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Amyloid Formation by the Model Protein Muscle Acylphosphatase is Accelerated by Heparin and Heparan Sulphate Through a Scaffolding-based Mechanism

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Amyloid formation is the hallmark of many diseases. The propensity of a protein to aggregate depends on a number of biological factors like the presence of sulphated polysaccharides termed as glycosaminoglycans (GAGs). Here we assessed whether the polymeric nature of GAGs is responsible for their protein aggregation-promoting effect. We studied the effect of different monosaccharide derivatives, featuring the main characteristics of heparin and heparan sulphate (HS) building blocks, on the aggregation kinetics of human muscle acylphosphatase (mAcP), a useful model protein for these studies. We observed that while heparin and HS changed the mAcP aggregation kinetic profile, the monosaccharide derivatives had no effect, whatever their concentration could be and both when they are studied separately or in combination. In contrast, heparin fragments with six or more monosaccharides reproduced the effects of HS and in part those of heparin. We conclude that the effect of heparin and HS on protein aggregation arises from the clustering and regular distribution of their composing units on a polymeric structure. We propose a model in which heparin and HS promote mAcP aggregation through a scaffolding-based mechanism, in which the regularly spaced sulphate moieties of the polymer interact with mAcP molecules increasing their local concentration and facilitating their orientation.

Key words: amyloidosis, biological polymers, extracellular matrix, glycosaminoglycan, protein folding and aggregation.

Abbreviations: GAGs, glycosaminoglycans; GlcA, β-D-glucuronic acid; GlcNAc, *N*-acetyl-α-D-glucosamine; GlcNAc-6S, *N*-acetyl-α-D-glucosamine-6-*O*-sulphate; GlcNS, *N*-sulpho-α-D-glucosamine; GlcNS-6S, *N*-sulpho-α-D-glucosamine-6-*O*-sulphate; GST, glutathione S-transferase; HS, heparan sulphate; IdoA-2S, 2-*O*-sulpho-α-L-iduronic acid; mAcP, human muscle acylphosphatase; TFE, 2,2,2-trifluoroethanol; ThT, thioflavin T.

The conversion of peptides and proteins from their native, soluble states into extracellular fibrillar assemblies, generally termed as amyloid fibrils, is the hallmark of a number of human diseases, including Alzheimer's disease, systemic amyloidoses and type II diabetes (*1*, *2*). In these diseases, the resulting fibrillar aggregates feature a characteristic cross-β structure and share the ability to bind specific diagnostic dyes such as Congo red and thioflavin T (ThT) (*2*). Amyloid fibril formation results in a toxic gain-of-function mechanism although it is likely, in many diseases at least, that the oligomeric species that form before the fibrils are the real pathogenic species (*3*, *4*).

It is well established that many biological factors are susceptible to modulate amyloid fibril formation *in vivo*, rendering this process remarkably more complex than actually observed in studies performed *in vitro*, where such factors are generally absent. These factors include protein expression levels, local protein concentration, macromolecular crowding or the presence of specific cofactors like metal ions, phospholipids, glycosaminoglycans (GAGs), collagen fibres, the serum amyloid P component, etc. (*5–12*). The study of the mechanisms by which these biological determinants influence amyloid fibril formation is of fundamental relevance for our understanding of the molecular basis of amyloid-associated diseases and the development of future therapeutics (*12*).

GAGs are linear unbranched polysaccharides built up from repetitive disaccharide units. They are extremely abundant in the human body where they exist either in free form or covalently linked to a protein core as proteoglycans. GAGs are synthesized in the Golgi apparatus and released in the extracellular space, where they fulfil a large variety of biological roles, both structural and functional (*13*). It is well-known that GAGs have a profound influence on amyloidogenesis *in vivo*. Different GAGs have been found to be intimately associated with amyloid fibrils *in vivo* (*7*). In particular, heparan sulphate (HS) has been found to be associated with all

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amyloid deposits so far analysed (*7*). This interaction is not passive, as GAGs have been found to accelerate amyloid formation by a variety of proteins that form extracellular amyloid deposits in pathological conditions (*14–20*). GAGs have also been found to increase the resistance of fibrils to depolymerization (*21*) or proteolytic degradation (*22*) *in vitro*, and to modulate the cytotoxicity of amyloid fibrils in cellular models (*23*, *24*).

The interactions between GAGs and amyloidogenic proteins are mainly electrostatic, as they are shielded by high ionic strengths (*25*, *26*). These electrostatic interactions are of relative low specificity but of high affinity, with binding constants ranging from the nanomolar to the micromolar order (*14*, *27*, *28*). The sulphate moieties present on the GAG molecule play a central role (*14*, *20*, *29*), with the GAG–protein interactions probably taking place between the negatively charged sulphate groups of the GAG and the basic residues of the polypeptide chain (*14*, *20*, *30*, *31*). Apart from the electrostatic component, the molecular basis of the GAG–protein interaction that leads to the GAG-induced amyloid aggregation is largely unknown.

Human muscle acylphosphatase (mAcP) has proven to be a useful model system to study amyloid formation *in vitro* (*32–39*). It is a relatively simple α/β protein of 98 residues that lack complicating factors, such as disulphide bridges or bound factors (*40*). Under destabilizing conditions, mAcP forms readily well-defined amyloid-like protein aggregates characterized by a fibrillar morphology as detected with electron microscopy, an extensive β-sheet structure as revealed by circular dichroism and infrared spectroscopy, and typical tinctorial properties like ThT binding or Congo red birefringence under cross-polarized light (*32*). mAcP aggregates are therefore structurally similar to those that are extracted from patients or reproduced *in vitro* from disease-related proteins. Amyloid formation starts from an unfolded form of mAcP (*33*, *34*, *37*), and for this reason amyloid formation has been triggered in these studies using moderate concentrations of 2,2,2-trifluoroethanol (TFE) to populate initially a denatured state enriched with α-helical structure (*35*, *36*, *38*, *39*). Recently, mAcP has also been used to study the effect of different polyanions on protein aggregation *in vitro* (*28*). Polyanions such as ATP, DNA and the GAG heparin were found to increase dramatically the aggregation rate of mAcP and to stabilize the resulting aggregates (*28*). Both phenomena were dependent on the net charge and size of the polyanion (*28*). Magnesium or calcium ions were found to inhibit the interactions between polyanions and mAcP, suggesting that they are electrostatic in nature (*28*). The heparin-related, low-sulphated HS has also been found to promote aggregation of mAcP (*41*). Therefore, the interaction between mAcP and GAGs such as heparin and HS recapitulates the main characteristics of the effects of GAGs on protein aggregation *in vitro*.

In this work, we take advantage of the model composed by mAcP, on the one hand, and heparin and HS on the other, to investigate the mechanism by which the sulphate groups present on these GAGs promote amyloid formation by mAcP. In particular, we assess whether the various sulphate groups present with a high density and a regular spatial distribution in the polysaccharide chain act in a synergistic and cooperative way. To this aim, we tested the effect of different monosaccharide derivatives of heparin and HS and of different heparin fragments with various lengths on the kinetics of mAcP aggregation. By comparing the effects of such monosaccharides and oligosaccharides with those of heparin and HS, and by correlating the observed differences with the different chemical nature of the molecules used, we show that heparin and HS promote mAcP aggregation through a scaffolding-based mechanism.

MATERIALS AND METHODS

Materials—Heparin sodium salt from porcine intestinal mucosa (molecular weight 18,000, ref. H3149), *N*-acetyl-α-D-glucosamine (GlcNAc, ref. A8625), β-D-glucuronic acid (GlcA, ref. G8645), *N*-acetyl-α-D-glucosamine-6-*O*-sulphate (GlcNAc-6S, ref. A7661) and *N*-sulpho-α-D-glucosamine (GlcNS, ref. G7889) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Heparin fragments with 2 [2-*O*-sulpho-β-L-iduronic acid (IdoA-2S)-GlcNS-6S, ref. HD001], 6 (ref. HO06), 12 (ref. HO12) and 18 (ref. HO18) monosaccharide residues were purchased from Iduron (Manchester, UK). The iduronic acid at the non-reducing end of the oligosaccharides has a C4-C5 double bond as a result of the endolytic action of bacterial heparinases.

Each component was dissolved and diluted in H_2O at a concentration of 100 μg/ml, divided in aliquots and the stock solutions were kept at −20°C. Each aliquot was used only once after thawing.

Protein production and purification—The mAcP variant used here has the mutation C21S, which avoids the complexity arising from the presence of a free thiol group (*42*). Protein expression and purification were carried out as previously described (*43*). Briefly, the protein was expressed from a pGEX-2T vector in *Escherichia coli* cytoplasm as a protein fused to glutathione S-transferase (GST), then purified by affinity chromatography using a glutathione–agarose resin from Sigma-Aldrich. The GST was removed by on-column thrombin digestion and the resulting protein solution buffer exchanged into 50 mM acetate buffer, pH 5.5, using centrifugal filter devices from Millipore (Billerica, MA, USA). Protein purification was assessed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrospray ionization mass spectrometry.

mAcP aggregation—Aggregation of mAcP was triggered as previously described (*35*). Briefly, the reaction was started by mixing a solution containing the native protein with another containing TFE. The final conditions after dilution were 0.4 mg/ml mAcP (36 μM), 25% (v/v) TFE, 50 mM acetate buffer, pH 5.5, 25°C. When aggregation was performed in the presence of an additive (heparin, HS, monosaccharide derivatives or heparin fragments), a defined quantity of the additive stock solution was added in the TFE-containing solution before mixing with the protein-containing solution. In all cases, the protein-containing solution was centrifuged at 20,000*g* for 5 min before mixing and the protein concentration was measured by UV absorption using an

extinction coefficient value at 280 nm (ε_{280}) of 1.49 ml mg^{-1} cm^{-1}.

ThT assay—At different incubation times after the initiation of the aggregation reaction, aliquots of 60 µl were mixed with 440 µl of 10 mM phosphate buffer, pH 6.0, containing 25 µM ThT (Sigma-Aldrich). The steady state fluorescence of the resulting samples was measured at 25°C using a 2 × 10-mm path length cuvette and a Perkin-Elmer LS-55 fluorimeter (Wellesley, MA, USA) equipped with a thermostated cell compartment attached to a Haake F8 water bath (Karlsruhe, Germany). The excitation and emission wavelengths were 440 and 485 nm, respectively. The dead time of the experiment before the first point can be recorded is ~30 s.

All the fluorescent values were blank substracted. When aggregation was performed in the presence of a defined concentration of heparin, HS, monosaccharide derivatives or heparin fragments, the same concentration was added to the blank to exclude a direct effect of the additive alone on ThT fluorescence. Plots of blank-subtracted fluorescence values versus time were analysed with a procedure of best fit, using the following single exponential function:

$$F(t) = F_\infty - (F_\infty - F_0) \times \exp(-k_{agg} \times t) \quad (1)$$

where F_0, F(t) and F_∞ are the fluorescence intensities at time 0, t and ∞, respectively, k_{agg} is the apparent aggregation rate constant. F_0, F_∞ and k_{agg} were used as floating parameters in the procedure of best fit. Blank-subtracted fluorescence values were then normalized using:

$$F_{norm}(t) = 100 \times (F(t) - F_0)/(F_\infty - F_0) \quad (2)$$

where $F_{norm}(t)$ is the normalized fluorescence intensity at time t (ranging from 0 to 100%) and the F_∞ and F_0 parameters have the same meaning as in Equation (1).

RESULTS

HS is the most abundant and ubiquitous GAG of the body. The polysaccharide chain of HS contains alternating sulphated and non-sulphated domains, each containing 5–10 repeated disaccharide units (Fig. 1A). In the non-sulphated domain, the most common disaccharide unit is composed by GlcA bound to GlcNAc; whereas in the sulphated domain, the most common disaccharide unit is composed of IdoA-2S bound to *N*-sulpho-α-D-glucosamine-6-*O*-sulphate (GlcNS-6S). Heparin is a related highly sulphated form of HS, composed only of sulphated domains, in which the IdoA-2S and GlcNS-6S disaccharide unit is by far the most represented one (Fig. 1A).

To assess whether the various negatively charged groups present in the polysaccharide chain of heparin and HS are able to accelerate mAcP aggregation when dissected from the polymeric structure of the GAGs or whether they need to be clustered in one polymer, four different GAG-derived molecules were tested: GlcA, GlcNAc, GlcNAc-6S and GlcNS (Fig. 1B). These monosaccharide derivatives recapitulate the different monosaccharides composing heparin and HS. Moreover, the

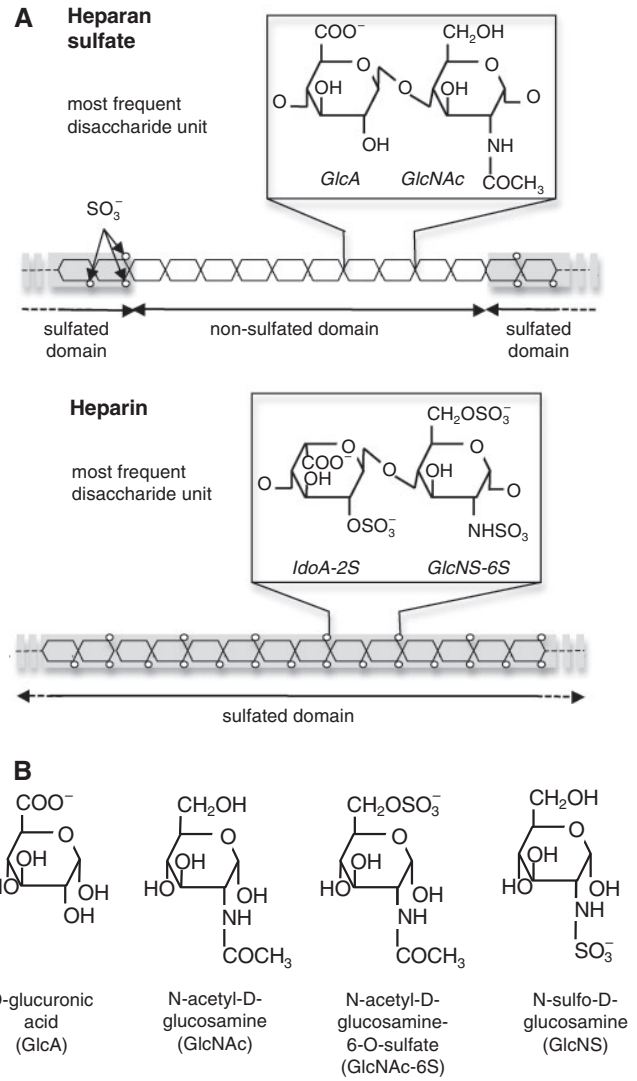

Fig. 1. **Primary structure of HS and heparin and the monosaccharide derivatives used in this study.** (A) Domain organization of HS and heparin and structure of the most represented disaccharide units present in HS and heparin. (B) Structural formulas of the four monosaccharide derivatives used in this study.

four monosaccharide derivatives have one carboxylate group, no charge, one *O*-sulphated group and one *N*-sulphated group, respectively, each located in the chemical context of the heparin/HS chain. This allows the various negative groups present on heparin and HS to be investigated separately.

We first tested the effect of heparin and HS on the kinetics of mAcP aggregation. mAcP was incubated at a protein concentration of 36 µM (0.4 mg/ml), in 25% (v/v) TFE, 50 mM acetate buffer, pH 5.5, 25°C, with heparin concentrations ranging from 0 to 0.28 µM (5 µg/ml). At regular time intervals, aliquots were withdrawn to carry out the ThT fluorescence assay. In the absence of heparin, the time course of ThT fluorescence follows single exponential kinetics, with a rate constant k_{agg} of 1.11 (±0.07) × 10^{-3} s^{-1}, similar within experimental error

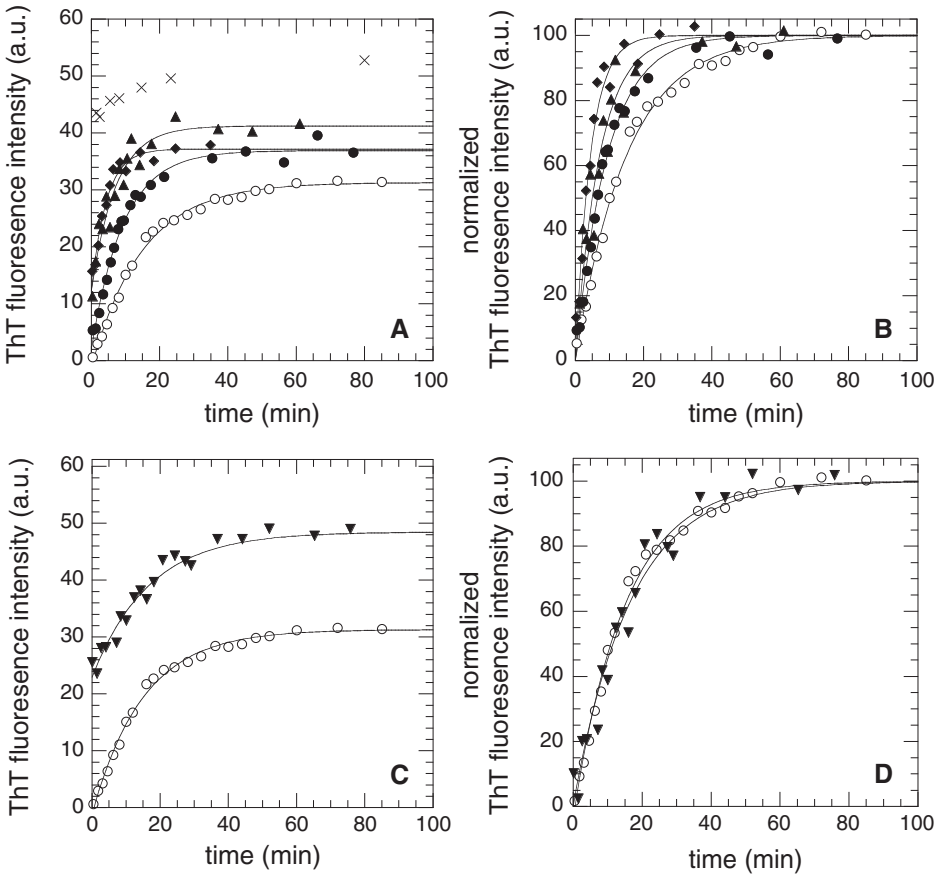

Fig. 2. **Time courses of mAcP aggregation in the presence and absence of heparin (A and B) and HS (C and D).** Kinetic traces were obtained at the following heparin and HS concentrations: empty circles, no added heparin or HS; closed circles, 1 μg/ml (0.06 μM) heparin; triangles, 2 μg/ml (0.11 μM) heparin; diamonds, 3 μg/ml (0.17 μM) heparin; crosses, 5 μg/ml (0.28 μM) heparin; inverted triangles, 7.5 μg/ml (0.56 μM) HS.

to that obtained previously under identical conditions (*28*, *35*, *44*). The addition of increasing concentrations of heparin caused increases of the k_{agg} value, of the final intensity of ThT fluorescence and of the initial ThT fluorescence intensity detected before the observed exponential phase, in a concentration-dependent manner (Fig. 2A and B; Table 1). It is noteworthy that heparin is already efficient at very low concentrations, with a 1 : 600 molar ratio having a significant effect on protein aggregation (0.06 μM heparin for 36 μM mAcP). At the highest heparin concentration tested (0.28 μM), the increase in the ThT signal after 30 s was too high, and the difference between the final and starting signal was too small to follow the aggregation kinetics accurately (Fig. 2A). The increase in the ThT fluorescence was not due to a direct interaction between ThT and heparin, as heparin alone had no effect on ThT fluorescence.

The experiment was repeated under the same conditions described above, in the absence of heparin and in the presence of 0.56 μM HS (7.5 μg/ml). HS was found to cause an increase of both the initial and final ThT fluoresce intensities without affecting the k_{agg} value (Fig. 2C and D; Table 1). The reason for this behaviour is discussed elsewhere (*41*).

mAcP was then allowed to aggregate in the presence of different concentrations of GlcA, GlcNAc, GlcNAc-6S and GlcNS. The effect of each monosaccharide derivative was tested at four different concentrations, spanning three orders of magnitude and ranging from 1.7 μM to 1.7 mM. The lowest concentration of monosaccharide derivative used (1.7 μM) corresponds to a molar concentration of the related monosaccharide residue in a heparin solution of 0.06 μM, i.e. the smallest concentration of heparin used in this study (Table 1, column 3). The second lowest concentration of monosaccharide derivative used (17 μM) corresponds to a molar concentration of the related monosaccharide residue in a heparin solution of 0.56 μM, which is beyond the range used in our experiments. It corresponds to a molar concentration of the related monosaccharide residue in a HS solution of 0.56 μM, which is that used in our experiments (Table 1, column 3). The two other concentrations of monosaccharide used, 170 μM and 1.7 mM, correspond to molar concentrations of the same monosaccharide residue in heparin solutions of 5.6 and 56 μM, respectively. In a previous work where mAcP aggregation was studied using the same experimental conditions, equivalent concentrations of heparin were found to accelerate significantly the aggregation process (*28*). Importantly, the

Table 1. **Measured parameters of mAcP aggregation.**[a]

Additive	Concentration (μM)	Concentration of monosaccharide (μM)	Concentration of SO_3^- (μM)	k_{agg} (10^{-3} s^{-1})[b]	Initial ThT fluorescence (AU)[c]	Final ThT fluorescence (AU)[c]
None				1.11 ± 0.07	0 ± 0	32 ± 1
Heparin[d]	0.06	3.4	5	2.0 ± 0.2	4 ± 1	37 ± 1
	0.11	6.6	10	2.4 ± 0.2	9 ± 1	38 ± 1
	0.17	10.0	15	3.7 ± 0.3	13 ± 1	42 ± 2
	0.28	16.6	25	ND	42 ± 2	53 ± 1
HS[d]	0.56	34.6	26	1.11 ± 0.07	23 ± 1	48 ± 1
GlcA	1.7	1.7	0	1.20 ± 0.02	0 ± 0	35 ± 3
	17	17	0	1.1 ± 0.1	0 ± 0	36 ± 2
	170	170	0	1.21 ± 0.06	1 ± 1	34 ± 3
	1700	1700	0	1.45 ± 0.01	0 ± 0	36 ± 1
GlcNAc	1.7	1.7	0	1.1 ± 0.2	0 ± 0	38 ± 1
	17	17	0	0.9 ± 0.2	1 ± 1	37 ± 2
	170	170	0	0.88 ± 0.07	1 ± 1	38 ± 1
	1700	1700	0	0.8 ± 0.2	0 ± 0	35 ± 3
GlcNAc-6S	1.7	1.7	1.7	1.23 ± 0.03	1 ± 1	36 ± 2
	17	17	17	0.92 ± 0.09	0 ± 0	36 ± 2
	170	170	170	0.9 ± 0.1	0 ± 0	35 ± 2
	1700	1700	1700	1.1 ± 0.2	0 ± 0	33 ± 1
GlcNS	1.7	1.7	1.7	0.9 ± 0.1	1 ± 1	35 ± 1
	17	17	17	1.2 ± 0.1	0 ± 0	35 ± 1
	170	170	170	1.44 ± 0.08	0 ± 0	32 ± 1
	1700	1700	1700	0.90 ± 0.05	0 ± 0	36 ± 2
Mixture[e]	170	170	127	1.2 ± 0.3	2 ± 1	31 ± 1
2-mer[f]	5.0	10.0	15	1.22 ± 0.09	3 ± 1.5	46 ± 2.2
6-mer[f]	1.67	10.0	15	1.49 ± 0.02	29 ± 0.4	51 ± 5.2
12-mer[f]	0.83	10.0	15	1.26 ± 0.02	24 ± 1.4	50 ± 2.4
18-mer[f]	0.56	10.0	15	0.99 ± 0.05	14 ± 3.7	38.3 ± 0.7

[a]Aggregation was studied at a protein concentration of 36 μM, in 25% (v/v) TFE, 50 mM acetate buffer, pH 5.5, 25°C. [b]k_{agg}, rate constant of aggregation, obtained by analysing the ThT fluorescence time course using a procedure of best fitting and a single exponential function [Equation (1)]. For the aggregation of mAcP in the absence of any additive, the indicated mean and associated SE values follow 23 independent experiments, 14 of them having been published previously (*28*, *35*, *44*). In the other cases, each entry gives the mean and associated SE values of two to six independent experiments. [c]ThT fluorescence intensity at 485 nm (excitation at 440 nm). Experimental errors correspond to SEs. [d]Heparin and HS lengths of 60 monosaccharide residues were used in our experiments. [e]The exact composition of the mixture was 42.5 μM GlcNAc, 42.5 μM GlcNS and 85 μM GlcNAc-6S. [f]Heparin fragments containing 2 (2-mer), 6 (6-mer), 12 (12-mer) and 18 (18-mer) monosaccharide residues.

highest concentration of monosaccharide derivative used exceeds by two orders of magnitude the equivalent concentration of the same monosaccharide type at the highest concentrations of heparin and HS used here (Table 1, column 3).

None of monosaccharide derivatives had any effect on mAcP aggregation, at any of the concentrations tested (Figs 3 and 6; Table 1). Neither the k_{agg} value nor the initial or final intensities of ThT fluorescence appeared to be significantly affected by the presence of these derivatives (Figs 3 and 6; Table 1). The k_{agg} values of mAcP aggregation in the presence of monosaccharide derivatives were equal or even lower than in their absence, as noticed for GlcNAc at high concentrations (Fig. 6; Table 1). The intensities of ThT fluorescence were unchanged, both after 30 s and at the apparent plateau (Table 1).

To test whether the effect of heparin on mAcP aggregation was due to a synergic effect between different monosaccharide derivatives that were individually inactive, mAcP was allowed to aggregate in the presence of a mixture of monosaccharide derivatives. This mixture contained GlcNAc, GlcNAc-6S and GlcNS in the proportions 1 : 2 : 1, for a total monosaccharide concentration of 170 μM. The respective concentrations used for the different monosaccharide derivatives mimic the number and types (*N*- or *O*-sulphatation) of sulphate moieties present on a standard disaccharide unit of heparin. The total monosaccharide concentration corresponds to one of the highest monosaccharide concentrations used in the experiments described above in which the derivatives were tested separately, and is one order of magnitude higher than the monosaccharide concentration present in the most concentrated heparin solution used here. The results show that the mixture of monosaccharide derivatives has absolutely no effect on mAcP aggregation kinetics (Figs 4 and 6; Table 1). Overall, these results imply that the various monosaccharide derivatives, and the various negatively charged moieties present on them, accelerate mAcP aggregation and change the ThT fluorescence intensities at the beginning and at the end of the observed exponential phase only when they are clustered on the same macromolecule. Hence, the aggregation-inducing capacities of heparin and HS arise from their polymeric nature (see Discussion below).

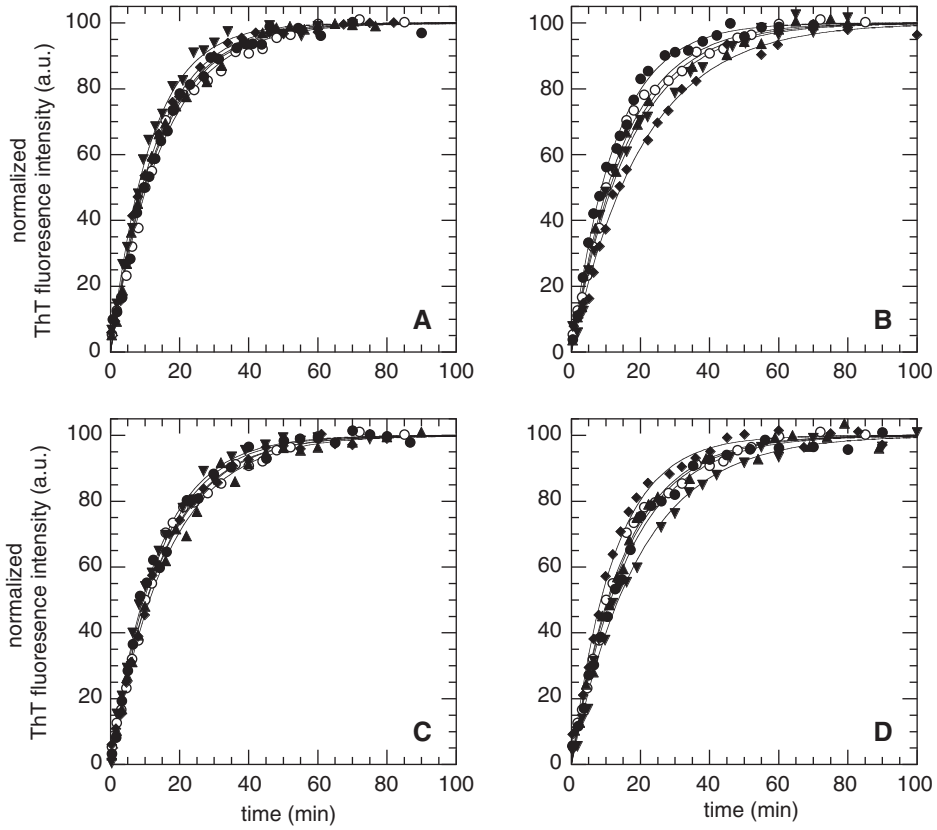

Fig. 3. **Time courses of mAcP aggregation in the presence and absence of monosaccharide derivatives.** (A) GlcA; (B) GlcNAc; (C) GlcNAc-6S; and (D) GlcNS. Each panel reports mAcP aggregation without monosaccharide derivative (empty circles), with 1.7 μM (closed circles), 17 μM (triangles), 170 μM (diamonds) and 1700 μM (inverted triangles) monosaccharide derivative.

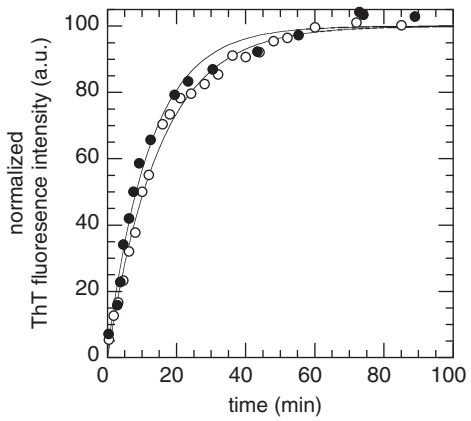

Fig. 4. **Time courses of mAcP aggregation in the presence and absence of a mixture of monosaccharide derivatives.** Traces of mAcP aggregation without monosaccharide derivative (empty circles), and with a mixture of GlcNAc (42.5 μM), GlcNS (42.5 μM) and GlcNAc-6S (85 μM) (closed circles) are reported.

To test the minimal lengths of the heparin and HS chains necessary to produce their effects on mAcP amyloid aggregation, we have monitored the time course of mAcP aggregation in the presence of heparin fragments of various lengths. These include compounds with 2, 6, 12 and 18 monosaccharide units and are called 2-, 6-, 12- and 18-mer, respectively. They all contain alternating IdoA-2S and GlcNS-6S residues, similarly to heparin (e.g. the 2-mer contains one IdoA-2S and one GlcNS-6S residue). In these experiments, mAcP was incubated under the same conditions described above, in the presence of either 5.0 μM 2-mer, 1.67 μM 6-mer, 0.83 μM 12-mer or 0.56 μM 18-mer, to achieve in all cases a total monosaccharide concentration of 10.0 μM. The 2-mer did not have any effect on the k_{agg} value and on the initial ThT fluorescence intensity, causing just a small increase of the final ThT fluorescence (Figs 5 and 6; Table 1). The 6-, 12- and 18-mer induced increases of both the initial and final ThT fluorescence intensities, without affecting the k_{agg} value (Figs 5 and 6; Table 1). These data indicate that while a length of two monosaccharide residues is insufficient to cause any change in the aggregation time courses, longer heparin fragments with 6, 12 and 18 units can reproduce the effects of HS. Both HS and heparin fragments can indeed increase the initial and final ThT fluorescence intensity without affecting the rate of the exponential change observed after the initial ThT fluorescence jump (compare Fig. 2C and 5A). This is consistent with the notion that the HS chain contains alternating sulphated and non-sulphated domains, where each sulphated

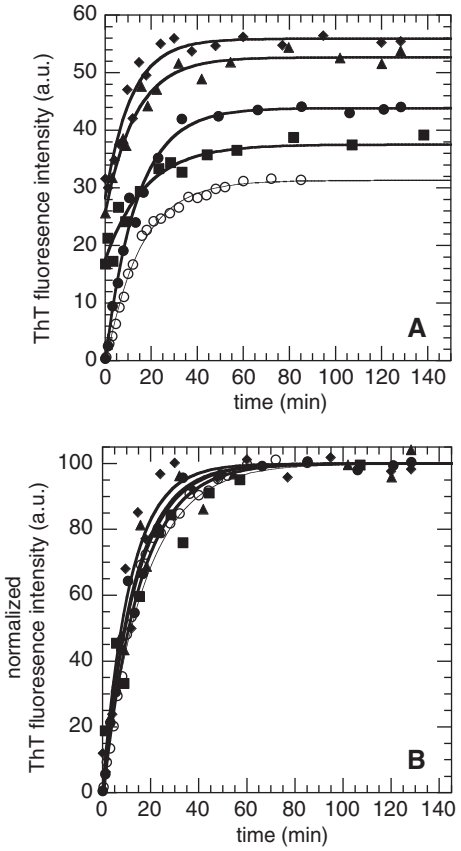

Fig. 5. **Time courses of mAcP aggregation in the presence of heparin fragments.** Empty circles, no additive; filled circles, 5.0 μM 2-mer; diamonds, 1.67 μM 6-mer; triangles, 0.83 μM 12-mer; and squares, 0.56 μM 18-mer. Such concentrations correspond to 10 μM monosaccharide for all fragments tested.

stretch has a length of about 5–10 monosaccharide residues each (see Discussion below). However, even heparin fragments with 18 monosaccharide units cannot reproduce the effect of whole-length heparin on the rate constant of mAcP aggregation.

DISCUSSION

The aim of this work was to evaluate whether the various sulphate moieties present on heparin and HS can promote mAcP aggregation either when they are freely circulating in solution, but still in the chemical context of the constituent monosaccharides, or only through a synergistic mechanism when they are clustered on the polymeric structure of the GAG. The results strongly indicate that none of the negative moieties present on heparin and HS can affect amyloid formation by mAcP when present free in solution. Heparin had a complex effect on mAcP aggregation, increasing (i) the rate constant of aggregation, k_{agg}, (ii) the final ThT fluorescence emission and (iii) the initial ThT fluorescence, all phenomena being concentration dependent. HS also caused an increase of the initial and final ThT fluorescence, without affecting the rate constant of aggregation. The four monosaccharide derivatives had no effect on either factor, even at molar concentrations of 1.7 mM, which is ~100-fold higher than the equivalent concentration of constituent monosaccharide in the heparin macromolecule at the highest heparin concentration used. The absence of any effect persists when the monosaccharide derivatives are mixed to reconstitute the composition of *O*- and *N*-sulphate groups present on the heparin disaccharide unit.

When short fragments of heparin were used, we could not detect any effect for the 2-mer (i.e. the heparin constituent disaccharide), but we found that the 6-, 12- and 18-mer could reproduce the effect of HS, causing an increase of both the initial and final ThT fluorescence at the extremes of the observed exponential phase. Since the HS polymer is composed of highly sulphated stretches of 5–10 monosaccharide units alternated by non-sulphated segments, with the former being similar in structure and sulphate composition to heparin, the heparin fragments containing 6, 12 and 18 units can have an effect similar to HS where such stretches are in isolation. Neither HS, nor the heparin fragments could induce an increase of the rate of the observed exponential phase, indicating that in this case the long, highly polymeric structure of heparin is a required factor. This analysis allows limits to be fixed for both effects: a length between two and six sulphated monosaccharide residues is required to cause the final and initial increases of the ThT fluorescence, whereas a length exceeding 18 monosaccharide residues is required to have an acceleration of the observed exponential phase.

Fraser *et al*. (*45*) previously reported qualitative differences on the effects of full-length GAGs and some of its saccharide derivatives on fibril formation. However, the authors used only unique concentrations of protein and GAG, and studied parameters difficult to interpret like the extent of lateral aggregation (*45*). Suk *et al*. (*20*) reported a molecular weight-dependent accelerating effect on the aggregation of the 173–243 fragment of D187N gelsolin when using different heparin oligosaccharides, ranging from a 4-mer to 5 kDa heparin fragments.

The observed effect of heparin and HS on the initial and final fluorescence intensity of the ThT probe could have different grounds. The increase in the ThT fluorescence intensity at plateau can be attributed to neither a higher quantity of fibrils formed as most of the mAcP molecules aggregate even in the absence of GAGs (*28*), nor to significant differences in the fibril morphology, since mAcP fibrils formed in the absence and presence of heparin have similar characteristics in electron microscopy and circular dichroism (*28*). Therefore, the higher ThT fluorescence values observed in the presence of heparin are likely to arise from a higher degree of rigidity within the aggregates (*46*, *47*). The higher ThT signal observed at the beginning of the observed exponential phase in the presence of heparin or HS is likely to arise from the presence of ThT-positive aggregates already present after 30 s. These phenomena are described elsewhere (*41*).

The effect of a specific GAG on the aggregation kinetics could be influenced by the concentrations of

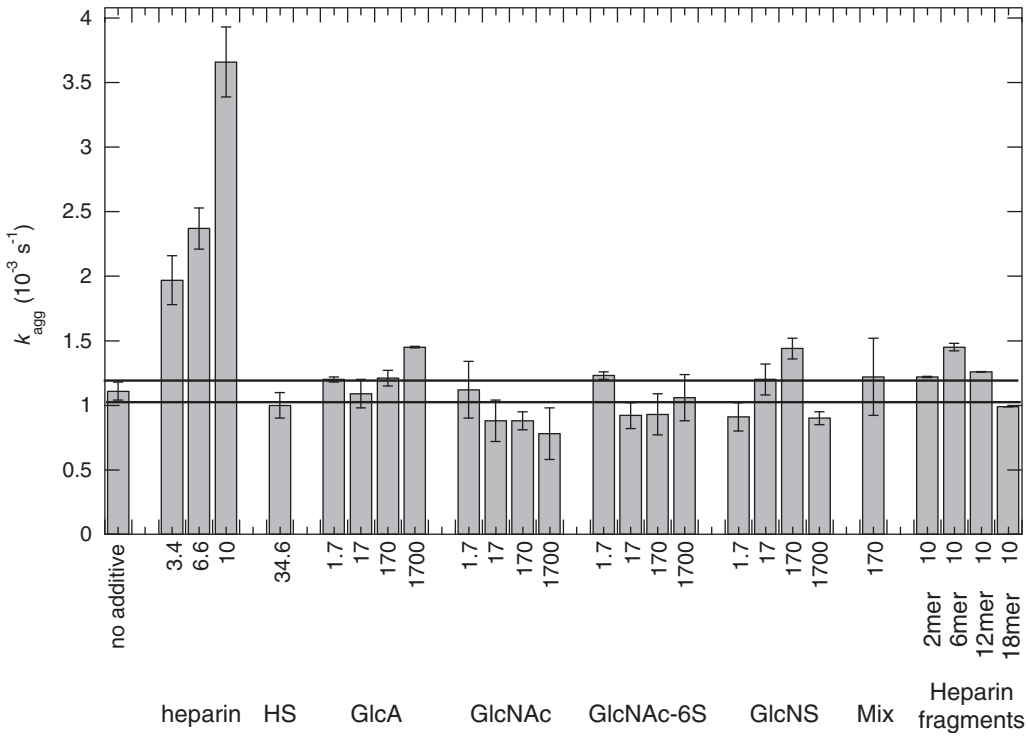

Fig. 6. **Effect of heparin, HS, monosaccharide derivatives and heparin fragments on the rate constant of mAcP aggregation (k_{agg}).** The numbers below the bars indicate the equivalent monosaccharide concentrations used in the experiments. The three monosaccharide concentrations indicated here for heparin correspond to heparin concentrations of 1 μg/ml (0.06 μM), 2 μg/ml (0.11 μM) and 3 μg/ml (0.17 μM), respectively. The exact composition of the mixture was the following: 42.5 μM GlcNAc, 42.5 μM GlcNS and 85 μM GlcNAc-6S. The four monosaccharide concentrations indicated here for heparin fragments correspond to concentrations of 5.0 μM (2-mer), 1.67 μM (6-mer), 0.83 μM (12-mer) and 0.56 μM (18-mer) heparin fragment. Values on the *y*-axis correspond to the mean and associated SE of k_{agg} reported in Table 1. The two horizontal lines indicate the mean value of k_{agg} in the absence of any additive ± 1 SE.

both protein and GAG (*28*, *31*, Monsellier, E. *et al*., unpublished data), or by the sulphation state of the GAG (*14*, *20*, *29*). We tested for each monosaccharide four different concentrations, within a range spanning three orders of magnitude (from 1.7 μM to 1.7 mM). Importantly, this range includes the equivalent concentrations of the constituent monosaccharides (Table 1, column 3) and of sulphate moieties (Table 1, column 4) under conditions in which heparin and HS effectively influence mAcP aggregation. Thus, the differential effects of heparin/HS and monosaccharide derivatives on the aggregation kinetics of mAcP do not originate from different sulphation states or different concentrations of the molecular species. These differential effects could also arise from a synergic cooperation between the different sulphate moieties present on heparin and HS, for example, between the *N*- and *O*-sulphates. However, a mixture of GlcNAc, GlcNAc-6S and GlcNS, in proportions that mimic the *N*- and *O*-sulphation composition of a heparin or HS disaccharide, had no effect on the aggregation kinetics of mAcP. Therefore, the differential effects of heparin/HS and monosaccharide derivatives on mAcP aggregation probably originate from their different chemical nature, which is polymeric in the former and monomeric in the latter. The observation that heparin fragments having a length comparable with that of the sulphated stretches in HS can reproduce the effect of HS but not of high molecular weight heparin further confirms that the effect of heparin and HS on mAcP aggregation is due to the polymeric nature of these GAGs.

In the sulphated domains of HS and in heparin, monosaccharide residues are aligned into a linear polymer, in which the sulphate moieties are regularly distributed along a constant pattern (Fig. 1A) (*48*). In contrast, in the two sulphate-containing monosaccharide derivatives used in this study, the various sulphate groups are freely circulating in solution, implying that they are not clustered together in one restricted region of space and do not have a regular distribution. Considering that the effect of GAGs on protein aggregation is primarily mediated by sulphate groups (*14*, *20*, *29*, Monsellier, E. *et al*., unpublished data), it is likely that the regular arrangement and high density of the sulphate moieties along the polymer are responsible for the heparin/HS-induced effects on mAcP aggregation. Very interestingly, a similar conclusion has been drawn for the 'protein side' of the equation. In fact, Dobson and colleagues (*28*) observed that the interaction between heparin and fibrillar mAcP was stronger than the interaction between heparin and the native form of the protein. It was thus suggested that a higher density of positive charges in the fibrillar form of the protein accounts for the higher affinity for heparin (*28*).

CONCLUSIONS

Overall, the data presented here indicate that negative charges present in heparin and HS promote mAcP aggregation only when they are clustered and regularly distributed on the polymeric structure of these GAGs. A possible mechanism describing the aggregation-promoting effect of heparin that accounts for our and previously published results is described in Fig. 7. The unstructured monomer of mAcP binds to the heparin molecule through ionic interactions between the positive charges of the protein and the negatively charged sulphate moieties of the GAG. The regular distribution and high density of sulphate groups along the heparin molecule allows a similar orientation of the sulphate-bound protein molecules. This could have two important consequences, both resulting in facilitated aggregation: an increase of the local concentration of the protein molecules and an induced orientation of the various protein molecules. The latter is *per se* aggregation inducing as fibrils tend to have a regular structure in which the various protein molecules form in-register parallel β-strands (*2*). Once formed, the fibrils remain associated with the heparin molecule. Strikingly, a similar mechanism has been proposed for the acceleration of the copper–zinc superoxide dismutase aggregation by DNA (*49*). Therefore, this template-based acceleration of protein aggregation could possibly be a general characteristic of negatively charged biological polymers.

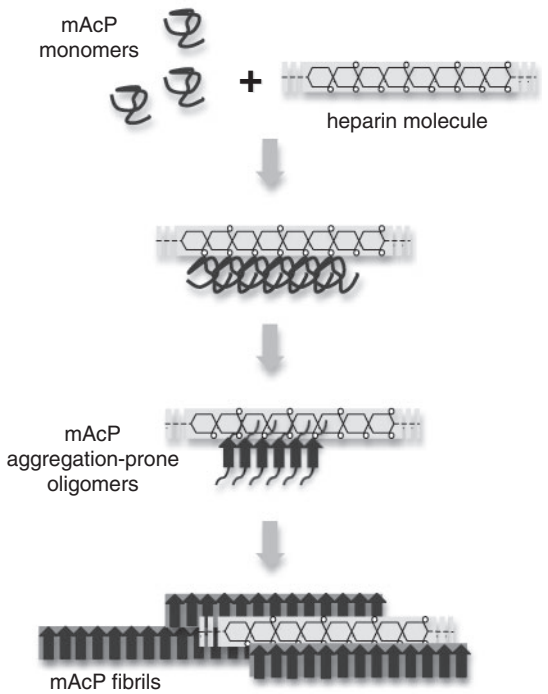

Fig. 7. **Proposed mechanism of scaffolding-based acceleration of mAcP aggregation by heparin.** In this model, mAcP monomers interact with the numerous regularly spaced sulphate moieties on the heparin molecule. This interaction leads to an increase of local protein concentration and/or a change of the mAcP molecules' orientation, with both factors promoting oligomerization and fibril formation. Heparin remains associated to the fibrils.

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CONFLICT OF INTEREST

None declared.

REFERENCES

1. Westermark, P., Benson, M.D., Buxbaum, J.N., Cohen, A.S., Frangione, B., Ikeda, S., Masters, C.L., Merlini, G., Saraiva, M.J., Sipe, J.D., and Nomenclature Committee of the International Society of Amyloidosis, (2005) Amyloid: toward terminology clarification. Report from the Nomenclature Committee of the International Society of Amyloidosis. *Amyloid* **12**, 1–4
2. Chiti, F. and Dobson, C.M. (2006) Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.* **75**, 333–366
3. Merlini, G. and Westermark, P. (2004) The systemic amyloidoses: clearer understanding of the molecular mechanisms offers hope for more effective therapies. *J. Intern. Med.* **255**, 159–178
4. Walsh, D.M. and Selkoe, D.J. (2007) A beta oligomers - a decade of discovery. *J. Neurochem.* **101**, 1172–1184
5. Pepys, M.B. (2001) Pathogensis diagnosis and treatment of systemic amyloidosis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **356**, 203–210
6. Uversky, V.N., Li, J., and Fink, A.L. (2001) Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular link between Parkinson's disease and heavy metal exposure. *J. Biol. Chem.* **276**, 44284–44296
7. Ancsin, J.B. (2003) Amyloidogenesis: historical and modern observations point to heparan sulfate proteoglycans as a major culprit. *Amyloid* **10**, 67–79
8. Ellis, R.J. (2003) Protein misassembly: macromolecular crowding and molecular chaperones. *Adv. Exp. Med. Biol.* **594**, 1–13
9. Gorbenko, G.P. and Kinnunen, P.K. (2006) The role of lipid-protein interactions in amyloid-type protein fibril formation. *Chem. Phys. Lipids* **141**, 72–82
10. Relini, A., Canale, C., De Stefano, S., Rolandi, R., Giorgetti, S., Stoppini, M., Rossi, A., Fogolari, F., Corazza, A., Esposito, G., Gliozzi, A., and Bellotti, V. (2006) Collagen plays an active role in the aggregation of beta2-microglobulin under physiopathological conditions of dialysis-related amyloidosis. *J. Biol. Chem.* **281**, 16521–16529
11. Tartaglia, G.G., Pechmann, S., Dobson, C.M., and Vendruscolo, M. (2007) Life on the edge: a link between gene expression levels and aggregation rates of human proteins. *Trends Biochem. Sci.* **32**, 204–206
12. Bellotti, V. and Chiti, F. (2008) Amyloidogenesis in its biological environment: challenging a fundamental issue in protein misfolding diseases. *Curr. Opin. Struct. Biol.* **18**, 771–779
13. Raman, R., Sasisekharan, V., and Sasisekharan, R. (2005) Structural insights into biological roles of protein-glycosaminoglycan interactions. *Chem. Biol.* **12**, 267–277
14. Castillo, G.M., Cummings, J.A., Yang, W., Judge, M.E., Sheardown, M.J., Rimvall, K., Hansen, J.B., and Snow, A.D. (1998) Sulfate content and specific glycosaminoglycan backbone of perlecan are critical for perlecan's

enhancement of islet amyloid polypeptide (amylin) fibril formation. *Diabetes* **47**, 612–620
15. Takase, K. (1998) Reactions of denatured proteins with other cellular components to form insoluble aggregates and protection by lactoferrin. *FEBS Lett.* **441**, 271–274
16. McLaurin, J., Franklin, T., Zhang, X., Deng, J., and Fraser, P.E. (1999) Interactions of Alzheimer amyloid-beta peptides with glycosaminoglycans effects on fibril nucleation and growth. *Eur. J. Biochem.* **266**, 1101–1110
17. Wong, C., Xiong, L.W., Horiuchi, M., Raymond, L., Wehrly, K., Chesebro, B., and Caughey, B. (2001) Sulfated glycans and elevated temperature stimulate PrP(Sc)-dependent cell-free formation of protease-resistant prion protein. *EMBO J.* **20**, 377–386
18. Yamamoto, S., Yamaguchi, I., Hasegawa, K., Tsutsumi, S., Goto, Y., Gejyo, F., and Naiki, H. (2004) Glycosaminoglycans enhance the trifluoroethanol-induced extension of beta 2-microglobulin-related amyloid fibrils at a neutral pH. *J. Am. Soc. Nephrol.* **15**, 126–133
19. McLaughlin, R.W., De Stigter, J.K., Sikkink, L.A., Baden, E.M., and Ramirez-Alvarado, M. (2006) The effects of sodium sulfate, glycosaminoglycans, and Congo red on the structure, stability, and amyloid formation of an immunoglobulin light-chain protein. *Protein Sci.* **15**, 1710–1722
20. Suk, J.Y., Zhang, F., Balch, W.E., Linhardt, R.J., and Kelly, J.W. (2006) Heparin accelerates gelsolin amyloidogenesis. *Biochemistry* **45**, 2234–2242
21. Borysik, A.J., Morten, I.J., Radford, S.E., and Hewitt, E.W. (2007) Specific glycosaminoglycans promote unseeded amyloid formation from beta2-microglobulin under physiological conditions. *Kidney Int.* **72**, 174–181
22. Gupta-Bansal, R., Frederickson, R.C., and Brunden, K.R. (1995) Proteoglycan-mediated inhibition of A beta proteolysis. A potential cause of senile plaque accumulation. *J. Biol. Chem.* **270**, 18666–18671
23. McLaurin, J., Franklin, T., Kuhns, W.J., and Fraser, P.E. (1999) A sulfated proteoglycan aggregation factor mediates amyloid-beta peptide fibril formation and neurotoxicity. *Amyloid* **6**, 233–243
24. Bergamaschini, L., Donarini, C., Rossi, E., De Luigi, A., Vergani, C., and De Simoni, M.G. (2002) Heparin attenuates cytotoxic and inflammatory activity of Alzheimer amyloid-beta in vitro. *Neurobiol. Aging* **23**, 531–536
25. Caughey, B., Brown, K., Raymond, G.J., Katzenstein, G.E., and Thresher, W. (1994) Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and congo red. *J. Virol.* **68**, 2135–2141
26. Friedhoff, P., Schneider, A., Mandelkow, E.M., and Mandelkow, E. (1998) Rapid assembly of Alzheimer-like paired helical filaments from microtubule-associated protein tau monitored by fluorescence in solution. *Biochemistry* **37**, 10223–10230
27. Narindrasorasak, S., Lowery, D., Gonzalez-DeWhitt, P., Poorman, R.A., Greenberg, B., and Kisilevsky, R. (1991) High affinity interactions between the Alzheimer's beta-amyloid precursor proteins and the basement membrane form of heparan sulfate proteoglycan. *J. Biol. Chem.* **266**, 12878–12883
28. Calamai, M., Kumita, J.R., Mifsud, J., Parrini, C., Ramazzotti, M., Ramponi, G., Taddei, N., Chiti, F., and Dobson, C.M. (2006) Nature and significance of the interactions between amyloid fibrils and biological polyelectrolytes. *Biochemistry* **45**, 12806–12815
29. Castillo, G.M., Lukito, W., Wight, T.N., and Snow, A.D. (1999) The sulfate moieties of glycosaminoglycans are critical for the enhancement of beta-amyloid protein fibril formation. *J. Neurochem.* **72**, 1681–1687
30. Ancsin, J.B. and Kisilevsky, R. (1999) The heparin/heparan sulfate-binding site on apo-serum amyloid A. Implications for the therapeutic intervention of amyloidosis. *J. Biol. Chem.* **274**, 7172–7181
31. Cohlberg, J.A., Li, J., Uversky, V.N., and Fink, A.L. (2002) Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from alpha-synuclein in vitro. *Biochemistry* **41**, 1502–1511
32. Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C.M. (1999) Designing conditions for in vitro formation of amyloid protofilaments and fibrils. *Proc. Natl Acad. Sci. USA* **96**, 3590–3594
33. Chiti, F., Taddei, N., Bucciantini, M., White, P., Ramponi., G., and Dobson, C.M. (2000) Mutational analysis of the propensity for amyloid formation by a globular protein. *EMBO J.* **19**, 1441–1449
34. Chiti, F., Taddei, N., Stefani, M., Dobson, C.M., and Ramponi, G. (2001) Reduction of the amyloidogenicity of a protein by specific binding of ligands to the native conformation. *Protein Sci.* **10**, 879–886
35. Chiti, F., Taddei, N., Baroni, F., Capanni, C., Stefani, M., Ramponi, G., and Dobson, C.M. (2002) Kinetic partitioning of protein folding and aggregation. *Nat. Struct. Biol.* **9**, 137–143
36. Chiti, F., Stefani, M., Taddei, N., Ramponi, G., and Dobson, C.M. (2003) Rationalization of the effects of mutations on peptide and protein aggregation rates. *Nature* **424**, 805–808
37. Calamai, M., Chiti, F., and Dobson, C.M. (2005) Amyloid fibril formation can proceed from different conformations of a partially unfolded protein. *Biophys. J.* **89**, 4201–4210
38. Calamai, M., Canale, C., Relini, A., Stefani, M., Chiti, F., and Dobson, C.M. (2005) Reversal of protein aggregation provides evidence for multiple aggregated states. *J. Mol. Biol.* **346**, 603–616
39. Calamai, M., Tartaglia, G.G., Vendruscolo, M., Chiti, F., and Dobson, C.M. (2008) Mutational analysis of the aggregation-prone and disaggregation-prone regions of acylphosphatase. *J. Mol. Biol.* **387**, 965–974
40. Pastore, A., Saudek, V., Ramponi, G., and Williams, R.J. (1992) Three-dimensional structure of acylphosphatase. Refinement and structure analysis. *J. Mol. Biol.* **224**, 427–440
41. Motamedi-Shad, N., Monsellier, E., Torrassa, S., Relini, A., and Chiti, F. (2009) Kinetic analysis of amyloid formation in the presence of heparan sulfate. Faster unfolding and change of pathway. *J. Mol. Biol.* (in press)
42. van Nuland, N.A., Chiti, F., Taddei, N., Raugei, G., Ramponi, G., and Dobson, C.M. (1998) Slow folding of muscle acylphosphatase in the absence of intermediates. *J. Mol. Biol.* **283**, 883–891
43. Modesti, A., Taddei, N., Bucciantini, M., Stefani, M., Colombini, B., Raugei, G., and Ramponi, G. (1995) Expression, purification, and characterization of acylphosphatase muscular isoenzyme as fusion protein with glutathione S-transferase. *Protein Expr. Purif.* **6**, 799–805
44. Bemporad, F., Taddei, N., Stefani, M., and Chiti, F. (2006) Assessing the role of aromatic residues in the amyloid aggregation of human muscle acylphosphatase. *Protein Sci.* **15**, 862–870
45. Fraser, P.E., Darabie, A.A., and McLaurin, J.A. (2001) Amyloid-beta interactions with chondroitin sulfate-derived monosaccharides and disaccharides. Implications for drug development. *J. Biol. Chem.* **276**, 6412–6419
46. Krebs, M.R., Bromley, E.H., and Donald, A.M. (2005) The binding of thioflavin-T to amyloid fibrils: localisation and implications. *J. Struct. Biol.* **149**, 30–37
47. Stsiapura, V.I., Maskevich, A.A., Kuzmitsky, V.A., Uversky, V.N., Kuznetsova, I.M., and Turoverov, K.K. (2008) Thioflavin T as a molecular rotor: fluorescent properties of thioflavin T in solvents with different viscosity. *J. Phys. Chem. B* **112**, 15893–15902
48. Rabenstein, D.L. (2002) Heparin and heparan sulfate: structure and function. *Nat. Prod. Rep.* **19**, 312–331
49. Jiang, W., Han, Y., Zhou, R., Zhang, L., and Liu, C. (2007) DNA is a template for accelerating the aggregation of copper, zinc superoxide dismutase. *Biochemistry* **46**, 5911–5923