Supporting Information

2,5-Anhydro-d-mannose end-functionalised chitin oligomers activated by dioxyamines or dihydrazides as precursors of diblock oligosaccharides

Ingrid Vikøren Mo, Marianne Øksnes Dalheim, Finn L. Aachmann, Christophe Schatz, Bjørn E. Christensen

Contents

S1 Preparation and characterization of A\textsubscript{n}M oligomers..............................................................2
S2 Conjugation of A\textsubscript{n}M oligomers to ADH and PDHA studied by time course NMR ................3
S3 Kinetic modelling of the reductive amination reaction.................................................................5
S4 Modelling of A\textsubscript{n}M conjugation reactions ........................................................................7
S5 Spontaneous decomposition of reducing agents........................................................................8
S6 Reduction of A\textsubscript{n}M oligomers.................................................................................................10
S7 Reduction of A\textsubscript{n}M conjugates............................................................................................14
S8 Modelling of A\textsubscript{n}M reduction reactions ........................................................................16
S9 Optimisation of preparative protocols .........................................................................................16
  Statistical distribution of mono-, di- and unsubstituted ADH and PDHA ................................16
  Minimisation of disubstituted ADH or PDHA ..............................................................................19
  Optimisation of reduction conditions for PDHA conjugates .......................................................20
  Preparation of A\textsubscript{n}M conjugates using optimised protocols ............................................20
S10 2D NMR characterization of the reduced and purified A\textsubscript{2}M-PDHA ........................................22
S11 Preparation of chitin-b-chitin diblocks from activated chitin oligomers (A\textsubscript{n}M-ADH) ............25
S12 Preparation of chitin-b-chitin diblocks using a sub-stoichiometric amount of ADH or PDHA 28
S13 Preparation and characterization of dextran (Dext\textsubscript{m}) oligomers ............................................32
S14 Conjugation of Dext\textsubscript{m} oligomers to ADH and PDHA studied by time course NMR ........34
S15 Preparation of chitin-b-dextran diblocks ....................................................................................36
S16 References ....................................................................................................................................41
S1 Preparation and characterization of AₙM oligomers

Chitin oligomers of the type AₙM, where Aₙ represent uninterrupted sequences of N-acetyl D-glucosamine (A) residues with a 2,5-anhydro-D-mannose (M) residue at the reducing end, were prepared by nitrous acid (HONO) depolymerisation of chitosan (Fₐ = 0.48), using an excess HONO (1.3 equivalents) to the fraction of D-glucosamine (D) residues. The excess of HONO converts all the D residues into M residues, whereas A residues are unaffected, providing solely AₙM oligomers. The water-soluble oligomers (DP < 10) were fractionated by preparative gel filtration chromatography (GFC) to obtain purified low molecular weight AₙM oligomers of specific DPs (Figure S1a). The chitosan with Fₐ = 0.48 provided mainly trimers (A₂M) and dimers (AM) (Figure S1). Separation of the oligomer mixture by analytical GFC (Figure S1b) showed shoulder peaks for all the oligomer fractions in the chromatogram. Without further investigation, shoulder peaks were suggested to be caused by the alternative forms of M (discussed below).

![Figure S1: a) preparative and b) analytical GFC fractionation of the mixture of water-soluble AₙM (n = 1-8) oligomers obtained by nitrous acid degradation of chitosan (Fₐ = 0.48) using an excess (1.3x) of nitrous acid.](image)

Oligomers were purified (by dialysis), freeze-dried and characterized by ¹H-NMR (Figure S2). The ¹H-NMR spectra were annotated according to literature. The doublet at approximately 4.9 ppm was assigned to the gem-diol (hydrated aldehyde) of the reducing end M residue. Additional doublets in the anomeric region (4.95-5.1 ppm) were assigned to alternative forms of the as M residue suggested in the literature by Lindberg et al. Purified AₙM oligomers were slightly polydisperse due to the incomplete baseline separation by the preparative GFC (Figure S1a). The polydispersity of the oligomers was confirmed by the ¹H-NMR characterization of A₄M and A₂M in Figure S2a and S2b, respectively, where the integral of H1, A was higher than expected relative to the sum of the integrals of H1, M and H1, M’ (expected ratio 4:1 and 2:1 for A₄M and A₂M, respectively).
Figure S2: $^1$H-NMR characterization of fractionated and purified a) A$_n$M and b) A$_2$M in D$_2$O including annotations and integrals (600 MHz). M’ refers to alternative (minor) forms of M (see S2).

S2 Conjugation of A$_n$M oligomers to ADH and PDHA studied by time course NMR

Conjugation of A$_n$M oligomers to ADH and PDHA was monitored by time course NMR as described earlier. In brief, conjugation reactions were performed in deuterated NaAc-buffer (with TSP added as internal standard) at pH 3.0, 4.0 or 5.0 using 2 equivalents of ADH or PDHA relative to the concentration of oligomers (20.1 mM). $^1$H-NMR spectra were recorded at specific time points (Figure S3 and S4) and yields of conjugates were determined by integration of the obtained spectra. Minor resonances close to the major resonances from the hydrazones/oximes (H1, M E or Z) were attributed to the conjugation of oligomers with alternative forms of the M residue (H1, M’ E or Z) (assigned in Figure S3 and S4). Both the major and minor resonances from the E and Z hydrazones or oximes were integrated to obtain the yield of conjugates. Due to overlapping resonances (e.g. H2, M Z overlapping with H1, M (gem-diol) in Figure S4), yields could not be calculated from the sum of integrals from the H1 reducing end proton resonances in each individual spectrum. Due to the slight polydispersity of the oligomers, the yields could not be calculated from the integrals of the resonances resulting from
the oligomers. Therefore, the integral of the resonance from the internal standard (TSP, 0 ppm) was used as reference to calculate yields. As the conjugates were reduced in a subsequent step (see below) the integral value of the TSP resonance was related to the integrals of the resonances from the completely reduced conjugates (for each individual reaction mixture). The TSP integral value was set to this specific value in all the spectra obtained during the time course NMR study for the conjugation and reduction to calculate the yields of conjugates and subsequently reduced secondary amine conjugates during the reactions. The yields were calculated based on the assumption of 100 % yield of reduced secondary amine conjugates. Integrals of overlapping resonances were calculated by subtraction.

Figure S3: $^1$H-NMR spectra obtained at defined time points for the conjugation reaction with A$_2$M (20.1 mM) and 2 equivalents ADH at pH 4.0, RT.
**S3 Kinetic modelling of the reductive amination reaction**

The reductive amination of oligosaccharides with different amines (e.g. oxyamines (PDHA) or hydrazides (ADH)) is comprised of several individual reactions with independent rates and rate constants. The overall reaction involves the conjugation (amination) of the oligosaccharide, where E- and Z- oximes or hydrazones (Schiff bases) are formed for oxyamines or hydrazides, respectively. For oligosaccharides where the reducing end aldehyde is in equilibrium with a hemiacetal (normal reducing end), the acyclic Schiff bases are in equilibrium with cyclic N-glycosides (e.g. N-pyranosides). By adding a reducing agent, the Schiff bases will be irreversibly reduced forming secondary amine conjugates. Irreversible reduction of oligosaccharides by the reducing agent will prevent the reductive amination reaction from going to completion. The general reaction scheme is shown in Figure S5.
Figure S5: General reaction scheme for the reductive amination of oligosaccharides with normal reducing ends including assigned rate constants for each independent reaction involved. Reversible reactions are described by two rate constants (forward and reverse), whereas irreversible reactions are described by one rate constant (the scheme indicates the assumption that reduction of E and Z had the same rate constant \(k_5\)).

When considering the reactions to be *first order* with respect to each reactant, reaction rates can be determined by the following equations

\[
\begin{align*}
\frac{d[A]}{dt} &= -k_1[A][B] + k_{-1}[E] - k_2[A][B] + k_{-2}[Z] - k_6[A][R] \\
\frac{d[B]}{dt} &= -k_1[A][B] + k_{-1}[E] - k_2[A][B] + k_{-2}[Z] \\
\frac{d[R]}{dt} &= -k_5[E][R] - k_5[Z][R] - k_6[A][R] \\
\frac{d[E]}{dt} &= k_1[A][B] - k_{-1}[E] - k_3[E] + k_{-3}[N] - k_5[E][R] \\
\frac{d[Z]}{dt} &= k_2[A][B] - k_{-2}[Z] - k_4[Z] + k_{-4}[N] - k_5[Z][R] \\
\frac{d[N]}{dt} &= k_3[E] - k_{-3}[N] + k_4[Z] - k_{-4}[N] \\
\frac{d[A^*]}{dt} &= k_6[A][R]
\end{align*}
\]

The concentration of each reactant or product at specific time points, \([X]_t\), can be obtained from the reaction rates by the following equation

\[
[X]_t = [X]_{t-\Delta t} + \frac{d[X]}{dt} \Delta t
\]
where, $t$ is the time, and $\Delta t$ is the time difference from last modelled time point. The numeric modelling was carried out using Excel, generally substituting differentials of the type $d[X]/dt$ with $\Delta[X]/\Delta t$. From starting concentrations $[A]_0$, $[B]_0$ etc, the concentrations at successive time increments $t_{i+1} = t_i + \Delta t$ were inductively calculated as $[X]_{i+1} = [X]_i + (\Delta[X]/\Delta t)\Delta t$. The time interval ($\Delta t$) was chosen sufficiently small to result in a simulation which did not further change when choosing an even smaller time interval. All reactions were modelled using this approach, and the model was fitted to the experimental data by adjusting the rate constants to give the minimum sum of squares.

In the special case of reductive amination with chitin or chitosan oligosaccharides prepared by nitrous acid degradation, only Schiff bases (oximes/hydrazone) can be formed. Hence, the general reaction scheme for these reactions is simplified as showed in Figure S6.

**Figure S6:** General reaction scheme for the reductive amination of oligosaccharides with M residue at the reducing end including assigned rate constants for each independent reaction involved.

**S4 Modelling of $A_nM$ conjugation reactions**

Conjugation reactions (no reducing agent present) with $A_nM$ oligomers were studied by time course NMR (S2). The experimental data obtained for the conjugation reactions were fitted using the model described in S3 (based on the reaction scheme presented in Figure S6). Examples of the data fitting for the conjugation of $A_2M$ oligomers to ADH and PDHA (2 equivalents) at pH 4.0 are given in Figure S7.
S5 Spontaneous decomposition of reducing agents

The decomposition of α-picoline borane (PB) and sodium cyanoborohydride (NaCNBH$_3$) in 500 mM deuterated NaAc-buffer, pH 4.0, was monitored by NMR. The two reducing agents have distinct resonances in the $^1$H-NMR spectrum and hence, their decomposition was studied. In contrast to NaCNBH$_3$, which is completely dissolved in the buffer, PB has low initial solubility, but dissolves slowly over time (observed by increased intensity of the resonances over time).

The protons of the pyridine ring in PB give resonances with chemical shifts in the range 7.2 to 8.7 ppm, whereas the protons of the methyl group give one resonance with a chemical shift of approximately 2.6 ppm (Figure S8). The protons of the -BH$_3$ group are not visible in the $^1$H-NMR spectrum due to negative chemical shifts. When PB is oxidised (o), the proton resonances are moved slightly downfield as seen in Figure S8. The relative reductive power (%) for PB over time was calculated by relating the intensity of the protons resulting from the reduced form of PB (r) to the total amount of dissolved PB (r+o) (Figure S8).

In contrast to PB, the proton resonances of the -BH$_3$ group in NaCNBH$_3$ are within the NMR scale (0 – 8 ppm). NaCNBH$_3$ lacks other protons and hence, it was not possible to study the oxidation of this reducing agent. Therefore, the decomposition was related to the reduced intensity of the resonances from the protons in the -BH$_3$ group relative to an impurity in the buffer (Figure S9). As the first spectrum was obtained after one hour, the intensity of the resonances at t = 0 was obtained by extrapolation. The relative reductive power (%) for NaCNBH$_3$ was related to the decomposition of the reducing agent. By comparing the change in relative reductive power (%) for the two reducing agents
over time (Figure S10), NaCNBH$_3$ was shown to decompose approximately 20 times faster than PB in the buffer.

Figure S8: Spontaneous decomposition of PB in deuterated acetate buffer (500 mM, pH 4.0, RT) monitored by time course NMR.

Figure S9: Spontaneous decomposition of NaCNBH$_3$ in deuterated NaAc-buffer (500 mM, pH 4.0, RT) monitored by time course NMR.
**S6 Reduction of AₙM oligomers**

Reduction of AₙM oligomers was performed by adding 3 equivalents reducing agent (PB or NaCNBH₃) to oligomers dissolved in deuterated NaAc-buffer. The course of the reduction was studied by monitoring the disappearance of reducing end gem-diol resonances (H1, M and H1, M'). Resonance intensities were related to the internal standard (TSP). Reduction of AₙM oligomers by PB was studied at pH 3.0, 4.0 or 5.0 (Figure S11-S13) or by NaCNBH₃ at pH 4.0 (Figure S14).

For comparison, reduction of AA oligomers (normal reducing end) was studied using 3 equivalents PB (Figure S15) or NaCNBH₃ (Figure S16) at pH 4.0. In contrast to the AₙM oligomers, no detectable reduction in the intensity of the reducing end resonances for AA was observed. However, the relative ratio of the α- to β- reducing end resonances gradually changed (Figure S15). For this experiment AA was dissolved in the buffer shortly before PB was added and the first spectrum was obtained.
Figure S11: $^1$H-NMR spectra obtained at defined time points for the reduction of A$_2$M oligomers (20.1 mM) in deuterated acetate buffer, pH 3.0, RT using 3 equivalents PB (60.3 mM).

Figure S12: $^1$H-NMR spectra obtained at defined time points for the reduction of A$_2$M oligomers (20.1 mM) in deuterated acetate buffer, pH 4.0, RT using 3 equivalents PB (60.3 mM).
Figure S13: $^1$H-NMR spectra obtained at defined time points for the reduction of A$_2$M oligomers (20.1 mM) in deuterated acetate buffer, pH 5.0, RT using 3 equivalents PB (60.3 mM).

Figure S14: $^1$H-NMR spectra obtained at defined time points for the reduction of A$_3$M oligomers (20.1 mM) in deuterated acetate buffer, pH 4.0, RT using 3 equivalents NaCNBH$_3$ (60.3 mM).
**Figure S15:** $^1$H-NMR spectra obtained at defined time points for the reduction of AA oligomers (20.1 mM) in deuterated acetate buffer, pH 4.0, RT using 3 equivalents PB (60.3 mM).

**Figure S16:** $^1$H-NMR spectra obtained at defined time points for the reduction of AA oligomers (20.1 mM) in deuterated acetate buffer, pH 4.0, RT using 3 equivalents NaCNBH$_3$ (60.3 mM).
S7 Reduction of AₙM conjugates

Reduction of AₙM conjugates was studied by adding 3 equivalents reducing agent (PB or NaCNBH₃) to the equilibrium mixtures of conjugates (pH 3.0, 4.0 or 5.0). The course of the reduction was studied by monitoring the disappearance of hydrazone or oxime proton resonances and the appearance methylene proton resonances from the reduced secondary amine conjugates (at approximately 3 ppm) over time. Due to the overlap of the methylene proton resonances from the different forms of the M residue after reduction, the total resonance area (2.9 – 3.35 ppm) was integrated to give the yield of reduced conjugates. ¹H-NMR spectra obtained at different time points for the reduction of A₂M-ADH and A₂M-PDHA conjugates by 3 equivalents PB at pH 4.0 are given in Figure S17 and S18, respectively. The kinetics of the reduction is given in Figure S19 a and b, respectively. For the reduction of AₙM-PDHA conjugates, E-oxime resonances (H1, M E) overlapped with one of the resonances from the reduced form of PB (Figure S18). Hence, this integral was obtained by subtraction.

**Figure S17:** ¹H-NMR spectra obtained at defined time points for the reduction of A₂M-ADH conjugates (prepared using 2 equivalents ADH) by 3 equivalents (60.3 mM) PB at pH 4.0, RT. Resonances from the unreduced conjugate and unreacted oligomer are annotated in the first two spectra, whereas resonances from the reduced secondary amine conjugate are annotated in the last obtained spectrum (16.5 h).
Figure S18: $^1$H-NMR spectra obtained at defined time points for the reduction of A$_2$M-PDHA conjugates (prepared using 2 equivalents PDHA) by 3 equivalents (60.3 mM) PB at pH 4.0, RT. Resonances from the unreduced conjugate and unreacted oligomer are annotated in the first two spectra, whereas resonances from the reduced secondary amine conjugate are annotated in the last obtained spectrum (178 h).

Figure S19: Kinetics of the reaction obtained from the spectra in Figure S17 and S18 for the reduction of a) A$_2$M-ADH (hydrazone) conjugates and b) A$_2$M-PDHA (oxime) conjugates by 3 equivalents PB at pH 4.0, RT, respectively.
S8 Modelling of $A_nM$ reduction reactions

Using the rate constants and equilibrium yields obtained for the conjugation of $A_nM$ to 2 equivalents ADH or PDHA, the experimental data obtained for the reduction of conjugates (studied by time course NMR, S7) were fitted using the model described in S3 (based on the reaction scheme presented in Figure S6). Examples for the data fitting for the reduction $A_2M$-ADH or $A_2M$-PDHA conjugates using 3 equivalents PB at pH 4.0 (RT) are given in Figure S20 a and b, respectively. The addition of reducing agent to the equilibrium mixture of conjugates was set as $t = 0$. Due to overlapping resonances and low resolution in the NMR spectra, the yield of unreacted oligomers ($[A_nM]$ in the model) was not monitored. However, the model also predicts the consumption of unreacted oligomers based on the yield of conjugates and the rate of reduction (Figure S20).

![Figure S20](image)

Figure S20: Model fitted to the experimental data obtained for reduction of $A_2M$-ADH (a) and $A_2M$-PDHA (b) conjugates using 3 equivalents PB at pH 4.0, RT.

S9 Optimisation of preparative protocols

Statistical distribution of mono-, di- and unsubstituted ADH and PDHA

The relative amount of unsubstituted, monosubstituted and disubstituted ADH and PDHA can be calculated assuming the reactivities of the two termini are identical. Such estimates are primarily intended to determine how much ADH or PDHA should be used for mono-substitution (activation) without producing too much disubstituted species.

Definitions and main relations:

- $[A_nM]_0$: Initial molar concentration of oligosaccharide
- $[L]_0$: Initial molar concentration of ADH or PDHA
[-NH₂]₀: Initial molar concentration of terminal amine

Let a be defined as the equivalence of linker (ADH or PDHA) relative to oligosaccharide before reaction. Hence:

\[ [L]₀ = a[AₙM]₀ \]

Since we treat each terminal amine as a separate and independent reactant we may further write:

\[ [ -NH₂]₀ = 2[L]₀ = 2a[AₙM]₀ \]

Let b be defined as the fraction of oligosaccharide that has become substituted. b is obtained directly from the equilibrium yields listed in Table 1. Hence, b[AₙM]₀ becomes the molar concentration of substituted oligosaccharide, which must necessarily equal the molar concentration of substituted amine. The fraction of substituted amines (p) thus becomes:

\[ p = b \frac{[AₙM]₀}{[ -NH₂]₀} = b \frac{[AₙM]₀}{2[L]₀} = \frac{b}{2a} \]

Hence, p is a simple function of the fraction of substituted oligosaccharide (b) and the molar equivalence of linker (a). For statistical distributions fractions and probabilities are interchangeable. Hence:

The probability that both ends are substituted, fraction of disubstituted species (f_DS) = p²
The probability that none of the ends are substituted, fraction of unsubstituted species (f_US) = (1 – p)²
The probability that one end is substituted, fraction of monosubstituted species (f_MS) = 1 – p² – (1 – p)² = 2p – 2p² = 2p(1-p)

b is obtained directly from the equilibrium yields given in Table 1. As an example, the reaction between A₂M and 2 equivalents of ADH (a = 2) at pH 4.0 is considered. The equilibrium yield of substituted oligosaccharides obtained by NMR was 87 % and hence, b = 0.87. Then p becomes:

\[ p = \frac{0.87}{4} = 0.22 \]

and f_DS is (0.22)² = 0.0484 = 5 %. When 10 equivalents of ADH is used (i.e. a = 10) and we assume b = 0.87, f_DS is reduced to 0.002 = 0.2 %. Examples of the statistical distribution of mono-, di- and unsubstituted species with different equivalents of linker (a) and fractions of substituted oligomer (b) are given in Figure S21-S23.
Figure S21: Statistical distribution of mono-, di- and unsubstituted species with different equivalents of linker (a) and b = 0.70.

Figure S22: Statistical distribution of mono-, di- and unsubstituted species with different equivalents of linker (a) and b = 0.87.
Figure S23: Statistical distribution of mono-, di- and unsubstituted species with different equivalents of linker ($a$) and $b = 0.99$.

Minimisation of disubstituted ADH or PDHA

As shown above the statistical amount of disubstituted ADH or PDHA is significant (5-6%) when 2 equivalents are used, especially for high reaction yields, but decreases below 1% for 10 equivalents. This was qualitatively verified by GFC fractionation of $A_{n}M$-PDHA conjugates prepared with 2 or 10 equivalents of PDHA, respectively (Figure S24). A significant decrease in disubstituted species was observed when 10 equivalents were used. Hence, a large excess ($\geq 10$ equivalents) is necessary to minimise the amount of disubstituted species.

Figure S24: GFC fractionation of the reaction mixtures obtained for the conjugation of $A_{n}M$ oligomers to 2 or 10 equivalents (2x or 10x, respectively) PDHA under otherwise standard conditions (20.1 mM oligomer, pH 4.0, RT).
Optimisation of reduction conditions for PDHA conjugates

The reduction of PDHA conjugates was optimised by varying the concentration of reducing agent (PB). However, due to the low solubility of PB in the buffer, reduction of A₅M-PDHA conjugates (equilibrium mixture with 10 equivalents PDHA) using 20 equivalents PB was performed in deuterated buffer in a separate vial (not in the NMR tube). The reaction was performed on a shaking device to increase the collision frequency of undissolved reducing agent and conjugates. NMR spectra of the dissolved phase of the reaction mixture was obtained after 24 and 48 hours revealing complete reduction after 48 hours.

Preparation of AₙM conjugates using optimised protocols

Reduced AₙM conjugates (A₅M-ADH and A₅M-PDHA) were prepared using optimised protocols. In brief, conjugation was carried out for 6 hours at RT using 10 equivalents ADH and PDHA at pH 4.0. For ADH conjugates, reduction was performed for 24 hours at RT by adding 3 equivalents PB to the equilibrium mixture, whereas for the PDHA conjugates, 20 equivalents PB were added to the equilibrium mixture and the reduction was performed for 48 hours at RT to ensure complete reduction. Reaction mixtures were fractionated by GFC and purified conjugates were characterized by ¹H-NMR. Fractionation of reduced A₅M-ADH and A₅M-PDHA conjugates is shown in Figure S25. Fractionation of the A₅M oligomer mixture is included in the figure for comparison. Small amounts of disubstituted ADH/PDHA were formed in both reactions. The relative amount of disubstituted species seemed higher than obtained for the optimised protocol using A₃M oligomers (Figure S24), which may be a result of higher reactivity of oligomers with higher DP and the higher fraction of substituted amine (equilibrium yield of conjugates). In addition, conjugates with shorter and longer AₙM oligomers were present in the reaction mixture reflecting a slight polydispersity of the starting oligomer (A₅M), due to the lower resolution of the preparative GFC system. However, pure A₅M-ADH/PDHA conjugates were obtained after fractionation, verified by the ¹H-NMR characterization of A₅M-ADH and A₅M-PDHA given in Figure S26 and S27, respectively.
**Figure S25:** GFC fractionation of the reaction mixtures obtained for the preparation of AₓM-ADH and AₓM-PDHA conjugates using optimised conditions. Fractionation of the mixture of AₓM oligomers is included for comparison.

**Figure S26:** ¹H-NMR spectrum of the purified AₓM-ADH conjugate (D₂O, 300K, 600 MHz)
S10 2D NMR characterization of the reduced and purified A$_2$M-PDHA

The reduced and purified A$_2$M-PDHA conjugate was characterized by homo- and heteronuclear NMR correlation experiments. The conjugate was dissolved in D$_2$O and the NMR analysis was carried out using the 800 MHz spectrometer in a 3 mm NMR tube. Resonances were assigned by starting at the anomeric proton signal and then following the proton-proton connectivity using TOCSY, DQF-COSY/IP-COSY, $^{13}$C H2BC and $^{13}$C HSQC-$[^{1}H,^{1}H]$ TOCSY spectra. $^{13}$C-HSQC was used for assigning the carbon chemical shifts. The $^{13}$C HMBC spectrum provided information of connections between the sugars. One of the alternative forms of the M residue was structurally elucidated to be 3,5-anhydro-D-mannose. The following designations are used in the spectra displayed in Figure S28 and S29 and Table S1: A1 (N-acetyglucosamine (A) residue at the non-reducing end), A2 (middle N-acetyglucosamine (A) residue), M1 (2,5-anhydro-D-mannose (M) residue), M2 (3,5-anhydro-D-mannose (M') residue), -Ac (N-acetyl groups of A residues) a, b and c (unique identified methylene groups of PDHA). H/C# refers to the proton-carbon pairs with the ring carbon number for the monosaccharides. TSP was used for chemical shift reference. Chemical shifts are reported in Table S1. The structure of the A$_2$M-PDHA conjugate is included in Figure S29.
Figure S28: $^1$H-NMR spectrum of the reduced and purified A$_2$M-PDHA conjugate recorded at 298 K. Designations as described above.
Figure S29: $^{13}$C HSQC spectrum of the reduced and purified A$_2$M-PDHA conjugate recorded at 298 K. Designations as described above.

Table S1: Chemical shift assignment for the reduced and purified A$_2$M-PDHA conjugate at 298 K. The chemical shifts are related to TSP. Designations as described above.

| Structural unit | Assignment |
|-----------------|------------|
|                 | H-1; C-1  | H-2; C-2  | H-3; C-3  | H-4; C-4  | H-5; C-5  | H-6; C-6  | Ac-H; C |
| A1              | 4.60,104.2| 3.75;57.5 | 3.59;76.2 | 3.49;72.4 | 3.51;78.6 | 3.93,3.77;63.2 | 2.09; 24.8 |
| A2              | 4.56;103.4| 3.77;58.2 | 3.74;74.9 | 3.98;84.7 | 3.56;77.2 | 3.88,3.69;62.9 | 2.05; 24.8 |
| M1              | 3.14,3.02;54.8 | 4.11;83.1 | 4.20;80.5 | 4.12;88.3 | 3.98;84.7 | 3.73,3.69;64.0 | - |
| M2              | 3.25,3.07;52.2 | 4.16;80.9 | 4.36;78.4 | 3.62;82.3 | n.d | n.d | - |
| PDHA            | 3.82,72.9 | 1.88;29.6 | 3.81;75.6 | - | - | - |

a, b, c: Not determined.
S11 Preparation of chitin-β-chitin diblocks from activated chitin oligomers (A₄M-ADH)

Chitin-β-chitin diblocks were prepared by reacting A₄M-ADH conjugates (reduced and purified) with A₄M oligomers in an equimolar molar ratio. The conjugation of the second block (A₄M) was monitored by NMR (as described in S2). The kinetics was compared to simulated values for the corresponding reaction using rate constants obtained for the conjugation of A₂M and A₅M to free ADH. The results are summarised in Table S2 and kinetic plots are given in Figure S30. The comparison suggests the second conjugation proceeds somewhat faster than the first.

Table S2: Kinetic parameters obtained from the modelling of the reaction of A₄M with an equimolar proportion of A₄M-ADH. Simulated parameters for the corresponding reactions with equimolar proportions of oligomers and amines (0.5 equivalents ADH) using rate constants obtained for the conjugation of A₂M and A₅M to free ADH are given in italics.

| Equivalents | A₄M-ADH | A₂M ADH | A₅M ADH |
|-------------|---------|---------|---------|
| A₄M         | 1       | 0.5     | 0.5     |
| pH          | 4.0     | 4.0     | 4.0     |
| k₁ [h⁻¹]   | 7.3 x 10⁻² | 1.8 x 10⁻² | 3.0 x 10⁻² |
| k₂ [h⁻¹]   | 1.5 x 10⁻¹ | 2.0 x 10⁻¹ | 3.5 x 10⁻¹ |
| k₃ [h⁻¹]   | 1.1 x 10⁻² | 2.5 x 10⁻³ | 4.0 x 10⁻³ |
| t₀.5 [h]   | 0.35    | 0.91    | 0.54    |
| t₀.9 [h]   | 1.57    | 3.34    | 1.96    |
| Equilibrium yield [%] | 74 | 51 | 50 |

Figure S30: Kinetics of the reaction of A₄M with an equimolar proportion of A₄M-ADH. Simulated data for the corresponding reactions of A₂M and A₅M with free ADH and PDHA (0.5 equivalents, equimolar proportions of oligomers and amines) is included for comparison.

Reduction of the obtained equilibrium mixture was performed using 3 equivalents PB for 24 hours (RT). The reaction mixture was fractionated by GFC (Figure S31) and main products were purified.

¹H-NMR characterization of the main fraction (Figure S32) confirmed formation of the completely reduced A₄M-ADH-MA₄ diblock. Due to slight polydispersity of the A₄M oligomer (containing
oligomers of lower and higher DP), some longer and shorter diblocks were consequently formed. ¹H-NMR characterization of the fraction holding unreacted A₄M oligomers and A₄M-ADH conjugates (Figure S33) revealed complete reduction of the unreacted oligomers (no H1, M reducing end resonances). Hence, the fast reduction of oligomers prevented the diblock formation from going to completion. By integration of the chromatogram (Figure S31), the chitin-b-chitin diblock (A₄M-ADH-MAₙ) area was found to accounted for 70 % of the total area, whereas unreacted conjugates (A₄M-ADH) and unreacted oligomers (AₙM) accounted for the resisting 30 % of the area. By assuming an equimolar ratio of oligomers and conjugates, the weight yield of diblocks in the reaction was 82.5 %.

| Time (min) | Diblock area (Area) | Conjugate/oligomer area (Area) |
|------------|---------------------|-------------------------------|
| 440-685    | 17.9                | 7.6                           |
| 685-822    | 70.2                | 29.8                          |

**Figure S31:** GFC fractionation of the reaction mixture obtained for the preparation of chitin diblocks by reacting A₄M-ADH with A₄M oligomers in an equimolar ratio. Fractionation of the mixture of AₙM oligomers is included for comparison.
Figure S32: $^1$H-NMR spectrum of the purified $A_4M$-PDHA-MA$_4$ diblock fraction ($D_2O$, 300K, 600 MHz) from the chromatogram in Figure S31.

Figure S33: $^1$H-NMR spectrum of the $A_4M$-PDHA/$A_4M$ (reduced) fraction ($D_2O$, 300K, 600 MHz) from the chromatogram in Figure S31.
S12 Preparation of chitin-β-chitin diblocks using a sub-stoichiometric amount of ADH or PDHA

Chitin-β-chitin diblocks were prepared using \( A_{2}M \) oligomers and a sub-stoichiometric amount of ADH or PDHA (0.5 equivalents). The conjugation of oligomers was monitored by NMR. The kinetics was compared to simulated values using rate constants obtained for the conjugation of \( A_{2}M \) to free ADH or PDHA (2 equivalents). The results are summarised in Table S3 and the kinetic plots are given in Figure S34.

**Table S3:** Kinetic parameters obtained from the modelling of the reaction of \( A_{2}M \) with 0.5 equivalents of ADH or PDHA (equimolar proportions of oligomers and amines). Simulated parameters for the corresponding reactions using rate constants obtained for the conjugation of \( A_{2}M \) to free ADH or PDHA are given in italics.

| Equivalents | A+B ↔ E | A+B ↔ Z | A+B ↔ E + Z | Equilibrium yield [%] |
|-------------|---------|---------|-------------|----------------------|
| \( A_{2}M \) | B        | pH      | \( k_{1} \) [h\(^{-1}\)] | \( k_{2} \) [h\(^{-1}\)] | \( t_{0.5} \) [h] | \( t_{0.9} \) [h] |                  |
| ADH 0.5 | 4.0 | 4.0x10\(^{-1}\) | 9.0x10\(^{-1}\) | 5.8x10\(^{-2}\) | 9.0x10\(^{-1}\) | 0.06 | 0.28 | 73 |
| PDHA 0.5 | 4.0 | 1.2x10\(^{-1}\) | 2.0x10\(^{-1}\) | 1.8x10\(^{-2}\) | 2.5x10\(^{-3}\) | 2.0x10\(^{-1}\) | 0.22 | 1.21 | 51 |

**Figure S34:** Kinetics of the reactions of \( A_{2}M \) with 0.5 equivalents of **a)** ADH and **b)** PDHA. Simulated data for the corresponding reactions of \( A_{2}M \) with free ADH and PDHA (0.5 equivalents, equimolar proportions of oligomers and amines) is included for comparison.

Reduction of the equilibrium mixtures was performed using 3 equivalents PB for 24 hours or 20 equivalents PB for 48 hours at room temperature for the reaction with ADH and PDHA, respectively. Reaction mixtures were fractionated by GFC (Figure S35) and the main fractions were purified and characterized by \(^1\)H-NMR (Figure S36 – S40). Products from the ADH reaction were completely reduced (no hydrazone resonances in the spectra) whereas minor oxime resonances were observed in
the products from the PDHA reaction, indicating incomplete reduction. Impurity of the A\textsubscript{2}M oligomers gave additional fractions in the GFC chromatograms obtained for both reactions. The yield of diblocks was obtained by integrating the chromatograms (Figure S35) as described above. The weight yield of diblocks was 74 % and 87 % for the reaction with ADH and PDHA, respectively. The yield of diblocks corresponds well to the statistical amount of diblocks expected (73 % for ADH and 88 % for PDHA). Unreacted oligomers were completely reduced (no H\textsubscript{1}, M resonances observed in the $^1$H-NMR spectra), confirming the fast reduction of A\textsubscript{n}M oligomers preventing the diblock formation from going to completion.

**Figure S35:** GFC fractionation of the reaction mixture obtained for the preparation of chitin diblocks by reacting A\textsubscript{2}M oligomers with a sub stoichiometric amount (0.5 equivalents) of a) ADH or b) PDHA. Fractionation of the mixture of A\textsubscript{n}M oligomers is included for comparison.
**Figure S36:** $^1$H-NMR spectrum of the purified A$_2$M-ADH-MA$_2$ diblock fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S35a.

**Figure S37:** $^1$H-NMR spectrum of the A$_2$M-ADH fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S35a.
Figure S38: $^1$H-NMR spectrum of the A$_2$M (reduced) fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S35a.

Figure S39: $^1$H-NMR spectrum of the purified A$_2$M-PDHA-MA$_2$ diblock fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S35b.
S13 Preparation and characterization of dextran (Dext$_{m}$) oligomers

Dextran (Dext$_{m}$) oligomers were prepared by acid hydrolysis of dextran (Dextran T-2000, $M_w = 2,000,000$). $^1$H-NMR characterisation of Dextran T-2000 is given in Figure S41. The degree of branching was estimated from the integral of the H1 resonance of internal glucose residues in the main chain and the H1 of the glucose residues at the branching points (BP) to 3.6 %. The degradation mixture was fractionated by preparative GFC to obtain isolated Dext$_{m}$ oligomers (Figure S42a). The isolated Dext$_{m}$ oligomer (DP = 6) was characterized by $^1$H-NMR (Figure S43) and fractionated by analytical GFC (Figure S42b) to show the slight polydispersity of the oligomer. The degree of branching of the oligomer was estimated to approximately 0.8 % showing that the branches are more rapidly hydrolysed than the linkages in the main chain.
Figure S41: $^1$H-NMR characterization of Dextran T-2000 (D$_2$O, 300K, 600 MHz).

Figure S42: a) Preparative GFC fractionation of the mixture of Dext$_m$ oligomers b) analytical GFC fractionation of isolated Dext$_6$. Fractionation of the mixture of Dext$_m$ oligomers is included for comparison.
Figure S43: $^1$H-NMR spectrum of the isolated Dext$_6$ oligomer (in D$_2$O, 300 K, 600 MHz) obtained from the preparative GFC fractionation in Figure S42a.

S14 Conjugation of Dext$_m$ oligomers to ADH and PDHA studied by time course NMR

Conjugation of Dext$_5$ oligomers (DP = 5) to ADH and PDHA (2 equivalents) at pH 4.0, RT was monitored by NMR (Figure S44 and S45, respectively). Here, the reducing end of dextran (Glc, normal reducing end), governs the conjugation and hence, the acyclic hydrazones and oximes are in equilibrium with cyclic N-glycosides. As previously shown$^5$, dextran formed almost exclusively N-pyranosides with ADH, whereas it formed N-pyranosides in addition to E- and Z-oximes with PDHA. The equilibrium yield of conjugates (E-/Z-hydrazones or oximes + N-pyranosides) obtained for the reactions was 35% for Dext$_5$ with ADH and 87% for Dext$_5$ with PDHA. The experimental data obtained in the conjugation reactions were fitted using the model described in S3 (based on the reaction scheme presented in Figure S5). The data fitting for the conjugation Dext$_5$ oligomers to ADH and PDHA (2 equivalents) at pH 4.0 (RT) are given in Figure S46. Rates ($t_{0.5}$ and $t_{0.9}$) and equilibrium yields for the total conjugation reaction are given in Table S4.
Figure S44: $^1$H-NMR spectra obtained at defined time points for the conjugation reaction with Dext$_5$ (20.1 mM) and 2 equivalents ADH at pH 4.0, RT.

Figure S45: $^1$H-NMR spectra obtained at defined time points for the conjugation reaction with Dext$_5$ (20.1 mM) and 2 equivalents PDHA at pH 4.0, RT.
Figure S46: a) Model fitted to the experimental data obtained for the conjugation of Dext₅ to ADH (2 equivalents) at pH 4.0, RT. b) Model fitted to the experimental data obtained for the conjugation of Dext₅ to PDHA (2 equivalents) at pH 4.0, RT.

Table S4: Kinetic parameters obtained from the modelling of the reaction of Dext₅ with 2 equivalents ADH or PDHA.

| Equivalents | A   | B   | B   | pH | t₀.₅ [h] | t₀.₉ [h] | Equilibrium yield [%] |
|-------------|-----|-----|-----|-----|---------|---------|----------------------|
| Dext₅       | ADH | 2   | 4.0 | 1.74| 5.84    |          | 35                   |
| Dext₅       | PDHA| 2   | 4.0 | 2.38| 8.54    |          | 87                   |

S15 Preparation of chitin-β-dextran diblocks

Chitin-β-dextran diblocks were prepared by reacting A₅M-ADH or A₅M-PDHA conjugates (reduced and purified) with dextran oligomers of DP = 6 (Dext₆) in an equimolar ratio. The conjugation of the dextran block was monitored by NMR (as described in S2) and combined equilibrium yields of 15 and 66% were obtained for the conjugation to A₅M-ADH or A₅M-PDHA, respectively. The kinetics was (as above) compared to simulated values using rate constants (k₁, k⁻¹ etc) obtained for the conjugation of Dext₅ to free ADH or PDHA. The results are summarised in Table S5 and the kinetic plots are given in Figure S47. Compared to the model, it appears the second conjugation is faster and gives higher yields than the first.
Table S5: Kinetic parameters obtained from the modelling of the reaction of Dext₆ with an equimolar proportion of A₅M-ADH or A₅M-PDHA. Simulated parameters for the corresponding reactions with equimolar proportions of oligomers and amine using rate constants obtained for the conjugation of Dext₅ to free ADH or PDHA are given in italics.

| Equivalents | A     | B     | pH | t₀.5 [h] | t₀.9 [h] | Equilibrium yield [%] |
|-------------|-------|-------|----|---------|---------|-----------------------|
| Dext₆       | A₅M-ADH | 1     | 4.0 | 1.72    | 5.74    | 15                    |
| Dext₅       | ADH   | 0.5   | 4.0 | 2.27    | 7.54    | 11                    |
| Dext₆       | A₅M-PDHA | 1   | 4.0 | 2.07    | 8.36    | 66                    |
| Dext₅       | PDHA  | 0.5   | 4.0 | 5.76    | 21.30   | 51                    |

Figure S47: Kinetics of the reactions of Dex₆ with equimolar amounts of a) A₅M-ADH and b) A₅M-PDHA. Simulated data for the corresponding reactions of Dex₅ with free ADH and PDHA (0.5 equivalents) is included for comparison.

Reduction of the equilibrium mixtures with chitin-β-dextran diblocks was performed at 40 °C using 20 equivalents PB due to the slow reduction of conjugates with normal reducing ends. Reduction was terminated after 72 and 144 hours for the preparation A₅M-PDHA-Dext₆ and A₅M-ADH-Dext₆ diblocks, respectively, due to the slower reduction of ADH conjugates. The reaction mixtures were fractionated by GFC (Figure S48), and main fractions were purified and characterized by ¹H-NMR (Figure S49-S53). As purified A₅M conjugates were used for the diblock preparation, the integral for the H1, A resonance was set to 5 in all the ¹H-NMR spectra. The yield of diblocks was obtained by integrating the chromatograms (Figure S48) as described above. Due to the slight polydispersity of the Dext₆ oligomer, some longer and shorter diblocks were also formed. The weight yield of diblocks was 85 and 92 % for chitin-β-dextran diblocks with ADH and PDHA, respectively. The amount of remaining unreduced diblock could not be accurately determined because the resonance
corresponding to the unreduced \(N\)-pyranoside conjugates overlaps with other resonances in the spectra. However, the integral for the secondary amine resonance balanced the integrals of the resonances from both chitin and dextran, suggesting close to complete reduction of diblocks.

Approximately 40 % the unreacted dextran oligomers from the reaction with \(A_5M\)-ADH conjugates (20 equivalents PB, 40 °C) were reduced after 144 hours (6 days) (Figure S51). Hence, the dextran oligomers are reduced by PB with a much slower rate than the \(A_nM\) oligomers. The slow reduction of dextran oligomers also explains the higher yield of diblocks obtained compared to the low amination yield, as the dextran oligomers can react further after addition of reducing agent.

**Figure S48:** GFC fractionation of the reaction mixture obtained for the preparation of diblocks by reacting Dext\(_6\) oligomers with a) \(A_5M\)-ADH or b) \(A_5M\)-PDHA conjugates in an equimolar ratio. Fractionation of the Dext\(_6\) oligomers and the purified conjugates are included for comparison.
Figure S49: $^1$H-NMR spectrum of the A$_5$M-ADH-Dext$_6$ fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S48a.

Figure S50: $^1$H-NMR spectrum of the A$_5$M-ADH-Dext$_5$/A$_5$M-ADH fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S48a.
Figure S51: $^1$H-NMR spectrum of the Dext$_6$ fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S48a.

Figure S52: $^1$H-NMR spectrum of the A$_3$M-PDHA-Dext$_6$ fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S48b.
Figure S53: $^1$H-NMR spectrum of the A$_5$M-PDHA-Dext$_5$/A$_5$M-PDHA fraction (D$_2$O, 300K, 600 MHz) from the chromatogram in Figure S48b.

References

1. Allan, G. G.; Peyron, M., Molecular-Weight Manipulation of Chitosan .1. Kinetics of Depolymerization by Nitrous-Acid. Carbohydr. Res. 1995, 277, (2), 257-272.

2. Tømmeraas, K.; Vårum, K. M.; Christensen, B. E.; Smidsrød, O., Preparation and characterisation of oligosaccharides produced by nitrous acid depolymerisation of chitosans. Carbohydr. Res. 2001, 333, (2), 137-144.

3. Sugiyama, H.; Hisamichi, K.; Sakai, K.; Usui, T.; Ishiyama, J.-I.; Kudo, H.; Ito, H.; Senda, Y., The conformational study of chitin and chitosan oligomers in solution. Bioorg. Med. Chem. 2001, 9, (2), 211-216.

4. Lindberg, B.; Lönnren, J.; Svensson, S., Specific Degradation of Polysaccharides. In Advances in Carbohydrate Chemistry and Biochemistry, Tipson, R. S.; Derek, H., Eds. Academic Press: 1975; Vol. Volume 31, pp 185-240.
5. Mo, I. V.; Feng, Y.; Dalheim, M. Ø.; Solberg, A.; Aachmann, F. L.; Schatz, C.; Christensen, B. E., Activation of enzymatically produced chitooligosaccharides by dioxyamines and dihydrazides. *Carbohydr. Polym.* **2020**, *232*, 115748.

6. van Dijk-Wolthuis, W. N. E.; Franssen, O.; Talsma, H.; van Steenbergen, M. J.; Kettenes-van den Bosch, J. J.; Hennink, W. E., Synthesis, Characterization, and Polymerization of Glycidyl Methacrylate Derivatized Dextran. *Macromolecules* **1995**, *28*, (18), 6317-6322.