AUTHOR MANUSCRIPT OF ARTICLE PUBLISHED IN *CARBOHYDRATE POLYMERS*

Volume 267, 1 September 2021, 118193, DOI: 10.1016/j.carbpol.2021.118193

Functionalisation of the non-reducing end of chitin: A new approach to form complex block polysaccharides and soluble chitin-based block polymers

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Bullet points

- Chitin oligomers prepared by nitrous acid degradation of chitosan only possess a single vicinal diol prone to periodate oxidation at the non-reducing end (NRE) residue
- Both aldehydes formed at the NRE can subsequently be activated by dioxyamines or dihydrazides
- The NRE aldehydes are equally or more reactive towards dioxyamines and dihydrazides than the pending aldehyde of the 2,5-anhydro-D-mannose (M) reducing end residue
- The free end of the dioxyamines/dihydrazides after conjugation can react with aldehydes from the same (intra) or from a different oligosaccharide (inter)
- NRE functionalised and activated chitin oligomers can act as precursors for block polysaccharides or form water-soluble chitin-based block polymers

Abstract

Most polysaccharides used in polysaccharide-based block copolymers are attached to the second block through the reducing end, due to the few and highly polysaccharide specific non-reducing end (NRE) functionalisation methods available. Chitin oligomers, prepared by nitrous acid degradation of chitosan (A_nM) can, however, be selectively oxidised by periodate since they only possess a single vicinal diol in the NRE residue. Here, we show that both aldehydes formed after oxidation are highly reactive towards activation by bifunctional oxyamines and hydrazides. The reactivity was shown to be even higher than for the aldehyde of the already reactive M reducing end residue of such oligomers. Fully activated oxidised A_nM oligomers was obtained using an excess of bifunctional linkers, enabling the preparation of more complex block polysaccharides such as ABA- or ABC-type. A sub-stochiometric amount of linkers resulted in conjugation of the blocks through the reducing and NRE to form a discrete distribution of 'polymerised' A_nM oligomers. Such chitin-based block polymers were, in contrast to chitins of the same chain lengths, water-soluble.

Introduction

AB-type diblock polysaccharides represent a new class of engineered block polysaccharides, utilising only natural polysaccharide blocks[1, 2]. Coupling of the polysaccharide blocks through their reducing ends, result in antiparallel chains. Preparation of more complex block polysaccharides, such as linear ABA or ABC triblock copolymers, requires reactions at the non-reducing end (NRE) of the B block and only a few

polysaccharide specific methods for functionalisation of the NRE have been published. However, NRE reactivity has been demonstrated for dextran[3] and lyase degraded heparin[4] after intermediate functionalisation steps.

Periodate oxidation of polysaccharides converts vicinal diols (and closely related structures) to the corresponding dialdehydes. Hence, the ability of different polysaccharides to be oxidised depends on their chemical composition and linkage geometry[5]. In the special case of fully *N*-acetylated chitin oligomers prepared by nitrous acid degradation of chitosan (A_nM)[6], only the non-reducing end residue contains oxidisable, vicinal diols (Figure 1a). Therefore, periodate oxidation of such oligomers should in theory provide oligomers with two aldehydes (C3 and C4) in the NRE residue, in addition to the highly reactive pending aldehyde of the 2,5-anhydro-D-mannose (M) residue (Figure 1b). Only a few studies describing the functionalisation of the NRE of chitin by periodate oxidation is reported in the literature[7-9], and to our knowledge, subsequent reactions with the aldehydes have not been investigated. Hence, it is unknown whether both the aldehydes at the NRE are reactive, but the question is pertinent as only one of the dialdehydes obtained by lateral periodate oxidation of alginate has shown to react with amines through reductive amination[10, 11].

A_nM oligosaccharides have previously been subjected to reducing end activation by a dioxyamine (PDHA) and a dihydrazide (ADH) to form precursors for AB-type block polysaccharides[12]. Such activated A_nM oligomers were used to prepare A_nM-*b*-dextran diblocks. Reaction of *oxidised* A_nM oligomers with such dioxyamines or dihydrazides may form a range of new complex structures. In the present work we first study the reaction of periodate oxidised A_nM with an excess PDHA or ADH using the previously developed two-step reductive amination protocol[12] to assess the relative reactivities of aldehydes at the reducing and non-reducing end (Figure 1c). Secondly, we reacted oxidised A_nM oligomers with a sub-stoichiometric amount of PDHA to obtain a discrete distribution of a new type of water-soluble chitin block polysaccharides composed of a progressively increasing number of A_nM blocks reacted through both ends. The introduction of a dialdehyde at the NRE combined with the highly reactive aldehyde at the reducing of A_nM oligomers enables the preparation of a range of new glycoconjugates, including complex block polysaccharides.

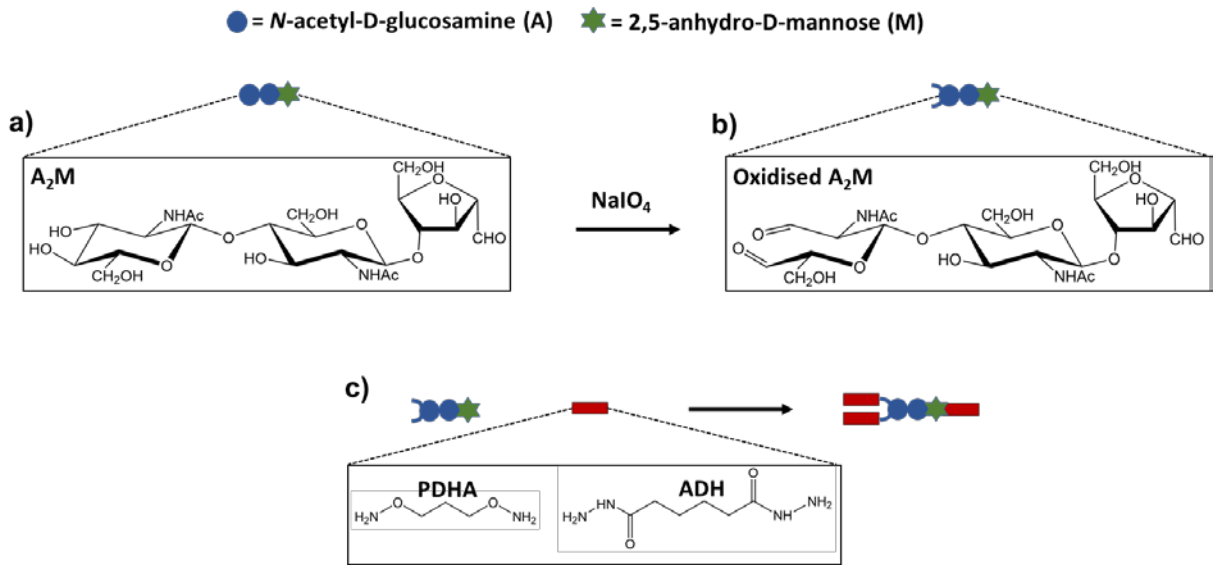

Figure 1: a) chemical structure of an A_2M oligomer, **b)** chemical structure of a periodate oxidised A_2M oligomer, **c)** conjugation of PDHA or ADH to the reducing and non-reducing end of oxidised A_2M oligomer.

Materials and methods

Materials

High molecular weight chitosan (F_A = 0.48, [η] = 1210 mL/g) was obtained from Advanced Biopolymers ABC. Adipic acid dihydrazide (ADH), O,O'-1,3-propanediylbishydroxylamine dihydrochloride (PDHA) and 2-methylpyridine borane complex (α-Picoline borane, PB) were obtained from Sigma-Aldrich. All other chemicals were obtained from commercial sources and were of analytical grade.

Gel filtration chromatography (GFC)

Preparative and analytical gel filtration chromatography (GFC) were used for fractionation of chitin oligosaccharides and fractionation of products, respectively, as described earlier[12]. In brief, both systems were composed of Superdex 30 columns (BPG 140/950 (140 x 950 mm) and HiLoad 26/600 (26 x 600 mm), respectively) connected in series, continuously eluting ammonium acetate (AmAc) buffer (0.15 M, pH 4.5 and 0.1 M, pH 6.9, respectively). Fractionation was monitored on-line using a refractive index (RI) detector and fractions were collected and pooled according to elution times. The pooled fractions were reduced to appropriate volumes, dialyzed (MWCO = 100-500 Da) against ultrapure Milli-Q (MQ) water until the measured conductivity of the water was < 2 µS/cm and freeze-dried or freeze-dried directly without dialysis to remove the volatile mobile phase (AmAc-buffer).

NMR spectroscopy

Water-soluble samples for NMR characterization were dissolved in D_2O (450-600 µL, approx.10 mg/mL). For some samples, 1% deuterated sodium 3-(trimethylsilyl)-propionate (TSP-d_4, 3 µL) was added as an internal standard. Water-insoluble samples were dissolved in deuterated hexafluoro isopropanol (HFIP-d_2).

Characterization was performed by obtaining 1D ^{1}H NMR spectra at 300K on a Bruker Ascend 14.1 Tesla 600 MHz equipped with Avance III HD electronics and a 5 mm Z-gradient CP-TCI cryogenic probe. All spectra were recorded, processed and analysed using TopSpin 3.5pl7 software (Bruker BioSpin).

Mass spectrometry (MS)

MS characterisation was performed using flow injection analysis (FIA) coupled with quadrupole time of flight (gTOF) MS as described earlier[13]. The mass range was set to 50-2000 Da which limited the characterisation of larger structures.

Preparation of chitin oligomers by nitrous acid degradation

Chitin oligomers (A_nM) were prepared by nitrous acid (HONO) degradation as described earlier[12]. In brief, chitosan (F_A = 0.48) was degraded using an excess of HONO (1.3 moles of HONO per mole GlcN) overnight in the dark at 4 °C. Washing and centrifugation steps were performed to remove the insoluble fraction, assumed to be water-insoluble chitin oligomers. The water-soluble low molecular weight chitin oligomers were fractionated according to degree of polymerization (DP) by GFC (DP < 10). Isolated oligomers were purified by dialysis, freeze-dried and characterized by ^{1}H-NMR.

Periodate oxidation

Chitin oligomers (A_nM) or chitin oligomer conjugates (A_6M-PDHA or A_2M-ADH-MA_2) (10 mM) were dissolved in MQ-water. Oxygen was removed by bubbling the solution with N_2 gas for 10 minutes. Freshy prepared and N_2 bubbled $NaIO_4$ (100 mM) was added to give a final molar ratio of 2:1 or 4:1 ($NaIO_4$: A_nM or conjugates). Reactions were performed in the dark at 4 °C for 24 hours on a shaking device. Ethylene glycol (10 molar equivalents) was added to the reaction mixture and stirred for 30 minutes to terminate the reaction. The reaction mixture was dialyzed (MWCO = 100-500 Da) against MQ-water until the measured conductivity was < 2 µS/cm and freeze-dried.

Conjugation of oxidised AnM oligomers to ADH and PDHA

Oxidised A_nM oligomers (6.5 mM) and ADH or PDHA (0.5 – 10 equivalents) were dissolved in NaAc-buffer (500 mM, pH 4.0) and reacted for 24 hours at room temperature. PB (20 equivalents) was added to the reaction mixture, and the reduction was allowed to proceed for 72 hours at room temperature. The reaction mixtures were dialysed (MWCO = 100-500 Da) against 0.05 M NaCl until the partly insoluble PB dissolved and subsequently freeze-dried. The mixtures were further fractionated by GFC. Isolated fractions were purified by direct freeze-drying and characterised by NMR or MS.

Results and discussion

Periodate oxidation of A_nM oligomers

A_nM oligomers, where n corresponds to DP-1, were prepared by nitrous acid depolymerisation as described earlier[12]. The water-soluble oligomers (n = 0-8) were isolated by GFC fractionation to obtain oligomers of specific DP. Initially, A_2M oligomers were oxidised using 2 equivalents of periodate, purified by dialysis and characterised by ^{1}H-NMR (Figure 2b). New resonances appeared in the anomeric region (4.8-5.6 ppm), but the spectrum was otherwise too complex for a complete determination of structure and degree of oxidation. The high complexity can be attributed to inter- or intra-residue hemiacetals readily formed with the dialdehydes after oxidation of the NRE [14, 15]. Such hemiacetals are acid stable and difficult to structurally elucidate. A reduced intensity was observed for the resonance resulting from H1, M (gem-diol) relative to other resonances in the spectrum after oxidation. The reduced intensity may be caused by the formation of inter-residue hemiacetals with the aldehydes in the NRE. The M residue seemed otherwise unaltered as predicted from its structure. Hence, the oxidation only took place at the NRE. Oxidation with 4 equivalents of periodate gave essentially the same result, however, with an even lower intensity of the H1, M resonance suggesting a higher degree of oxidation and more inter-residue hemiacetals formed (Figure 2c). Unless otherwise stated, oligomers oxidised with 2 equivalents periodate were used in the following experiments.

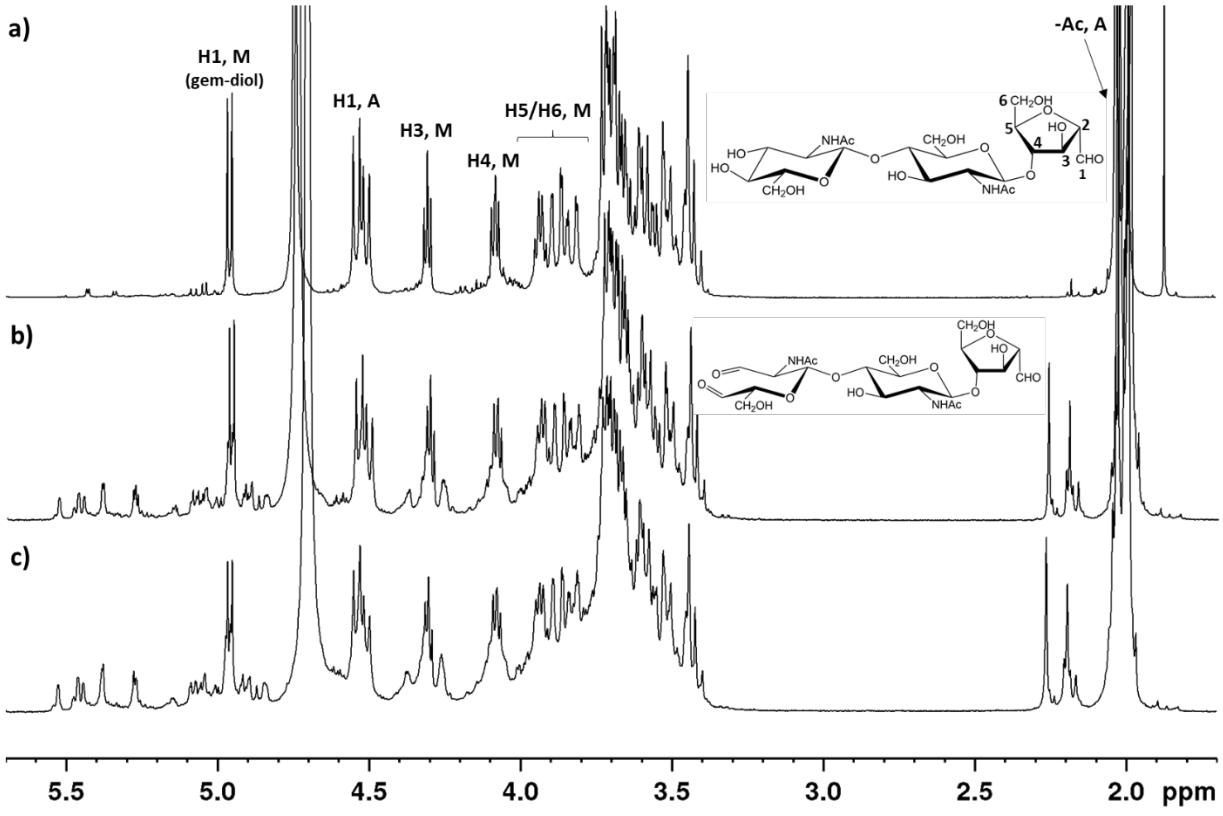

Figure 2: ^{1}H-NMR characterisation of **a)** A_2M (unoxidised) **b)** oxidised A_2M prepared using 2 equivalents $NaIO_4$ **c)** oxidised A_2M prepared using 4 equivalents $NaIO_4$.

Activation of oxidised A_2M oligomers by PDHA and ADH

Even though oxidised A_nM preparations were rather complex mixtures, we decided to proceed without further purification as this most likely could be better obtained after conjugation to PDHA or ADH. Thus, oxidised A_2M oligomers were reacted with 10 equivalents PDHA or ADH using the two-step reductive amination protocols recently developed for A_nM oligomers[12]. Interestingly, the two reaction systems behaved very differently. With ADH an insoluble fraction was formed, while this was not observed for PDHA. The reaction mixtures (soluble fractions) were characterized by ^{1}H-NMR (Supporting information, S1). Compared to the corresponding conjugates prepared with unoxidised oligomers, the NMR spectra of the reaction mixtures were more complex. However, resonances resulting from the characteristic secondary amine protons (around 3.0 ppm) appeared, indicating the formation of conjugates. Importantly, the H1 resonances for unreacted M disappeared, confirming complete reaction at the reducing end, as expected[12]. Incomplete reduction of conjugates formed with PDHA was indicated by the presence of resonances in the Schiff base area (6-8 ppm). Conjugates formed with ADH were in contrast completely reduced (Supporting information, S1). The reaction mixtures were further fractionated by GFC (Figure 3). Purified fractions were characterised by ^{1}H-NMR and MS (Supporting information, S2 and S3 for PDHA and ADH, respectively).

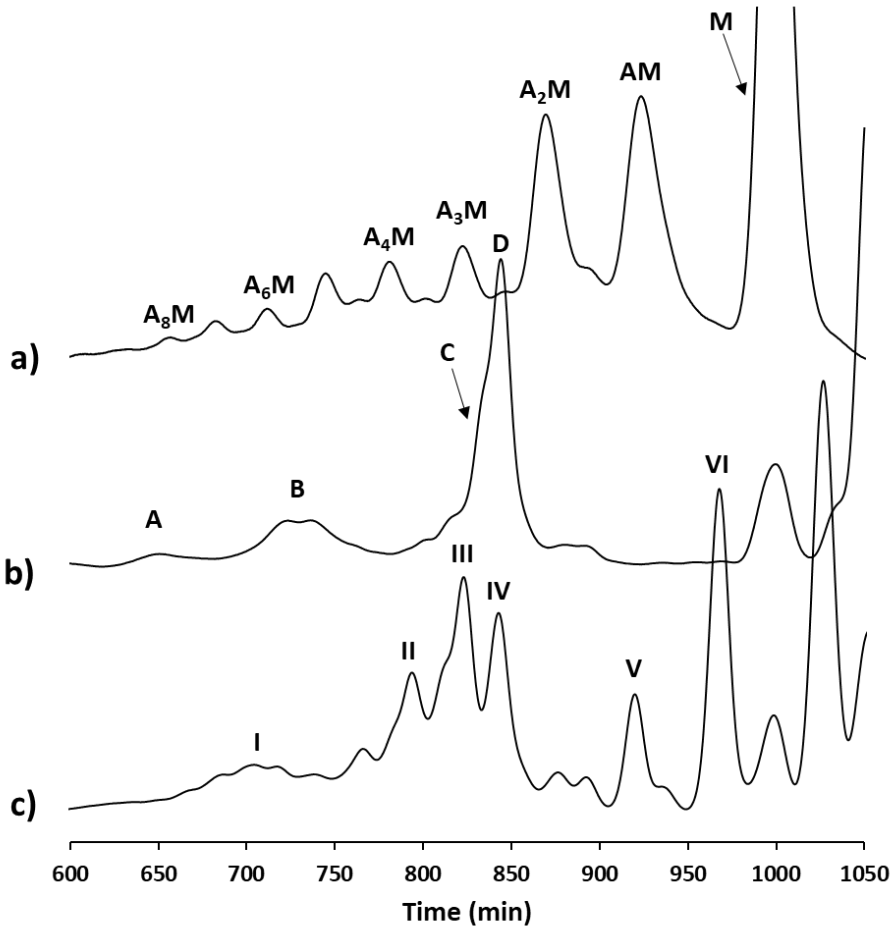

Figure 3: Analytical GFC fractionation of **a)** a standard mixture of A_nM oligomers for GFC calibration, **b)** the mixture following reaction of oxidised A_2M with 10 equivalents PDHA + PB and c**)** the water-soluble fraction from the reaction of oxidised A_2M with 10 equivalents ADH + PB.

The mixture obtained with PDHA resulted in a major fraction with an elution time corresponding to PDHA-activated A_2M (Fraction D, Figure 3b), with a shoulder fraction (collected separately) with slightly shorter elution time (Fraction C). Both fractions were shown to contain oligomers and PDHA linked by secondary amine or Schiff base bonds by ^{1}H-NMR and MS (Supporting information, S2). One oxidised A_2M oligomer can in principle enable the formation of eight different conjugates with diamines (such as PDHA and ADH) as illustrated for PDHA in Figure 4 (**2-9**). MS was used for determining their presence. **1**, **5**, **6** and **9** (Figure 4) were identified in fraction C and D. **5** and **6** have the same mass and cannot be distinguished by MS. Fraction C consisted predominantly of **9**, which was the structure aimed for when using an excess of the diamine linkers. The presence of **9** confirms the reactivity of both aldehydes at the NRE. This is in contrast to lateral periodate oxidation of alginate, where only one of the aldehydes is reactive towards substitution[10, 16]. The absence of conjugates containing unreacted aldehydes (**2-4**, **7** and **8),** indicates high reactivity of all

aldehydes, including the possibility to rapidly form intramolecular (cyclic) structures. The presence of **1** confirmed incomplete oxidation of the oligomers under the given conditions.

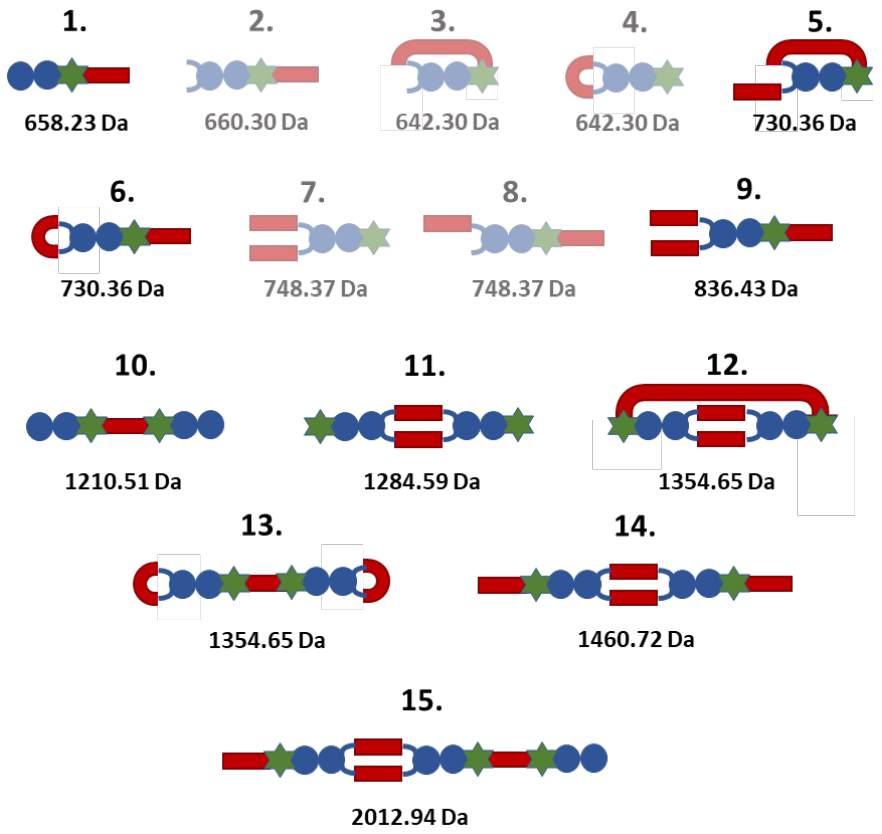

Figure 4: Graphical presentation of possible structures formed when unoxidised and oxidised A_2M oligomers were reacted with an excess PDHA and the theoretical mass of each structure (fully reduced). For structures with one oligomer (**1-9**) all possible combinations are presented (transparent structures were not identified). For products with two or three oligomers (**10-14** and **15**, respectively), only the identified structures are presented. The symbols used are defined in Figure 1.

The presence of fractions corresponding to a hexamer and a nonamer (Fraction A and B, respectively in Figure 3b based on the calibration with A_nM oligomers in Figure 3c) indicated conjugation of PDHA to both the RE and NRE of the oligomers to form chitin block structures. ^{1}H-NMR characterisation confirmed the presence of oligomer and PDHA linked by Schiff base or secondary amine bonds (Supporting information, S2). Due to the incomplete oxidation, the block structures were composed of a mixture of oxidised and unoxidised A_2M oligomers. MS characterisation revealed a complex mixture of structures due to the multiple ways of combining oligomers and PDHA. For the block structures composed of two oligomers (Fraction B, Figure 3b), diblocks containing unoxidised oligomers linked by a single PDHA were identified (**10** in Figure 5). Interestingly, diblocks composed of two oxidised oligomers linked by two

PDHA molecules through the NRE (**11**) were also identified. Their presence suggests similar or even higher reactivities of the NRE aldehydes compared to the RE aldehyde. In the opposite case, one would expect predominantly diblocks attached only at the reducing ends. Importantly, such diblocks could not be identified. Diblocks with intramolecular (cyclic) structures (**12** or **13**) were also detected, in addition to fully substituted diblocks attached through the NRE (**14**). For the block structures composed of three oligomers (Fraction A, Figure 3b) we identified block structures composed of two oxidised oligomers and one unoxidised oligomer linked through the RE and NRE (**15**). Details regarding the ^{1}H-NMR and MS characterisation are given in Supporting information, S2. The presence of non-reduced Schiff bases, especially for conjugates linked through the NRE suggest the need for longer reduction times and/or higher concentrations of PB for PDHA based reactions with oxidised oligomers.

Fractionation of the water-soluble fraction obtained with ADH revealed a more complex mixture of structures (Figure 3c). The three main fractions (II, III and IV) were confirmed to contain oligomers and ADH linked secondary amine bonds by ^{1}H-NMR (Supporting information, S3). Fraction IV (Figure 3c) was mainly composed of A_2M-ADH conjugates formed with unoxidised oligomers (**16**, Figure 5), confirming incomplete oxidation discussed above. The presence of intramolecular cyclic structures (**17** or **18**) in fraction III, analogous to those observed with PDHA, confirmed the high reactivity of all the aldehydes. Structures containing multiple ('polymerised') ADH (**19** or **20**) were identified in fraction II, in line with previous experiments with ADH[13]. Fraction VI and V contained free 'polymerised' ADH (**21** and **22**, respectively). In contrast to the PDHA case, complete reduction was obtained for all the conjugates with ADH. This supports our previous observations of faster reduction of hydrazones compared to oximes with PB[12].

The water-insoluble fraction was dissolved in hexafluoro isopropanol (HFIP) and shown to only contain 'polymerised' ADH by ^{1}H-NMR (Supporting information, S4). The mechanism for the polymerisation is unknown but seems to be enhanced by the pre-treatment of the oligomers by periodate due to the extensive formation of 'polymerised' insoluble ADH formed after only 96 hours at RT. In a corresponding reaction where 'polymerised' ADH was observed, the reaction proceeded for 40 days at RT without forming an insoluble fraction. The reactions were performed under the same conditions only differing in reaction time and type of oligomer (*N,N'*-diacetyl chitobiose (AA) instead of oxidised A_2M)[13]. Oxidised oligomers were only purified by dialysis prior to activation and traces of periodate or ethylene glycol may have enhanced the 'polymerisation' mechanism. We therefore assume that a more thorough purification by e.g. GFC fractionation of the oligomers prior to activation will reduce the formation of 'polymerised' ADH.

Fully ADH activated oxidised A_2M conjugates (corresponding to **9** in Figure 4) were not identified in the reaction mixture, in contrast to the reaction with PDHA were a large fraction of this structure was formed. This can be explained by the fast and extensive formation of 'polymerised' ADH.

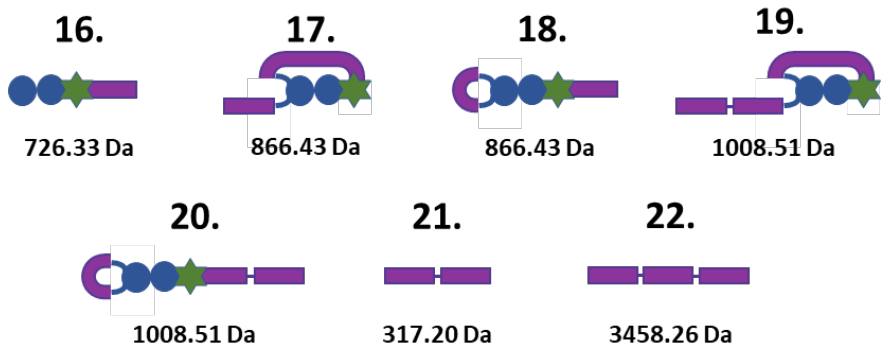

Figure 5: Graphical presentation of identified structures from the reaction of oxidised (and unoxidised) A_2M oligomers with an excess ADH and the mass of each structure (fully reduced).

Although reactions of oxidised A_nM oligomers with an excess of PDHA or ADH results in a range of different structures, including longer block structures (**11- 15**), the results demonstrate that both aldehydes at the NRE react avidly. This forms an excellent basis for further reactions towards A_nM-based block polysaccharides. Even though ADH reacted readily with the oxidised oligomers, further experiments were performed with PDHA to circumvent the complexing factor of ADH 'polymerisation'.

Soluble A_2M-based block polymers

Based on the results from the initial conjugation experiment with oxidised A_2M and PDHA, we attempted to form longer chitin block polymers by conjugating oxidised A_2M using a sub-stoichiometric amount of PDHA (0.5 equivalents). The resulting reaction mixture was completely water soluble. ^{1}H-NMR characterisation confirmed the presence of secondary amines (Supporting information, S5). The reaction mixture was subsequently fractionated by GFC (Figure 6a).

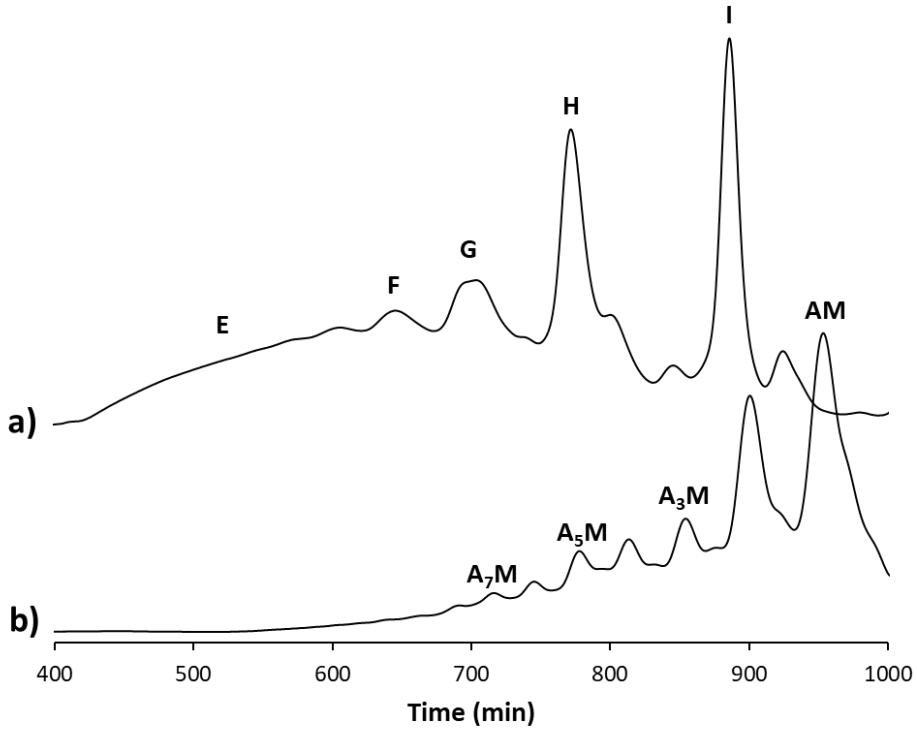

Figure 6: a) GFC fractionation following reaction of oxidised A_2M with 0.5 equivalents PDHA. **b)** fractionation of a standard mixture of A_nM oligomers for GFC calibration.

The reaction resulted in the formation of longer chitin block polymers (up to > DP 24 based on calibration with A_nM oligomers). Interestingly, all fractions were completely water-soluble, whereas A_nM oligomers only are water-soluble up to DP 9 (Figure 6b). Fractions (E-I) were purified and characterised by ^{1}H-NMR and MS (Supporting information, S6). Fraction I contained oxidised oligomers activated with one PDHA molecule forming intramolecular cyclic structures (**3** or **4**, Figure 4). This fraction also contained a large portion of reduced unreacted A_nM oligomers. Interestingly, no unreacted *oxidised* oligomers were detected, confirming that the NRE aldehydes are more reactive than the M aldehyde. Fractions G and H corresponded to fraction A and B in Figure 3 with short chitin block structures (DP 6 and 9, respectively). However, none of the structures were fully substituted with PDHA as a consequence of the lower equivalence of PDHA used. Due to limitation in the mass detection for larger ions by the MS method, identification of specific block structures in fraction E and F was difficult. However, the obtained ^{1}H-NMR spectra were identical to the spectrum obtained for fraction G, indicating that these are longer block polymers with otherwise similar structure.

The large amount of reduced unreacted unoxidised oligomers indicates slow kinetics for the periodate oxidation of the A_nM oligomers. Less unreacted oligomers were obtained when oxidised A_2M were prepared using 4 equivalents periodate and reacted under the same conditions (Supporting information, S7). Hence, the degree of oxidation can be increased by using a higher excess of periodate and/or longer reaction (oxidation) time.

Soluble chitin-based block polymers using longer oxidised A_nM oligomers

Oxidised A_5M oligomers were reacted with 0.5 equivalents PDHA and fractionated by GFC (Figure 7a). The resulting chromatogram showed a series of distinct fractions with DP (by extrapolation of the GFC calibration) corresponding to block structures composed of multiple oxidised A_5M blocks linked by PDHA.

Both the unfractionated sample and the fractions were soluble in water (buffer) even though A_5M oligomers are close to the solubility limit for chitin. The longest soluble block polymers detected had an apparent DP above 36 (Figure 7). ^{1}H-NMR characterisation confirmed the presence of oligomers linked through both the RE and NRE by PDHA in all fractions (Supporting information, S8). Due to limitation in mass detection for larger ions by the MS method and the complex mixture of structures formed, the possibilities for precise structure determination of the longer block polymers was limited. However, MS characterisation of the fraction eluting at approx. same time as the parent oligomer turned out to be a mixture of unoxidised oligomers and oxidised oligomers activated with a single PDHA molecule forming an intramolecular cyclic structure, confirming the high reactivity of the NRE aldehydes. Hence, the oxidised A_5M oligomers behaved in the same way as the oxidised A_2M. We therefore assume the longer block structures to be of the same composition as the structures identified above.

We suggest that the increased solubility is primarily associated with the flexibility of the PDHA linker, contributing to conformational entropy of the block structures as compared to homogenous chitin chains By increasing the degree of oxidation of the A_nM oligomers and optimising the conjugation protocol, this method can be used to prepare a new type of soluble chitin block polymers of longer chain length with potential for pharmaceutical and biomaterial applications.

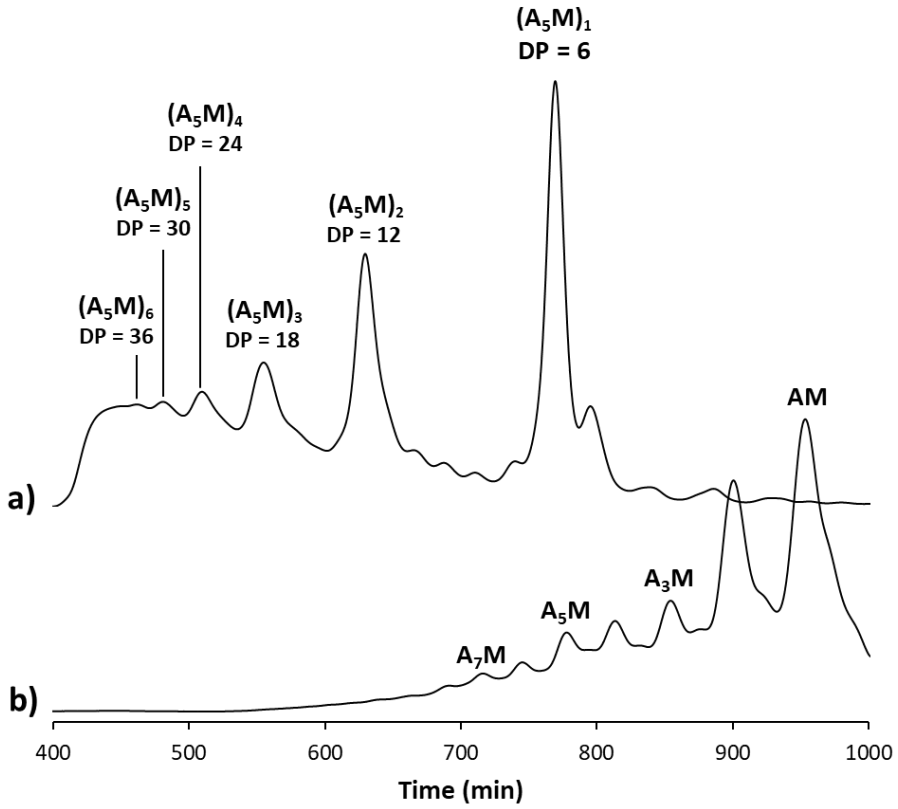

Figure 7: a) GFC fractionation following reaction of oxidised A_5M, prepared using 2 equivalents periodate, with 0.5 equivalents PDHA **b)** fractionation of a standard mixture of A_nM oligomers for GFC calibration.

An alternative approach to NRE functionalisation of chitin oligomers

Reaction with periodate oxidised A_nM oligomers resulted in a complex mixture of structures. We therefore investigated the oxidation of chitin conjugates (A_nM-ADH/PDHA) as an alternative approach to functionalise the NRE of chitin oligomers. This approach requires stability of the secondary amine linkage between the oligomers and PDHA or ADH throughout the oxidation step. A_nM conjugates (A_6M-PDHA and the diblock A_2M-ADH-MA_2) were oxidised using 2 equivalents periodate. ^{1}H-NMR characterisation after dialysis showed complete disappearance of the resonances resulting from the secondary amines and a drastic change in the proton resonances resulting from the reducing end M residue (Figure 8 for A_6M-PDHA and Supporting information, S9 for A_2M-ADH-MA_2). Hence, the results indicate that the secondary amine linkages are destroyed during the oxidation. Interestingly, a minor resonance from the reducing end reappeared after the treatment with periodate for the A_6M-PDHA conjugate (Figure 8c). This was not observed for the periodate treatment of A_2M-ADH-MA_2 (Supporting information, S9), suggesting different

mechanisms for the degradation of the conjugates. In any case, this approach is unfortunately not applicable for the preparation of precursors for ABA- or ABC-type block polysaccharides.

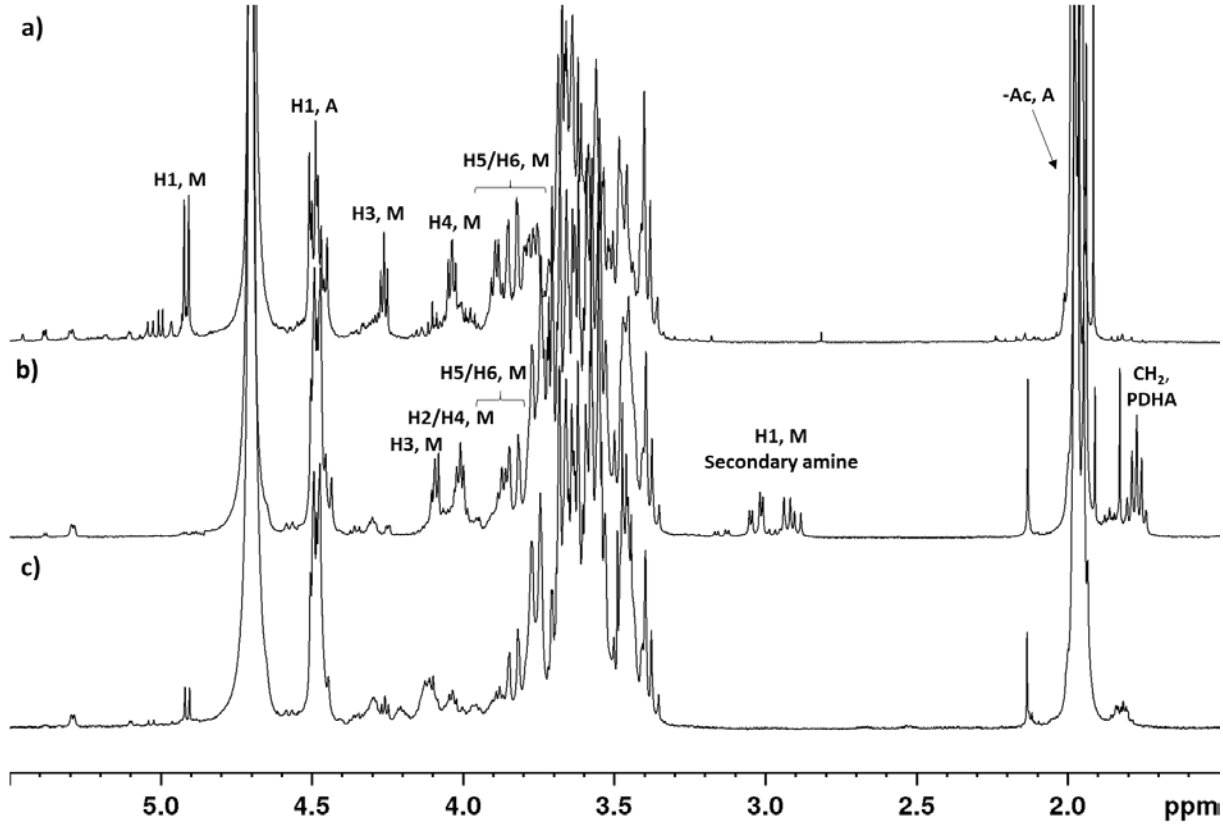

Figure 8: ^{1}H-NMR characterisation of **a)** a purified A_3M oligomer, **b)** the purified A_6M-PDHA conjugate prior to oxidation and **c)** the resulting mixture after oxidation and dialysis.

Conclusions

The vicinal diol in the NRE end residue of A_nM oligomers can be selectively oxidised by periodate, forming the corresponding dialdehydes adding to the aldehyde of the M reducing end residue. The dialdehydes are even more reactive towards dioxyamines and dihydrazides than the already reactive aldehyde of the M residue. Reactions with an excess of these divalent linkers can provide A_nM oligomers fully substituted at the RE and NRE. Such oxidised oligomers are therefore potential precursors for a range of new glycoconjugates, including complex block polysaccharides. The latter was demonstrated by the formation of a discrete distribution of 'polymerised' A_nM oligomers through reactions with sub-stoichiometric amounts of linkers. Importantly, these block polysaccharides were, in contrast to chitins of the same chain lengths, water-soluble. Such soluble chitin-based polymers may be relevant for biomedical and biomaterial applications.

Acknowledgements

This work was supported by a grant from the Norwegian University of Science and Technology to I. V. Mo. Rangnhild Bardal Roness is thanked for running some preliminary experiments with periodate oxidised A_nM oligomers during the work with her master thesis (2015-2017). Kåre Kristiansen is thanked for running the MS analysis.

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Supporting information

Functionalisation of the non-reducing end of chitin by selective periodate oxidation: A new approach to form complex block polysaccharides and water-soluble chitin-based block polymers

Ingrid Vikøren Mo, Christophe Schatz, Bjørn E. Christensen

Contents

S1 Characterisation of reaction mixtures with oxidised A_2M oligomers and an excess PDHA or ADH

The reaction mixtures formed when oxidised A_2M oligomers were reacted with 10 equivalents PDHA using the two-pot reductive amination procedure (pH 4.0, 20 equivalents PB added after 24 hours, with a total reduction time of 72 hours) and the water-soluble fraction from the corresponding experiment with ADH were characterized by ^{1}H-NMR and compared to purified A_2M-PDHA and A_2M-ADH conjugates prepared using unoxidised oligomers (Figure S1 and S2, respectively). Compared to the latter, the ^{1}H-NMR spectra of the reaction mixtures formed with oxidised A_2M oligomers were more complex. However, resonances resulting from the characteristic secondary amine protons (around 3.0 ppm) were present in the samples, confirming formation of conjugates. The resonances were, however, more complex than for the conjugates with unoxidised A_2M oligomers suggesting more than one type of conjugates formed. Incomplete reduction of conjugates formed with PDHA (Figure S1) was observed by the presence of resonances in the Schiff base area (6-8 ppm). Conjugates formed with ADH were in contrast completely reduced (Figure S2).

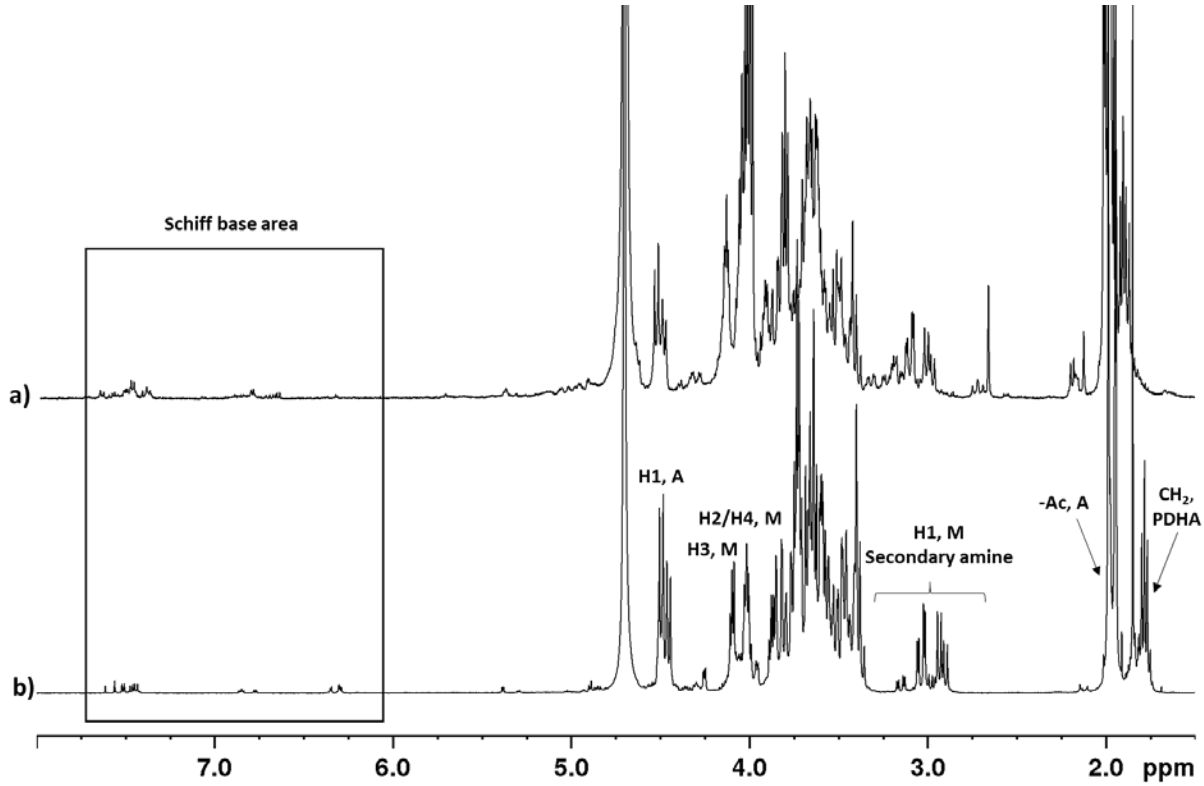


Figure S1: ^{1}H-NMR characterisation of **a)** the mixture formed upon reaction of oxidised A_2M oligomers with 10 equivalents PDHA and **b)** the purified A_2M-PDHA conjugate with annotations of identified resonances[1].

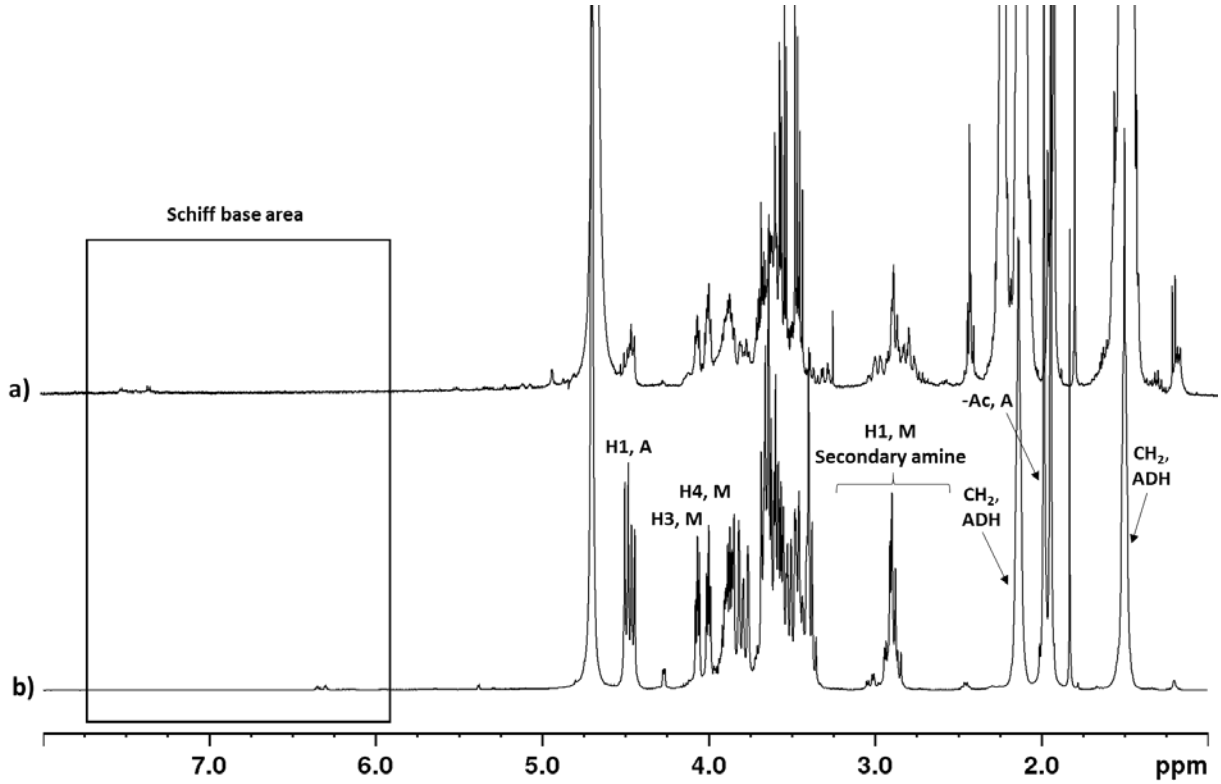


Figure S2: ^{1}H-NMR characterisation of **a)** the mixture formed upon reaction of oxidised A_2M oligomers with 10 equivalents ADH, **b)** purified A_2M-ADH conjugate with annotations of identified resonances[1].

S2 Characterisation of fractions from the reaction with oxidised A_2M oligomers and an excess PDHA

The reaction mixture obtained when oxidised A_2M oligomers were reacted with 10 equivalents PDHA was fractionated by GFC (Figure S3). Collected fractions (A-D) were purified and characterised by ^{1}H-NMR and MS. The ^{1}H-NMR characterisations of the fractions are given in Figure S4-S7. The structures identified by MS and their theoretical mass (Da) are included in the figures. The incomplete reduction of Schiff bases did, however, influence the observed mass of some of the structures. The results from the MS characterisation of the fractions are presented in Table S1.

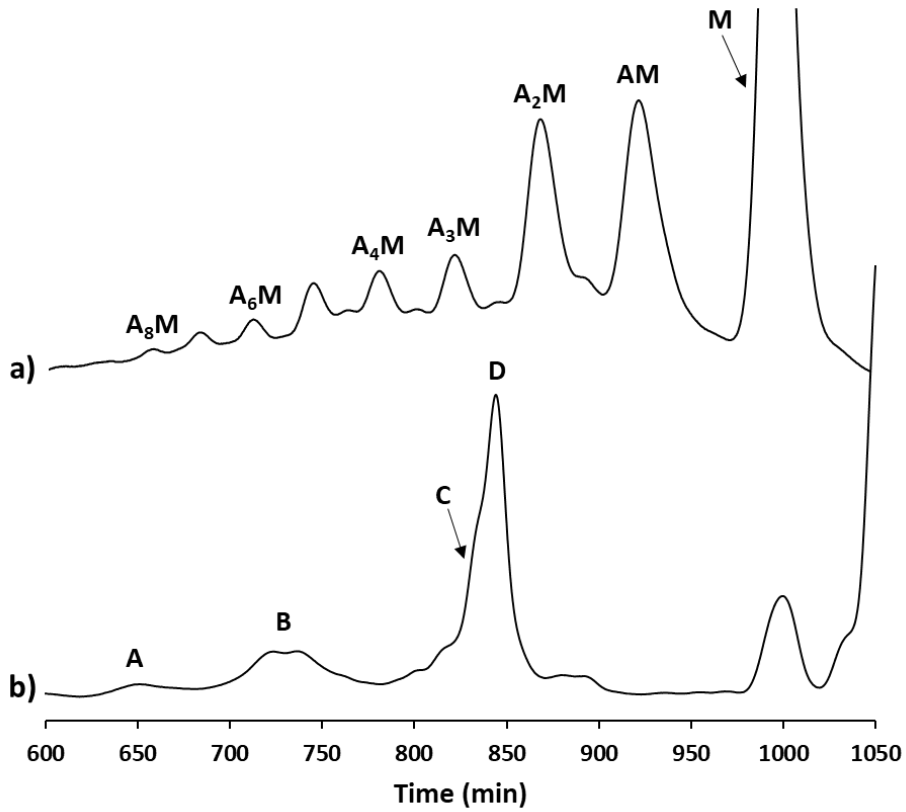


Figure S3: GFC fractionation of **a)** a standard mixture of A_nM oligomers for calibration and **b)** the reaction mixture obtained for oxidised A_2M oligomers reacted with 10 equivalents PDHA using the two-pot reductive amination protocol.

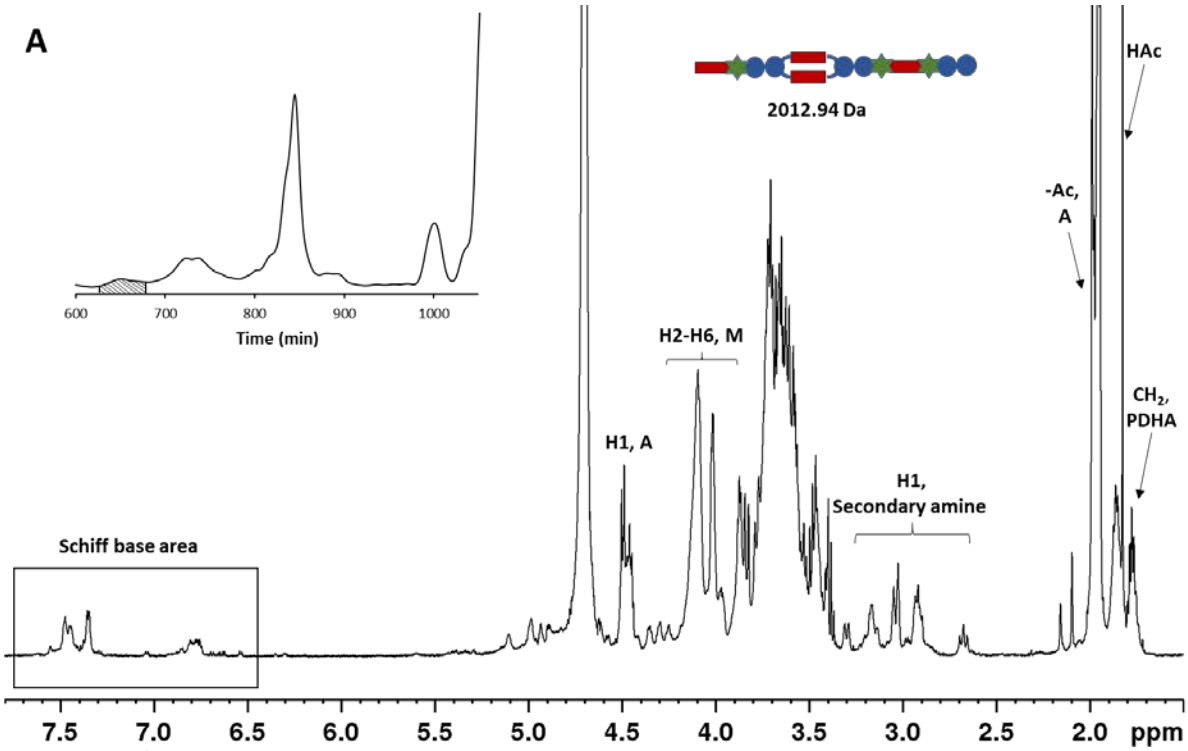


Figure S4: [1]H-NMR characterisation of fraction A from Figure S3. The structure identified by MS is included in the figure.

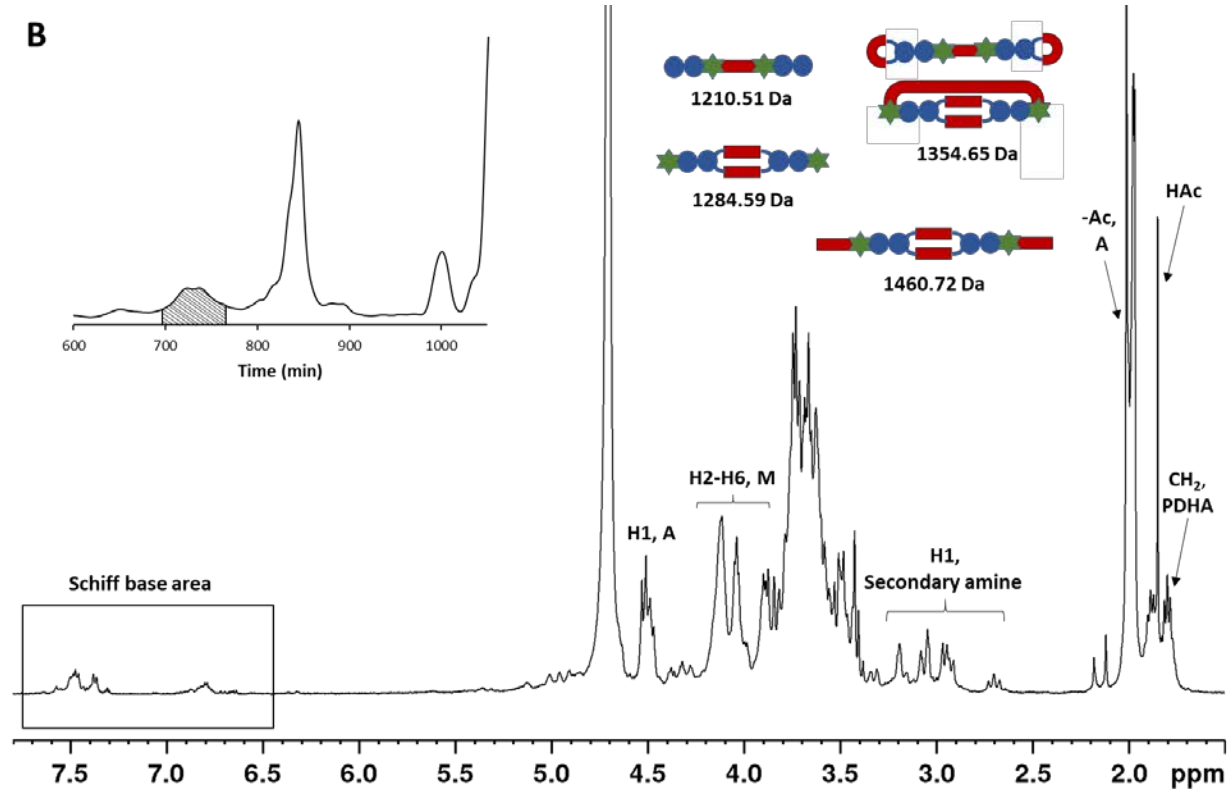


Figure S5: [1]H-NMR characterisation of fraction B from Figure S3. The structures identified by MS are included in the figure.

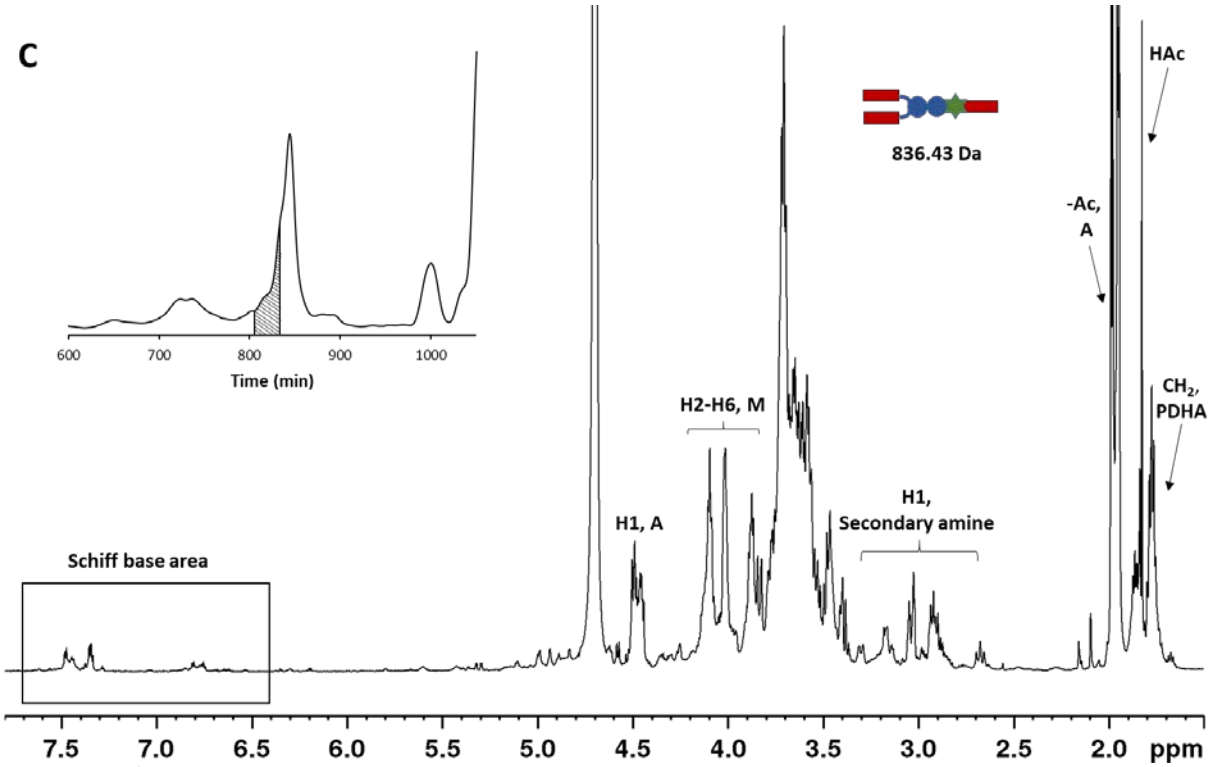


Figure S6: [1]H-NMR characterisation of fraction C from Figure S3. The structure identified by MS is included in the figure.

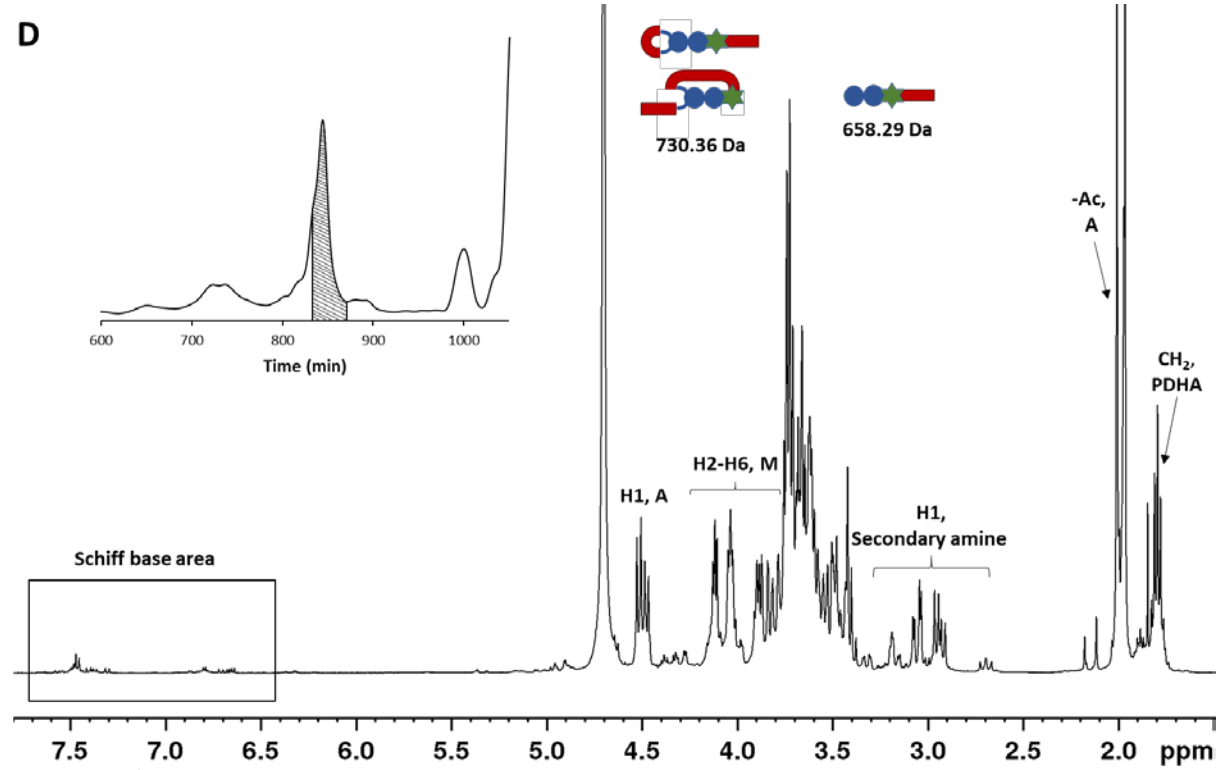
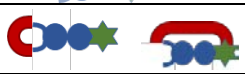
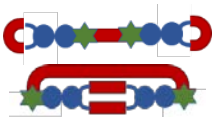


Figure S7: [1]H-NMR characterisation of fraction D from Figure S3. The structures identified by MS are included in the figure.

Table S1: Results of the MS characterisation of fractions obtained in the reactions with oxidised A_2M oligomers and 10 (fraction A-D) or 0.5 equivalents (fraction E-I) PDHA. Structures were either identified as fully reduced or with x number (#) of Schiff bases (oximes) due to incomplete reduction. The *m/z* ions in **bold** represent ions identified in the fractions. Semi-transparent structures were not identified in any of the fractions. Fractions E-I are discussed in chapter S6.

Structure	Fraction	Theoretical mass (Da)	# of Schiff bases*	*m/z*			
				$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+2H]^{2+}$
		*568.21*	-	*569.21*	*591.20*	*607.17*	
	I	570.23	-	571.24	**593.22**	609.19	
	D	658.29	-	**659.30**	**681.28**	697.25	
		*660.30*	-	*661.31*	*683.29*	*699.26*	
	I	642.30	-	643.31	665.29	681.26	
		640.28	1	**641.29**	**663.27**	679.24	
	D	730.36	-	**731.37**	**753.35**	**769.32**	
		726.33	2	**727.34**	**749.32**	**765.29**	
		*748.37*	-	*742.38*	*764.36*	*780.33*	
	C/D	836.43	-	837.44	859.42	**875.39**	
		834.42	1	**835.43**	857.41	**873.38**	
		832.40	2	**833.41**	855.39	871.36	
	B/H	1210.51	-	**1211.52**	**1233.50**	1249.47	
		1208.49	1	**1209.50**	**1231.49**	**1247.46**	
	B/H	1284.59	-	1285.60	1307.58	1323.55	643.30
		1282.58	1	**1283.59**	1305.57	1321.54	642.30
		1280.56	2	**1281.57**	**1303.55**	**1319.52**	641.29
		1278.54	3	1279.55	**1301.53**	1317.50	640.28
	B/H	1354.65	-	1355.66	1377.64	1393.61	678.33
		1352.63	1	**1353.64**	1375.62	1391.59	677.32
		1350.61	2	**1351.62**	1373.60	1389.57	676.31
		1346.58	4	1347.59	**1369.57**	1385.54	674.30
	B	1460.72	-	1461.73	1483.71	1499.68	731.37
		1458.70	1	1459.71	1481.69	1497.66	730.36
		1456.68	2	**1457.69**	**1479.67**	**1495.64**	729.35
		1454.66	3	**1455.68**	1477.65	1493.62	728.34
		1452.64	4	**1453.67**	1475.63	1491.60	727.33
	G	1924.87	-	1925.88	1947.86	1963.83	**963.44**
		1922.86	1	**1923.87**	1945.85	1961.82	**962.44**
	A	2012.94	-	2013.95	2035.93	2051.90	1007.48
		2008.90	2	2009.91	2031.89	2047.86	**1005.46**

* Number (#) of Schiff bases (oximes) in the structure that were not reduced

S3 Characterisation of fractions from the reaction with oxidised A_2M oligomers and an excess ADH (water-soluble fraction)

The water-soluble fraction from the reaction of oxidised A_2M oligomers and 10 equivalents ADH was separated by GFC (Figure S8). Collected fractions (I.-VI.) were purified and characterised by ^{1}H-NMR and MS. The ^{1}H-NMR characterisations of the fractions are given in Figure S9-S14. The structures identified by MS and their theoretical mass (Da) are included in the figures. All structures were completely reduced as in contrast to the structures from the corresponding reaction with PDHA. The results from the MS characterisation are presented in Table S2.

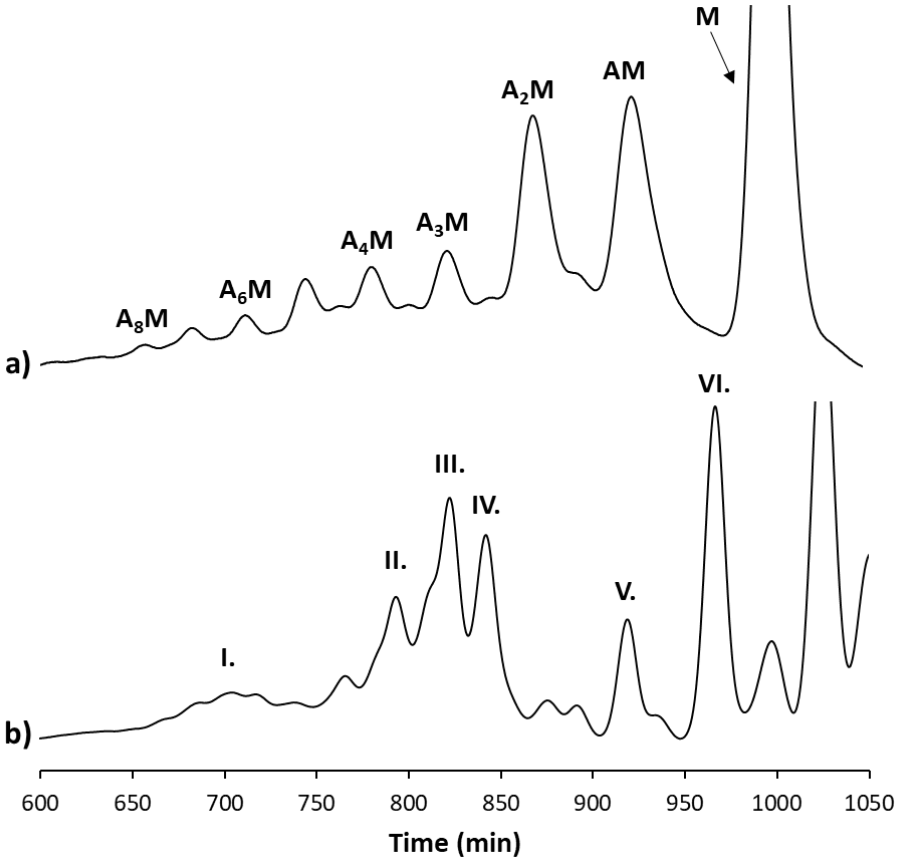


Figure S8: GFC fractionation of **a)** a standard mixture of A_nM oligomers for calibration and **b)** the water-soluble fraction obtained for oxidised A_2M oligomers reacted with 10 equivalents ADH using the two-pot reductive amination protocol.

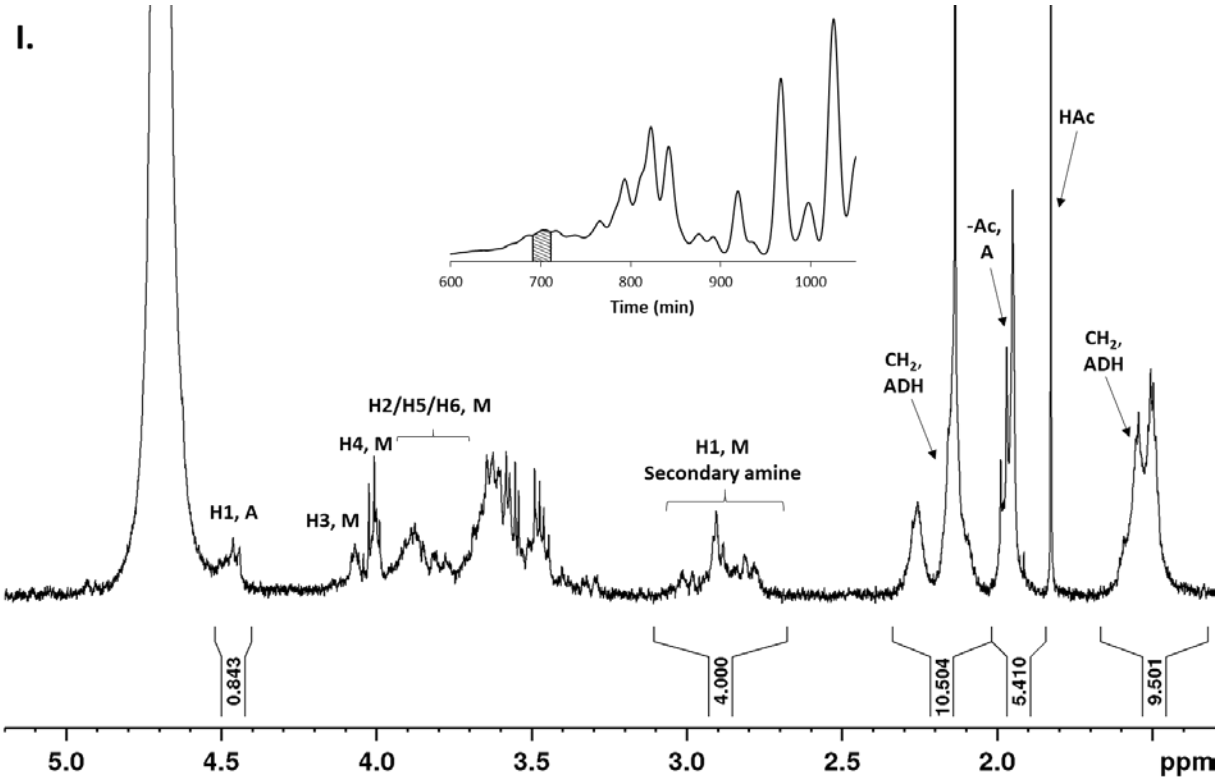


Figure S9: [1]H-NMR characterisation of fraction I. from Figure S8.

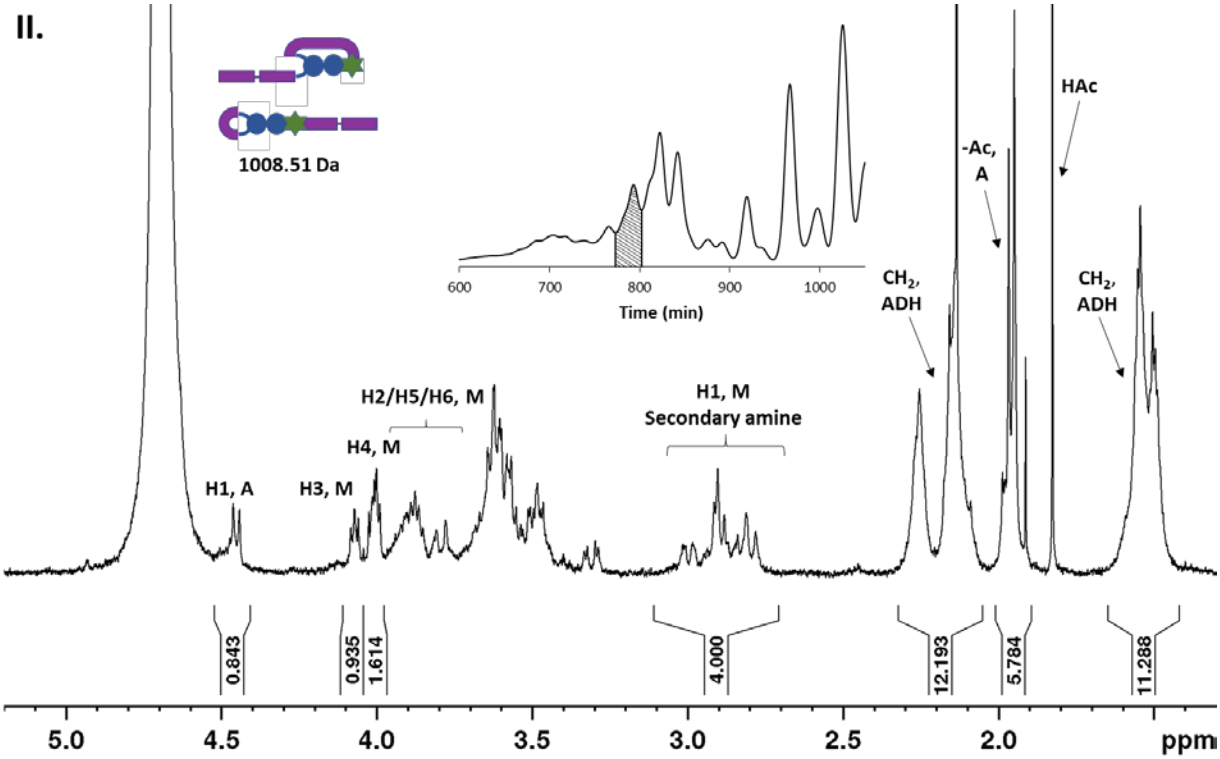


Figure S10: [1]H-NMR characterisation of fraction II. from Figure S8. The structures identified by MS are included in the figure.

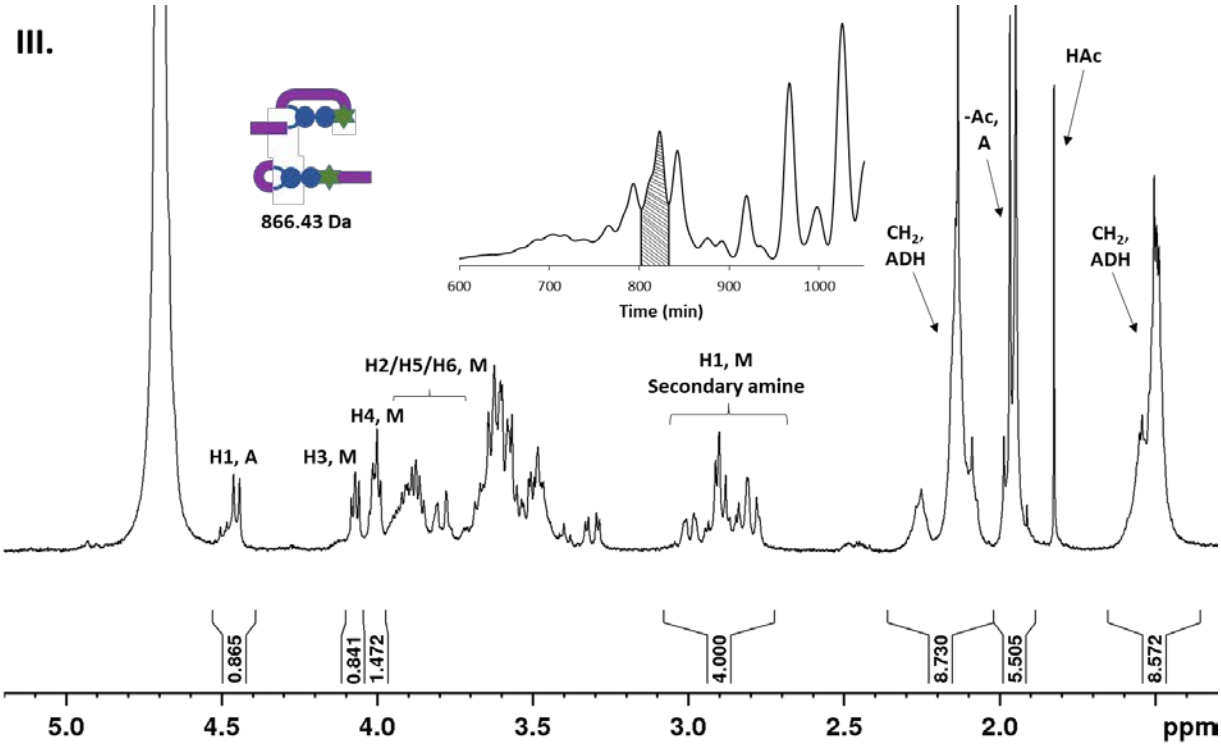


Figure S11: [1]H-NMR characterisation of fraction III. from Figure S8. The structures identified by MS are included in the figure.

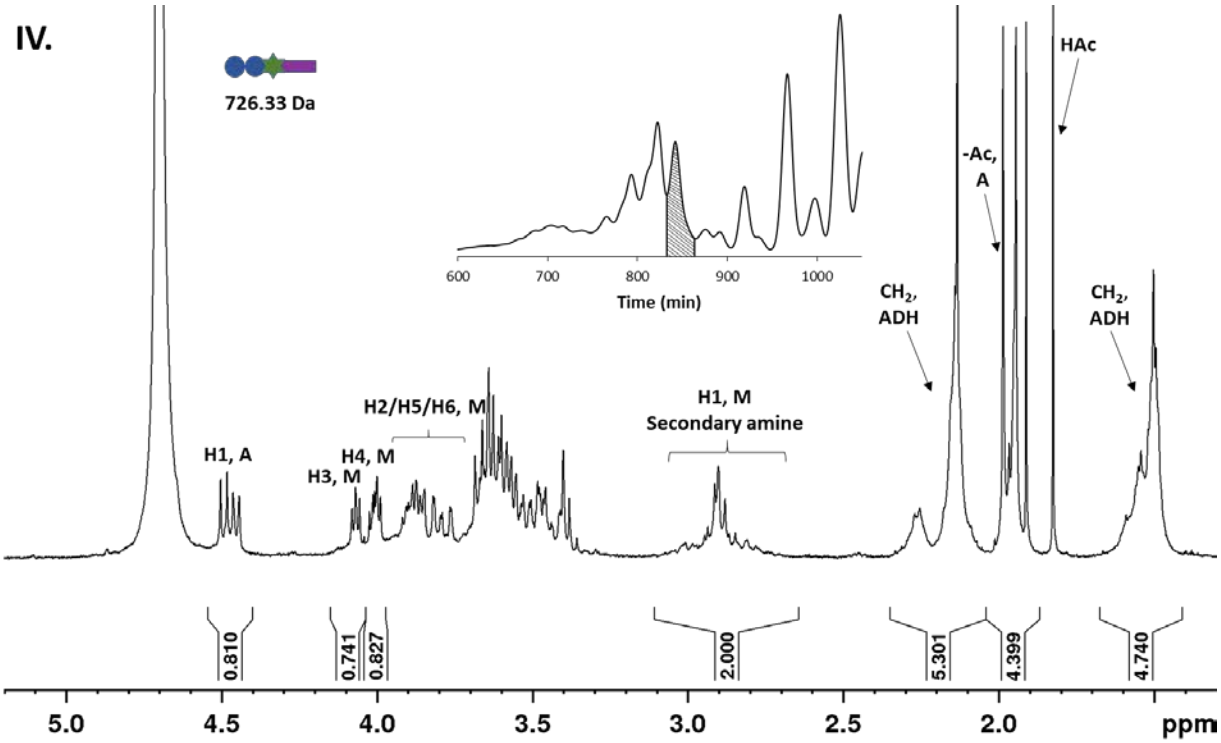


Figure S12: [1]H-NMR characterisation of fraction IV. from Figure S8. The structure identified by MS is included in the figure.

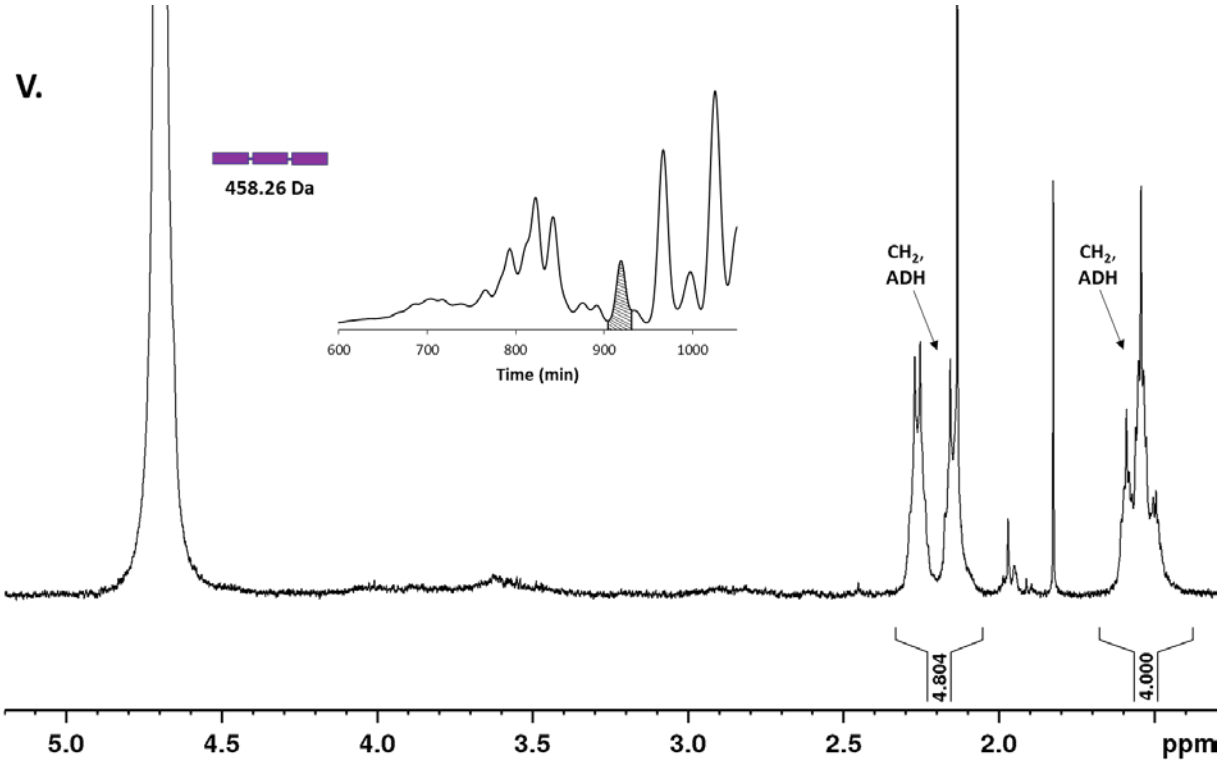


Figure S13: [1]H-NMR characterisation of fraction V. from Figure S8. The structure identified by MS is included in the figure.

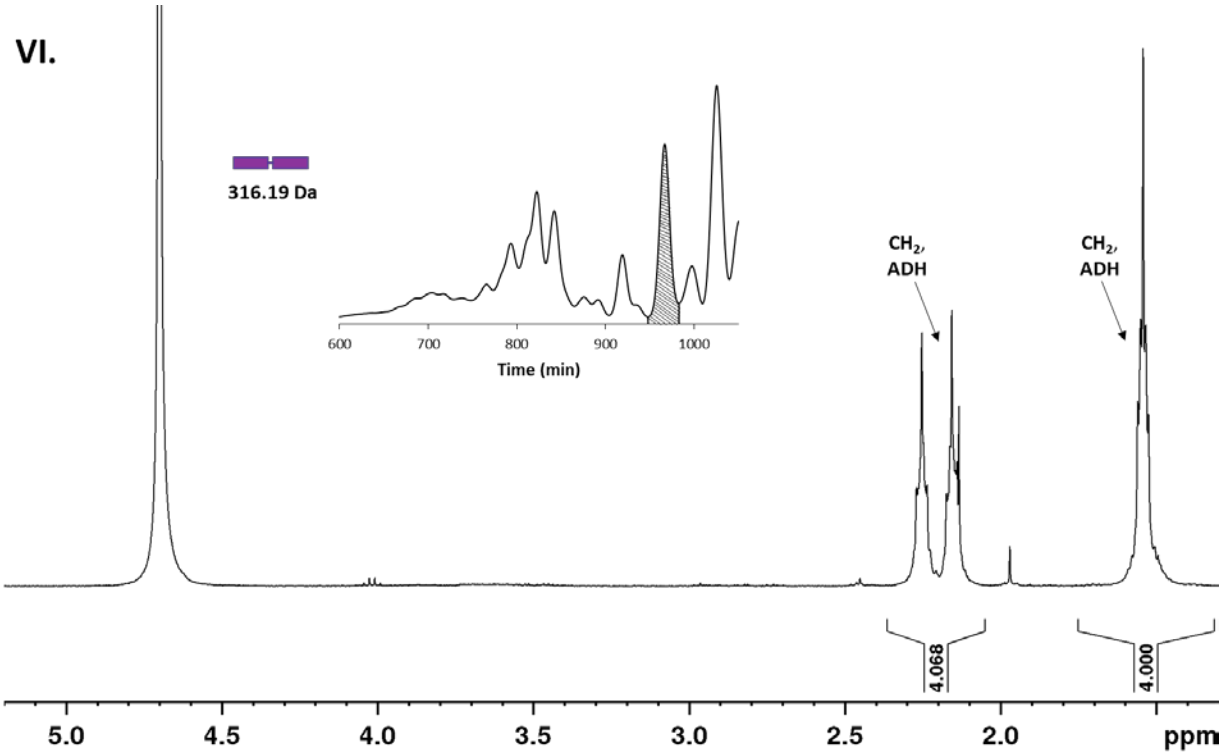
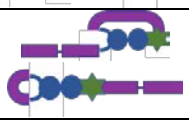


Figure S14: [1]H-NMR characterisation of fraction VI. from Figure S8. The structure identified by MS is included in the figure.

Table S2: Results of the MS characterisation of fractions obtained in the reaction with oxidised A_2M oligomers and 10 equivalents ADH (fraction II.-.VI.). All structures identified were fully reduced. The *m/z* ions in **bold** represent ions identified in the fractions.

Structure	Fraction	Theoretical mass (Da)	*m/z*			
			$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+2H]^{2+}$
	VI.	316.19	**317.20**	**339.18**	**355.15**	
	V.	458.26	**459.27**	**481.25**	**497.22**	
	IV.	726.33	**727.34**	**749.32**	**765.29**	
	III.	866.43	**867.44**	**889.42**	905.39	
	II.	1008.51	**1009.52**	**1031.50**	**1047.47**	**505.26**

S4 Characterisation of the water-insoluble fraction from the reaction with oxidised A_2M oligomers and an excess ADH

The water-insoluble fraction from the reaction of oxidised A_2M oligomers with 10 equivalents ADH was dissolved in hexafluoro isopropanol (HFIP) and characterised by ^{1}H-NMR (Figure S15a). Characterisation of A_2M and ADH dissolved in HFIP is included for comparison (Figure S15b and c, respectively). Only resonances from the protons of ADH was observed in the spectrum obtained for the insoluble fraction, and hence, the sample only contained polymerized ADH.

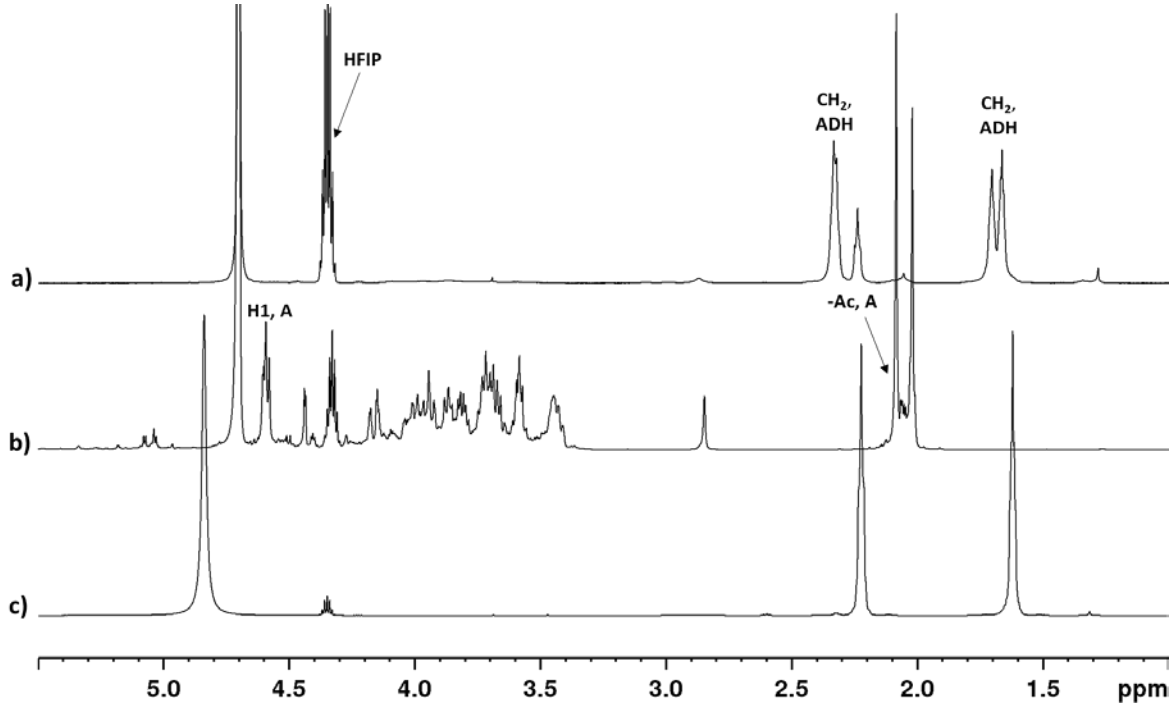


Figure S15: ^{1}H-NMR characterisation of **a)** the insoluble fraction from the reaction of oxidised A_2M oligomers and 10 equivalents ADH, **b)** A_2M and **c)** ADH in HFIP.

S5 Characterisation of the reaction mixture formed with oxidised A_2M oligomers and a sub-stoichiometric amount of PDHA

The reaction mixture obtained for oxidised A_2M oligomers and 0.5 equivalents PDHA was characterized by ^{1}H-NMR and compared to the characterisation of the mixture formed with 10 equivalents PDHA and the purified A_2M-PDHA conjugate (Figure S16). Compared to latter, the NMR spectra of the reaction mixtures formed with oxidised A_2M oligomers were more complex. However, resonances resulting from the characteristic secondary amine protons (around 3.0 ppm) were present in the sample, confirming formation of conjugates. The resonances were, however, more complex than for the conjugates with unoxidised oligomers suggesting more than one type of conjugates formed. Compared to the reaction mixture formed with 10 equivalents PDHA (Figure S16b), the sub-stoichiometric amount of PDHA resulted in less defined proton resonances and higher intensity of resonances in the Schiff base area (6-8 ppm), indicating cyclic structures and more unreduced conjugates, respectively.

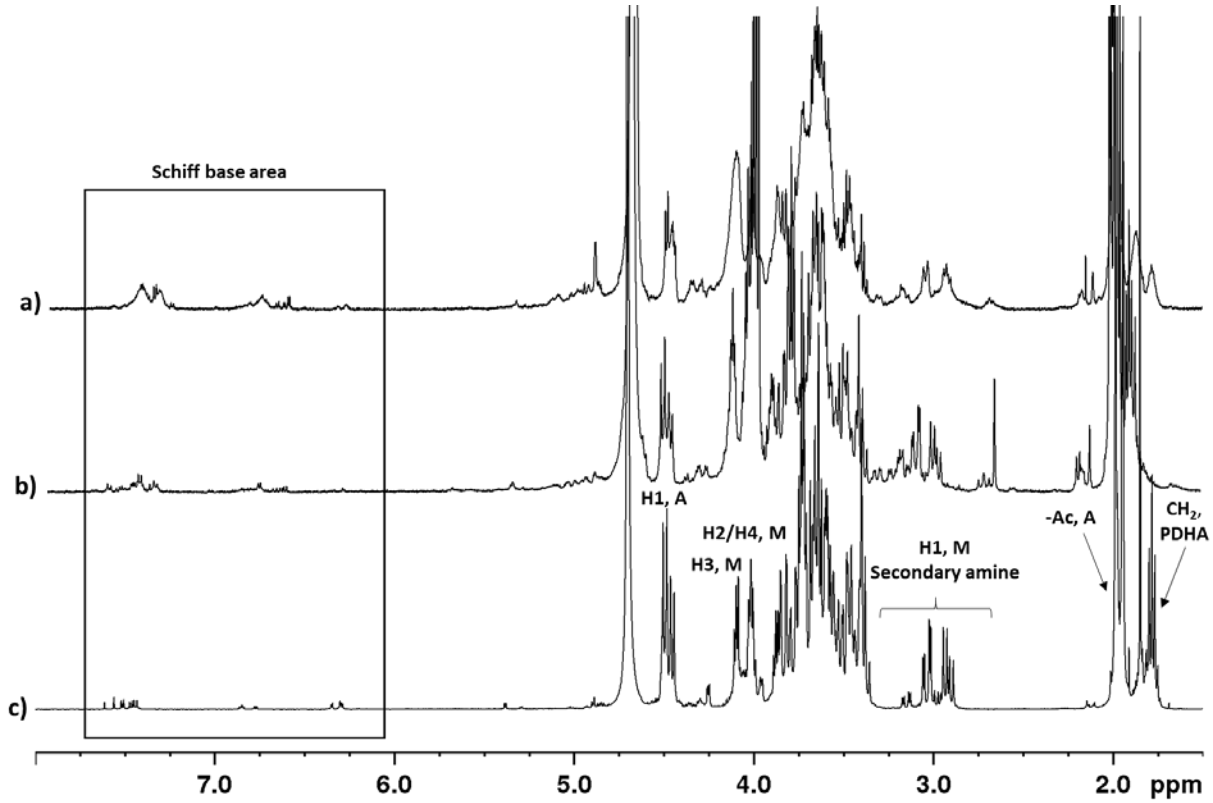


Figure S16: ^{1}H-NMR characterisation of **a)** the reaction mixture formed with oxidised A_2M oligomers and a sub-stochiometric amount of PDHA (0.5 equivalents), **b)** the reaction mixture formed with oxidised A_2M an excess of PDHA (10 equivalents) and **c)** the purified A_2M-PDHA conjugate with annotations of identified resonances

S6 Characterisation of fractions from the reaction with oxidised A_2M and a sub-stoichiometric amount PDHA

The reaction mixture obtained when oxidised A_2M oligomers were reacted with 0.5 equivalents PDHA was fractionated by GFC (Figure S17). Collected fractions (E-I) were purified and characterised by ^{1}H-NMR and MS. The ^{1}H-NMR characterisations of the fractions are given in Figure S18-S22. The structures identified by MS and their theoretical mass (Da) are included in the figures. The incomplete reduction of Schiff bases did, however, influence the observed mass of some of the structures. The results from the MS characterisation of the fractions are included in Table S1.

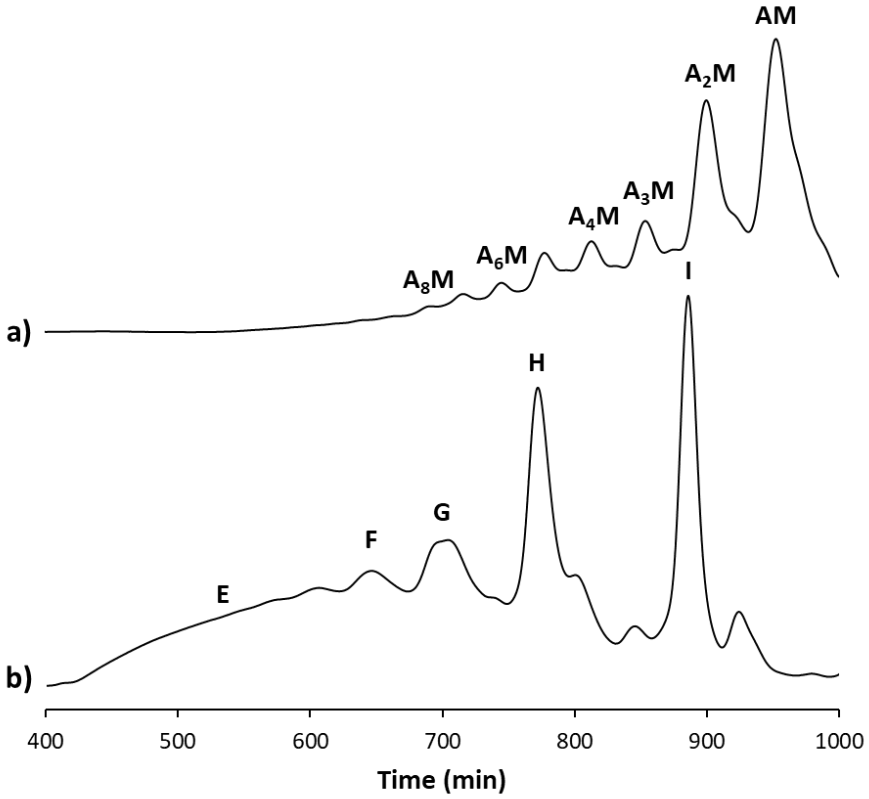


Figure S17: GFC fractionation of **a)** a standard mixture of A_nM oligomers for calibration and **b)** the reaction mixture obtained for oxidised A_2M oligomers reacted with 0.5 equivalents PDHA using the two-pot reductive amination protocol.

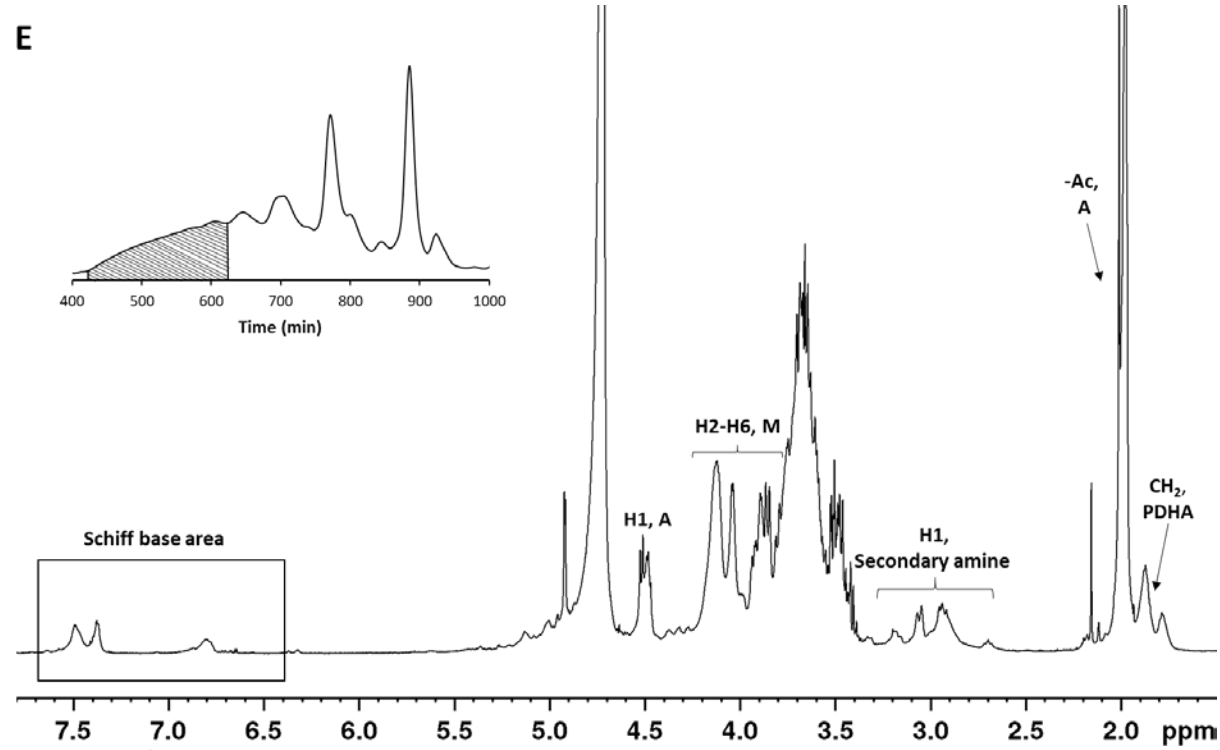


Figure S18: ^{1}H-NMR characterisation of fraction E from Figure S17.

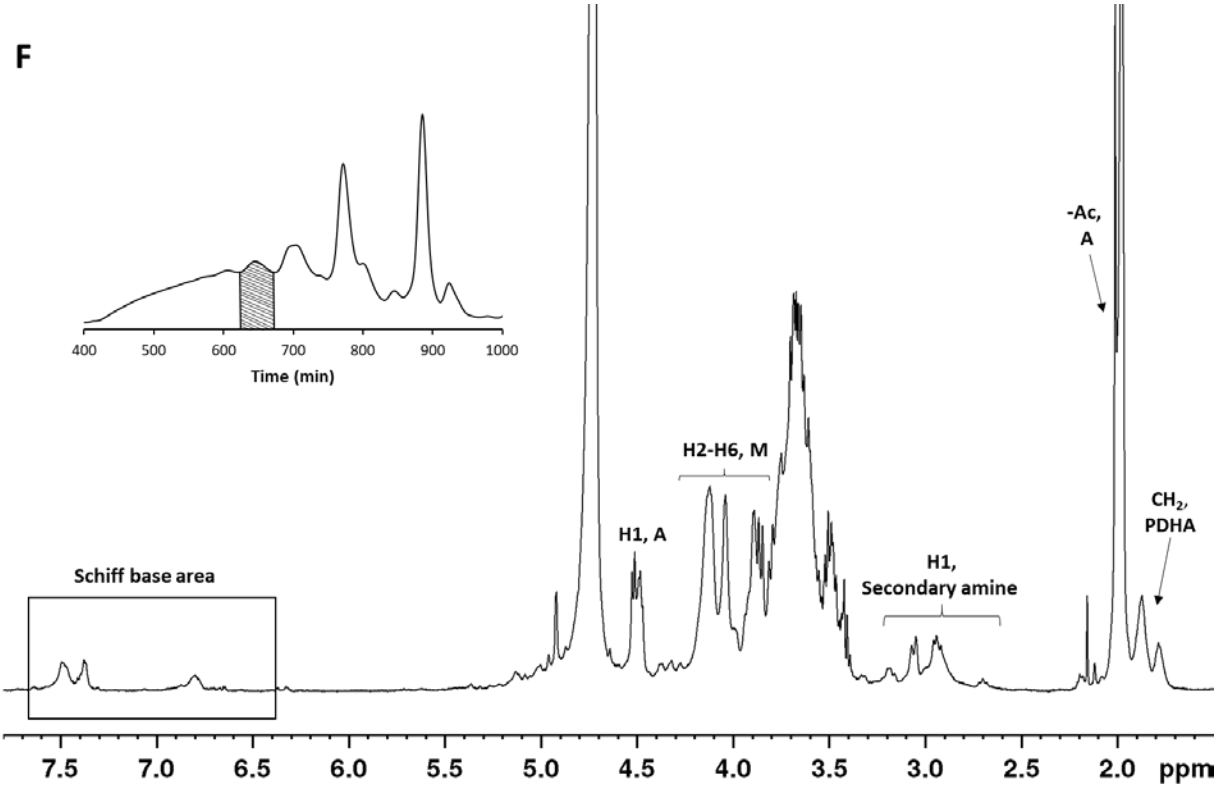


Figure S19: ^{1}H-NMR characterisation of fraction F from Figure S17.

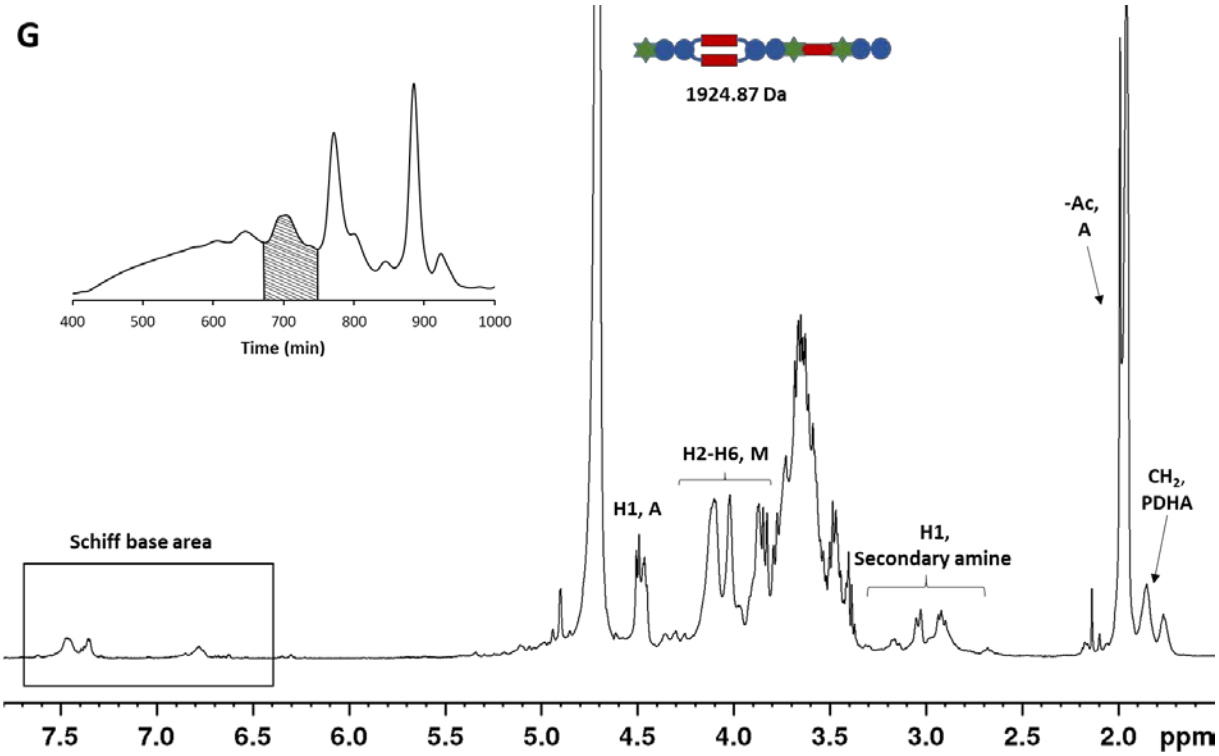


Figure S20: ^{1}H-NMR characterisation of fraction G from Figure S17. The structure identified by MS is included in the figure.

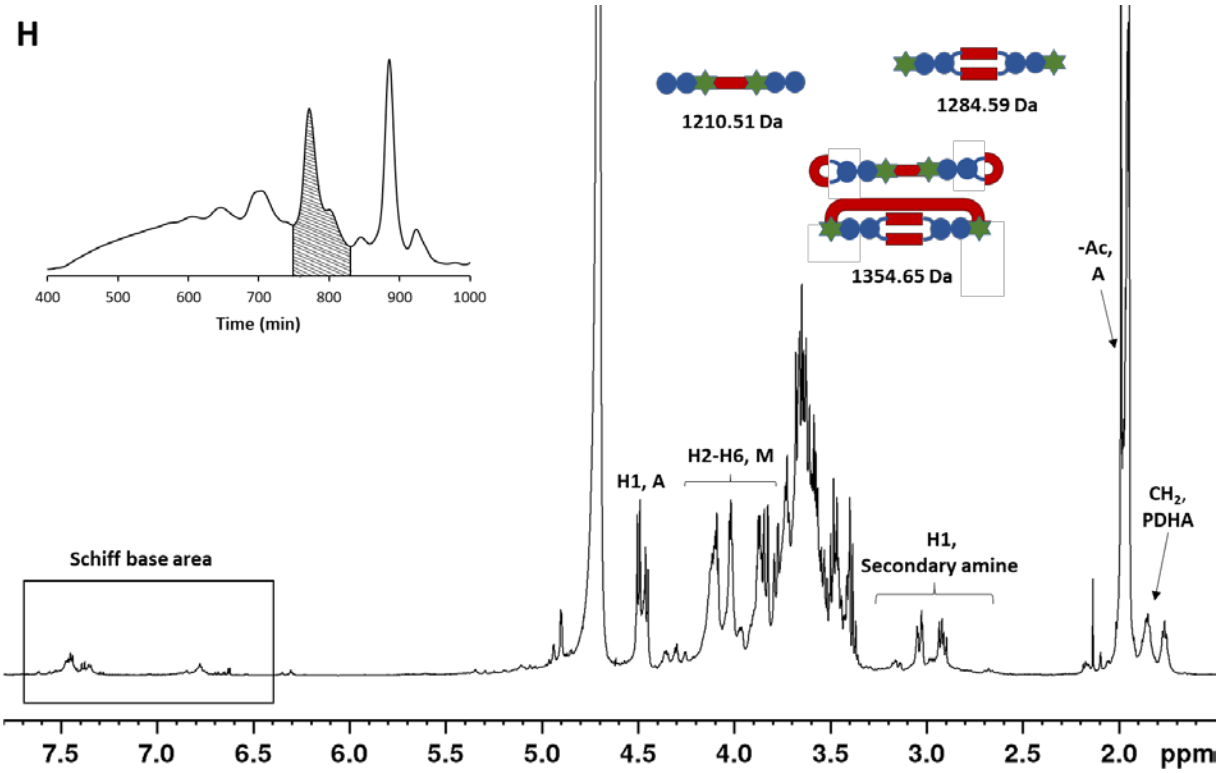


Figure S21: ^{1}H-NMR characterisation of fraction H from Figure S17. The structures identified by MS are included in the figure.

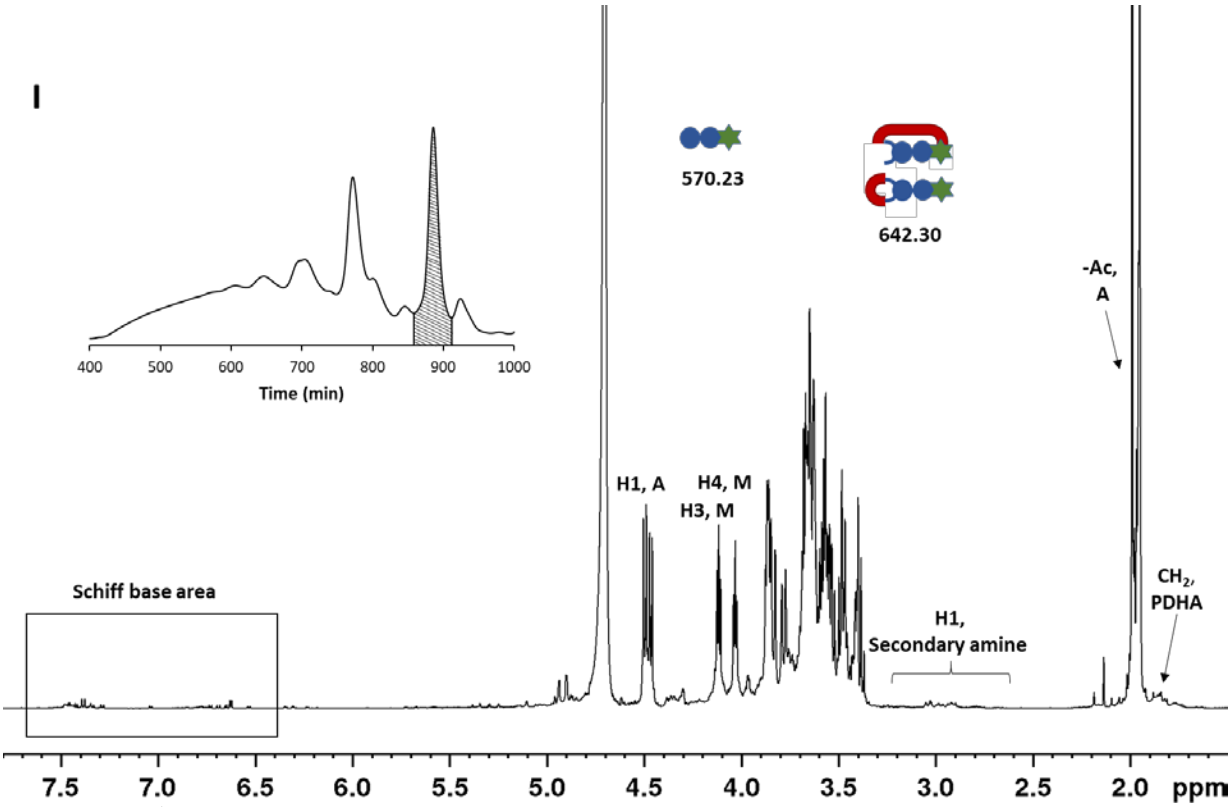


Figure S22: ^{1}H-NMR characterisation of fraction I from Figure S17. The structures identified by MS are included in the figure.

S7 Reaction of oxidised A_2M oligomers, obtained using 4 equivalents periodate, with a sub-stoichiometric amount of PDHA

Oxidised A_2M oligomers, obtained using 4 equivalents periodate, were reacted with 0.5 equivalents PDHA. GFC fractionation of the reaction mixture was compared to the fractionation of the reaction mixture obtained when oxidised A_2M oligomers, obtained using 2 equivalents periodate, were reacted with PDHA under the same conditions (Figure S23). The fractionation revealed a smaller relative fraction of unreacted oligomers when a higher concentration of periodate was used, indirectly confirming that a higher degree of oxidation was obtained using 4 equivalents periodate. The results also confirm that oxidised oligomers react more rapidly than unoxidised oligomers.

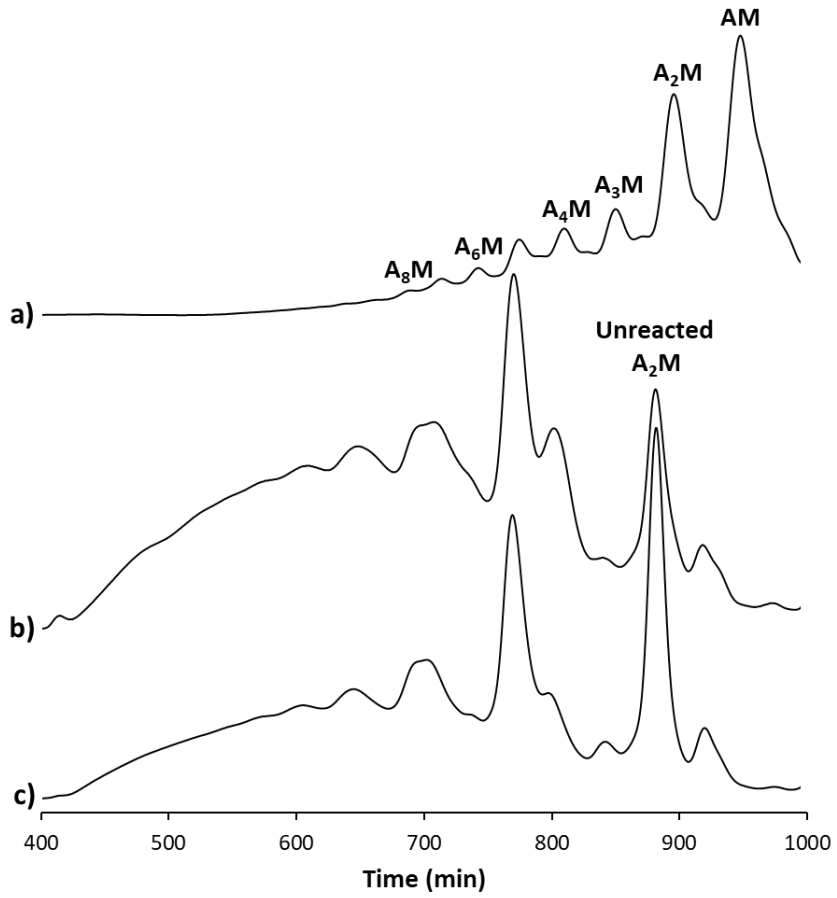


Figure S23: GFC fractionation of **a)** a standard mixture of A_nM oligomers for calibration, **b)** the reaction mixture obtained for A_2M oligomers, oxidised with 4 equivalents periodate, reacted with 0.5 equivalents PDHA and **c)** the reaction mixture obtained for A_2M oligomers, oxidised with 2 equivalents periodate, reacted with 0.5 equivalents PDHA using the two-pot reductive amination protocol.

S8 Characterisation of fractions from the reaction with oxidised A_5M and a sub-stoichiometric amount PDHA

The reaction mixture obtained when oxidised A_5M oligomers were reacted with 0.5 equivalents PDHA was fractionated by GFC (Figure S24). Collected fractions were purified and characterised by ^{1}H-NMR (and MS). The ^{1}H-NMR characterisations of the fractions are given in Figure S25 (main fraction) and Figure S26 (the remaining fractions). Structures identified by MS in the main fraction are included in Figure S25. The composition of the polymerised block structures was not identified due to limitations in the MS method for mass detection of larger ions.

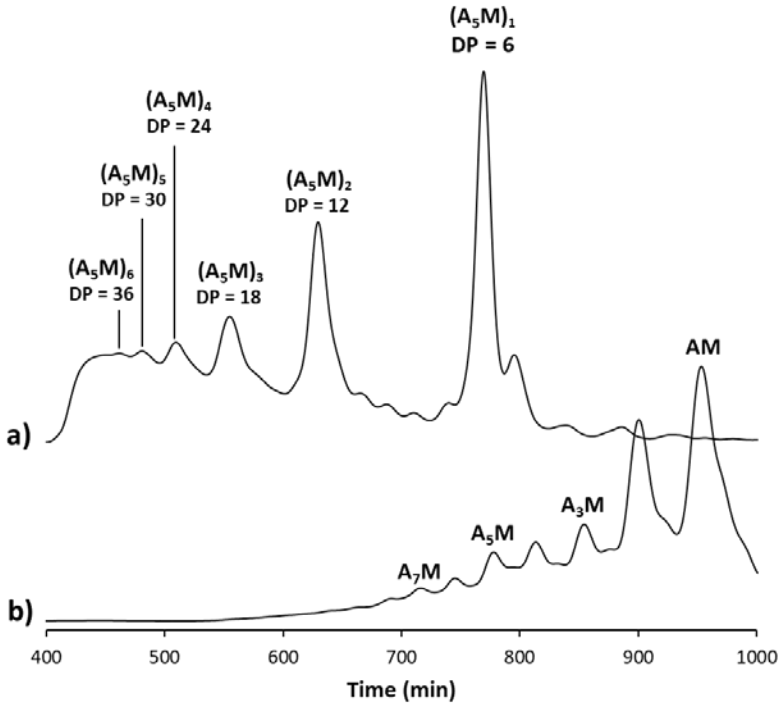


Figure S24: GFC fractionation of **a)** the reaction mixture obtained for oxidised A_5M oligomers reacted with 0.5 equivalents PDHA using the two-pot reductive amination protocol and **b)** a standard mixture of A_nM oligomers for calibration.

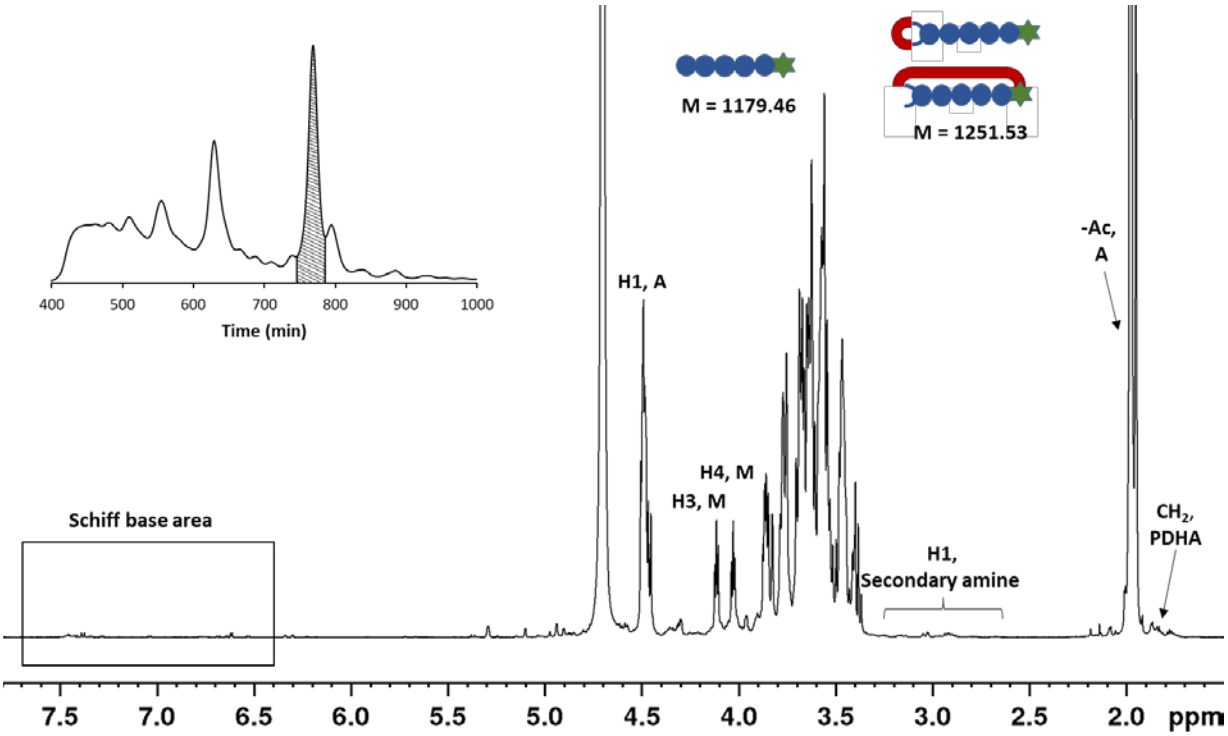


Figure S25: ^{1}H-NMR characterisation of the main fraction from Figure S24. The structures identified by MS are included in the figure.

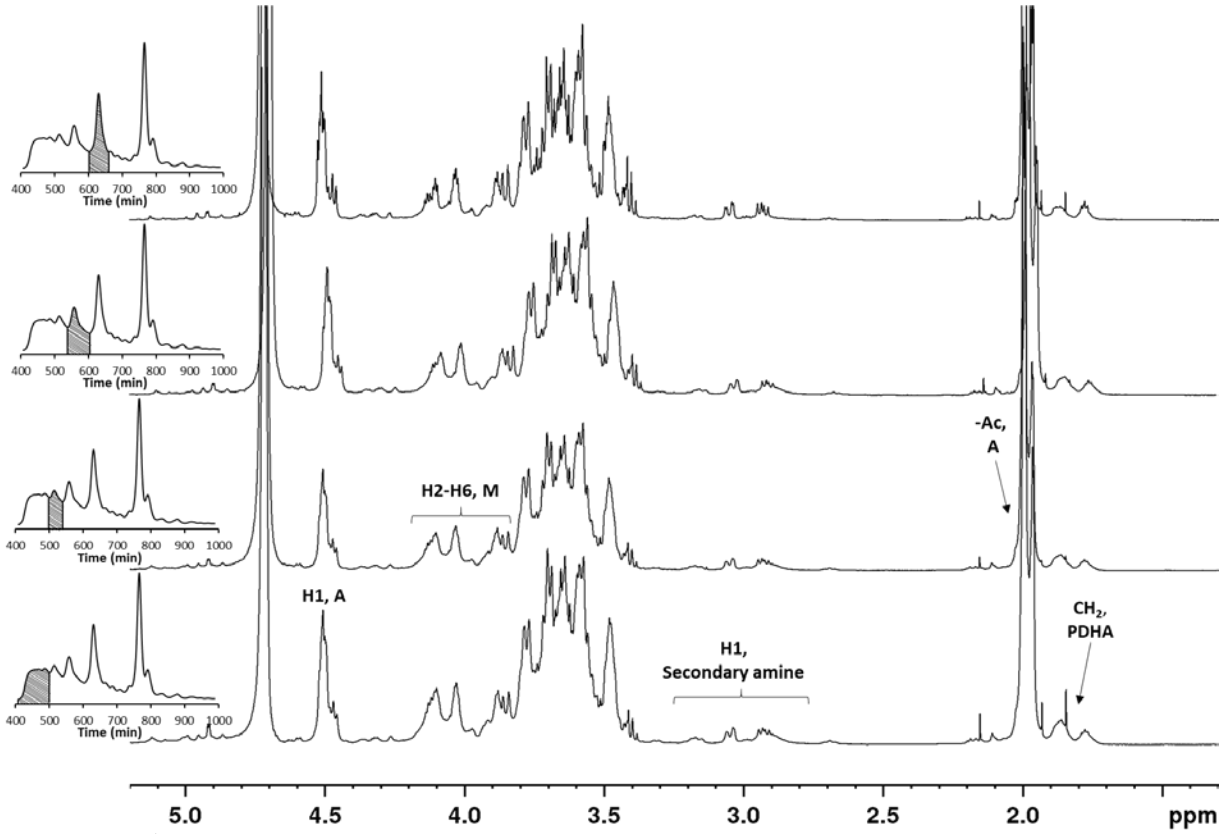


Figure S26: ^{1}H-NMR characterisation of fractions from Figure S24.

S9 Periodate oxidation of A_2M-ADH-MA_2 diblocks

The A_2M-ADH-MA_2 diblock was subjected to oxidation using 2 equivalents periodate. ^{1}H-NMR characterisation after purification by dialysis is given in Figure S27, revealing complete disappearance of the resonances resulting from the secondary amines and a drastic change in the proton resonances resulting from the reducing end M residue. Hence, the results indicate that the secondary amine linkages of the diblock are destroyed during the periodate oxidation.

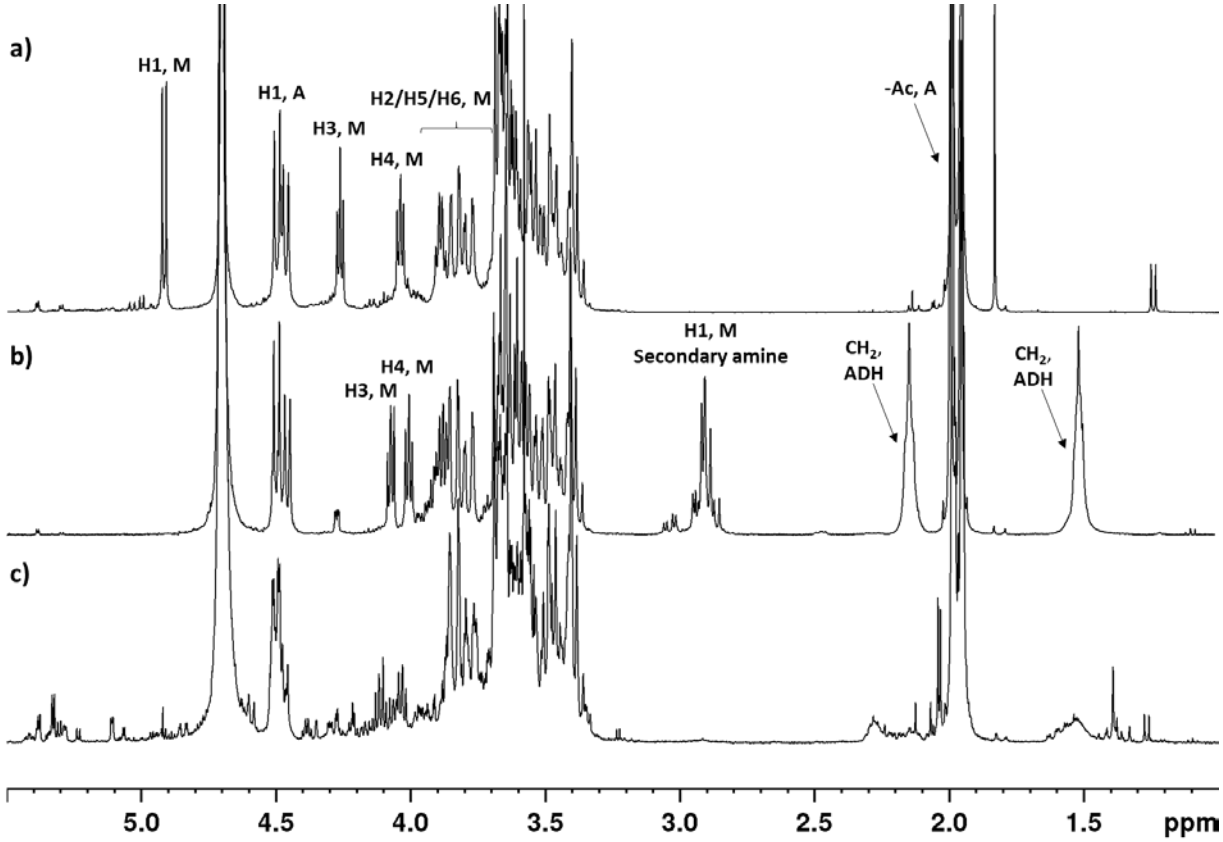


Figure S27: ^{1}H-NMR characterisation of **a)** a purified A_2M oligomer, **b)** the purified A_2M-ADH-MA_2 diblock prior to oxidation and **c)** the resulting mixture after oxidation and dialysis.

S10 References

1. Mo, I. V.; Dalheim, M. Ø.; Aachmann, F. L.; Schatz, C.; Christensen, B. E., 2,5-anhydro-D-mannose end-functionalized chitin oligomers activated by dioxyamines or dihydrazides as precursors of diblock oligosaccharides. *Biomacromolecules* **2020,** *21*, 2884-2895.