Synthesis of Polysaccharide-*b*-PEG Block Copolymers by Oxime Click.

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Electronic Supplementary Information

Contents:

1. Materials and methods

Materials

Hyaluronic acid [HA 6kDa (Mn 6.00 10^3, PDI 1.23), HA 9kDa (Mn 9.36 10^3, PDI 1.28) and HA 54kDa (Mn 5.43 10^4, PDI 1.22) were purchased from Lifecore Biomedical LLC (USA). Molecular weight provided by the company was measured by GPC-MALS. All other reagents were purchased form Sigma-Aldrich. The molecular weights form the 2 batches of dextran (~6kDa, ~and ~70kDa) were measured by GPC with dextran standards (see below). The degree of acetylation of the chitosan (Aldrich low molecular weight) was determined by NMR.[1] Dialysis membranes with molecular weight cut off of 50 kDa (regenerated cellulose) and 100 kDa (cellulose ester) were purchased from Spectrum Laboratories. Ultrafiltration membranes (regenerated cellulose) were purchased from Millipore.

Gel permeation Chromatography (GPC)

GPC measurements were performed on set of three columns: PSS suprema precolumn (10 µm, 8×50 mm), PL-aquagel-OH Mixed (8 µm, 8×300 mm) and PLaquagel-OH-30 (8 µm, 8×300 mm), with refractive index detection (RI-Detector 8110, Bischoff). The system was kept at 35ºC. GPC was measured at an elution rate of 1 mL/min. The eluent was 0,1M NaN_3, 0,01M NaH_2PO_4, pH=7.5, or the same solution with a 20% MeOH to allow the elution of MeO-PEG-ONH_2 and assure the purity of the block copolymers. For the measurments of chitosan block copolymers 0,1M NaN_3, 0,01M NaH_2PO_4, the pH was decreased to 2.5 by adittion of 0.1 M HCl. Four dextran calibration were performed (pH 7.5 and 3 with or without MeOH) and used to determine the apparent molecular weights and PDI of the block copolymers.

GPC-multi angle light scattering (GPC-MALS)

The same set of columns coupled to a multiangle light scattering detector was used to determine the absolute molecular weights of the depolymerised chitosans. 0.1M NaN_3, 0,01M NaH_2PO_4, pH=2.5 was used as eluent at a flow rate of 1.0 mL min^{-1}: An Agilent Technologies 1200 Series detector was used for refractive index and a Wyatt HELEOS MALS detector equipped with a 632.8 nm He−Ne for laser light scattering . The refractive index increments of the different polymers in the same eluent at 25 °C were measured using a PSS DnDc-2010/620 differential refractometer.

MALDI-TOF MS

MALDI-TOF-MS were carried out on a Bruker Daltonics Reflex III instrument equipped with an N_2 Laser (337 nm) and an acceleration voltage of 20 kV in positive reflectron mode was used. Sample preparation was done according to the "dried-droplet" method. In detail, matrix [either 2-(4-Hydroxyphenylazo)benzoic acid (DCTB) or 2-(4-Hydroxyphenylazo) benzoic acid (HABA), conc. 20 mg / mL)], analyte (conc. 10 mg / mL) and salt (LiTMS, 10 mg / mL) were separately dissolved in THF, subsequently mixed in a ratio of 20 : 5 : 1 µL. Finally, 1.5 µL of the resulting mixture was placed on the MALDI plate. Commercial MeO-PEG-OH, molecular weights were: PEG 2k (Matrix DCTB) M_n 1953 PDI 1.00, Mp [M+Na]+; PEG 5kDa (Matrix HABA-LiTMS) M_n 5241, PDI 1.00, Mp [M+Li]+.

NMR spectroscopy

NMR spectra were recorded at Bruker Avance 300 MHz, in D_2O, 2% DCl in D_2O, or $CDCl_3$. Chemical shifts are reported in ppm (δ units) downfield from internal tetramethylsilane ($CDCl_3$), or 3-(trimethylsilyl)-propionic acid-d_4 (D_2O).

2. Fractionation of dextrans

Commercial dextran (1g) was dissolved in deionised water and fractionated by ultrafiltration with regenerated cellulose membranes and freeze-dried. Dextran 3kDa (Mn 3.07 10^3, PDI 1.81) was ultrafiltered with 5kDa molecular weight cut off membrane and dextran 40kDa (M_n 3.99 10^4 (PDI 1.51) was ultrafiltered with 10kDa molecular weight cut off membrane. The procedure lead to dextrans with lower PDIs as analyzed by GPC (0,1M NaN_3, 0,01M NaH_2PO_4, pH=7.5, 20%MeOH): dextran 6kDa (Mn 6.17 10^3, PDI 1.27, 0.5 g) and dextran 50kDa (Mn 4.88 10^4, PDI 1.35, 0.43 g).

3. N-Deacetylation of CS

Finely grounded CS was suspended in aqueous 40% (w/v) NaOH at 333 K under a N_2 stream. After 45 min of stirring, the reaction mixture was filtered and thoroughly washed with deionized water (333 K). The resulting powdery residue was dried overnight under vacuum. This process was repeated 4 times until a DA of 1.0 %was obtained.[2] The product was then dissolved in 0.5% (w/v) AcOH (5 g/L) and dialyzed against water to obtain the CS acetic acid salt. Molecular weight of the product was determined by GPC-MALS M_n 53600, PDI 1.40. ^{1}H-NMR δ (2% DCl, 293K): 4.92 (s, H1 of GluNH2) 3.38-4.34 (m, H2 of GluNAc, H3-H6), 3.18 (s, H2 of $GluNH_2$), 2.09 (s, AcOH), 2.08 (s, NAc).

4. Depolymerization of CS combined with fractionation by ultrafiltration

N-Deacetylated CS was depolymerized by addition of nitrous acid.[3,4,5] Briefly, commercial CS was dissolved (10 g/L) in AcOH 0.5% under magnetic stirring at rt. When CS was completely dissolved, 1M $NaNO_2$ was added dropwise. After overnight stirring the crude reaction was fractionated by ultrafiltration with regenerated cellulose membranes. Adjusting the amount of $NaNO_2$ added and the molecular weight cut off membrane, CSs with a low PDI and molecular weights of 4 and 10 kDa were obtained: CS-4kDa: M_n 3.700, PDI 1.32 and CS-10kDa: Mn 10700, PDI 1.26, as determined by GPC-MALLS.^{1}H-NMR δ (D_2O, 293K): 9.52 [m, H1 of 2,5-anhydro-D-mannose end group (M-unit) free aldehyde], 8.05-7.97 (m, H1 of M-unit shiff base), 5.10 (d, J = 5.25, H1 of M-unit gem diol), 4.92 (s,

H1 of $GluNH_2$), 4.45 (m, H3 of M-unit), 4.22 (m, H4 of M-unit), 4.13 [m, H5], 4.34-3.38 (m, H2 of GluNAc, H3-H6), 3.18 (s, H2 of $GluNH_2$), 2.09 (s, AcOH), 2.08 (s, NAc).

5. Synthesis of MeO-PEG-ONH_2

MeO-PEG-ONH_2 (2kDa and 5kDA) was prepared by a two step procedure with commercial MeO-PEG-OH as starting material following a reported procedure with slight modifications.[6] MeO-PEG-OH (5 g, 2 or 5kDa), N-Hydroxyphthalimide (3 Eq.) and tryphenyl phosphine diisopropyl azodicarboxylate (3 Eq.) were mixed in dried CH_2CL_2 (25 mL) under N_2 atmosphere. To this mixture diisopropyl azodicarboxylate (3 Eq.) was added dropwise leading to a red to yellow color change in the reaction and the complete dissolution of the reagents after *ca*. 30 minutes. The reaction was stirred at rt for 14 h, before being evaporated. The resulting crude product was dissolved in CH_2Cl_2 (10 mL), precipitated by the addition of Et_2O (~500 mL), and filtered to give MeO-PEG-Phthalimide (MeO-PEG-NHP) as a white powder (98%). The ^{1}H-NMR showed the complete modification of the end group ^{1}H-NMR (300 MHz, $CDCl_3$, 293K) δ: 7.81 (ddd, J = 26.9, 5.5, 3.1 Hz, 4H), 4.44 – 4.33 (m, 2H), 4.02 – 3.80 (m, 3H), 3.75 – 3.39 (m, PEG CH_2), 3.38 (s, 3H).

The MeO-PEG-ONH_2 was obtained cleaving the phthalimide group by addition of hydrazine hydrate (3 Eq.) to a solution of the MeO-PEG-NHP product dissolved in CH_2Cl_2 (25 mL). The solution was stirred rapidly for 1 h, during which a white precipitate was formed. The crude reaction was then filtered over glass wool concentrated under reduced pressure up to 5 mL CH_2Cl_2, precipitated by the addition of Et_2O (~500 mL), and filtered to give MeO-PEG-ONH_2 (97%) as a white powder. ^{1}H-NMR (300 MHz, $CDCl_3$, 293K) δ: 4.02 – 3.80 (m, 3H), 3.75 – 3.39 (m, PEG CH_2), 3.38 (s, 3H). MALDI-TOF MS MeO-PEG-ONH_2 PEG 2kDa (Matrix HABA-LiTMS) m/z: Mn, 2113, PDI Mw 2145, Mp [M+Li]+; PEG 5kDa (Matrix HABA-LiTMS) m/z: Mn, 5318 Mw 5364, Mp [M+Li]+.

6. Optimization of Oxime Click reaction conditions

Oxime Click reaction of PEG and Glucosamine

MeO-PEG-ONH_2 2kDa (50 mg) and Glucosamine (53 mg, 10 Eq.) were mixed in a citric acid based buffer solution at pH 3 (4mL) or a 1/1 mixture of the same buffer and AcN (4mL). The reaction was stirred at room temperature or 45ºC. Aliquots of the reaction were taken at different times (see table S1) concentrated under reduced pressure and freeze dried. The reaction was followed by in ^{1}H NMR. The methoxy end group was integrated to the N-CH protons (E and Z) at 7.64 (d, J= 4.33) and 7.04 (d. J=6.22) ppm. Results are shown in Table S1.

Table S1. Conversion of the end group of PEG to GluN end modified PEG (%)

AcN %	Temp. (ºC)	24h	4 days
0%	20	26%	38%
80%	45	34%	51%
50%	45	55%	69%
50%	20	10%	--

Oxime Click reaction of PEG and low molecular weight dextran

Commercial dextran (50 mg, Mn 3.07 10^3, PDI 1.81) was dissolved in buffer solution at pH 3 (2 mL). MeO-PEG-ONH_2 2 kDa (10 Eq.) was dissolved in DMSO. Both solutions were mixed and stirred at 45ºC for 24 h. The reaction mixture was then added into excess dioxane and freeze dried. The anomeric dextran signal (4.97 ppm, d, J=3.13)[7] was integrated to the N-CH protons (E and Z) at 7.63 (d, J=6.35) and 7.04 (d. J=6.22) ppm. Results are shown in Table S2.

Table S2. Conversion of the end group of PEG to GluN end modified PEG (%)

Solvent mixture	PEG Eq.	12h	24h	48h
Buffer pH 3/ AcN 40%	2	52	63	64
Buffer pH 5/ DMSO 50%	2	16	43	57
Buffer pH 5/ AcN 40%	2	N. C	26	42
DMSO	2	N. C	N.C	--
Buffer pH 3 10%/ DMSO	2	N. C	N.C	--
Buffer pH 3/ DMSO 50%	1	40	42	--
	1.5	53	57	--
	2	65	70	--
	3	70	83	--
	5	--	99	99
	10	--	99	99

7. General synthetic procedure for the polysaccharide block copolymers

General procedure for the synthesis of dextran-b-PEG and HA-b-PEG

Dextran-(6kDa or 50kDa) or HA (6kDa, 9kDa and 54kDa) was dissolved in citric acid buffer solution at pH 3 (50 mg/mL). MeO-PEG-ONH_2 (2 or 5kDa, 5 Eq.) was dissolved in DMSO (50 mg/mL). Both solutions were mixed and stirred at 45ºC. After 24h the reaction was added into excess dioxane and freeze dried. The white foam obtained was dissolved in water and excess ethanol was added. The milky (high molecular weights) or opalescent (dextran-6kDa) solution obtained was dialyzed against ethanol until the excess MeO-PEG-ONH_2 was eliminated as observed by GPC (0.1 $MNaN_3$, 0.01 M, NaH_2PO4 /20% MeOH, pH 7.4). A molecular weight cut off of 50 kDa was used to remove PEG 2kDa and 100 kDa to remove PEG 5kDa. After dialysis the product suspended in ethanol was dissolved by addition of water, the ethanol was concentrated under reduced pressure and the water solution of the block copolymer finally freeze dried to obtain a white foam.

General procedure for the synthesis of CS-b-PEG

CS was dissolved in AcOH 0.5% w/v, (pH 3, and 50 mg/mL). MeO-PEG-ONH_2 (2 or 5kDa, 5 Eq.) was dissolved was dissolved in DMSO (50 mg/mL). Both solutions were mixed and stirred at 45ºC. Both solutions were mixed and stirred at 45ºC. After 24h the reaction was added into excess dioxane and freeze dried. The white foam obtained was dissolved in AcOH 0.5% and excess ethanol was added. The opalescent solution obtained was dialyzed against ethanol until the excess MeO-PEG-ONH_2 was eliminated as observed by GPC (0.1 $MNaN_3$, 0.01 M, NaH_2PO4 /20% MeOH, pH 2.5 by addition of diluted HCl). A molecular weight cut off of 50 kDa was used to remove PEG 2kDa and 100 kDa to remove PEG 5kDa. After dialysis the product suspended in ethanol was dissolved by addition of water (and a small amount of AcOH 0.5% in the case of CS 53kDa), the ethanol was concentrated under reduced pressure and the water solution of the block copolymer finally freeze dried to obtain a white foam. In the synthesis of CS-*b*-PEG with CS 54kDa the dialysis was substituted by ultrafiltration with a cellulose membrane of 30kDa molecular weight cut off.

8. ^{1}H NMR Characterization of the block copolymers

Dextran-b-PEG

^{1}H-NMR (300 MHz, D_2O, 293K) δ: 7.60 (d, *J* = 6.3 Hz, oxime N-C**H**), 6.95 (d, *J* = 6.4 Hz, oxime N-CH), 4.98 (d, *J* = 3.2 Hz, dextran H1), 4.43 (t, *J* = 6.5 Hz, 2H dextran-PEG junction), 4.34 – 4.21 (m, C**H_2**NO PEG), 4.16 – 3.41 (m, H2-H6 protons of HA, CH_2 of PEG), 3.38 (s, CH_3O PEG).

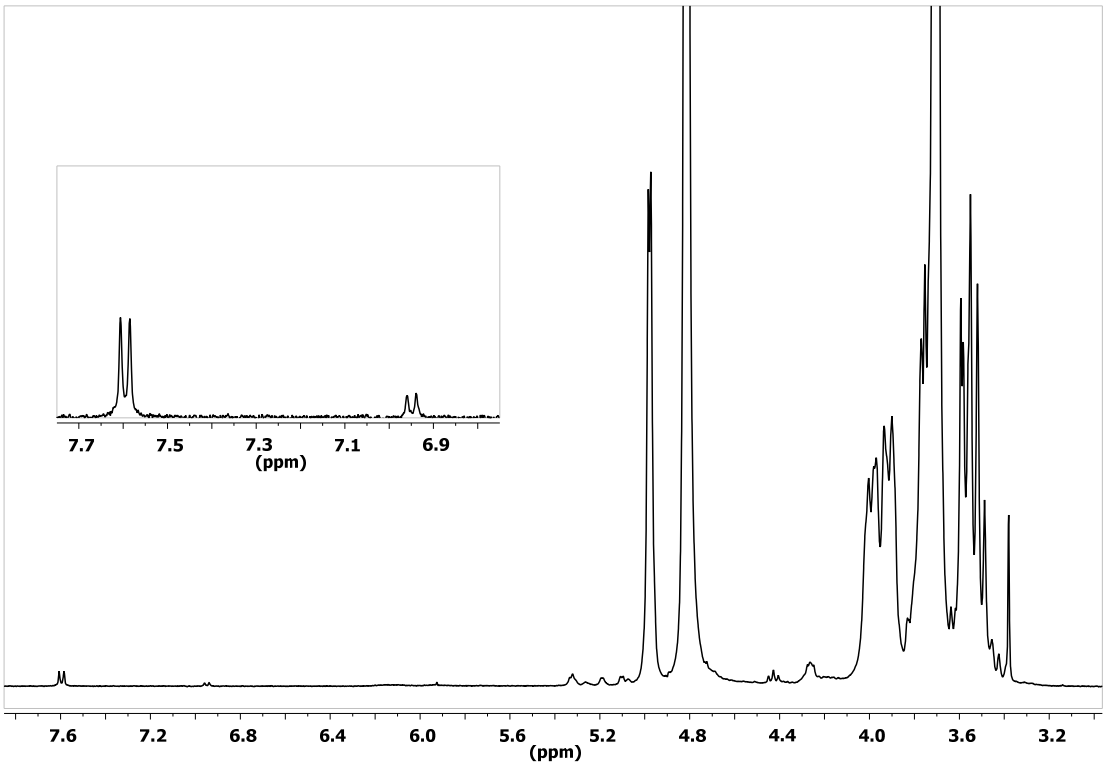

Fig. S1. Dextran (6kDa)-*b*-PEG (2kDa)^{1}H-NMR (300 MHz, D_2O, 293K)

Hyaluronic acid-b-PEG

^{1}H-NMR (300 MHz, D_2O, 293K) δ: 7.66 (d, J = 5.40 Hz, oxime N-C**H**), 7.59 (d, J = 5.86 Hz, oxime N-C**H**), 6.94-6.88 (m, oxime N-C**H**), 4.67 – 4.35 (m, HA anomeric proton H1 of glucuronic acid and N-acetylglucosamine units), 4.26-4.08 (m, HA-PEG junction), 4.08 – 3.21 (m, H2-H6 protons of HA, CH_3O and CH_2 of PEG), 2.01 (s, CH_3 of N-acetylglucosamine).

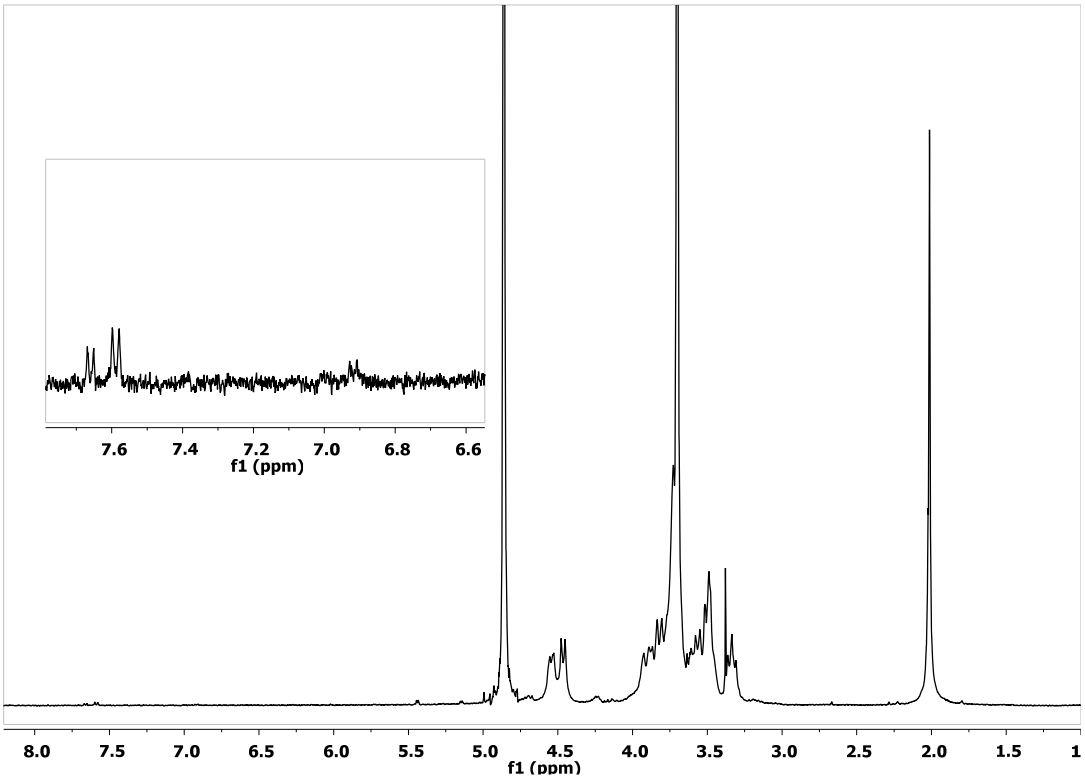

Fig. S2. HA (6kDa)-*b*-PEG (2kDa) ^{1}H-NMR (300 MHz, D_2O, 293K)

Chitosan-b-PEG

^{1}H-NMR (300 MHz, 2% DCl in D_2O, 313K): δ 4.91 (d, J = 8.1 Hz, H1 glucosamine), 4.16 – 3.43 (m, H2 of GluNAc, H3-H6 chitosan, CH_3O and CH_2 of PEG), 3.38 (s), 3.20 (s, H2 of $GluNH_2$), 2.08 (s, NAc).

^{1}H-NMR (300 MHz, D_2O, 293K, CS 4kDa): δ 7.92 (br s, oxime N-C**H** linked to 2,5-anhydro-D-mannose), 7.70-7.50 (m, oxime N-C**H** linked to glucosamine), 4.78-7.60 (m, J = 8.1 Hz, H1 glucosamine), 4.36-4.22 (m, HA-PEG junction), 4.05 – 3.43 (m, H2 of GluNAc, H3-H6 chitosan, and CH_2 of PEG), 3.38 (s, CH_3O of PEG), 3.20 (s, H2 of $GluNH_2$), 2.08 (s, NAc).

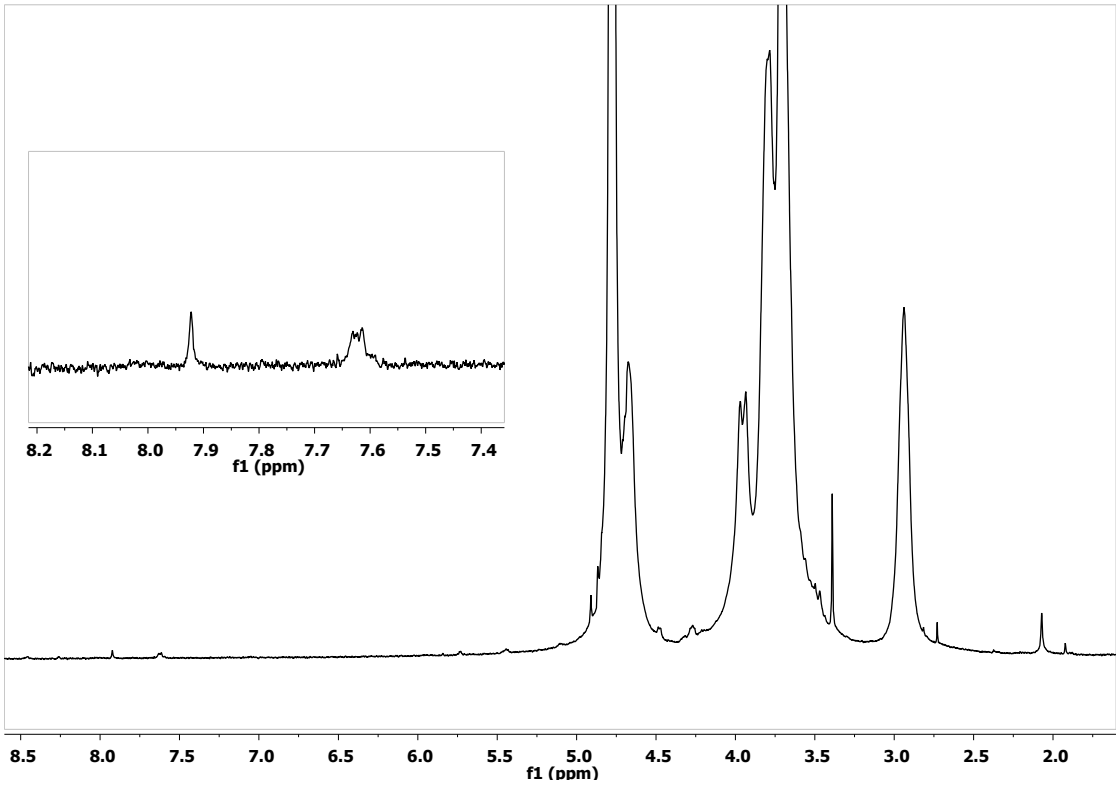

Fig. S3. CS (10kDa)-*b*-PEG (2kDa) ^{1}H-NMR (300 MHz, D_2O, 293K)

Block copolymer composition

Composition of the block copolymers was determined by integration of the appropriate signals in the ^{1}H NMR spectra (256 scans, 7 seconds of recovery delay) according to the DP of the polysaccharide (GPC or GPC-MALS) and the PEG (MALDI-TOF-MS). The solvent was D_2O for dextran and hyaluronic acid, and 2% DCl in D_2O for chitosan. Intervals of integration: Dex-*b*-PEG: 4.90-5.05 ppm (H1 of dextran) and 3.20-4.20 ppm (H2-H6 protons of dextran, CH_3O and methylene protons of PEG) and/or oxime N-CH protons (7.45-7.70 and 6.87-7.02 ppm, E and Z). HA-*b*-PEG: 4.32-4.62 ppm (HA anomeric proton H1 of D-glucuronic acid and N-acetylglucosamine units) and 3.15-4.10 ppm (H2-H6 protons of HA, CH_3O and methylene protons of PEG) and/or oxime N-CH protons [7.45-7.70 ppm and

7.30-7.39 ppm, E and Z]. CS-*b*-PEG: 2.90-3.10 ppm (H2 proton of glucosamine), 3.13-4.02 (H3-H6 glucosamine, CH_3O and methylene protons of PEG).

9. Apparent molecular weight distributions of the block copolymers

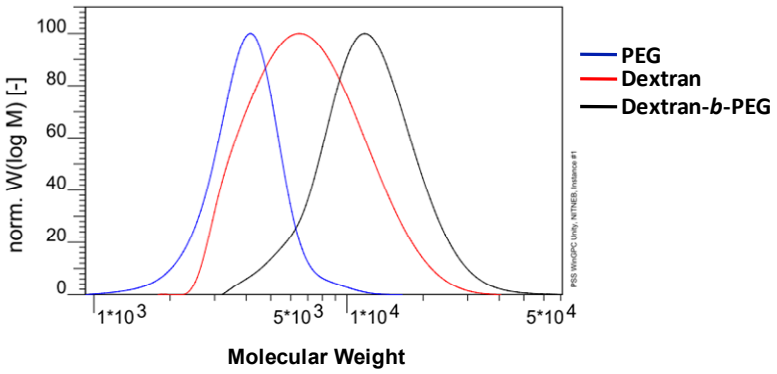

Fig. S4. Dextran (6kDa)-*b*-PEG(2kDa) apparent molecular weight distribution compared to PEG and dextran precursors (0.1 M NaN_3, 0.01 M NaH_2PO4 / 20% MeOH)

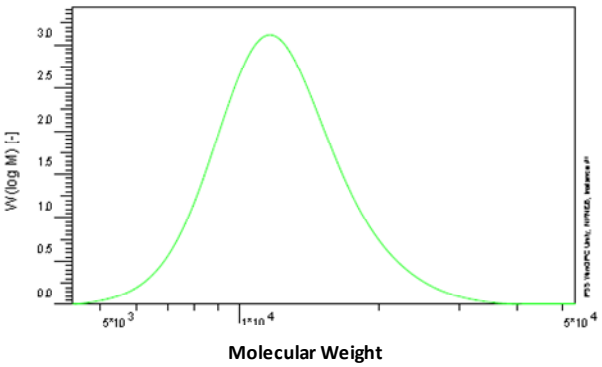

Fig. S5. Dextran (6kDa)-*b*-PEG(5kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4 / 20% MeOH)

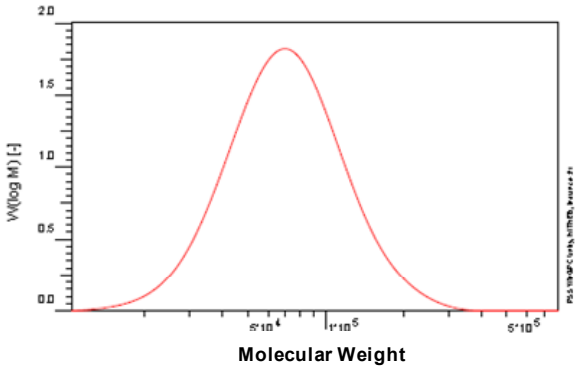

Fig. S6. Dextran (49kDa)-*b*-PEG(5kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4 / 20% MeOH)

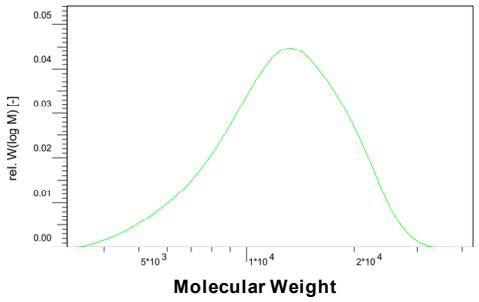

Fig. S7. Hyaluronic acid (6kDa)-*b*-PEG(2kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4 / 20% MeOH)

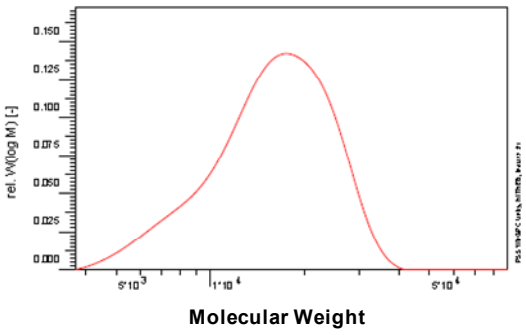

Fig. S8. Hyaluronic acid (6kDa)-*b*-PEG (5kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4 / 20% MeOH)

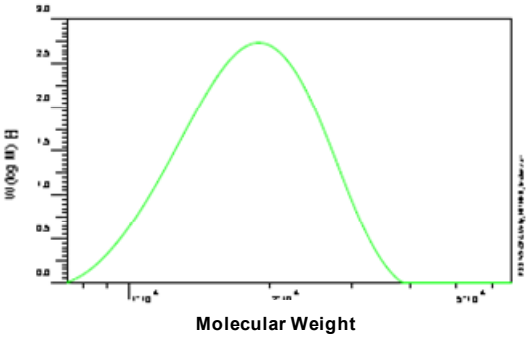

Fig. S9. Hyaluronic acid (9kDa)-*b*-PEG (2kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4 / 20% MeOH)

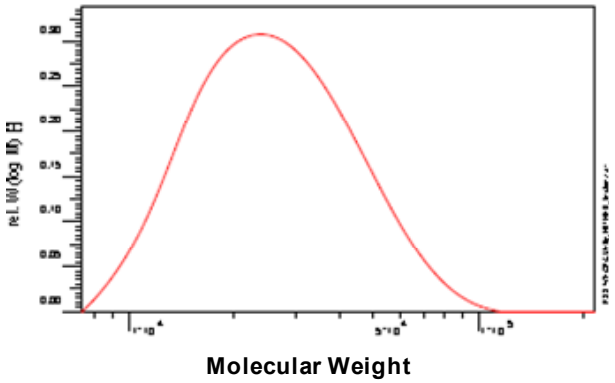

Fig. S10. Hyaluronic acid (9kDa)-*b*-PEG (5kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4 / 20% MeOH)

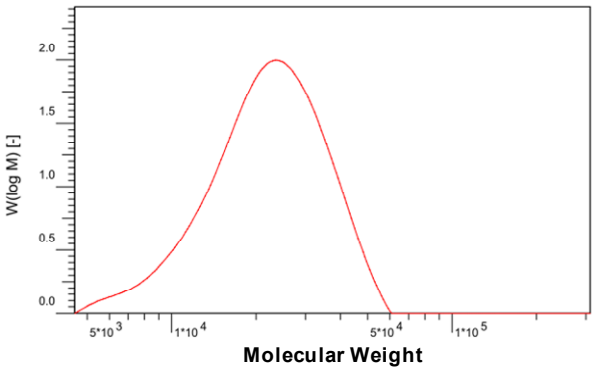

Fig. S11. Hyaluronic acid (54kDa)-*b*-PEG (2kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M, NaH_2PO4 /20% MeOH)

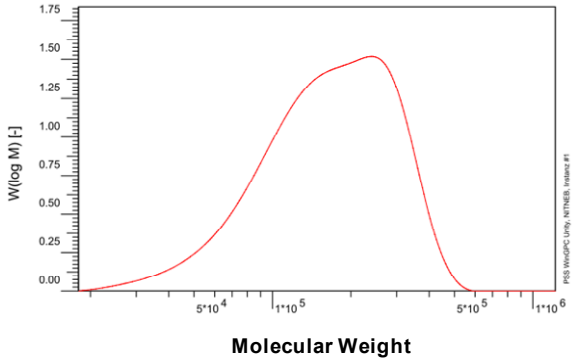

Fig. S12. Hyaluronic acid (54kDa)-*b*-PEG (2kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4)

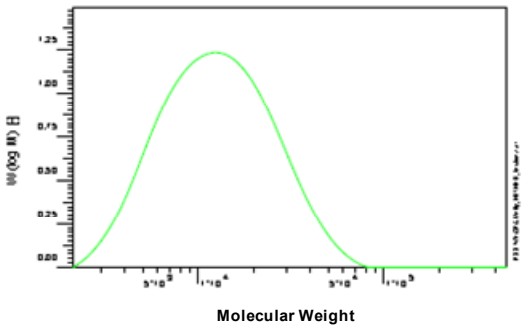

Fig. S13. Chitosan (4kDa)-*b*-PEG (2kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4, 20% MeOH)

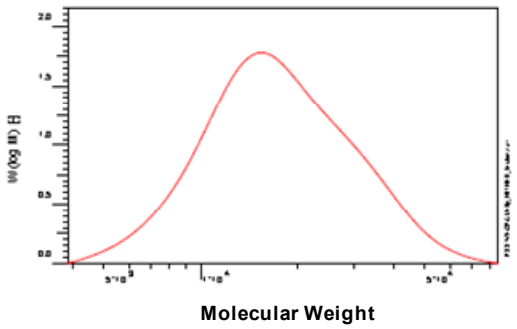

Fig. S14. Chitosan (4kDa)-*b*-PEG (5kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4, 20% MeOH)

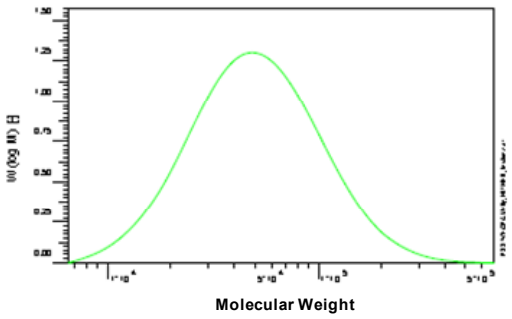

Fig. S15. Chitosan (10kDa)-*b*-PEG (2kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4, 20% MeOH)

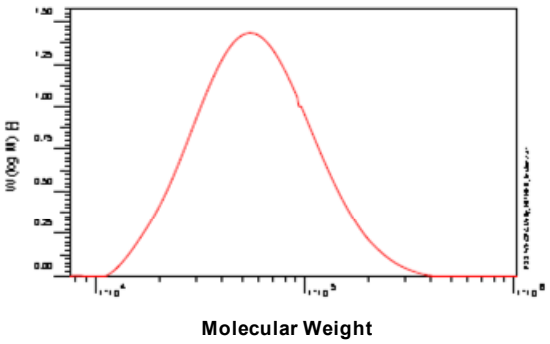

Fig. S16. Chitosan (10kDa)-*b*-PEG (5kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4, 20% MeOH)

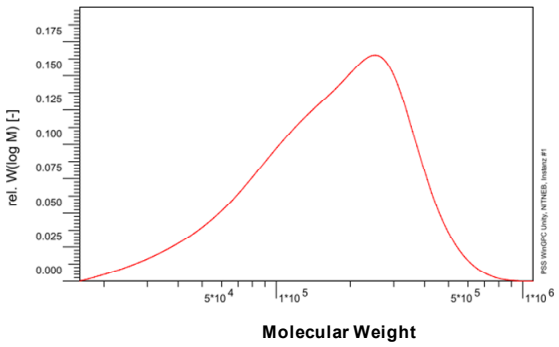

Fig. S17 Chitosan (54kDa)-*b*-PEG (2kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M NaH_2PO4)

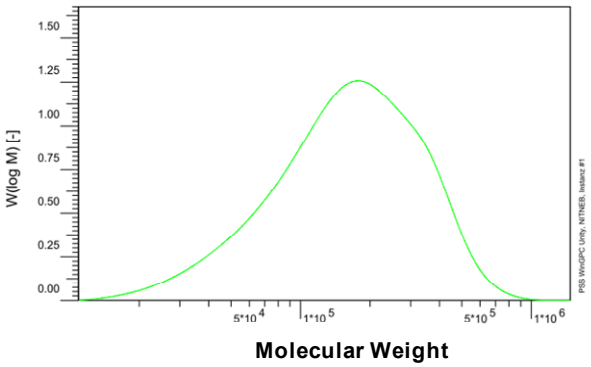

Fig. S17 Chitosan (54kDa)-*b*-PEG (5kDa) apparent molecular weight distribution (0.1 M NaN_3, 0.01 M, NaH_2PO4)

9. Oxime stability studies

The stability of the dex-*b*-PEG (dextran 6 kDa, PEG 2kDa) was studied by the integration of the oxime N-CH protons (7.45-7.70), in a ^{1}H-NMR spectra in D_2O, at 293 K. A 10 mg/mL solution of the block copolymer at different DCl concentrations was prepared and NMR spectra (256 NS relaxation delay 7s) at increasing times were measured. Sample was kept at ambient temperature between measurements. The pD was determined with pH indicator strips. Data are shown in Table S3.

Table S1. Oxime stability as afuction of the pD.

pD	Time (h)	Remaining oxime %
1	2	60%
	6	40%
	24	>1%
2	6	50%
	24	27%
	55	>1%
3	6	100%
	24	100%

55	100%

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