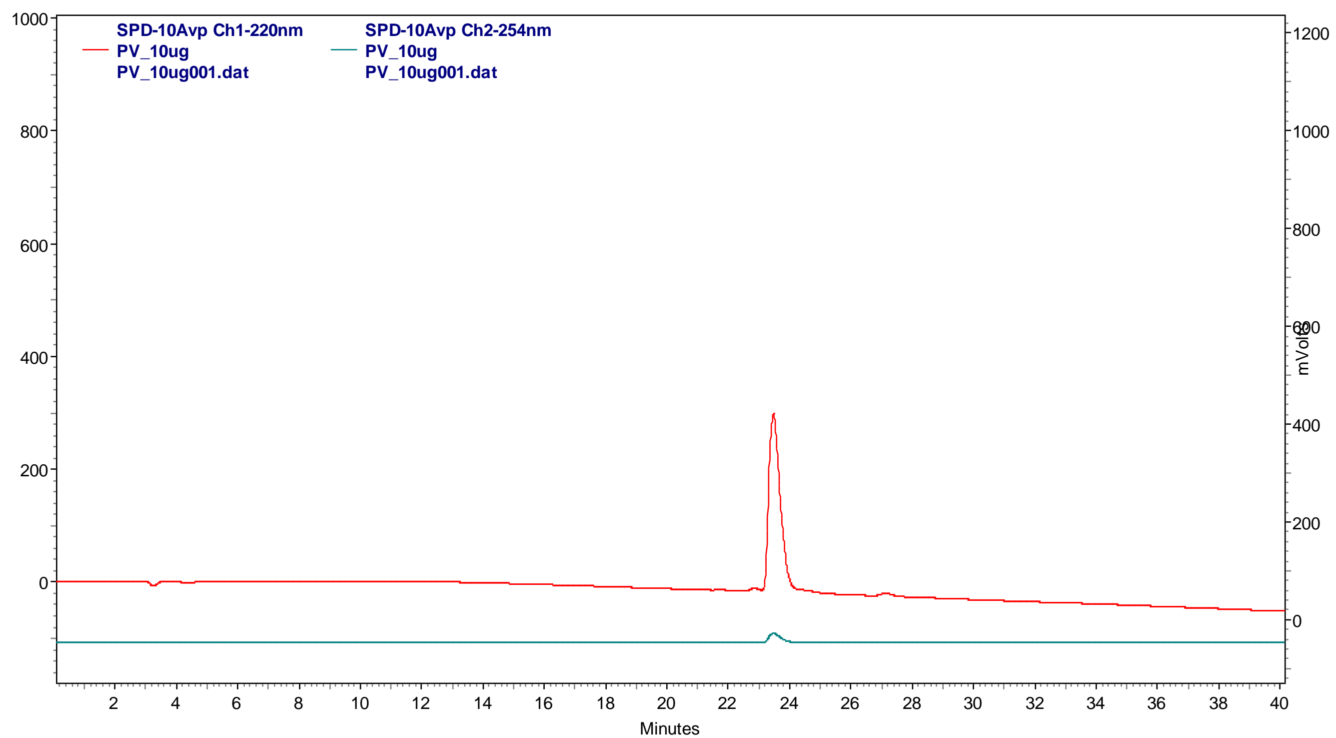


Figure S1. RP-HPLC chromatogram of 10 µg the PV peptide.

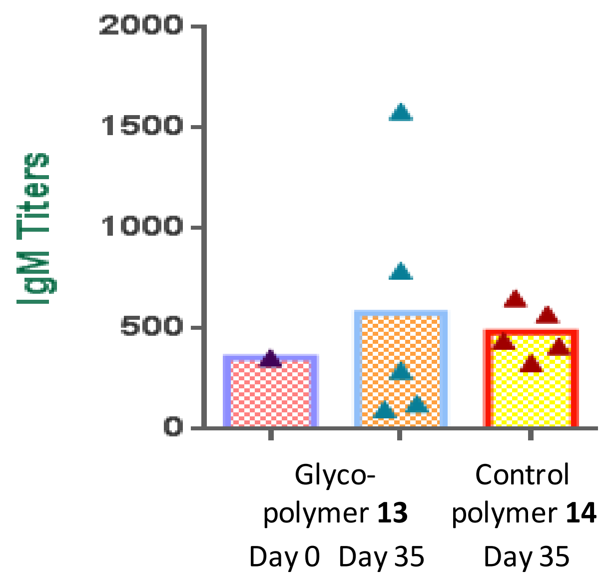


Figure S2. Anti-Tn IgM titers on days 0, and 35 from mice immunized with glyco-polymer **13** and the anti-Tn IgM titers on day 35 from mice receiving control polymer **14**.

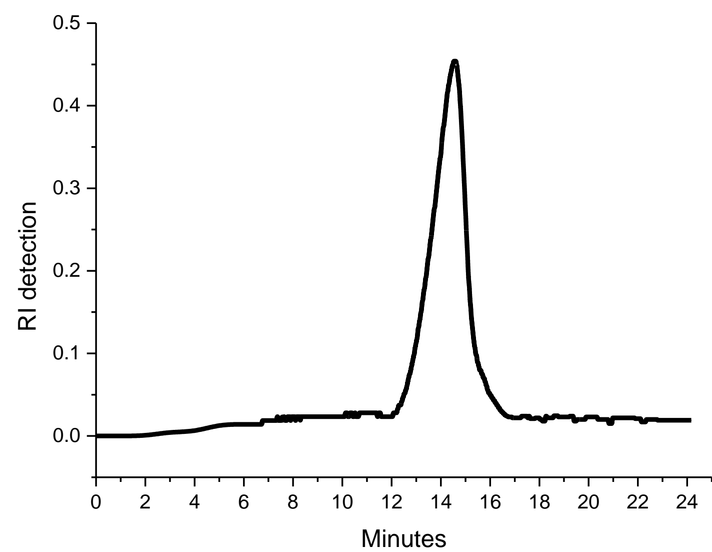
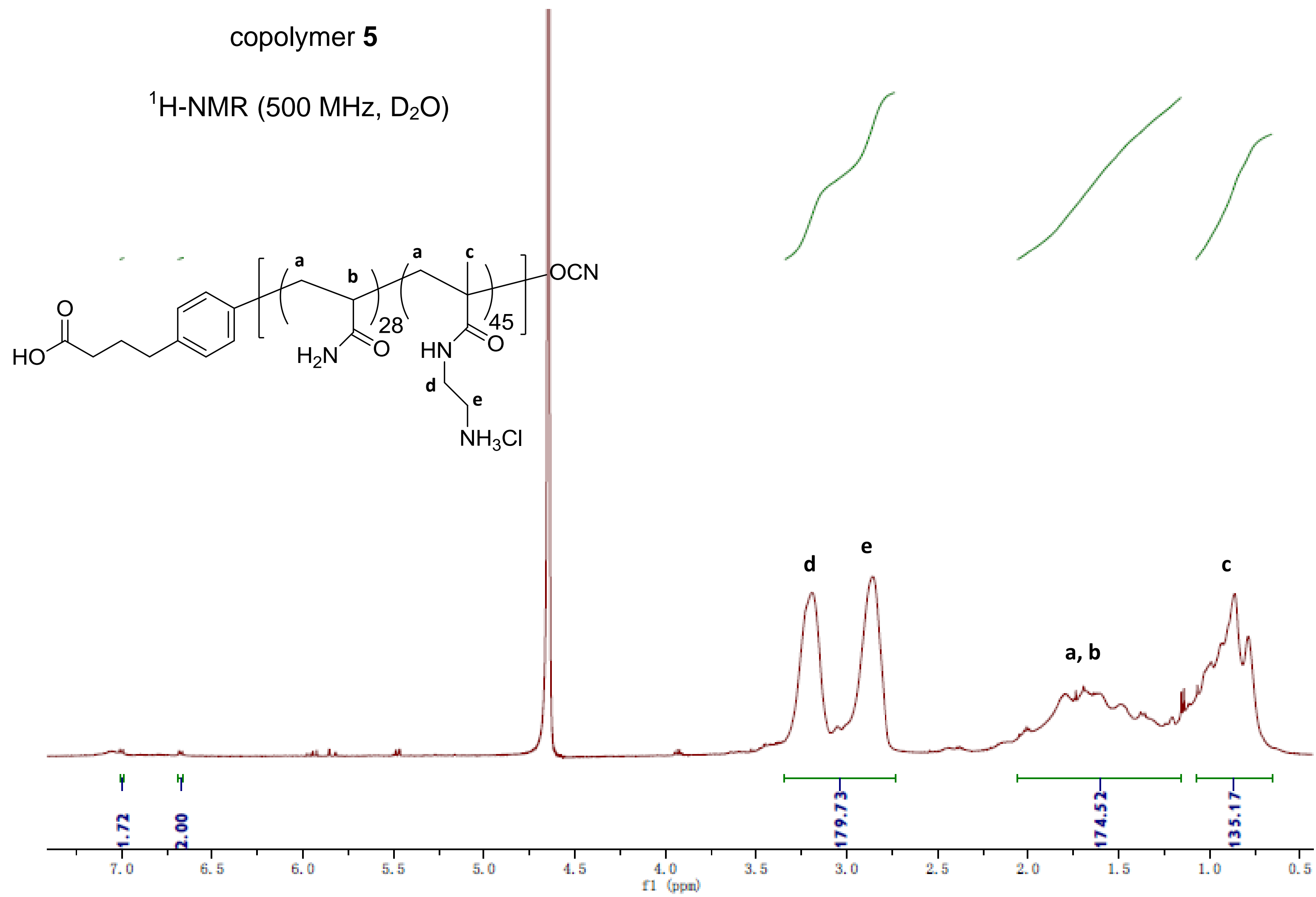
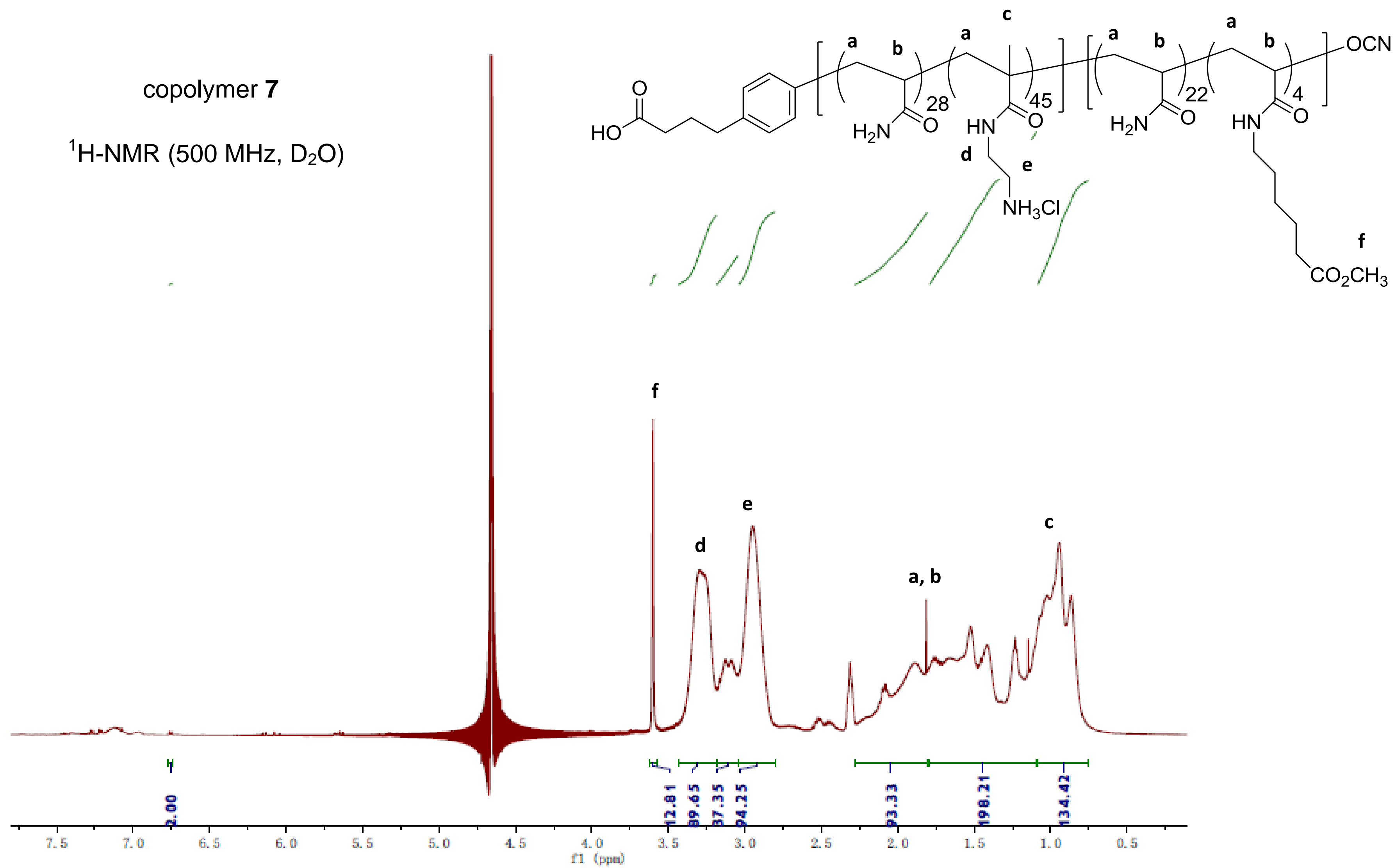
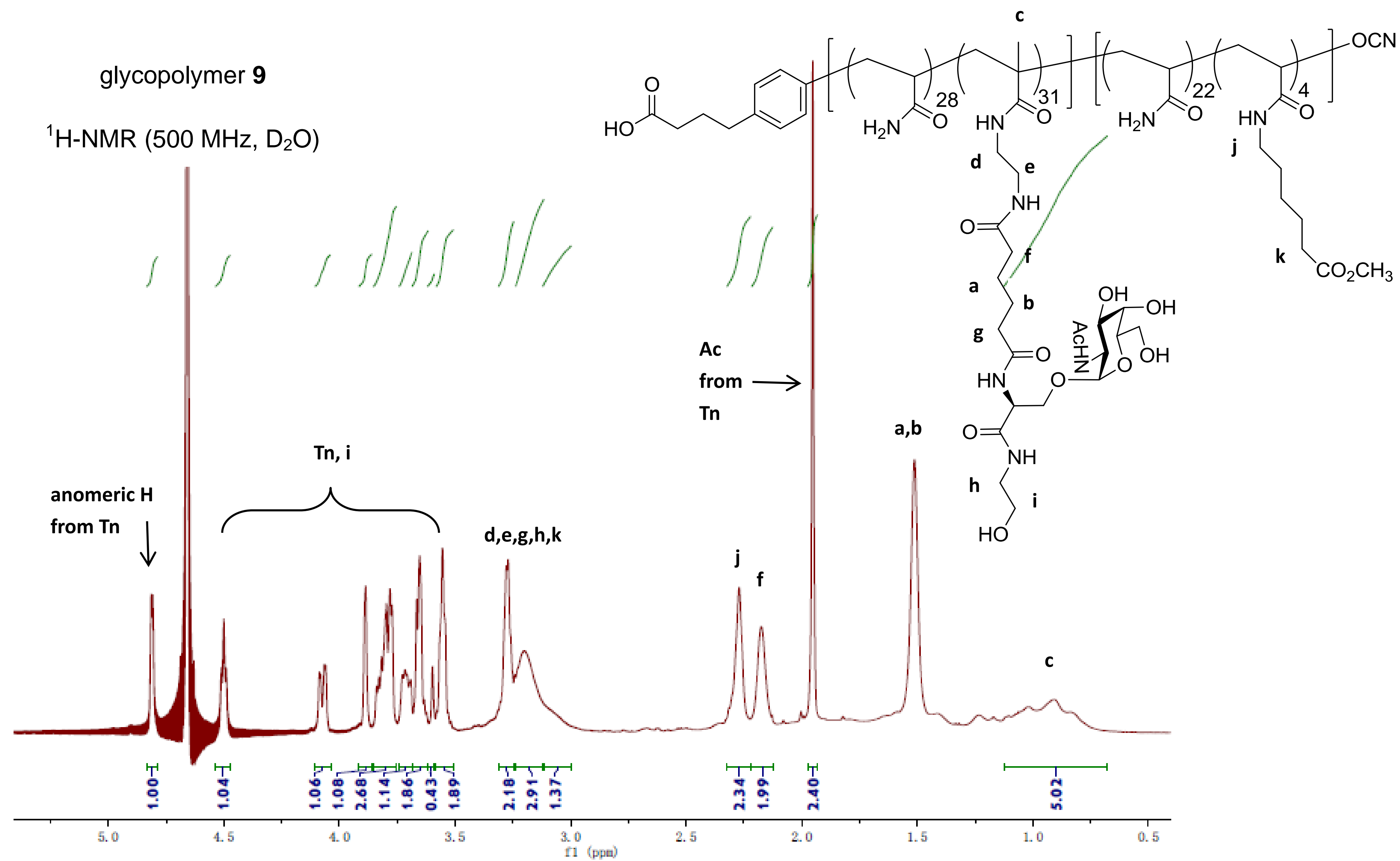


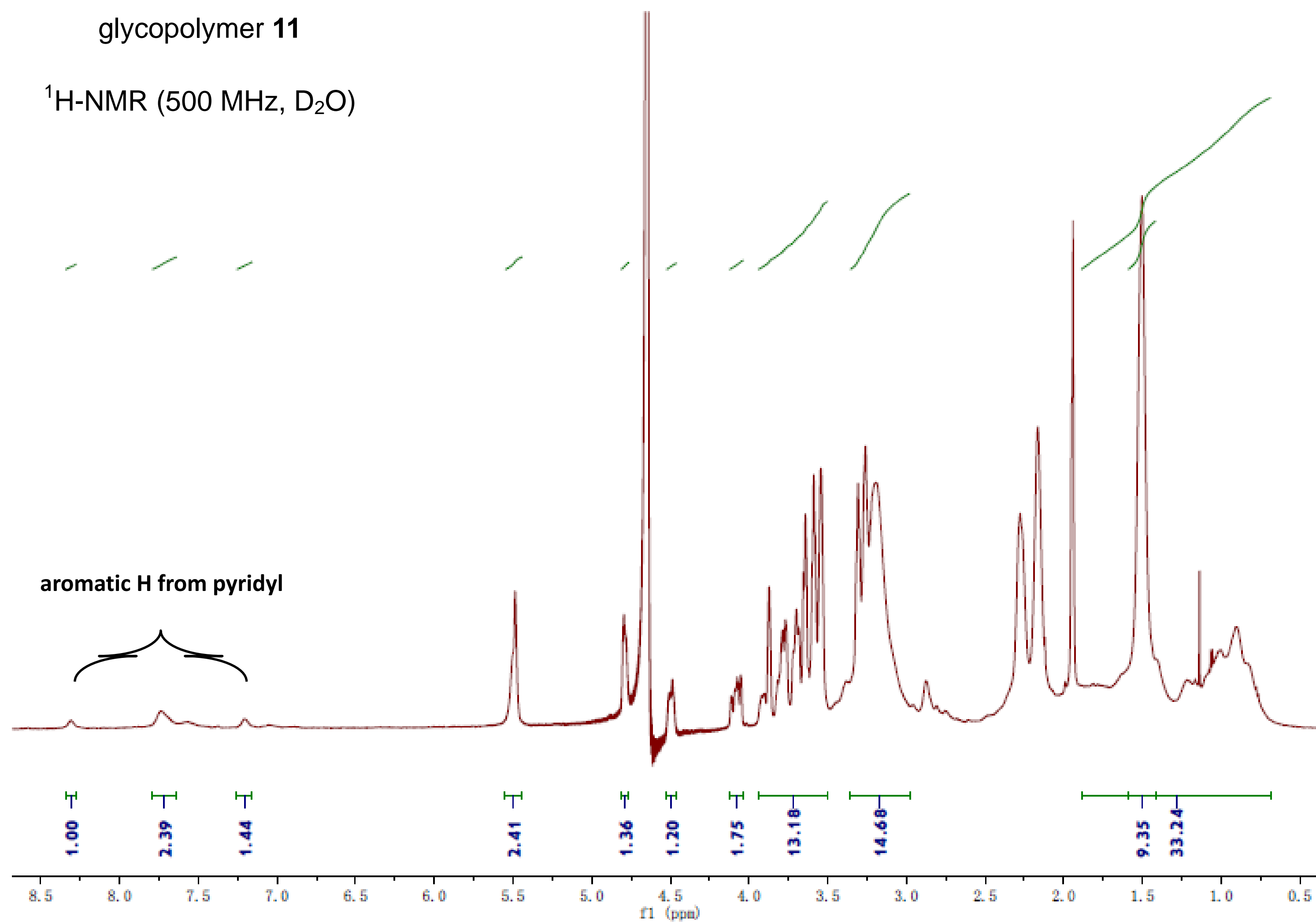
Figure S3. GPC trace of the phenyl acetic acid derivative of copolymer **7**. (Eluent: DMF)

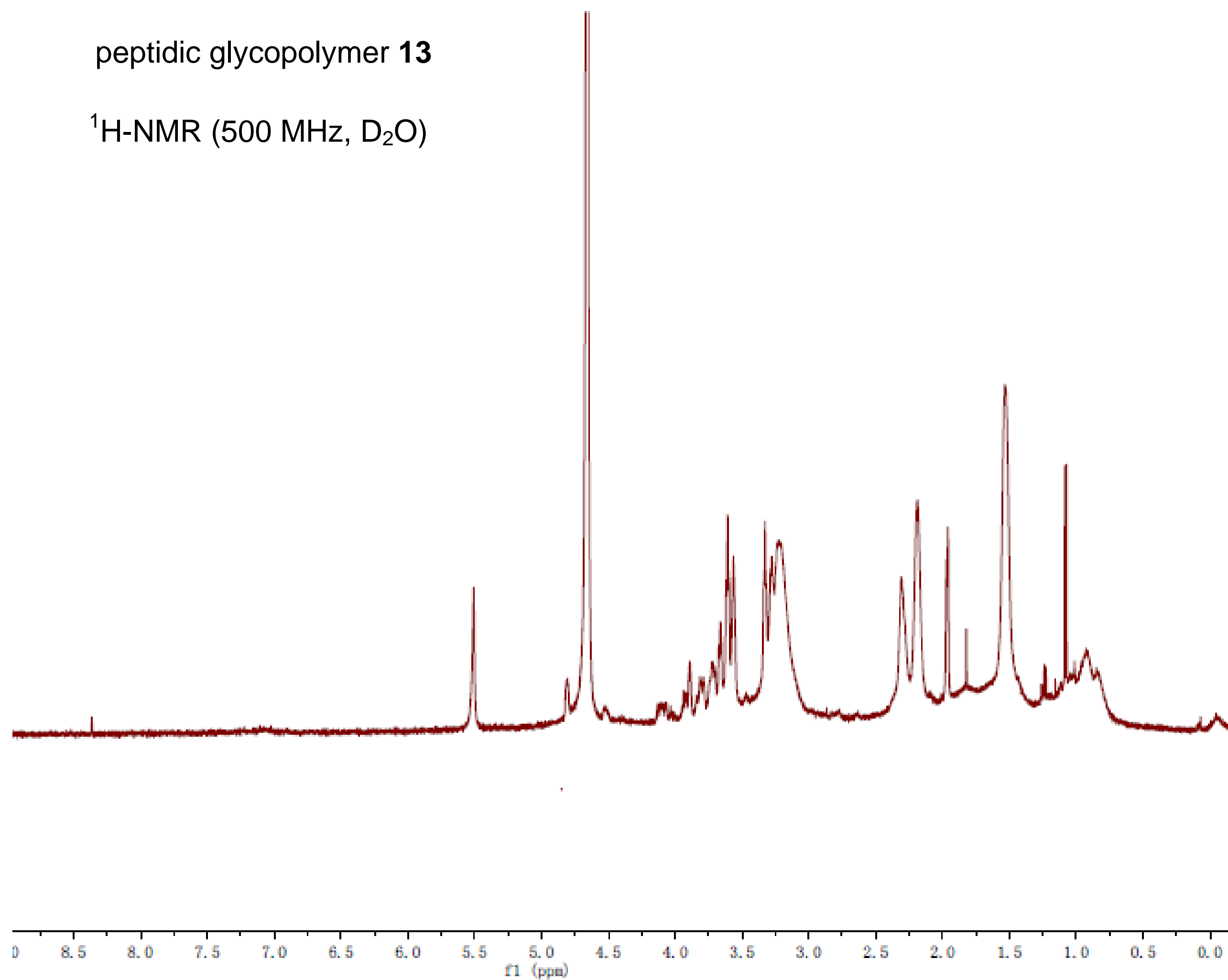


copolymer 5
^{1}H-NMR (500 MHz, D_2O)
HO
O
a
b
a
c
28
45
OCN
H_2N
O
HN
O
d
e
NH_3Cl
d
e
a, b
c
1.72
2.00
179.73
174.52
135.17
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)


copolymer 7
^{1}H-NMR (500 MHz, D$_2$O)
HO
O
H$_2$N
O
28
HN
O
45
d
e
NH$_3$Cl
H$_2$N
O
22
HN
O
4
OCN
f
CO$_2$CH$_3$
a
b
c
a, b
2.00
12.81
89.65
87.35
94.25
93.33
198.21
134.42
f1 (ppm)


glycopolymer 9
1H-NMR (500 MHz, D2O)
anomeric H
from Tn
Tn, i
d,e,g,h,k
Ac
from
Tn
j
f
a,b
c
1.00
1.04
1.06
1.08
2.68
1.14
1.86
0.43
1.89
2.18
2.91
1.37
2.34
1.99
2.40
5.02
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)
HO
O
28
31
22
4
OCN
H2N
HN
j
d
e
NH
k
CO2CH3
a
b
g
HN
h
i
OH
AcHN


glycopolymer **11**
^{1}H-NMR (500 MHz, D_2O)
aromatic H from pyridyl
1.00
2.39
1.44
2.41
1.36
1.20
1.75
13.18
14.68
9.35
33.24
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)



peptidic glycopolymer **13**
^{1}H-NMR (500 MHz, D_2O)
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

References:

1. J.-M. Moon, B.-S. Kim, Y.-S. Choi, J.-O. Lee, T. Nakahara and K. Yoshinaga, *Macromol. Res.*, 2010, **18**, 793.
2. Z. Yin, M. Comellas-Aragones, S. Chowdhury, P. Bentley, K. Kaczanowska, L. BenMohamed, J. C. Gildersleeve, M. G. Finn and X. Huang, *ACS Chem. Biol.*, 2013, **8**, 1253.